Journal of Pharmaceutical Investigation
Vol. 41, No. 5, 273-278 (2011)

Pharmacokinetic Interaction between Warfarin and Efonidipine in Rats

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(Received July 5, 2011 · Revised July 26, 2011 · Accepted September 5, 2011)

ABSTRACT − The aim of this study was to investigate the effect of efonidipine on the pharmacokinetics of warfarin after oral and intravenous administration of warfarin in rats. Warfarin was administered orally (0.2 mg/kg) or intravenously (0.05 mg/kg) without or with oral administration of efonidipine (1 or 3 mg/kg) in rats. The effect of efonidipine on the cytochrome P450 (CYP) 3A4 activity was also evaluated. Efonidipine inhibited CYP3A4 enzyme activity with 50% inhibition concentration (IC_{50}) of 0.08 μM. Compared to those in the oral control group (warfarin without efonidipine), the area under the plasma concentration–time curve (AUC) of warfarin was significantly greater (1 mg/kg, $P<0.05$; 3 mg/kg, $P<0.01$) by 25.9-59.0%, and the peak plasma concentration (C_{max}) was significantly higher (3 mg/kg, $P<0.05$) by 26.2% after oral administration of warfarin with efonidipine, respectively. The total body clearance of warfarin was significantly (3 mg/kg, $P<0.05$) decreased by efonidifine. Consequently, the relative bioavailability of warfarin was increased by 1.26- to 1.59-fold and the absolute bioavailability of warfarin with efonidipine was significantly greater by 59.7-75.4 % compared to that in the control group (47.4%). In contrast, efonidipine had no effect on any pharmacokinetic parameters of warfarin given intravenously. Therefore, the enhanced oral bioavailability of warfarin may be due to inhibition of CYP 3A4-mediated metabolism in the intestine and/or liver and to reduction of total body celarance rather than renal elimination, resulting in reducing first-pass metabolism by efonidipine.

Key words − Warfarin, Efonidipine, Pharmacokinetics, Bioavailability, CYP 3A, Rats

Efonidipine is a novel dihydropyridine calcium antagonist and is a powerful vasodilator with considerably less negative inotropic action than any other dihydropyridine derivative (Nakabeppu et al., 1996; Masuda et al., 1990); its antihypertensive action is very slow in onset and long-lasting (Tamura et al., 1991). Previous studies have shown that, in contrast to other dihydropyridines, efonidipine decreases the heart rate in patients with essential hypertension (Tanaka et al., 2007; Shimizu et al., 2003). In general, 1,4-dihydropyridine calcium antagonists are inhibitors of CYP3A4; however, there are few reports about CYP enzyme activity for efonidipine. Therefore, we evaluated the inhibition of CYP enzyme activity by efonidipine using CYP inhibition assays.

Warfarin is the most extensively used oral anticoagulant for the prevention and treatment of thromboembolic complications in cardiovascular diseases such as atrial fibrillation, venous thrombosis and pulmonary embolism (Hirsh et al., 1998). Warfarin's anticoagulant effect is due to its interference with the cyclic interconversion of vitamin K and its 2, 3 epoxide, and to its limitation of the synthesis of the vitamin K-dependent clotting factors, II, VII, IX and X (Scordo et al., 2002; Wallin et al., 2002). Warfarin is readily absorbed from the gastrointestinal tract, extensively bound to plasma proteins. Warfarin is used as a racemic mixture of roughly equal amounts of R and S enantiomers yet S-warfarin has been reported to be more potent (Mungall, 1985). Warfarin is metabolized by CYPs and is converted to inactive metabolites through selective hydroxylation (Holford, 1986). R-warfarin is metabolized primarily by CYP3A4 to 10-hydroxywarfarin, by CYP1A2 to 6- and 8-hydroxywarfarin. On the other hand, S-warfarin is metabolized primarily by CYP2C9 to 7-hydroxywarfarin. Potential warfarin-drug interactions could occur with any of a very wide range of drugs that are metabolized by these CYPs, and a number of such interactions have been reported (Kaminsky and Zhang, 1997).

