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Inhibition on Human Liver Cytochrome P450 3A4 by Constituents of Fennel (*Foeniculum vulgare*): Identification and Characterization of a Mechanism-Based Inactivator

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Fennel, a seed of *Foeniculum vulgare*, is used as a culinary spice and traditional medicine. The methanolic extract of fennel showed a characteristic of mechanism-based inactivation on erythromycin N-demethylation mediated by human liver microsomal cytochrome P450 3A4 (CYP3A4). The present study was conducted to identify the fennel constituent having the inhibition. Thirteen compounds have been isolated from a methanol extract of fennel and tested for their inhibition on CYP3A4. Among them, 5-methoxypsoralen (5-MOP) showed the strongest inhibition with an IC_{50} value of 18.3 μM and a mixed type of inhibition. In addition, with the preincubation time of 20 min only 5-MOP showed preincubation time dependency; the IC_{50} value decreased from 18.3 μM with a preincubation time of 0 min to 4.6 μM with a preincubation time of 20 min. Further investigation on 5-MOP showed the characteristics of time-dependent inhibition, requirement of NADPH, lack of protecting effect of nucleophiles, and recovery of CYP3A4 activity by the competitive inhibitor. This result suggests that the inhibitory activity of CYP3A4 by 5-MOP was a mechanism-based inactivation. The kinetic parameter for mechanism-based inactivation was characterized by a K_I value of 15.0 μM and a k_{inact} value of 0.098 min^{-1}.

KEYWORDS: Fennel; *Foeniculum vulgare*; cytochrome P450 3A4; CYP3A4; mechanism-based inactivation; 5-methoxypsoralen

INTRODUCTION

Cytochrome P450s (CYPs) are the principal enzymes for oxidative metabolism of drugs and other xenobiotic in many mammalian tissues. Although the primary site of drug metabolism is the liver, metabolism can also occur in the intestines, blood, and other tissues (*1*). The metabolism of drugs is divided in two phases. The first involves phase I enzymes responsible for drug oxidation, reduction, or hydrolysis. The second involves phase II enzymes responsible for drug conjugation of the phase I metabolite with the water-solubilizing endogenous moiety. The CYP isoenzymes are responsible for most of the phase I metabolism processes (*2*). There are approximately 650 CYPs, which are organized into 96 families, and among them 35 CYPs are described in humans. Families 1, 2, and 3 appear to be responsible for the metabolism of drugs and therefore are potential sites for drug interactions (*3*). Of these CYPs, CYP3A4 is the most abundant P450 isoform in human liver, has very broad substrate specificity, and is believed to be responsible for the metabolism of >50% of all clinically used drugs (*4*).

Inhibition of the prominent drug-metabolizing enzyme CYP3A4 is the major mechanism underlying numerous drug−drug interactions. The inhibition often causes an accumulation of drugs during chronic dosing to result in an increase of the apparent half-life with the dose. Thus, potential interaction with co-administered drugs can cause serious side effects in clinical aspects. However, there are times when these interactions may be exploited. The inhibitor can be used as a drug-sparing agent when taken concomitantly with conventional medicines, to decrease the dosage and financial cost of expensive drug regimens. The mechanism of CYP inhibition is divided into three categories: (a) reversible, (b) quasi-irreversible, and (c) irreversible inhibition (*5*). In the case of irreversible inhibition, the inactivator first binds to and is then catalytically activated by a target enzyme to a reactive intermediate that covalently binds to heme and/or protein in the enzyme active site, resulting in an irreversible loss of enzymatic activity. Thus, this specific type of inhibitor is also called a "mechanism-based inactivator". The mechanism-based inactivators can completely inactivate

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drug-metabolizing enzyme and cause serious adverse effects, which persist even after withdrawal of the inhibitors. Of course, the gene encoding the inactivated enzyme will produce the new enzyme, but this process must take several days or, at least, several hours to recover the enzyme activity to the sufficient level (*6*). During the process, lack of the drug-metabolizing enzyme increases the potential toxicity of the used drug.

Potential drug−herb interactions through inhibition of CYP also have a great amount of attention. A number of in vitro and/or in vivo studies showed that combinations of drug and herbal medicine can cause an interaction through inhibition of CYPs. The most widely studied in a natural product is grapefruit juice, which has been found to increase the bioavailability and/or to prolong the metabolic elimination of many drugs (*7*). Furanocoumarins isolated from grapefruit juice showed the mechanism-based inactivation on CYP3A4 (*8*). Methylenedioxyphenyl lignans from *Piper cubeba* (*9*) and kaempferol derivatives from *Zingiber aromaticum* (*10*) have also been reported as mechanism-based inhibitors of CYP3A4 as have been serpentine from *Catharanthus roseus* (*11*) and alkamides from *Piper nigrum* on CYP2D6 (*12*).

