Department of Food and Drug[1], College of Pharmacy[2], College of Medicine[3], Chosun University, Gwangju, Republic of Korea

Effects of licochalcon A on the pharmacokinetics of losartan and its active metabolite, EXP-3174, in rats

J.S. Choi[1], J.S. Choi[2], D.H. Choi[3]

Received March 11, 2013, accepted April 12, 2013

Prof. Dong-Hyun Choi, College of Medicine, Chosun University, Susuk-dong, Dong-Gu, Gwangju 501-759, Republic of Korea
dhchoi@chosun.ac.kr

Pharmazie 68: 882–888 (2013) *doi: 10.1691/ph.2013.3600*

Losartan and licochalcon A interact with cytochrome P-450 (CYP) enzymes and P-glycoprotein (P-gp), and the increase in the use of health supplements may result in licochalcon A being taken concomitantly with losartan to treat or prevent cardiovascular diseases as a combination therapy. The effect of licochalcon A, a natural flavonoid, on the pharmacokinetics of losartan and its active metabolite, EXP-3174, was investigated in rats. Pharmacokinetic parameters of losartan and EXP-3174 were determined after oral administration of losartan (9 mg/kg) to rats in the presence or absence of licochalcon A (0.5, 2.5 and 10 mg/kg). The effect of licochalcon A on P-glycoprotein (P-gp) as well as CYP3A4 and 2C9 activities was also evaluated. Licochalcon A inhibited CYP3A4 and CYP2C9 enzyme activities with 50% inhibition concentrations (IC_{50}) of 2.0 and 0.1 μM, respectively. In addition, licochalcon A significantly enhanced the cellular accumulation of rhodamine-123 in a concentration-dependent manner in MCF-7/ADR cells overexpressing P-gp. The pharmacokinetic parameters of losartan were significantly altered by licochalcon A. Licochalcon A (2.5 mg/kg or 10 mg/kg) increased $AUC_{0-\infty}$ of losartan by 33.4–63.2% and C_{max} of losartan by 34.0–62.8%. The total body clearance (CL/F) was significantly decreased (2.5 mg/kg, $p < 0.05$; 10 mg/kg, $p < 0.01$) by licochalcon A. Consequently, the absolute bioavailability of losartan in the presence of licochalcon A increased significantly (2.5 mg/kg, $p < 0.05$; 10 mg/kg, $p < 0.01$) compared to that in the control group. The relative bioavailability (R.B.) of losartan was 1.15- to 1.63-fold greater than that of the control group. However, there was no significant change in T_{max} and $t_{1/2}$ of losartan in the presence of licochalcon A. Licochalcon A (10 mg/kg) increased the $AUC_{0-\infty}$ of EXP-3174 but this was not significant. Furthermore, concurrent use of licochalcon A (10 mg/kg) significantly decreased the metabolite-parent AUC ratio (M.R.) by 20%, suggesting that licochalcon A inhibited the CYP-mediated metabolism of losartan to its active metabolite, EXP-3174. In conclusion, the enhanced oral bioavailability of losartan in the presence of licochalcon A may mainly result from decreased P-gp-mediated efflux transporter in the small intestine and from the inhibition of CYP 3A- and CYP2C9-mediated metabolism in the small intestine and liver and/or from the reduction of total body clearance of losartan by licochalcon A.

1. Introduction

Following oral administration, losartan is rapidly absorbed, reaching maximum concentrations 1–2 hours post-dose, but it has low and highly variable oral bioavailabilities (12.1–66.6%) (Lo et al. 1995). Losartan is metabolized to an active metabolite, EXP-3174 which is about 10-fold more potent than its parent drug. Losartan is the prototype of a new class of orally active and long-lasting selective antagonists of angiotensin II receptors for the treatment of hypertension (Diez 2006; McIntyre et al. 1997; Inagami et al. 1992). Some *in vitro* and *in vivo* studies have indicated that losartan is metabolized to EXP-3174 mainly by cytochrome P450 (CYP) 2C9 and 3A4 enzymes (Yun et al. 1995; Meadowcroft et al. 1999; Kaukonen et al. 1998; McCrea et al. 1999). Furthermore, Soldner et al. (2000) suggested that losartan is a substrate of P-glycoprotein (P-gp). Since losartan is a substrate of both CYP enzymes and P-gp, the modulation of CYP and P-gp activities may cause significant changes in the pharmacokinetic profiles of losartan and its active metabolite, EXP-3174 (Zaidenstein et al. 2001; Kobayashi et al. 2008; Choi et al. (2010)).

Licochalcone A is the main active component in the licorice species *Glycyrrhiza inflate* (Rafi et al. 2002). Licochalcone A also has a wide range of biological and pharmacological activities, including antioxidant, superoxide scavenging (Haraguchi et al. 1998) and anti-leishmanial activities, effects on the function of parasite mitochondria (Zhai et al. 1999), antimalarial activities both *in vitro* and *in vivo* (Chen et al. 1994), and anti-tumor activities in cancer cells (Fu et al. 2004; Shibata 1994; De Vincenzo et al. 2000). Kwon et al. (2008) demonstrated that licochalcone A exerts anti-inflammatory effects by suppressing nuclear factor-kB (NF-kB) and activator protein-1 (AP-1) signaling. Kim et al. (2010) provided the first evidence that licochalcone A can inhibit the angiogenesis *in vitro* and *in vivo* as well as tumor growth.

