Regular Article

Mechanism-Based Inactivation of Human Liver Microsomal CYP3A4 by Rutaecarpine and Limonin from Evodia Fruit Extract

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Summary: Evodia fruit (Evodiae Fructus) is used as a herbal medicine prepared from the matured fruit of the Evodia rutaecarpa Bentham or Evodia officinalis Dode, of the Rutaceae plant family. An extract of Evodia fruit in the presence of NADPH was shown to inhibit human liver microsomal erythromycin N-demethylation activity, mediated by cytochrome P450 3A4 (CYP3A4), in a preincubation-time dependent manner. The present study was conducted to identify components of Evodia fruit extract having preincubation-time dependent inhibitory effects on CYP3A4 by analyzing human liver microsomal erythromycin N-demethylation activity. Rutaecarpine, a major component of Evodia fruit, and limonin caused the most dramatic decrease in residual CYP3A4 activity (IC50 before and after 20 min preincubation with: rutaecarpine, >100 μM and 1.4 μM; limonin, 23.5 μM and 1.8 μM, respectively). Furthermore, rutaecarpine and limonin were identified as mechanism-based inhibitors of CYP3A4 from the following observations: 1) The inhibitory effects of rutaecarpine and limonin on CYP3A4 activity were dependent on the preincubation time, 2) The inhibition required NADPH, 3) The inhibition was depressed in the presence of the competitive CYP3A4 inhibitor, ketoconazole, 4) Dialysis resulted in no recovery of CYP3A4 activity. The kinetic parameters for inactivation $k_{inact}$ and $K_I$ were: 0.387 min$^{-1}$ and 107.7 μM for rutaecarpine, 0.266 min$^{-1}$ and 23.2 μM for limonin, respectively. These results indicate that rutaecarpine and limonin are mechanism-based inhibitors of CYP3A4.

Key words: Herbal medicines; Evodia fruit; CYP3A4; mechanism-based inhibition; inactivation

Introduction

Cytochrome P450 (CYP) enzymes are generally considered to be involved in 95% of drug-drug interactions associated with the metabolism of drugs. Furthermore, about 70% of drug interactions associated with CYP-mediated metabolism are the result of enzyme inhibition.1) Metabolism-dependent inhibitors are classified as reversible, quasi-irreversible or irreversible.2) Quasi-irreversible and irreversible metabolism-dependent inhibitors are referred to as suicidal or mechanism-based inhibitors. As the inhibition is irreversible, these compounds often cause serious adverse effects, especially in a clinical situation, since their effect can persist even after withdrawal of the inhibitor. Recovery of enzyme activity after irreversible inhibition can be accomplished by biosynthesis of the enzyme molecule to a sufficient level. The concomitant use of sorivudine and an anticancer fluorouracil
derivative is a well-known example of a drug interaction caused by an irreversible metabolism-dependent inhibition.\textsuperscript{9} Other examples include macrolide antibiotics such as erythromycin and troleandomycin\textsuperscript{9} (both CYP3A4 inhibitors) and furafylline\textsuperscript{5} (CYP1A2 inhibitor). It is therefore important to clarify the mechanism of enzyme inhibition in order to better understand possible drug interactions. This is particularly important for herbal medicines because of their complex composition.

We are currently carrying out a survey of possible interactions of herbal medicines with synthetic drugs. In total we have studied crude extracts of 78 herbal medicines for their inhibitory effects on CYP3A4 and CYP2D6, which are involved in the metabolism of numerous synthetic drugs.\textsuperscript{6} In a previous study, Evodia fruit extract caused the most dramatic decrease in residual CYP3A4 activity in a manner dependent on the preincubation time.\textsuperscript{7} These results indicate that an extract of Evodia fruit contain one or more mechanism-based inhibitors of CYP3A4. However, the identity of the inhibitor(s) in the extract is/are unknown.

Evodia fruit (\textit{Evodiae Fructus}) is a herbal medicine prepared from the matured fruit of the plant, \textit{Evodia rutaecarpa} Bentham or \textit{Evodia officinalis} Dode, of the Rutaceae family. It can act as a stimulant for the secretion of digestive enzymes to promote appetite, and give a diuretic and analgesic effect. Evodia fruit is widely used in Japan as a component of Kampo medicines, such as unkei-to, gosyuyu-to, toki-shigyakuka-gosyuyu-shokyou-to, and hensei-shinki-in, etc. In Asia, Evodia fruit is used as a single herbal medicine. Due to its widespread use, Evodia fruit is highly likely to be used in combination with various synthetic drugs.

The present study was conducted to identify CYP3A4 preincubation-time dependent inhibiting components from Evodia fruit extract by using an \textit{in vitro} human liver microsomal system. We have identified two components of the extract that irreversibly inactivate CYP3A4.

**Materials and Methods**

**Chemicals:** Powder prepared from the Evodia fruit extract was kindly provided by Tsumura Ltd. (Tokyo, Japan). The Evodia fruit extract powder was prepared by immersing the herb in distilled water and heating it to 95–100°C for 60 min, before filtering. The filtrate was evaporated under reduced pressure and then spray-dried to give the powder. \textit{[N-methyl-\textsuperscript{14}C]}Erythromycin (2.035 GBq/mmol; radiochemical purity >99\%) was purchased from American Radiochemicals Inc. (St. Louis, MO, USA). Erythromycin, ketoconazole, evodiamine, rutaecarpine, limonin and nomilin were purchased from Wako Pure Chemical Industries, Ltd. (Osaka, Japan). Troleandomycin was purchased from Sigma Chemical Co. (St. Louis, MO, USA). Nicotinamide adenine dinucleotide phosphate oxidized form (NADP\textsuperscript{+}), glucose 6-phosphate (G-6-P) and G-6-P dehydrogenase were purchased from Oriental Yeast Co., Ltd. (Tokyo, Japan). All other reagents and solvents were analytical grade and were supplied by Wako Pure Chemical Industries, Ltd.