In the previous investigation on the mechanism-based inhibition of 30 Indonesian medicinal plants, the methanol extract of *Foeniculum vulgare* inhibited CYP3A4 with a mechanism-based mode (*13*). *F. vulgare* (Umbelliferae), commonly known as fennel, is a small genus of annual, biennial, or perennial herbs distributed in central Europe and the Mediterranean region. In Indonesia, fennel has been used as culinary spice and traditional medicine for the treatment of digestive ailments, hypertension, dyspnea, and diaphoresis (*14*). However, no work has been reported on its CYP3A4 inhibitory constituents. In our continued interest, thus, we have examined the constituents of fennel and their inhibition on CYP3A4, which we report in this paper.

MATERIALS AND METHODS

Chemicals. [*O-methyl*-^{14}C]Erythromycin (55 mCi/mmol, radiochemical purity > 99%) was purchased from American Radiolabeled Chemicals, Inc. (St. Louis, MO). β-Nicotinamide adenine dinucleotide phosphate ($NADP^+$, oxidized form), glucose-6-phosphate (G-6-P), and G-6-P dehydrogenase were purchased from Oriental Yeast Co., Ltd. (Tokyo, Japan). *N*-Acetyl-L-cysteine and glutathione reduced (GSH) were purchased from Wako Pure Chemical Industries, Ltd. (Osaka, Japan). All other chemicals and solvents were of the highest grade available. Human liver microsomes (HLM) were obtained from XenoTech, LCC (Lenexa, KS) and stored at –80 °C prior to use.

Plant Material. Seeds of fennel (*Foeniculum vulgare*) were obtained at GORO traditional market, Jakarta, Indonesia, in May 2002 and identified by Drs. Sucipto (PJ. Bintang Terang Lestari, Traditional Medicine Supplier, Jakarta, Indonesia). A voucher sample (TMPW22274) is preserved in the Museum for Materia Medica, Analytical Research Center for Ethnomedicines, Institute of Natural Medicine, University of Toyama, Toyama, Japan.

Extraction and Isolation of Compounds. The air-dried seeds of fennel (1 kg) were crushed and extracted with MeOH. Evaporation of MeOH under reduced pressure followed by lyophilization gave a MeOH extract (124 g). The MeOH extract (20.1 g) was subjected to silica gel column chromatography with an n-hexane/EtOAc solvent system to yield eight fractions. Further separation by repeated column chromatography with $CHCl_3$/MeOH or n-hexane/EtOAc solvent system and normal-or reverse-phase preparative TCL with n-hexane/EtOAc or CH_3CN/MeOH/H_2O gave 13 compounds (**Figure 1**).

CYP3A4 Inhibition Assay. Inhibitory activity on the metabolism mediated by CYP3A4 was determined using a radiometric measurement of [^{14}C]formaldehyde formed by the reaction with [*N-methyl*-^{14}C]erythromycin as a substrate (*15*). Briefly, in disposable culture tubes (13 × 100 mm; Iwaki, Tokyo, Japan) containing phosphate buffer (pH 7.4), 50 μL of HLM (4 mg/mL), sample, and purified water were added in