We evaluated CYP enzymes activities and P-gp activity in the presence of licochalcone A using CYP inhibition assays and rhodamine-123 retention assays in P-gp-over-expressing

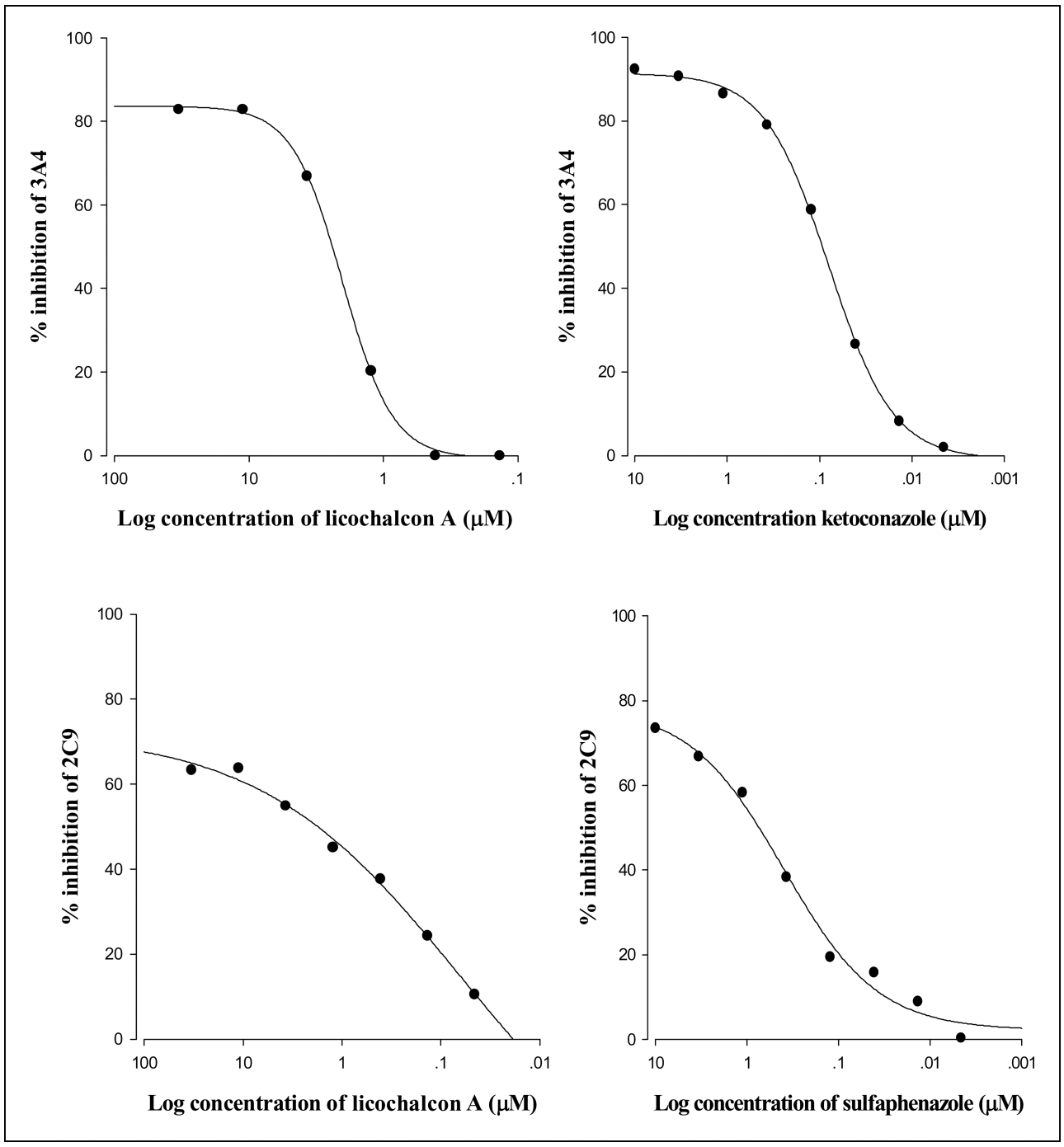

Fig. 1: Inhibitory effect of licochalcon A on CYP3A4 and 2C9 activity. Results are expressed as the percent of inhibition.

MCF-7/ADR cells. Licochalcone A is expected to change the bioavailability and pharmacokinetics of drugs that are substrates of P-gp and/or CYP3A4, if they are used concomitantly.

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

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

2. Investigations and results

2.1. Inhibition of CYP3A4 and 2C9

The inhibitory effect of licochalcon A on CYP3A4 and CYP2C9 activity is shown in Fig. 1. Licochalcon A inhibited CYP3A4 and CYP2C9 enzyme activity and the 50 % inhibition concentration (IC_{50}) values for licochalcon A against CYP3A4 and CYP2C9 activity were 2.0 and 0.1 μM, respectively.