**Human liver microsomes:** Human liver microsomes, prepared from 16 individuals (male, 11; female, 5), were obtained from Xeno Tech LLC (Lenexa, KS, USA) and stored at –80°C until use.

**Evodia fruit extract:** Powder prepared from Evodia fruit extract (1 g) was extracted with methanol as described previously.\textsuperscript{5} A 1 µL aliquot of methanol extract was equivalent to 0.33 mg of Evodia fruit extract powder.

**Fractionation of Evodia fruit extract:** A sample of the methanol soluble fraction of Evodia fruit extract (5 µL; equivalent to 1.65 mg of Evodia fruit extract powder) was subjected to HPLC. HPLC analysis was performed with a Mightsil RP-18 (4.6×250 mm; Kanto Chemical Co., Inc., Tokyo, Japan) using a model L-7000 system (Hitachi, Ltd., Tokyo, Japan). A gradient condition of solvent A (water) and solvent B (acetonitrile) was programmed as follows: 0 min, 10\% (B); 50 min, 90\% (B); 55 min, 90\% (B) [Condition 1]. The flow rate was set at 1 mL/min and the column temperature was set at 40°C. The eluate was monitored by the absorption at 254 nm, and fractions were collected every 5 min. An aliquot of each fraction (5 mL/fraction) was transferred to a 13×100 mm glass test tube and the solvent was removed by freeze-drying. A total of 11 fractions were analyzed.

**Isolation of CYP3A4 inhibitory components from Evodia fruit extract:** The methanol soluble fraction of Evodia fruit extract was repeatedly analyzed by HPLC with the mobile phase of Condition 1 as described above. Aliquots of Fractions 6 and 7 were collected separately. Both fractions were evaporated to dryness under a stream of nitrogen and each residue was dissolved in a small volume of methanol. The resulting solution was spotted on a thin-layer chromatography (TLC) plate (Kiesegel 60F254, 0.25 or 0.5 mm thickness, Merck, Ltd., Tokyo, Japan) and developed with the solvent CHCl\textsubscript{3}:diethyl ether:methanol (ratio 14:6:1). Five compounds were collected (three from fraction 6 and two from fraction 7).

**NMR and HPLC analysis of components:** \textsuperscript{1}H-NMR spectra were determined using a JEOL JNM-LA400 spectrometer (JEOL Ltd., Tokyo, Japan) in CDCl\textsubscript{3} with tetramethylsilane (TMS) as an internal standard. Chemical shifts are recorded as \textdelta\textsuperscript{-}values. Each component was analyzed by HPLC with the mobile phase of Condition 1 as described above.

**Inhibition of CYP3A4 by Evodia fruit extract:** A
methanol soluble fraction from Evodia fruit extract was preincubated with human liver microsomes in the presence of an NADPH-generating system (NADPH-gs) (0.5 mM NADPH, 5 mM MgCl₂, 5 mM G-6-P and 1 U/mL G-6-P DH). The composition of the reaction mixture was 100 mM potassium phosphate (pH 7.4), 50 μM EDTA, 0.4 mg/mL human liver microsomal protein, 55 μg of dried Evodia fruit extract/mL and 100 μM [N-methyl-¹⁴C]erythromycin. The final volume of the reaction mixture was 500 μL and the final methanol concentration was 1%. The reaction mixture except for NADPH-gs and [N-methyl-¹⁴C]erythromycin was equilibrated at 37°C for 5 min. [N-methyl-¹⁴C]Erythromycin was added to the reaction mixtures following preincubation for 0 and 20 min at 37°C after adding NADPH-gs. Microsomal erythromycin N-demethylation activity was determined according to the method described above.

Effect of NADPH, competitive inhibitor and nucleophiles: The effect of NADPH on CYP3A4 inactivation was determined by preincubating rutaecarpine (25 μM), limonin (50 μM) or DMSO (1%, vehicle control) with 4 mg/mL human liver microsomal protein for 0 and 20 min at 37°C in the presence or absence of NADPH-gs in a total volume of 500 μL of 100 mM potassium phosphate (pH 7.4) containing 50 μM EDTA. The effect of CYP3A4 competitive inhibitor on CYP3A4 inactivation was determined by preincubating rutaecarpine (25 μM), limonin (50 μM) or DMSO (1%, vehicle control) with 4 mg/mL human liver microsomal protein and NADPH-gs for 0 and 20 min at 37°C in the presence or absence of ketoconazole in a total volume of 500 μL of 100 mM potassium phosphate (pH 7.4) containing 50 μM EDTA. The effect of the nucleophile-trapping agent on CYP3A4 inactivation was determined by preincubating rutaecarpine (25 μM), limonin (50 μM) or DMSO (1%, vehicle control) with 4 mg/mL human liver microsomal protein and NADPH-gs for 0 and 20 min at 37°C in the presence or absence of glutathione (10 mM) or N-acetylcysteine (10 mM) in a total volume of 500 μL of 100 mM potassium phosphate (pH 7.4) containing 50 μM EDTA. A 50-μL aliquot was transferred to tubes containing 450-μL of 100 mM potassium phosphate (pH 7.4) containing 50 mM EDTA, [N-methyl-¹⁴C]erythromycin and NADPH-gs (enzyme assay mixture). Erythromycin N-demethylation activity was determined as described above. Values are presented as a percentage of the activity obtained from the sample at preincubation time 0 min.