However, there have been a few reports regarding the effects of calcium antagonist on the bioavailability or pharmacokinetics of warfarin in rats (Abernethy and Kaminsky, 1991; Stoysich et al., 1996). Moreover, efonidipine and warfarin could be prescribed for the prevention or treatment of thromboembolism and ischemic stroke in some patients with atrial fibrillation, valvular heart disease and a myocardial infarction as a combination therapy. Warfarin has a narrow therapeutic range and its efficacy can be influenced by drug-drug inter-

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DOI : 10.4333/KPS.2011.41.5.273

actions. Therefore, the present study aimed to investigate the effects of efonidipine on the CYP3A subfamily and pharmacokinetics of warfarin after oral and intravenous administration in rats.

Materials and Methods

Chemicals and apparatus

Warfarin, efonidipine and 7-ethoxycoumarin (internal standard for high-performance liquid chromatograph analysis of warfarin) were purchased from Sigma-Aldrich Co. (St. Louis, MO, USA). HPLC grade acetonitrile was a purchased from Merck Co. (Darmstadt, Germany). Other chemicals for this study were reagent grade.

The HPLC system used in this study was comprised of a Waters 1515 isocratic HPLC pump, a Waters 717 plus autosampler and a Waters™ 474 scanning fluorescence detector (Waters Co., Milford, MA, USA), a 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 micro centrifuge (Hitachi Co., Tokyo, Japan).

Animal experiments

Male Sprague-Dawley rats 7-8 weeks of age (weighing 270-300 g) were purchased from Dae Han Laboratory Animal Research Co. (Choongbuk, Republic of Korea) and given free access to a commercial rat chow diet (No. 322-7-1; Superfeed Co., Gangwon, Republic of Korea) and tap water. The animals were housed, two per cage, maintained at 22 ± 2°C and 50-60% relative humidity under a 12:12 h light-dark cycle. The rats were acclimated under these conditions for at least 1week. 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) and the Animal Care Committee of Chosun University (Gwangju, Republic of Korea) approved the protocol of this animal study. The rats were fasted for at least 24 h prior to beginning the experiments and had free access to tap water. Each animal was anaesthetized with light ether. The left femoral artery and vein were cannulated using polyethylene tubing (SP45, I.D. 0.58 mm, O.D. 0.96 mm; Natsume Seisakusho Co. LTD., Tokyo, Japan) for blood sampling and drug administration, respectively.

Oral and intravenous administration of warfarin

The rats were randomly divided into six groups (n = 6, each); an oral group (0.2 mg/kg of warfarin dissolved in water; homogenized at 36°C for 30 min; 1.0 mL/kg) without (control) or with 1 or 3 mg/kg of oral efonidipine, and an intravenous group (0.05 mg/kg of warfarin, dissolved in 0.9% NaCl-injectable solution; homogenized at 36°C for 30 min; 0.3 mL/kg) without (control) or with 1 or 3 mg/kg of oral efonidipine. Warfarin was administered orally using a gastric gavage tube, and efonidipine was orally administered 30 min prior to oral or intravenous administration of warfarin. Warfarin for intravenous administration was injected through the femoral vein within 0.5 min. A blood sample (0.3 mL) was collected into heparinized tubes from the femoral artery at 0 (control), 0.25, 0.5, 1, 2, 4, 8, 12, 24 and 36 h after intravenous infusion, and 1, 2, 3, 4, 5, 6, 8, 12, 24, 36, 48 and 72 h after oral administration. The blood samples were centrifuged (13,000 rpm, 5 min), and the plasma samples (0.15 mL) were stored at -40°C until HPLC analysis of warfarin. An approximately 1 mL of whole blood 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.