2.2. Rhodamine-123 retention assay

As shown in Fig. 2, accumulation of rhodamine-123, a P-gp substrate, was reduced in MCF-7/ADR cells overexpressing P-gp compared to that in MCF-7 cells lacking P-gp. The concurrent use of licochalcon A enhanced the cellular uptake

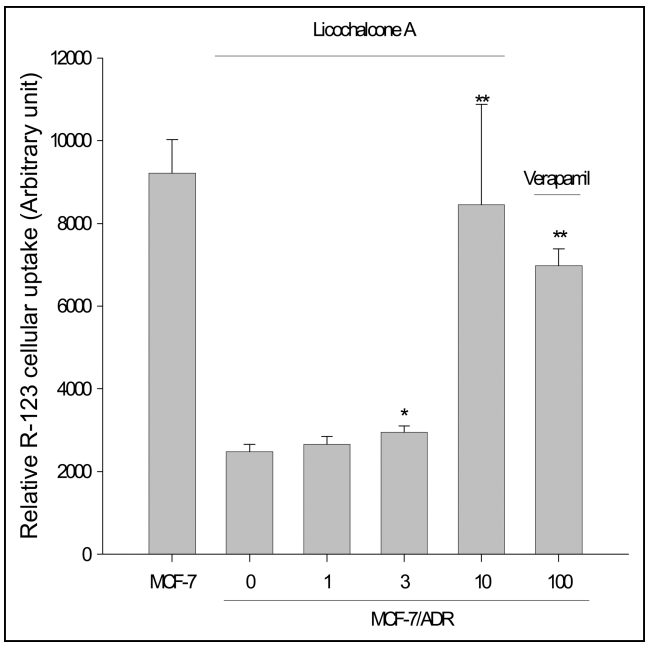

Fig. 2: Rhodamine-123 retention. MCF-7/ADR cells were preincubated with licochalcon A for 24 h. After incubation of MCF-7/ADR cells with 20 μM R-123 for 90 min. Data represents mean ± SD of 6 separate samples (significant versus control MCF-7 cells, * $p < 0.05$, ** $p < 0.01$).

of rhodamine-123 in a concentration-dependent manner and showed statistically significant ($p < 0.01$) increase over the concentration range of 3–10 μM. This result suggests that licochalcon A significantly inhibits P-gp activity.

2.3. Effects of licochalcon A on the pharmacokinetics of oral losartan

The plasma concentration-time profiles of losartan in the presence or absence of licochalcon A ase characterized in Fig. 3. The mean pharmacokinetic parameters of losartan are also summarized in Table 1.

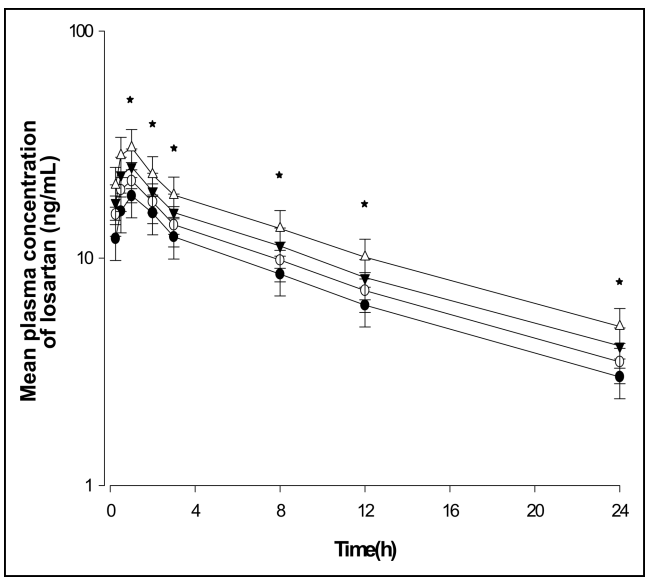

Fig. 3: Mean plasma concentration-time profiles of losartan after oral administration of losartan (9 mg/kg) to rats in the presence or absence of licochalcon A at doses of 0.5, 2.5 and 10 mg/kg. (Mean ± SD, n = 6). ●: Control (losartan 9 mg/kg, alone); ◯: co-administered with 0.5 mg/kg of licochalcon A; ▼: co-administered with 2.5 mg/kg of licochalcon A, Δ: co-administered with 10 mg/kg of licochalcon A. * $p < 0.05$.

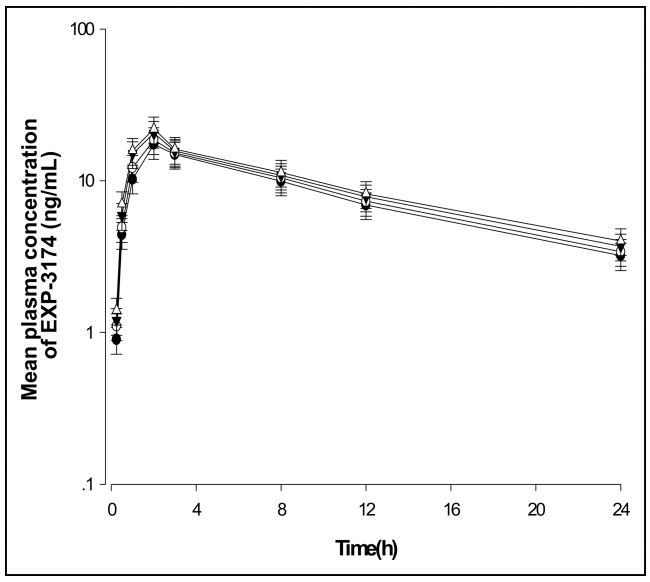

Fig. 4: Mean plasma concentration-time profiles of EXP-3174 after oral administration of losartan (9 mg/kg) to rats in the presence or absence of licochalcon A at doses of 0.5, 2.5 and 10 mg/kg. (Mean ± SD, n = 6). ●: Control (losartan 9 mg/kg, alone); ◯: co-administered with 0.5 mg/kg of licochalcon A; ▼: co-administered with 2.5 mg/kg of licochalcon A, △: co-administered with 10 mg/kg of licochalcon A.