Inhibition of CYP3A4 by the HPLC eluted fractions and the components from Evodia fruit extract: The HPLC eluted fractions and the components from Evodia fruit extract (evodiamine, rutaecarpine, limonin and nomilin) were preincubated with human liver microsomes in the presence of NADPH-gs. Evodiamine, rutaecarpine, limonin and nomilin were dissolved in DMSO. The composition of the reaction mixture was 100 mM potassium phosphate (pH 7.4), 50 μM EDTA, 0.4 mg/mL human liver microsomal protein, 0.5–50 μM the components from Evodia fruit extract and 100 μM [N-methyl-¹⁴C]erythromycin. The composition of each HPLC eluted fraction was the amount fractionated from 1.65 mg of Evodia fruit extract powder per 1 mL of reaction mixture. The final volume of the reaction mixture was 500 μL and the final DMSO concentration was 0.5%. The reaction mixture except for NADPH-gs and [N-methyl-¹⁴C]erythromycin was equilibrated at 37°C for 5 min. [N-methyl-¹⁴C]Erythromycin was added to the reaction mixtures following preincubation for 0 and 20 min at 37°C after adding NADPH-gs. Microsomal erythromycin N-demethylation activity was determined according to the method described above.

CYP3A4 inactivation experiments: Rutaecarpine (0, 6.25, 12.5, 25 and 50 μM) or limonin (0, 6.25, 12.5, 25 and 50 μM) were preincubated with 4 mg/mL human liver microsomal protein for 0, 5, 10, 20 and 30 min at 37°C in the presence of NADPH-gs in a total volume of 500 μL of 100 mM potassium phosphate (pH 7.4) containing 50 μM EDTA. The final DMSO concentration was 1%. A 50-μL aliquot was transferred to tubes containing 450-μL of 100 mM potassium phosphate (pH 7.4) containing 50 mM EDTA, [N-methyl-¹⁴C]erythromycin and NADPH-gs (enzyme assay mixture). Erythromycin N-demethylation activity was determined as described above. The remaining activity was calculated as a percentage of the activity obtained from the vehicle control samples. A logarithm of the percentage of the remaining activity was plotted against incubation time and the slope of the lines was obtained from linear regression analysis. The apparent inactivation rate
constants ($k_{\text{obs}}$) were taken from the slope of the line ($-k_{\text{obs}}$) (rutaecarpine, 0–10 min; limonin, 0–5 min). The maximal rate constant of inactivation ($k_{\text{inact}}$) and the inhibitor concentration required for half-maximal rate of inactivation ($K_i$) were calculated from the double reciprocal plots of $k_{\text{obs}}$ versus inhibitor concentration by linear regression analysis using the spreadsheet software Microsoft Excel 2000 SR-1 (Microsoft Co., Tokyo, Japan). The $y$-intercept is known to equal $1/k_{\text{inact}}$ while the $x$-intercept equals $-1/K_i$.

**Dialysis experiments:** Rutacarpine (25 μM), limonin (50 μM) or DMSO (1%, vehicle control) were incubated with 4 mg/mL human liver microsomal protein for 20 min at 37°C in the presence of NADPH-gs in a total volume of 500 μL of 100 mM potassium phosphate (pH 7.4) containing 50 μM EDTA. Subsequently, the incubation mixtures were transferred to a Slide-A-Lyzer mini-dialysis unit with a molecular weight cutoff of 10,000 (Pierce Chemical Co., Rockford, IL, USA). Dialysis was performed at 4°C for 16 hr in 2 liter of water. The samples were assayed for erythromycin N-demethylation activity as described above, before and after dialysis. Ketoconazole (1 μM), irreversible inhibitor, in place of rutaecarpine and limonin was also incubated. Values are presented as a percentage of the activity obtained from the vehicle control sample.

**Results**

**Preincubation time-dependent inhibition of CYP3A4 activity using Evodia fruit extract:** The remaining activity of human liver microsomes preincubated with the equivalent of 55 μg of Evodia fruit extract powder for up to 30 min in the presence of NADPH-gs is shown in Fig. 1. CYP3A4 activity in the microsomes preincubated with Evodia fruit extract decreased in a preincubation time-dependent manner. The residual activity in microsomes after 30 min preincubation with an extract of Evodia fruit extract was 22.3% of that for the 0 min sample. Troleandomycin, a known mechanism-based inhibitor of CYP3A4, caused a preincubation time-dependent decrease in CYP3A4 activity.2) The residual activity after 30 min preincubation with troleandomycin was 49.4%. In contrast, ketoconazole, a known competitive inhibitor of CYP3A4, caused no preincubation time-dependent decrease in the CYP3A4 activity.3)

**Isolation of CYP3A4 inhibitory components from Evodia fruit extract:** A typical HPLC chromatogram of the methanol soluble fraction of Evodia fruit extract is shown in Fig. 2A. The eluent from HPLC was fractionated and the inhibitory potential to CYP3A4 was investigated for each fraction. Two fractions of 25–30 min (Fraction 6) and 30–35 min (Fraction 7) showed strong preincubation-time dependent inhibition of CYP3A4 activity (Fig. 2B).

**Identification of the chemical structure of CYP3A4 inhibitory components:** Three isolated compounds (Peak 6–1, 6–2 and 6–3) from Fraction 6 and two (Peak 7–1 and 7–2) from Fraction 7 were subjected to structural analysis by 1H-NMR. The spectral data for peaks 6–1, 6–2, 6–3, 7–1 and 7–2 coincided with those reported for limonin,8) evodol,8) nomilin,9) evodiamine10) and rutaecarpine,10) respectively. The retention time of each peak corresponded to that determined for the relevant standard sample run under identical HPLC conditions. From these results, peaks 6–1, 6–2, 6–3, 7–1 and 7–2 were confirmed to be limonin, evodol, nomilin, evodiamine and rutaecarpine, respectively. The structural formulas of the five components from Evodia fruit extract are shown in Fig. 3.