HPLC assay

The plasma concentrations of warfarin were determined by a HPLC assay method reported by Zhu et al. (1999). Briefly, 50 μL of 7-ethoxycoumarin (2 μg/mL dissolved in methanol), 50 μL of methanol, 200 mL distilled water, 0.5 mL of 2 M hydrochloric acid, and 0.8 mL of diethyl ether were added to 0.15 mL of plasma sample. The mixture was then stirred for 10 min and centrifuged (13,000 rpm, 10 min). 0.7 mL of the organic layer was transferred to a clean test tube and evaporated under a gentle stream of nitrogen at 35°C. The residue was dissolved in 150 μL of the phosphate buffer. Next, 70 μL of the supernatant was injected into the HPLC system. Chromatographic separations were achieved using a guard column packed with C_{18} column (4×3.0 mm, 5 μm, Phenomenex), and a reversed-phase Luna® C_{18} column (4.6×150 mm, 5 μm, Phenomenex). The mobile phase was 10 mM phosphate buffer-methanol (50:50, v/v), which was run at a flow rate of 1.0 mL/min. Chromatography was performed at a temperature of 30°C that was set by an HPLC column temperature controller, while the UV detector was set to 300 nm. The retention times of warfarin and the internal standard were 16.7 and 9.1 min, respectively. The detection limit of warfarin in rat's plasma was 5 ng/mL. The coefficients of variation for warfarin were below 12.8%.

CYP inhibition assay

The inhibition assays of human CYP3A4 enzyme activity were performed in a multiwell plate using the CYP inhibition assay kit (BD Bioscience, San Jose, CA). Briefly, human CYP

enzymes were obtained from baculovirus-infected insect cells. CYP3A4 substrate (7-Benzyloxy-4-(trifluoromethyl)couamrin (BFC)) was incubated with or without test compounds in a reaction mixture containing 1 pmol of CYP3A4 enzyme and the 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 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) set at an excitation wavelength of 409 nm and an emission wavelength of 530 nm. Positive control (1 μM ketoconazole) was run on the same plate and produced 99% inhibition. All experiments were performed in duplicate, and the results are expressed as the percent of inhibition.

Pharmacokinetic analysis

The pharmacokinetic parameters were calculated using non-compartmental analysis (WinNonlin; software version 4.1; Pharsight Co., Mountain View, CA, USA). The elimination rate constant (K_{el}) was calculated by log-linear regression of warfarin concentration data during the elimination phase, and 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 warfarin were directly read from the experimental data. 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}. Total body clearance (CL) was calculated by Dose/AUC. The absolute bioavailability (A.B.) of warfarin was calculated by $AUC_{oral}/AUC_{IV} \times Dose_{IV}/Dose_{oral} \times 100$, and the relative bioavailability (R.B.) of warfarin was estimated by $AUC_{with\ efonidipine}/AUC_{control} \times 100$.

Statistical analysis

All data were expressed with their standard deviation (Mean ± SD). Statistical analysis was conducted using a one-way analysis of variance (ANOVA) followed by *a posteriori* testing with Dunnett's correction. Differences were considered significant at a level of $P<0.05$.

Results

Inhibition of CYP3A4

The inhibitory effect of efonidipine on CYP3A4 activity is shown in Fig. 1. Efonidipine inhibited CYP3A4 activity in a concentration-dependent manner. Efonidipine strongly inhibited CYP3A4 with an IC_{50} value of 0.08 μM.

Effect of efonidipine on the pharmacokinetics of warfarin after oral administration

The mean arterial plasma concentration–time profiles of warfarin after oral (0.2 mg/kg) administration of warfarin with or without efonidipine (1 and 3 mg/kg) are shown in Fig. 2. The relevant pharmacokinetic parameters of warfarin are also listed in Table I. The AUC of warfarin was significantly