Licochalcon A (2.5 or 10 mg/kg) significantly altered the pharmacokinetic parameters of losartan compared to those in the control group (given losartan alone). Licochalcon A significantly (2.5 mg/kg, $p < 0.05$; 10 mg/kg, $p < 0.01$) increased $AUC_{0-\infty}$ of losartan by 33.4–63.2% and C_{max} of losartan by 34.0–62.8%. The total body clearance (CL/F) was significantly decreased (2.5 mg/kg, $p < 0.05$; 10 mg/kg, $p < 0.01$) by licochalcon A. Consequently, the absolute bioavailability (A.B.) of losartan in the presence of licochalcon A was significantly (2.5 mg/kg, $p < 0.05$; 10 mg/kg, $p < 0.01$) higher than that in the control group. The relative bioavailability (R.B.) of losartan was 1.15- to 1.63-fold greater than that in the control group. However, there was no significant change in T_{max} and $t_{1/2}$ of losartan with licochalcon A. Given that losartan is a substrate of P-gp, CYP3A4 and 2C9, the enhanced bioavailability of losartan might be due to the inhibition of the CYP3A and CYP2C9-mediated metabolism of losartan in the small intestine and the liver and the inhibition of the P-gp efflux transporter in the small intestine and/or, the total body clearance of losartan by licochalcon A.

2.4. Effects of licochalcon A on the pharmacokinetics of EXP-3174

The plasma concentration-time profiles of EXP-3174, an active metabolite, in the presence of licochalcon A were increased compared to those in the control group (Fig. 4.) As shown in Table 2, licochalcon A significantly (10 mg/kg, $p < 0.05$) increased $AUC_{0-\infty}$ of losartan but this was not significant. The metabolite-parent AUC ratios decreased by 23% in the presence of licochalcon A compared to that in the control group, suggesting that licochalcon A inhibited the CYP-mediated metabolism of losartan. However, there was no significant change in C_{max}, T_{max} and $t_{1/2}$ of EXP-3174 with licochalcon A.

2.5. Effects of licochalcon A on the pharmacokinetics of intravenous losartan

Mean arterial plasma concentration-time profiles of losartan following intravenous administration of losartan (3 mg/kg) to

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

Parameters	Control	Losartan + Licochalcon A		
		0.5 mg/kg	2.5 mg/kg	10 mg/kg
$AUC_{0-\infty}$ (ng·h/mL)	225.0 ± 38.3	260.2 ± 44.2	300.1 ± 51.0*	367.3 ± 62.4**
C_{max} (ng/mL)	18.8 ± 3.4	21.9 ± 3.9	25.2 ± 4.5*	30.6 ± 5.5**
T_{max} (h)	0.92 ± 0.21	0.92 ± 0.21	1.17 ± 0.41	1.17 ± 0.41
CL/F (mL/hr/kg)	664.8 ± 126.3	577.3 ± 109.7	499.7 ± 94.9*	411.3 ± 78.1**
$t_{1/2}$ (h)	10.6 ± 2.1	10.8 ± 2.2	10.8 ± 2.2	11.0 ± 2.3
A.B. (%)	25.4 ± 4.8	33.3 ± 6.3	38.5 ± 7.3*	46.8 ± 8.9**
R.B. (%)	100	115	133	163

* $p < 0.05$, ** $p < 0.01$, significant difference compared to the control group given losartan alone.
$AUC_{0-\infty}$: area under the plasma concentration-time curve from 0 h to infinity; C_{max}: peak plasma concentration; T_{max}: time to reach C_{max}; CL/F, total body clearance; $t_{1/2}$: terminal half-life; A.B.: absolute bioavailability; R.B.: relative bioavailability.

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

Parameters	Control	Losartan + Licochalcon A		
		0.5 mg/kg	2.5 mg/kg	10 mg/kg
$AUC_{0-\infty}$ (ng ·h/mL)	231.1 ± 39.3	245.0 ± 41.7	264.1 ± 44.9	282.3 ± 48.1
C_{max} (ng/mL)	17.3 ± 3.1	18.5 ± 3.3	20.5 ± 3.7	21.9 ± 3.9
T_{max} (h)	1.67 ± 0.52	1.67 ± 0.52	1.83 ± 0.41	2.17 ± 0.41
$t_{1/2}$ (h)	9.5 ± 1.8	9.8 ± 1.9	10.2 ± 1.9	10.5 ± 2.1
R.B. (%)	100	106	114	122
M.R. (%)	1.00 ± 0.20	0.94 ± 0.19	0.88 ± 0.17	0.77 ± 0.14*

* $p < 0.05$, significant difference compared to the control group given losartan alone.
$AUC_{0-\infty}$: area under the plasma concentration-time curve from 0 h to infinity; C_{max}: peak plasma concentration; T_{max}: time to reach C_{max}; R.B.: relative bioavailability; M.R.: metabolite-parent ratio ($AUC_{EXP\text{-}3174}/AUC_{losartan}$).

rats in the presence or absence of licochalcon A (0.5, 2.5 and 10 mg/kg) are shown in Fig. 5 and the corresponding pharmacokinetic parameters were shown in Table 3.
The $AUC_{0-\infty}$ of losartan was increased, but was not statistically significant compared to that in the control. The $t_{1/2}$ of losartan was also prolonged, but this increase was not significant. The pharmacokinetics of intravenous losartan was not affected by the concurrent use of licochalcon A in contrast to those of oral losartan. Accordingly, the enhanced oral bioavailability in the presence of licochalcon A may be mainly due to the inhibition of CYP3A-and/or CYP2C9-mediated metabolism of losartan in the small intestine and/or liver rather than to the reduction of renal elimination of losartan by licochalcon A.