**Inhibitory effects of components from Evodia fruit extract on CYP3A4 activity:** Inhibitory effects of four components (evodiamine, rutaecarpine, limonin and nomilin) from Evodia fruit extract toward CYP3A4 were investigated to establish the potency of inhibition. Evodiamine, rutaecarpine, limonin and nomilin inhibited microsomal erythromycin N-demethylation with IC50 values of 19.6, >100, 23.5 and 5.0 μM, respectively (Fig. 4). The IC50 values for evodiamine, rutaecarpine, limonin and nomilin after 20 min preincubation in the presence of NADPH-gs were 12.0, 1.4, 1.8 and 2.3 μM, respectively. The IC50 values for rutaecarpine, limonin and nomilin after 20 min preincubation in the absence of NADPH-gs were similar to those without preincubation.

**Requirement of NADPH for CYP3A4 inactivation**
Fig. 2. HPLC chromatogram (A) of methanol soluble fraction of Evodia fruit extract and the inhibition (B) of human liver microsomal erythromycin N-demethylation activity by each fraction eluted from HPLC. Methanol fraction of Evodia fruit extract (1.65 mg equivalent to the powder) was injected into the HPLC. The eluate was monitored at 254 nm. Fractions were collected every 5 min. Human liver microsomes (0.4 mg protein/mL) were incubated with each HPLC fraction. Microsomal erythromycin N-demethylation activity was determined without preincubation (○) or after preincubation for 20 min (■) at 37°C in the presence of NADPH-gs, as described in Materials and Methods. Values are presented as an inhibitory percentage of the activity obtained from the vehicle control samples. Each column represents the means of duplicate experiments.

Fig. 3. Evodia fruit extract components investigated in the present study.
Fig. 4. Effect of preincubation on the inhibition of human liver microsomal erythromycin $N$-demethylation by evodiamine, rutaecarpine, limonin and nomilin. Human liver microsomes (0.4 mg protein/mL) were incubated with 0.5–50 μM of evodiamine, rutaecarpine, limonin and nomilin. Microsomal erythromycin $N$-demethylation activity was determined following preincubation for 0 min (▲) and 20 min (●) at 37°C in the presence of NADPH-gs, as described in Materials and Methods. Microsomal erythromycin $N$-demethylation activity was also determined following preincubation with rutaecarpine, limonin or nomilin for 20 min at 37°C in the absence of NADPH-gs (■). Values are presented as a percentage of the activity obtained from the vehicle control samples. Each symbol represents the means of duplicate experiments. IC$_{50}$ values were determined by nonlinear regression.

by rutaecarpine and limonin: The remaining activities in human liver microsomes preincubated with either 25 μM rutaecarpine or 50 μM limonin for 20 min in the presence or absence of NADPH-gs is shown in Fig. 5. The remaining activity in microsomes preincubated with rutaecarpine in the presence of NADPH-gs for 20 min was 43.6%. This residual activity was significantly greater (96.7%) if the preincubation was performed in the absence of NADPH-gs. Similarly, the remaining activity in microsomes preincubated with limonin in the presence of NADPH for 20 min was 18.4%, but 101.9% in the absence of NADPH-gs.

Effect of a CYP3A4 competitive inhibitor on CYP3A4 inactivation by rutaecarpine and limonin: The remaining CYP3A4 activity in human liver microsomes preincubated with either 25 μM rutaecarpine or 50 μM limonin for 20 min in the presence or absence of ketoconazole is shown in Fig. 6. In the microsomes preincubated with rutaecarpine in the presence of ketoconazole, the remaining CYP3A4 activity was 69.0%, which is higher than that observed (45.3%) in the absence of ketoconazole. Similarly, the remaining CYP3A4 activity in microsomes preincubated with

Fig. 5. Requirement for NADPH in the inhibition of human liver microsomal erythromycin $N$-demethylation by rutaecarpine and limonin. Human liver microsomes (4 mg protein/mL) were preincubated with 25 μM rutaecarpine, 50 μM limonin or 1% DMSO (vehicle control) for 0 and 20 min at 37°C in the presence or absence of NADPH-gs. A 50-μL aliquot was transferred to a 450-μL enzyme activity assay mixture and erythromycin $N$-demethylation activity was determined as described in Materials and Methods. Values are presented as a percentage of the activity obtained from the sample at preincubation time 0 min. Each column represents the mean of duplicate experiments.
Fig. 6. Effect of CYP3A4 competitive inhibitor on the inhibition of human liver microsomal erythromycin N-demethylation by rutaecarpine and limonin. Human liver microsomes (4 mg protein/mL) were preincubated with 25 μM rutaecarpine, 50 μM limonin or 1% DMSO (vehicle control) for 0 and 20 min at 37°C in the presence or absence of 1 μM ketoconazole. A 50-μL aliquot was transferred to a 450-μL enzyme activity assay mixture and erythromycin N-demethylation activity was determined as described in Materials and Methods. Values are presented as a percentage of the activity obtained from the sample at preincubation time 0 min. Each column represents the mean of duplicate experiments.

Fig. 7. Effect of a nucleophile on the inhibition of human liver microsomal erythromycin N-demethylation by rutaecarpine and limonin. Human liver microsomes (4 mg protein/mL) were preincubated with 25 μM rutaecarpine, 50 μM limonin or 1% DMSO (vehicle control) for 0 and 20 min at 37°C in the presence or absence of 10 mM glutathione or 10 mM N-acetylcysteine. A 50-μL aliquot was transferred to a 450-μL enzyme activity assay mixture and erythromycin N-demethylation activity was determined as described in Materials and Methods. Values are presented as a percentage of the activity obtained from the sample at preincubation time 0 min. Each column represents the mean of duplicate experiments.