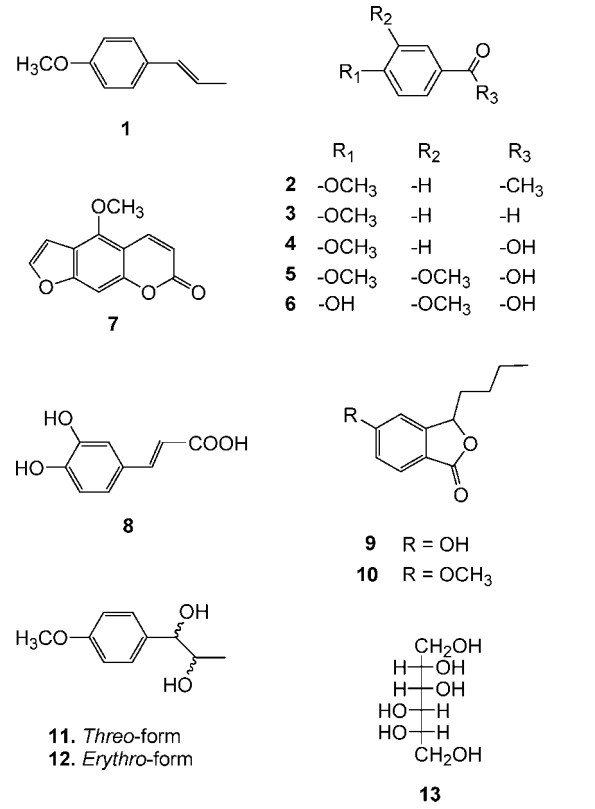

Figure 1. Structures of the compounds isolated from seeds of *Foeniculum vulgare*.

500 μL of total incubation volume. After a preincubation of 5 min in a shaking water bath at 37 °C, the reaction was initiated by the addition of 50 μL of NADPH-generating system (4.20 mg/mL of $NADP^+$ in a solution of 100 mM G-6-P, 100 mM $MgCl_2$, and 10 units/mL G-6-P dehydrogenase). Fifty microliters of substrate solution was added and incubated for 10 min in a shaking water bath at 37 °C. The reaction was stopped by the addition of 125 μL of 10% trichloroacetic acid (Nacalai Tesque, Inc., Kyoto, Japan), and the solution was centrifuged at 3000 rpm for 10 min at room temperature. The supernatant was applied to Envi-carb solid phase extraction columns (Supelco, Bellefonte, PA) and eluted with 2 volumes of ultrapure water (2 × 500 mL). After the addition of 10 mL of Clear-sol I (Nacalai Tesque, Inc.), the eluted radioactivity was quantified by liquid scintillation counting LS 6500 (Beckman, Fullerton, CA). Troleandomycin (Toronto Research Chemicals Inc., Toronto, Canada), ketoconazole, and MeOH were used as a positive control, negative control, and vehicle control, respectively. Correction was made for radioactivity eluted from control incubation in which HLM and NADPH-generating systems had been omitted. The assays were performed in duplicate for all test specimens, and remaining activity was analyzed using software product WinNonlin ver.3.1 (Pharsight Corp., Mountain View, CA).

Time-Dependent Inhibition Assay. Effect of preincubation time on inhibition was determined by preincubating sample with 4 mg/mL HLM in phosphate buffer (0.1 M, pH 7.4) for 0 or 20 min at 37 °C in the presence of NADPH. Substrate solution (50 μL) was added, and incubation was continued for 10 min. Addition of 125 μL of trichloroacetic acid to stop the reaction and activity was assayed as described above.

Effect of NADPH, Nucleophiles, and Competitive Inhibitor on Inhibition of CYP3A4. The effect of NADPH on CYP3A4 inactivation was determined by preincubating samples with 4 mg/mL HLM in phosphate buffer (0.1 M, pH 7.4) for 0, 5, 10, and 20 min at 37 °C in the presence and absence of NADPH. Fifty microliters of substrate solution and NADPH were added to 500 μL of the final solution, and incubation was continued for 10 min. The reaction was stopped by adding 125 μL of trichloroacetic acid, and the activity was assayed (as described above). The effects of nucleophiles were determined by