3. Discussion

P-gp is colocalized with CYP3A4 in the apical membrane of the intestine (Benet et al. 2003; Cummins et al. 2002), and they act synergistically in regulating the first-pass metabolism and bioavailability of many orally administered drugs.
Some studies have indicated that losartan is metabolized to EXP-3174 mainly by CYP2C9 and 3A4 enzymes (Yun et al. 1995; Meadowcroft et al. 1999; Kaukonen et al. 1998; McCrea et al. 1999). Furthermore, Soldner et al. (2000) suggested that losartan is a substrate of P-glycoprotein (P-gp). Considering that losartan is a substrate of both CYP enzymes and P-gp, the modulation of CYP and P-gp activities may cause significant changes in the pharmacokinetic profiles of losartan and its active metabolite, EXP-3174.
The inhibitory effect of licochalcon A against CYP3A4-mediated metabolism was confirmed by the employment of recombinant CYP3A4 enzyme. As shown in Fig. 1, licochalcon A exhibited inhibitory effects against CYP3A4-and CYP2C9-mediated metabolism with IC_{50} of 1.6 μM and 0.1 μM, respectively. Furthermore, the cell-based assay using rhodamine-123 indicated that licochalcon A (3–10 μM) significantly ($p < 0.01$) inhibited P-gp-mediated drug efflux (Fig. 2).

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

Parameter	Control	Losartan + Licochalcon A		
		0.5 mg/kg	2.5 mg/kg	10 mg/kg
$AUC_{0-\infty}$ (ng·h/mL)	296.1 ± 59.2	314.0 ± 62.8	331.3 ± 66.3	349.1 ± 69.8
CL/F (mL/hr/kg)	168.9 ± 62.8	159.3 ± 28.7	150.8 ± 27.1	142.4 ± 25.6
$t_{1/2}$ (h)	8.2 ± 1.3	8.4 ± 1.4	8.6 ± 1.5	8.7 ± 1.6
R.B. (%)	100	106	112	118

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

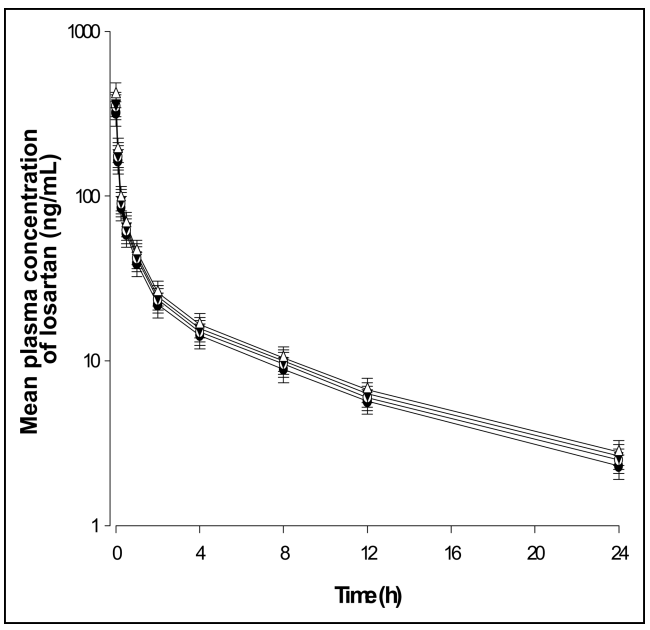

Fig. 5: Mean plasma concentration-time profiles of losartan after intravenous (3 mg/kg) administration to rats in the presence or absence of licochalcon A at doses of 0.5, 2.5 and 10 mg/kg. (Mean ± SD, n = 6). ●: Control (losartan 3 mg/kg, alone); ◯: co-administered with 0.5 mg/kg of licochalcon A; ▼: co-administered with 2.5 mg/kg of licochalcon A, △: co-administered with 10 mg/kg of licochalcon A.

Those results appeared to be consistent with the findings of previous studies (von Moltke et al. 2004; Kitagawa et al. 2005). These findings suggest that licochalcon A might effectively improve the bioavailability of losartan, a substrate of CYP2C9, CYP3A4 and P-gp. Therefore, the pharmacokinetic characteristics of losartan were evaluated in the absence and presence of licochalcon A in rats. CYP3A4 expressed in rats corresponds to the function of CYP3A4 in human (Kelly et al. 1999; Choi et al. 2010; Chung et al. 2009; Piao and Choi 2008; Cho et al. 2009; Zaidenstein et al. 2001). As summarized in Table 1, licochalcon A significantly increased $AUC_{0-\infty}$ of losartan by 33.4–63.2% and C_{max} of losartan by 34.0–62.8%. The total body clearance (CL/F) was significantly decreased (2.5 mg/kg, $p < 0.05$; 10 mg/kg, $p < 0.01$) by licochalcon A. The absolute bioavailability (A.B.) of losartan in the presence of licochalcon A was significantly ($p < 0.05$) higher than that in the control group. Licochalcon A inhibited CYP3A4 and CYP2C9 isozymes and P-gp activity in the present study. These results appeared to be consistent with the finding that myricetin, a CYP3A4, 2C9 and P-gp inhibitor significantly increased the $AUC_{0-\infty}$ and C_{max} of losartan (Choi et al. 2010). These results were also consistent with the findings of some previous studies (Chung et al. 2009; Piao and Choi 2008; Choi and Han 2005; Cho et al. 2009), in which epigallocatechin and hesperidin increased the bioavailability of verapamil (a substrate of CYP3A4 and P-gp) in rats, and in which morin and hesperidin increased $AUC_{0-\infty}$ and C_{max} of diltiazem (a substrate of CYP3A4 and P-gp) in rats. Piao et al. (2008) reported that morin significantly enhanced the bioavailability of nicardipine, perhaps through the inhibition of P-gp and intestinal metabolism by morin.