Fig. 8. Effect of dialysis on the inactivation of human liver microsomal erythromycin N-demethylation by rutaecarpine and limonin. Human liver microsomes (4 mg protein/mL) were preincubated with 25 μM rutaecarpine, 50 μM limonin or 1% DMSO (vehicle control) in the presence of NADPH-gs for 20 min at 37°C. A 500-μL aliquot was transferred to a Slide-A-Lyzer mini-dialysis unit and dialyzed for 16 hr at 4°C. A 50-μL aliquot was transferred to a 450-μL enzyme assay mixture and erythromycin N-demethylation activity was determined before and after dialysis as described under Materials and Methods. Values are presented as a percentage of the activity obtained from the vehicle control sample. Each column represents the mean of duplicate experiments.

Effect of a nucleophile on CYP3A4 inactivation by rutaecarpine and limonin: The remaining CYP3A4 activity in human liver microsomes preincubated with either 25 μM rutaecarpine or 50 μM limonin for 20 min in the presence or absence of a nucleophile (glutathione or N-acetylcysteine) is shown in Fig. 7. In microsomes preincubated with rutaecarpine, a slight increase in residual CYP3A4 activity (approximately 30%) was observed in the presence of glutathione or N-acetylcysteine. However, the CYP3A4 activity did not return to the control level. In microsomes preincubated with limonin, a slight increase in residual CYP3A4 activity (approximately 2-fold) was observed in the presence of glutathione or N-acetylcysteine.

Reversibility of inactivation by rutaecarpine and limonin on CYP3A4 activity: We investigated whether the inactivation of CYP3A4 mediated by rutaecarpine and limonin was reversible. Human liver microsomes were preincubated with rutaecarpine or limonin at 37°C for 20 min with NADPH-gs. The samples were then transferred to a mini-dialysis unit and dialyzed at 4°C for 16 hr. For comparison, a simultaneous experiment using ketoconazole, a known reversible inhibitor of CYP3A4, was also performed. As indicated in Fig. 8, recovery of CYP3A4 activity was not observed with rutaecarpine or limonin after dialysis, whereas complete recovery was observed for ketoconazole.

Inactivation kinetics of CYP3A4 activity by rutaecarpine and limonin: Human liver microsomal erythromycin N-demethylation activity was inactivated by rutaecarpine and limonin in time- and concentration-dependent manner in the presence of NADPH-gs (Fig. 9). The $k_{\text{inact}}$ and $K_i$ values were determined from
Fig. 9. Inactivation of human liver microsomal erythromycin N-demethylation by rutaecarpine (A, C) or limonin (B, D). Human liver microsomes (4 mg protein/mL) were preincubated with rutaecarpine or limonin at concentrations of 0 ( ●, vehicle control), 6.25 ( ▲), 12.5 ( ○), 25 ( △), and 50 μM ( ▲) in the presence of NADPH-generating system for 0, 5, 10, 20 and 30 min at 37°C. A 50-μL aliquot was transferred to a 450-μL enzyme assay mixture and erythromycin N-demethylation activity was determined as described under Materials and Methods. The log percentage of the control activity versus preincubation time is plotted (A, B). Values are presented as a percentage of the activity obtained from the vehicle control samples. Each symbol represents the mean of duplicate experiments. The reciprocal of the $k_{\text{inact}}$ value obtained from slopes of the line (0 to 10 min for rutaecarpine, 0 to 5 min for limonin) were plotted against the reciprocal of the rutaecarpine (C) or limonin (D) concentrations. The fitted line was produced by linear regression analysis of each point. The intercepts on the y- and x-axis of the fitted line represent $1/k_{\text{inact}}$ and $-1/K_I$, respectively.

double-reciprocal plots of $k_{\text{obs}}$ and inhibitor concentration. The $k_{\text{inact}}$ and $K_I$ values of rutaecarpine were 0.387 min$^{-1}$ and 107.7 μM, respectively. The $k_{\text{inact}}$ and $K_I$ values of limonin were 0.266 min$^{-1}$ and 23.2 μM, respectively.

Discussion

Preincubation of Evodia fruit extract with human liver microsomes in the presence of an NADPH-generating system resulted in inhibition of erythromycin N-demethylation activity, a CYP3A4-dependent activity. The inhibition was observed in a manner dependent on the preincubation time. The inhibition rates were higher than those attained with the irreversible inhibitor for CYP3A4, troleandomycin (Fig. 1). In contrast, ketoconazole, a known competitive inhibitor of CYP3A4 did not cause a significant preincubation time-dependent decrease in the CYP3A4 activity. Indeed, the level of inhibition of CYP3A4 decreased as the length of the preincubation period increased. This result suggests that ketoconazole is metabolized by the microsomes during the preincubation period, thereby lowering the level of inhibitor prior to the assay.

In the present study, we have analyzed the components of the extract and identified rutaecarpine and limonin as mechanism-based inhibitors for CYP3A4. The indole-quinazoline alkaloid, rutaecarpine, is a major component of Evodia fruit extract. Limonin is a compound having a furan ring and an epoxide moiety in its structure. Rutaecarpine and limonin were identified as mechanism-based inhibitors of CYP3A4 from the following observations: 1) The inhibitory effects of rutaecarpine and limonin on CYP3A4 activity were dependent on the preincubation time, 2) The inhibition required NADPH, 3) The inhibition was depressed in the presence of the competitive CYP3A4 inhibitor, ketoconazole, 4) Dialysis resulted in no recovery of CYP3A4 activity. Interestingly, inhibition by rutaecarpine was almost unaffected by the presence of a nucleophile (glutathione or N-acetylcysteine). In contrast, the inhibition of CYP3A4 activity mediated by limonin was partially alleviated by the presence of glutathione or N-acetylcysteine during the preincubation period. Since exogenous nucleophiles had some protective effect against inactivation of CYP3A4 mediated by limonin, the mechanism of inhibition may
be different from that of rutaecarpine. One possible explanation for this result is an enzymatic or non-enzymatic conjugation of limonin with glutathione or N-acetyl cysteine at the epoxide moiety. Further study is needed to verify this hypothesis.