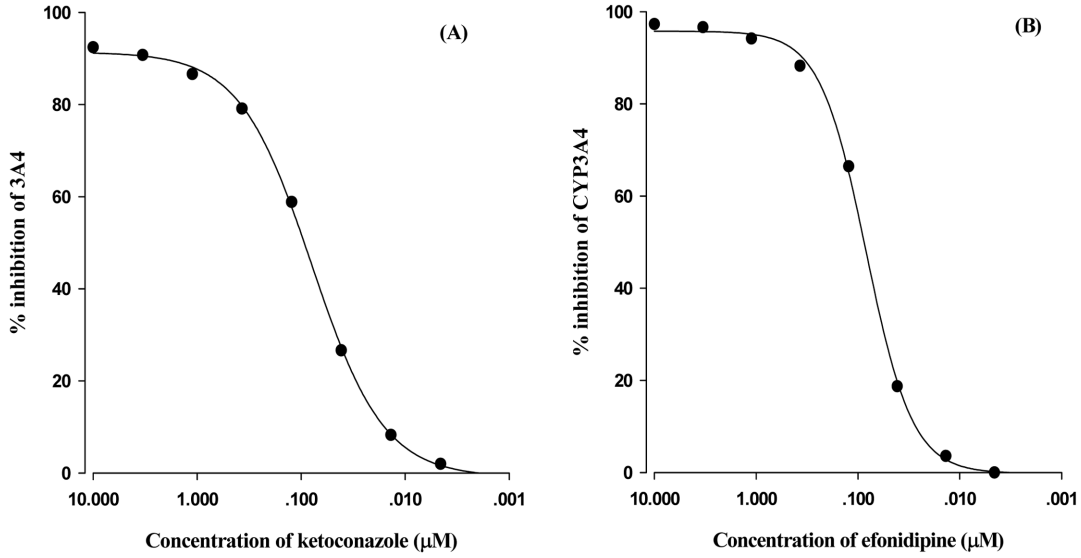

Figure 1. Inhibitory effects of ketoconazole (A) and efonidipine (B) on CYP3A4 activity. All experiments were performed in duplicate, and results were expressed as the percent of inhibition.

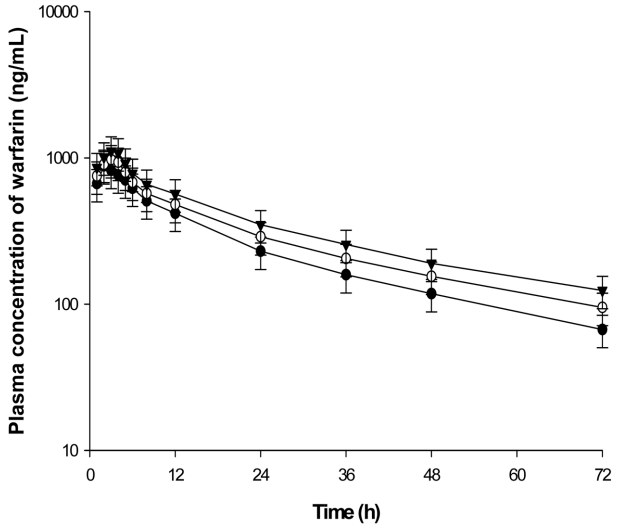

Figure 2. Mean arterial plasma concentration-time profiles of warfarin after oral administration of warfarin (0.2 mg/kg) without or with efonidipine (1 or 3 mg/kg) in rats. Bars represent the standard deviation (n = 6), (●) 0.2 mg/kg of oral warfarin, (○) with 1 mg/kg of efonidipine, (▼) with 3 mg/kg of efonidipine.

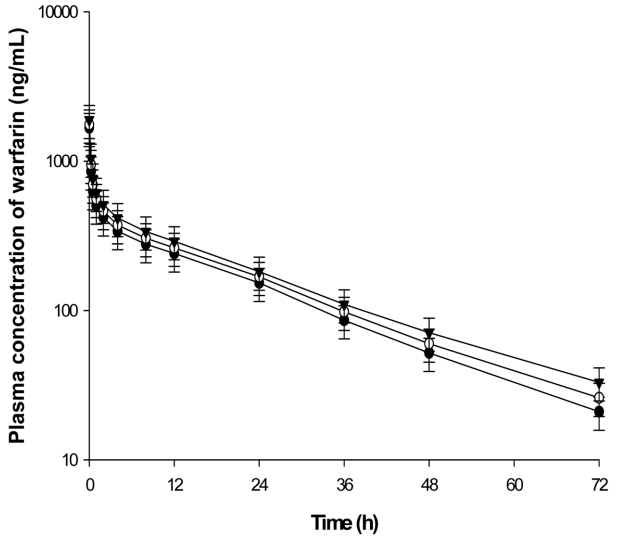

Figure 3. Mean arterial plasma concentration-time profiles of warfarin after intravenous administration of warfarin (0.05 mg/kg) without or with efonidipine (1 or 3 mg/kg) in rats. Bars represent the standard deviation (n = 6), (●) 0.05 mg/kg of i.v. warfarin, (○) with 1 mg/kg of efonidipine, (▼) with 3 mg/kg of efonidipine.