Our results also appeared to be consistent with the findings of some previous studies (Zaidenstein et al. 2001; Kobayashi et al. 2008; Choi et al. 2010). Zaidenstein et al. (2001) reported significant differences in the pharmacokinetic parameters of losartan and its metabolite EXP-3174 as a result of concomitant intake of grapefruit juice, a CYP3A4 inhibitor.

As summarized in Table 2, licochalcon A significantly decreased metabolite-parent AUC ratios (M.R.). This result was consistent with the findings of some previous studies (Zaidenstein et al. 2001; Kobayashi et al. 2008; Choi et al. 2010). The M.R., a characteristic of the magnitude of metabolic conversion, was significantly changed by the inhibition of CYP3A4-mediated metabolism of losartan after co-administration of grapefruit juice (Zaidenstein et al. 2001). Concurrent use of bucolome, a CYP2C9 inhibitor, significantly increased the AUC of losartan by inhibiting the metabolic conversion of losartan to EXP-3174 (Kobayashi et al. 2008). Epigallocatechin and curcumin significantly decreased M.R. of verapamil and tamoxifen, respectively (Chung et al. 2009; Cho et al. 2012). In conjunction with our present findings, those studies suggested that the combination of losartan and CYP (CYP2C9, CYP3A4) inhibitors could result in a significant pharmacokinetic drug interaction.

Although many CYP enzymes are present in the intestine, there is no evidence of significant oxidation of losartan by the enterocytes using either *in vitro* or *in situ* absorption models (Krieter et al. 1995). In contrast, studies have suggested that the active metabolite EXP-3174 detected in rats is most likely of hepatic origin (Stearns et al. 1995). In the present study, therefore, the decrease in the metabolite-parent AUC ratios (M.R.) of losartan might be mainly due to the inhibitory effect of licochalcon A on the first-pass metabolism in the small intestine and/or liver. Licochalcon A did not significantly change the pharmacokinetic parameters of intravenous administration of losartan (Table 3), suggesting that licochalcon A may improve the oral bioavailability of losartan by more increasing the absorption or reducing the intestinal metabolism of losartan through the inhibition of CYP3A and CYP2C9. Licochalcon A significantly enhanced the oral bioavailability of losartan in rats. The enhanced bioavailability of losartan might be mainly due to the inhibition of the CYP3A-and CYP2C9-mediated metabolism of losartan in the small intestine and/or liver and to the inhibition of P-gp efflux transporter in the small intestine, and/or to the reduction of total body clearance of losartan by licochalcon A. Therefore, concomitant use of licochalcon A with losartan may require close monitoring for potential drug interactions. The clinical importance of these findings should be further investigated in clinical trials.

4. Experimental

4.1. Chemicals and apparatus

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

4.2. Animal studies

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

4.3. Drug administration

Rats were divided into seven groups (n = 6 per each group) as follows: Group 1: losartan (9 mg/kg, p.o., control), Groups 2∼4: losartan (9 mg/kg, p.o.) with licochalcon A (0.4, 2 or 8 mg/kg, given orally at 30 min prior to the administration of losartan), and Groups 5–7: intravenous losartan (3 mg/kg)

with licochalcon A (0.4, 2 or 8 mg/kg, given orally at 30 min prior to the administration of losartan). Blood samples were collected from the femoral artery into heparinized tubes at 0.25, 0.5, 1, 2, 4, 8, 12 and 24 h after oral administration of losartan and at 0, 0.1, 0.25, 0.5, 1, 2, 4, 8, 12 and 24 h after intravenous administration of losartan. The blood samples were centrifuged at 13,000 rpm for 5 min and the obtained plasma samples were stored at −40 °C until analyzed by HPLC.

4.4. HPLC assay

The plasma concentrations of losartan were determined by the HPLC assay reported by Zarghi et al. (2005) with slight modification. Briefly, 50 μl of L-158.809 (0.2 μg/ml dissolved in methanol; an internal standard) and 0.5 ml of acetonitrile were added to a 0.2-ml aliquot of plasma sample in a polypropylene microtube. The mixture was then stirred for 5 min and centrifuged at 13,000 rpm for 10 min. A 0.5-ml aliquot of the organic layer was transferred into a clean test tube and evaporated under a gentle stream of nitrogen gas at 35 °C. The residue was reconstituted with 150 μl of the mobile phase and centrifuged at 13,000 rpm for 5 min and then a 70-μl aliquot of the supernatant was injected into the HPLC system. The HPLC system consisted of two solvent delivery pumps (Model LC-10AD, Shimadzu Co., Japan), a UV-Vis detector (Model SPD-10A), a system controller (Model SCL-10A), a degasser (Model DGU-12A) and an autoinjector (SIL-10AD). The UV detector was set at 215 nm. The stationary phase was a Kromasil KR 100–5C_8 column (5 μm, 4.6 × 250 mm, EKA Chemicals, Sweden) and the mobile phase was acetonitrile:0.01 M phosphate buffer (41:59 v/v, pH 2.5 adjusted with phosphoric acid). The retention times at a flow rate of 0.8 ml/min were as follows: 6.7 min for internal standard, 11.5 min for losartan and 17.1 min for EXP-3174. The lower limit of quantification for losartan and EXP-3174 in rat plasma was 5 ng/ml.