The chemical structure of rutaecarpine and limonin presumably play an important role in the inactivation of CYP3A4. In the case of rutaecarpine, a remarkable reduction in IC$_{50}$ for human liver microsomal erythromycin N-demethylation activity was observed after preincubation for 20 min (1.4 µM with preincubation vs >100 µM without any preincubation). In contrast evodiamine, which has a similar structure to rutaecarpine, displayed only a 39% reduction in IC$_{50}$ after preincubation for 20 min (12.0 µM with preincubation vs 19.6 µM without any preincubation). As seen in Fig. 3, evodiamine has a methyl group at the N-14 position and hydrogen at the C-13b position. These groups represent the only difference in the structure of rutaecarpine from that of evodiamine, suggesting that they play an important role in the mechanism-based inhibition mediated by rutaecarpine. Limonin has a furan ring in its structure. Intriguingly many furan ring-containing compounds such as methoxsalen,11) furafylline5) and L-754,39412) are mechanism-based inhibitors. Although the inhibitory mechanism for these compounds has not been fully elucidated, a similar mechanism may underlie the inactivation of CYP3A4 by limonin.

The kinetic parameters for the inactivation of CYP3A4 by rutaecarpine and limonin have been determined: $K_i$, 107.7 and 23.2 µM; $k_{inact}$, 0.387 and 0.266 min$^{-1}$, respectively. The inactivation kinetic constants of several compounds reported as mechanism-based inhibitors of CYP3A4 are listed in Table 1. The ratios of $k_{inact}/K_i$ for rutaecarpine (3.59) and limonin

<table>
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<tr>
<th>Compound</th>
<th>Enzyme source</th>
<th>Metabolic reaction</th>
<th>$k_{inact}$ (1/min)</th>
<th>$K_i$ (µM)</th>
<th>Ratio of $k_{inact}/K_i$ (min$^{-1}$·nM$^{-1}$)</th>
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<td>HLM</td>
<td>Testosterone 6β-OH</td>
<td>0.08</td>
<td>40.00</td>
<td>2*</td>
<td>Tassaneeyakul et al., 200027</td>
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<tr>
<td>Clarithromycin</td>
<td>HLM</td>
<td>Midazolam 1’-OH</td>
<td>0.0423</td>
<td>41.4</td>
<td>1.02*</td>
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</tr>
<tr>
<td>Clarithromycin</td>
<td>HLM</td>
<td>Midazolam 4-OH</td>
<td>0.0459</td>
<td>37.0</td>
<td>1.24*</td>
<td>Ito et al., 200320</td>
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<tr>
<td>Clarithromycin</td>
<td>HLM</td>
<td>Midazolam 1’-OH</td>
<td>0.072</td>
<td>5.49</td>
<td>13.11*</td>
<td>Mayhew et al., 200026</td>
</tr>
<tr>
<td>Delavirdine</td>
<td>HLM</td>
<td>Triazolam 1’-OH</td>
<td>0.59</td>
<td>21.6</td>
<td>27.31*</td>
<td>Voorman et al., 199829</td>
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<td>N-Desalkyverapamil</td>
<td>CYP3A4</td>
<td>Testosterone 6β-OH</td>
<td>0.07</td>
<td>7.93</td>
<td>8.52</td>
<td>Wang et al., 200424</td>
</tr>
<tr>
<td>N-Desmethyldiltiazem</td>
<td>HLM</td>
<td>Midazolam 1’-OH</td>
<td>0.027</td>
<td>0.77</td>
<td>35.06*</td>
<td>Mayhew et al., 200026</td>
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<tr>
<td>N-Desmethyltamoxifen</td>
<td>CYP3A4</td>
<td>Testosterone 6β-OH</td>
<td>0.08</td>
<td>2.6</td>
<td>30.77*</td>
<td>Zhao et al., 200230</td>
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<td>HLM</td>
<td>Testosterone 6β-OH</td>
<td>0.0495</td>
<td>35.0</td>
<td>1.41*</td>
<td>Masubuchi et al., 199923</td>
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<tr>
<td>6’, 7’-Dihydroxybergamottin</td>
<td>HLM</td>
<td>Testosterone 6β-OH</td>
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<td>5.56</td>
<td>10.79*</td>
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<td>6’, 7’-Dihydroxybergamottin</td>
<td>CYP3A4</td>
<td>Testosterone 6β-OH</td>
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<td>59</td>
<td>2.71*</td>
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<td>Erythromycin</td>
<td>HLM</td>
<td>Triazolam 1’-OH</td>
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<td>3.90*</td>
<td>Kanamitsu et al., 200026</td>
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<td>HLM</td>
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<td>3.16*</td>
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<td>CYP3A4</td>
<td>Triazolam 1’-OH</td>
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<td>19.1</td>
<td>9.06*</td>
<td>Mayhew et al., 200026</td>
</tr>
<tr>
<td>Erythromycin</td>
<td>CYP3A4</td>
<td>Triazolam 4-OH</td>
<td>0.097</td>
<td>18.9</td>
<td>5.13*</td>
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<tr>
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<td>HLM</td>
<td>Midazolam 1’-OH</td>
<td>0.0240</td>
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<td>1.90*</td>
<td>Ito et al., 200320</td>
</tr>
<tr>
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<td>HLM</td>
<td>Midazolam 4-OH</td>
<td>0.0256</td>
<td>14.1</td>
<td>1.82*</td>
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<tr>
<td>17a-Ethynylestradiol</td>
<td>CYP3A4</td>
<td>Testosterone 6β-OH</td>
<td>0.04</td>
<td>18</td>
<td>2.22*</td>
<td>Lin et al., 200221</td>
</tr>
<tr>
<td>Fluoxetine</td>
<td>HLM</td>
<td>Midazolam 1’-OH</td>
<td>0.017</td>
<td>5.26</td>
<td>3.23*</td>
<td>Mayhew et al., 200026</td>
</tr>
<tr>
<td>Gestodene</td>
<td>HLM</td>
<td>Nifedipine oxidation</td>
<td>0.39</td>
<td>46</td>
<td>8.48*</td>
<td>Guengerich et al., 199020</td>
</tr>
<tr>
<td>GF-I-1</td>
<td>HLM</td>
<td>Testosterone 6β-OH</td>
<td>0.05</td>
<td>0.31</td>
<td>161.29*</td>
<td>Tassaneeyakul et al., 200027</td>
</tr>
<tr>
<td>GF-I-4</td>
<td>HLM</td>
<td>Testosterone 6β-OH</td>
<td>0.05</td>
<td>0.13</td>
<td>384.62*</td>
<td>Tassaneeyakul et al., 200027</td>
</tr>
</tbody>
</table>