Table I. *Mean (±S.D.) pharmacokinetic parameters of warfarin after oral administration of warfarin (0.2 mg/kg) to rats with or without efonidipine*

Parameter	Control	Warfarin + efonidipine	
		1 mg/kg	3 mg/kg
AUC(ng·h/mL)	18703 ± 3251	23540 ± 4443*	29742 ± 6275**
C_{max}(ng/mL)	881 ± 153	971 ± 185	1112 ± 230*
T_{max}(h)	2.0 ± 0.63	2.3 ± 0.52	2.3 ± 0.52
$t_{1/2}$(h)	26.3 ± 4.6	28.4 ± 5.5	31.4 ± 6.6
CL/F(ml/min/kg)	10.6 ± 2.5	8.5 ± 2.2	6.7 ± 2.0*
A.B.(%)	47.4 ± 8.1	59.7 ± 11.8*	75.4 ± 15.7**
R.B.(%)	100	126	159

Mean ± S.D., n = 6. *P<0.05, **P<0.01, compared with the control group.
AUC: area under the plasma concentration–time curve from 0 h to time infinity, C_{max}: peak plasma concentration, T_{max}: time to reach peak concentration, $t_{1/2}$: terminal half-life, CL/F: total body clearance, A.B.: absolute bioavailability, R.B.: relative bioavailability.

Table II. *Mean (±S.D.) Pharmacokinetic parameters of warfarin after intravenous administration of warfarin (0.05 mg/kg) to rats with or without efonidipine*

Parameter	Control	Warfarin + efonidipine	
		1 mg/kg	3 mg/kg
AUC(ng·h/mL)	9851 ± 2209	10482 ± 2238	11073 ± 2543
CL(mL/min/kg)	5.3 ± 1.8	5.0 ± 1.6	4.8 ± 1.5
$t_{1/2}$(h)	18.5 ± 3.9	19.4 ± 4.4	19.7 ± 4.6
R.B.(%)	100	106	112

Mean ± S.D., n = 6.
AUC: area under the plasma concentration-time curve from time 0 to infinity, CL: total body clearance, $t_{1/2}$: terminal half-life, R.B.: relative bioavailability.

greater (1 mg/kg, P<0.05; 3 mg/kg, P<0.01) by 25.9-59.0%, and the C_{max} was significantly higher (3 mg/kg, P<0.05) by 26.2% after oral administration of warfarin with efonidipine. The total body clearance of warfarin was significantly (3 mg/kg, P<0.05) decreased by efonidifine. Consequently, the relative bioavailability (R.B.) of warfarin was increased 1.26- to 1.59-fold and the absolute bioavailability (A.B.) of warfarin with efonidipine was significantly greater by 59.7-75.4% compared to that in the control group (47.4%). There was no significant change in the half-life of warfarin and T_{max}.

Effect of efonidipine on the pharmacokinetics of warfarin after i.v. administration

The mean arterial plasma concentration–time profiles of warfarin after intravenous administration of warfarin (0.05 mg/kg) with or without efonidipine (1 or 3 mg/kg) are shown in Fig. 3. The relevant pharmacokinetic parameters of warfarin are listed in Table II. Efonidipine had no effect on the pharmacokinetic parameters of warfarin given intravenously although it exhibited a significant effect on the bioavailability of warfarin given orally, suggesting that CYP3A-mediated metabolism was inhibited by efonidipine, resulting in reducing intestinal or hepatic first-pass metabolism in rats.