4.5. CYP3A4 and 2C9 inhibition assay

The assays of inhibition of human CYP3A4 and 2C9 enzyme activities were performed in a multiwell plates using CYP inhibition assay kit (GENTEST, Woburn, MA) as described previously (Crespi et al. 1997). 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 licochalcon A in the enzyme/substrate-containing buffer with 1 pmol of P450 enzyme and a 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 (0.5 M Tris-buffer mixed with acetonitrile as a stop solution) after 45-min incubation. Metabolite concentrations were measured by a spectrofluorometer (Molecular Device, Sunnyvale, CA) at an excitation wavelength of 409 nm and an emission wavelength of 530 nm. Positive control (1 μM ketoconazole and 2 μM sulfaphenazole for CYP3A4 and 2C9, respectively) was run on the same plate and produced 99% inhibition. All experiments were performed in duplicate, and the results are expressed as the percentage of inhibition.

4.6. Rhodamine-123 retention assay

The procedures used for the Rho-123 retention assay were similar to a previously reported method (Han et al. 2008). MCF-7/ADR cells were seeded in 24-well plates. At 80% confluence, the cells were incubated in FBS-free DMEM for 18 h. The culture medium was changed to Hanks' balanced salt solution and the cells were incubated at 37 °C for 30 min. After incubation of the cells with 20 μM rhodamine-123 in the presence of licochalcon A (1, 3 and 10 μM) 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 lysis buffer. The rhodamine-123 fluorescence in the cell lysates was measured using excitation and emission wavelengths of 480 and 540 nm, respectively. Fluorescence values were normalized to the total protein content of each sample and presented as the ratio to control.

4.7. Pharmacokinetic analysis

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

4.8. Statistical analysis

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

References

Benet LZ, Cummins CL, Wu CY (2003) Transporter-enzyme interactions: implications for predicting drug-drug interactions from *in vitro* data. Curr Drug Metab 4: 393–398.

Chen M, Theander TG, Christensen SB, Hviid L, Zhai L, Kharazmi A (1994) Licochalcone, a new antimalarial agent, inhibits *in vitro* growth of the human malaria parasite plasmodium falciparum and protects mice from P. yoelii infection. Antimicrob Agents Chemother 38: 1470–1475.

Cho YA, Choi DH, Choi JS (2009) Effect of hesperidin on the oral pharmacokinetics of diltiazem and its main metabolite, desacetyldiltiazem, in rats. J Pharm Pharmacol 61: 825–829.

Choi DH, Li C, Choi JS (2010) Effects of myricetin, an antioxidant, on the pharmacokinetics of losartan and its active metabolite, EXP-3174, in rats: possible role of cytochrome P450 3A4, cytochrome P450 2C9 and P-glycoprotein inhibition by myricetin. J Pharm Pharmacol 62: 908–914.

Cho YA, Lee W, Choi JS (2012) Effects of curcumin on the pharmacokinetics of tamoxifen and its active metabolite, 4-hydroxytamoxifen, in rats: possible role of CYP3A4 and P-gp inhibition by curcumin. Pharmazie 67: 124–130.

Chung JH, Choi DH, Choi JS (2009) Effect of epigallocatechin gallate on the oral pharmacokinetics of verapamil in rats. Biopharm Drug Dispos 30: 90–93.

Crespi CL, Miller VP, Penman BW (1997) Microtiter plate assays for inhibition of human, drug-metabolizing cytochromes P450. Anal Biochem 248: 188–190.

Cummins CL, Jacobsen W, Benet LZ (2002) Unmasking the dynamic interplay between intestinal P-glycoprotein and CYP3A4. J Pharmacol Exp Ther 300: 1036–1045.

De Vincenzo R, Ferlini C, Distefano M, Gaggini C, Riva A, Bombardelli E, Morazzoni P, Valenti P, Belluti F, Ranelletti FO, Mancuso S, Scambia G (2000) *In vitro* evaluation of newly developed chalcone analogues in human cancer cells. Cancer Chemother Pharmacol 46: 305–312.

Diez J (2006) Review of the molecular pharmacology of losartan and its possible relevance to stroke prevention in patients with hypertension. Clin Ther 28: 832–848.

Fu Y, Hsieh TC, Guo J, Kunicki J, Lee MY, Darzynkiewicz Z, Wu JM (2004) Licochalcone-A, a novel flavonoid isolated from licorice root (Glycyrrhiza glabra), causes G2 and late-G1 arrests in androgen-independent PC-3 prostate cancer cells. Biochem Biophys Res Commun 322: 263–270.

Han CY, Cho KB, Choi HS, Han HK, Kang KW (2008) Role of FoxO1 activation in MDR1 expression in adriamycin-resistant breast cancer cells. Carcinogenesis 29: 1837–1844.

Haraguchi H, Ishikawa H, Mizutani K, Tamaru Y, Kinoshita T (1998) Antioxidative and superoxide scavenging activities of retrochalcones in glycyrrhiza inflate. Bioorg Med Chem 6: 339–347.

Inagami T, Iwai N, Sasaki K, Yamamo Y, Bardhan S, Chaki S, Guo DF, Furuta H (1992) Cloning, expression and regulation of angiotensin II receptors. J Hypertens 8: 713–716.