Table 1. Comparison of enzyme inactivation kinetic constants for the various mechanism-based inactivators of CYP3A4
(11.47) were not as high as those reported for mechanism-based inhibitors such as GF-I-1 (161.29), GF-I-4 (384.62), gomisin C (230.58), L-754,394 (216), Mibebradil (166.67), norverapamil (57.23–190.15), tamoxifen (200) and verapamil (15.31–215.49). The $k_{\text{inact}}/K_i$ ratio for limonin was similar to that of clarithromycin (13.11), 6',7'-desmethylbergamottin (10.79) and gestoden (8.48). The $k_{\text{inact}}/K_i$ ratio for rutaecarpine was similar to that of amiodarone (4.48), bergamottin (2), erythromycin (3.9–9.06) and fluoxetine (3.23).

The contents of rutaecarpine and limonin in Evodia fruit were reported to be about 1–13 mg/g [13-15] or 2–15 mg/g [15], respectively, depending on the production area and the time that the herb was harvested. However, the actual contents of rutaecarpine and limonin in Evodia fruit extract used in this study are not clear. Therefore, it is not possible to ascertain the actual intake of rutaecarpine and limonin in a clinical situation. Since the $K_i$ values of rutaecarpine and limonin are not particularly low (107.7 and 23.2 $\mu$M), it is necessary that the CYP3A4 enzyme is exposed to high concentrations of rutaecarpine and limonin in order to cause the drug interaction. CYP3A4 is a CYP isoform not only found in the liver, but also in the small intestine of humans. [18] It is thought that inhibition of CYP3A4 in the small intestine, rather than the liver, plays a more important role in drug interactions associated with the inhibition of CYP3A4 by grapefruit juice. [17,18] The ingestion of Evodia fruit presumably results in the small intestine being exposed to high concentrations of rutaecarpine and limonin. Therefore, it is possible that Evodia fruit caused drug interaction through the CYP3A4 enzyme expressed in small intestine. In the future, it is necessary to investigate the actual rutaecarpine and limonin contents in Evodia fruit extract. It is also necessary to take into consideration the effect of CYP isoforms other than CYP3A4, since rutaecarpine has potent inhibition to CYP1A1 and CYP1A2 (IC$_{50}$ values: 0.90 $\mu$M for CYP1A1 activity and 0.06 $\mu$M of CYP1A2 activity). [19]

In addition, limonin is also found in citrus fruits, especially in the pulp of lemons and limes (350–400 $\mu$g/g), pomelos (about 150 $\mu$g/g), red grapefruit (about 50 $\mu$g/g) and white grapefruit (about 10 $\mu$g/g). [20] It has been reported that limonin is present in large amounts in the fruit pericarp of lemons (about 4000 $\mu$g/g), limes (about 1500 $\mu$g/g) and red grapefruit (about 500 $\mu$g/g). [20] From the potency of the inhibitory effect of limonin on human liver microsomal testosterone 6β-hydroxylation activity (IC$_{50}$ = 100 $\mu$M), Fukuda et al. [21] have concluded that the component in grapefruit juice responsible for inhibition of CYP3A4 activity is a compound other than limonin. In their studies, however, the mechanism for the inhibition by limonin