Discussion

Warfarin is an anticoagulant that has been used to prevent thromboembolism including pulmonary embolism, cardiovascular disease and stroke. Scheduled monitoring and dosage adjustment are critical to maintain efficacy and to prevent bleeding events. Warfarin has a narrow therapeutic range and its efficacy can be influenced by drug-drug interactions, drug-food interactions, genetic factors and patient characteristics (Hirsh et al., 1998; Kaminsky and Zhang, 1997).

CYPs enzymes make a considerable contribution to the first-pass metabolism and oral bioavailability of many drugs. Moreover, induction or inhibition of CYPs may be responsible for significant drug and drug interactions (Cummins et al., 2002; Benet et al., 2003). Modulators of P-gp can enhance or limit the permeability of a number of therapeutic agents that are considered substrates of this efflux pump protein (Darvari and Boroujerdi, 2004). Therefore efonodipine, a dual inhibitor 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 bioavailability and thus could act synergistically to limit oral bioavailability of its substrates (Saeki et al., 1993; Wacher et al., 2001).

As shown in Fig. 1, efonidipine exhibited inhibitory effect against CYP3A4-mediated metabolism with the IC_{50} values of 0.08 μM. As efonidipine is an inhibitor of CYP3A4, concomitant use of the drug might play a role in the wide inter-individual variability in the response to drugs (Choi et al., 2006; Choi et al., 2008). Most calcium channel blockers (verapamil, nifedipine, diltiazem, barnidipine) also have inhibitory effect on the drug transporter P-gp, which mediates drug's intestinal absorption (Wacher et al., 2001; Yusa and Tsuruo, 1989). However, Harmsze et al. (2010) reported that efonidipine have poor inhibitory effects on the drug transporter P-gp.

Therefore, the pharmacokinetic characteristics of warfarin were evaluated in the absence and presence of efonidipine in rats. As CYP3A9 expressed in rat corresponds to the ortholog of CYP3A4 in human (Kelly et al., 1999), rat CYP3A2 is similar to human CYP3A4 (Bogaards et al., 2000; Guengerich et al., 1986). Human 3A4 and rat 3A1 have 73% of protein homology (Lewis, 1996). Rats were selected as an animal model in this study to evaluate the potential pharmacokinetic interactions mediated by CYP3A4, although there should be some difference in enzyme activity between rat and human (Cao et al., 2006).

Compared to those animals in the oral control group (only warfarin without efonidipine), the AUC of warfarin was significantly greater by 25.9-59.0%, and the C_{max} was significantly higher by 26.2% after oral administration of warfarin with efonidipine (Table I).

The total body clearance of warfarin was significantly (3 mg/kg, $P<0.05$) decreased by efonidifine. Consequently, the absolute bioavailability of warfarin with efonidipine was significantly greater by 59.7-75.4% compared to that in the control group (47.4%). Efonidipine could inhibit CYP3A4-mediated metabolism of warfarin, resulting in reducing intestinal or hepatic first-pass metabolism (Wallin et al., 2002; Abernethy and Kaminsky, 1991). These results were consistent with the results reported by Nishio et al. (2005).

These results are consistent with a report by Kim et al. (2006) showing that fluvastatin significantly increased the $AUC_{0-\infty}$ and C_{max} of warfarin. Lilja et al. (2005) also reported that gemfibrozil significantly increased the $AUC_{0-\infty}$ and C_{max} of warfarin. These results are consistent with our study.

In conclusion, the enhanced bioavailability of warfarin might be mainly due to reduction of first-pass metabolism via inhibition of the CYP3A4 in the small intestine and/or in the liver in rats.

Conclusions

Efonidipine enhanced the bioavailability of oral warfarin in this study. The enhanced bioavailability of warfarin might be mainly due to inhibition of the CYP3A4-mediated metabolism of warfarin in the small intestine and/or in the liver and to reduction of total body clearance by efonidipine. Since the present study has raised the awareness of potential drug interactions by concomitant use of efonidipine with warfarin, the clinical significance of this finding needs to be further evaluated in clinical studies.

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