Kaukonen KM, Olkkola KT, Neuvonen PJ (1998) Fluconazole but not itraconazole decreases the metabolism of losartan to E-3174. Eur J Clin Pharmacol 53: 445–449.

Kelly PA, Wang H, Napoli KL, Kahan BD, Strobel HW (1999) Metabolism of cyclosporine by cytochromes P450 3A9 and 3A4. Eur J Drug Metab Pharmacokinet 24: 321–328.

Kim YH, Shin EK, Kim DH, Lee HH, Park JH, Kim JK (2010) Antiangiogenic effect of licochalcone A. Biochem Pharmacol 80: 1152–1159.

Kitagawa S, Nabekura T, Takahashi T, Nakamura Y, Sakamoto H, Tano H, Hirai M, Tsukahara G (2005) Structure-activity relationships of the inhibitory effects of flavonoids on P-glycoprotein-mediated transport in KB-C2 cells. Biol Pharm Bull 28: 2274–2278.

Kobayashi M, Takagi M, Fukumoto K, Kato R, Tanaka K, Ueno K (2008) The effect of bucolome, a CYP2C9 inhibitor, on the pharmacokinetics of losartan. Drug Metab Pharmacokinet 23: 115–119.

Krieter PA, Colletti AE, Miller RR, Stearns RA (1995) Absorption and glucuronidation of the angiotensin II receptor antagonist losartan by the rat intestine. J Pharmacol Exp Ther 273: 816–822.

Kwon HS, Park JH, Kim DH, Kim YH, Park JH, Shin HK, Kim JK (2008) Licochalcone A isolated from licorice suppresses lipopolysaccharide-stimulated inflammatory reactions in RAW264.7 cells and endotoxin shock in mice. J Mol Med 86: 1287–1295.

Lo MW, Goldberg MR, McCrea JB, Lu H, Furtek CI, Bjornsson TD (1995) Pharmacokinetics of losartan, an angiotensin II receptor antagonist, and its active metabolite EXP3174 in humans. Clin Pharmacol Ther 58: 641–649.

McCrea JB, Cribb A, Rushmore T, Osborne B, Gillen L, Lo MW, Waldman S, Bjornsson T, Spielberg S, Goldberg MR (1999) Phenotypic and genotypic investigations of a healthy volunteer deficient in the conversion of losartan to its active metabolite E-3174. Clin Pharmacol Ther 65: 348–352.

McIntyre M, Caffe SE, Michalak RA, Reid JL (1997) Losartan, an orally active angiotensin (AT1) receptor antagonist: a review of its efficacy and safety in essential hypertension. Pharmacol Ther 74: 181–194.

Meadowcroft AM, Williamson KM, Patterson JH, Hinderliter AL, Pieper JA (1999) The effects of fluvastatin, a CYP2C9 inhibitor, on losartan pharmacokinetics in healthy volunteers. J Clin Pharmacol 39: 418–424.

Piao YJ, Choi JS (2008) Effects of morin on the pharmacokinetics of nicardipine after oral and intravenous administration of nicardipine in rats. J Pharm Pharmacol 60: 625–629.

Piao YJ, Choi JS (2008) Enhanced bioavailability of verapamil after oral administration with hesperidin in rats. Arch Pharm Res 31: 518–522.

Rafi MM, Rosen RT, Vassil A, Ho CT, Zhang H, Ghai G, Lambert G, DiPaola RS (2002) Modulation of bcl-2 and cytotoxicity by licochalcone-A, a novel estrogenic flavonoid. Anticancer Res 20: 2653–2658.

Shibata S (1994) Anti-tumorigenic chalcones. Stem Cells 12: 44–52.

Soldner A, Benet LZ, Mutschler E, Christians U (2000) Active transport of the angiotensin-II antagonist losartan and its main metabolite EXP 3174 across MDCK-MDR1 and caco-2 cell monolayers. Br J Pharmacol 129: 1235–1243.

Stearns RA, Chakravarty PK, Chen R, Chiu SH (1995) Biotransformation of losartan to its active carboxylic acid metabolite in human liver microsomes. Role of cytochrome P4502C and 3A subfamily members. Drug Metab Dispos 23: 207–215.

von Moltke LL, Weemhoff JL, Bedir E, Khan IA, Harmatz JS, Goldman P, Greenblatt D (2004) Inhibition of human cytochromes P450 by components of Ginkgo biloba. J Pharm Pharmacol 56: 1039–1044.

Yun CH, Lee HS, Lee H, Rho JK, Jeong HG, Guengerich FP (1995) Oxidation of the angiotensin II receptor antagonist losartan (DuP 753) in human liver microsomes. Role of cytochrome P4503A(4) in formation of the active metabolite EXP3174. Drug Metab Dispos 23: 285–289.

Zaidenstein R, Soback S, Gips M, Avni B, Dishi V, Weissgarten Y, Golik A, Scapa E (2001) Effect of grapefruit juice on the pharmacokinetics of losartan and its active metabolite E3174 in healthy volunteers. Ther Drug Monit 23: 369–373.

Zarghi A, Foroutan SM, Shafaati A, Khoddam A (2005) A rapid HPLC method for the determination of losartan in human plasma using a monolithic column. Arzneimittelforschung 55: 569–572.

Zhai L, Chen M, Blom J, Theander TG, Christensen SB, Kharazmi A (1999) The antileishmanial activity of novel oxygenated chalcones and their mechanism of action. J Antimicrob Chemother 43: 793–803.