<table>
<thead>
<tr>
<th>Compound</th>
<th>Enzyme source</th>
<th>Metabolic reaction</th>
<th>$k_{\text{inact}}$ (1/min)</th>
<th>$K_i$ (µM)</th>
<th>Ratio of $k_{\text{inact}}/K_i$ (min$^{-1}$·nM$^{-1}$)</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Glabridin</td>
<td>CYP3A4</td>
<td>7-BFC O-D</td>
<td>0.14</td>
<td>7</td>
<td>20$^{a}$</td>
<td>Kent et al., 2002$^{27}$</td>
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<tr>
<td>Gomisin C</td>
<td>HLM</td>
<td>Erythromycin N-DM</td>
<td>0.092</td>
<td>0.399</td>
<td>230.58$^{a}$</td>
<td>Iwata et al., 2004$^{16}$</td>
</tr>
<tr>
<td>L-754,394</td>
<td>HLM</td>
<td>Testosterone 6β-OH</td>
<td>1.62</td>
<td>7.5</td>
<td>216$^{a}$</td>
<td>Chiba et al., 1995$^{28}$</td>
</tr>
<tr>
<td>Limonin</td>
<td>HLM</td>
<td>Erythromycin N-DM</td>
<td>0.266</td>
<td>23.2</td>
<td>11.47$^{a}$</td>
<td>in the present study</td>
</tr>
<tr>
<td>Mifepristone (RU486)</td>
<td>CYP3A4</td>
<td>Testosterone 6β-OH</td>
<td>0.089</td>
<td>4.7</td>
<td>18.94$^{a}$</td>
<td>He et al., 1999$^{39}$</td>
</tr>
<tr>
<td>Mibebradil</td>
<td>HLM</td>
<td>Testosterone 6β-OH</td>
<td>0.4</td>
<td>2.4</td>
<td>166.67$^{a}$</td>
<td>Prueksaritanont et al., 1999$^{40}$</td>
</tr>
<tr>
<td>R-Norverapamil</td>
<td>HLM</td>
<td>Testosterone 6β-OH</td>
<td>0.17</td>
<td>2.97</td>
<td>57.23</td>
<td>Wang et al., 2004$^{41}$</td>
</tr>
<tr>
<td>S-Norverapamil</td>
<td>HLM</td>
<td>Testosterone 6β-OH</td>
<td>0.27</td>
<td>1.64</td>
<td>164.63</td>
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<tr>
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<td>Testosterone 6β-OH</td>
<td>0.21</td>
<td>2.11</td>
<td>97.45</td>
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<tr>
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<td>Testosterone 6β-OH</td>
<td>1.12</td>
<td>5.89</td>
<td>190.15</td>
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<tr>
<td>Rutaecarpine</td>
<td>HLM</td>
<td>Erythromycin N-DM</td>
<td>0.387</td>
<td>107.7</td>
<td>3.59$^{a}$</td>
<td>in the present study</td>
</tr>
<tr>
<td>Tamoxifen</td>
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<td>Testosterone 6β-OH</td>
<td>0.04</td>
<td>0.2</td>
<td>200$^{a}$</td>
<td>Zhao et al., 2002$^{50}$</td>
</tr>
<tr>
<td>R-Verapamil</td>
<td>HLM</td>
<td>Testosterone 6β-OH</td>
<td>0.08</td>
<td>5.10</td>
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<td>Wang et al., 2004$^{41}$</td>
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<td>Testosterone 6β-OH</td>
<td>0.13</td>
<td>1.39</td>
<td>93.53</td>
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<tr>
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<td>Testosterone 6β-OH</td>
<td>0.64</td>
<td>2.97</td>
<td>215.49</td>
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<tr>
<td>R,S-Verapamil</td>
<td>CYP3A4</td>
<td>Testosterone 6β-OH</td>
<td>0.43</td>
<td>4.57</td>
<td>94.09</td>
<td></td>
</tr>
</tbody>
</table>

$^{a}$ We calculated from the reported $k_{\text{inact}}$ and $K_i$ value.

HLM, Human liver microsomes; CYP3A4, recombinant CYP3A4; Erythromycin N-DM, Erythromycin N-demethylation activity; Testosterone 6β-OH, Testosterone 6β-hydroxylation activity; Midazolam 1' - or 4-OH, Midazolam 1' - or 4-hydroxylation activity; Triazolam 1' - or 4-OH, Triazolam 1' - or 4-hydroxylation activity, 7-BFC O-D, 7-Benzoylxy-4-(trifluoromethyl)coumarin O-demethylation activity.
has never been addressed. In the present study, we clearly showed that limonin inactivated CYP3A4. Therefore, it is likely that limonin is a part of causative component for drug interactions associated with grapefruit juice.

We surveyed the literature on the drug interaction between citrus fruits (lemon, lime and pomelo) except for grapefruit and synthetic drugs in clinical situation. In the results, two incidents concerning pomelo and lime have been reported. Egashira et al. have reported an incidence of abnormal elevation (about 2.5-fold) of blood tacrolimus level in a patient who was taking the drug after receiving a kidney transplant.22) The incidence was considered to be associated with consumption of pomelo (about 100 g). These workers have also shown that the inhibitory effects of pomelo are dependent on the preincubation time and that pomelo exerts only a limited effect on P-glycoprotein.23) It is likely that limonin was involved in the abnormal elevation of blood tacrolimus reported by Egashira et al.22) Another study reported a 2.7- to 3.6-fold increase in the area under the curve (AUC) for felodipine in some patients.24) Although it is not clear whether limonin involved in the abnormal elevation of blood tacrolimus reported by Egashira et al.22) Another study reported a 2.7- to 3.6-fold increase in the area under the curve (AUC) for felodipine in some patients (2 out of 8) who were taking the drug together with lime juice.24) Although it is not clear whether limonin involved in the drug interactions in these reported incidents, it is possible that limonin is involved in part. To our knowledge, no studies have reported drug interactions in association with the consumption of lemon or lemon juice. This is probably related to the limited amount of lemon in the diet.

As described above, rutaecarpine and limonin were identified as Evodia fruit extract components having mechanism-based inactivation effects on CYP3A4 activity. These results indicate that Evodia fruit, or a Kampo medicine (Chinese herbal and crude drug preparation) containing Evodia fruit, when administered in a concomitant manner with a synthetic drug that is metabolized by CYP3A4 can cause interactions with the drug based on its inhibitory effects of CYP3A4 activity. Further studies are needed to clarify the absorption of rutaecarpine and limonin, their stability in the small intestine and liver, and their contents in Evodia fruit and its extract, as well as to estimate the possibilities of drug interactions in clinical situations.

Acknowledgement: We thank Tepy Usia (Toyama Medical and Pharmaceutical University) for help with the NMR spectral analysis. We also thank Tetsuya Kaneko (Mitsubishi Chemical Safety Institute Ltd.) for skillful technical assistance.

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