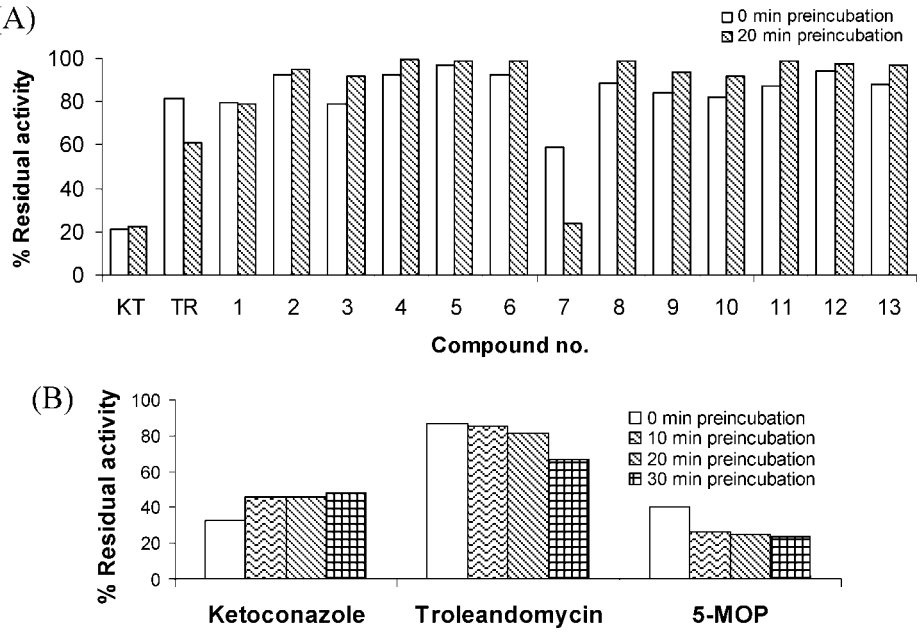

Figure 2. (**A**)Time-dependent inhibitory activity of the isolated compounds with preincubation times of 0 and 20 min. (**B**) Further investigation of inhibition by 5-MOP with preincubation times of 0, 10, 20, and 30 min at 37 °C as described under Materials and Methods (KT, ketoconazole, 0.5 μM; TR, troleandomycin, 10 μM). Each column represents the means of duplicate experiments.

preincubating HLM in phosphate buffer, inhibitor, and 5 mM nucleophile (GSH or *N*-acetylcysteine) in the presence of NADPH with the preincubation time of 0 and 20 min. The effect of the competitive inhibitor was determined in the same way as on nucloephiles using 0.5 μM ketoconazole with preincubation times of 0, 5, 10, and 20 min. Values are presented as a percentage of the activity observed at 0 min, which was arbitrarily set as 100%. To determine the type of inhibition, the compound (12.5, 25, 50, and 100 μM) was added to the reaction mixture with different concentrations of the substrate (25, 50, 75, and 100 μM). Lineweaver–Burk plots were used to determine the inhibitory type. All incubations were performed in duplicate, and the activity was assayed as described above.

Calculation of Kinetic Constants. The slopes obtained from linear regression of log percentage remaining activity versus time plots at each concentration were determined. The first-order inactivation constant (k_{app}) at each inactivator concentration was obtained by multiplying the slope obtained from the linear regression analysis by 2.303. The inactivation rate constant at infinite concentration of inhibitor (k_{inact}) and the inhibitor concentration required for half-maximal rate for inactivation (K_I) were calculated from the double-reciprocal plots of k_{app} versus sample concentration by linear regression analysis. The *y*-intercept is known to be equal to $1/k_{inact}$, whereas the *x*-intercept equals $-1/K_I$.

RESULTS AND DISCUSSION

Fennel (*F. vulgare*) is a popular spice and is also used as a traditional medicine in Asia (*22*). In a previous investigation, we observed the MeOH extract of fennel to inhibit CYP3A4 with characteristics of a mechanism-based inactivator (*13*). To determine the constituents responsible for the mechanism-based inactivation, we have isolated 13 compounds (**Figure 1**) from the methanolic extract of fennel and tested their CYP3A4 inhibitory activity. The isolated compounds were *trans*-anethole (**1**), methyl 4-methoxybenzoate (**2**), 4-methoxybenzaldehyde (**3**), 4-methoxybenzoic acid (**4**), 3,4-dimethoxybenzoic acid (**5**), 4-hydroxy-3-methoxybenzoic acid (**6**), 5-methoxypsoralen (**7**), caffeic acid (**8**), 3-butyl-5-hydroxyphthalate (**9**), 3-butyl-5-methoxyphthalate (**10**), *threo*-anethole diol (**11**), *erythro*-anethole diol (**12**), and D-mannitol (**13**) (**Figure 1**). The purity of each compound was checked by TLC and NMR spectrum, which did not show the presence of any impurity. The structures of the isolated compounds were confirmed by comparison of spectral data with those in the literature (*16*−*19*).

The activity of CYP3A4 was determined on the basis of erythromycin N-demethylation activity in human liver microsomes. Of the isolated compounds, 5-methoxypsoralen (5-MOP) was the strongest inhibitor with an IC_{50} value of 18.3 μM (3.9 μg/mL), whereas other constituents inhibited CYP3A4 with IC_{50} values of >100 μM. There are two possible types of inhibition by these compounds; direct inhibition or metabolism-dependent inhibition. Direct inhibition occurs when a drug inhibits CYP enzyme without requiring biotransformation (metabolism-independent) and metabolism-dependent inhibition occurs when a drug is converted to a metabolite to inhibit CYP enzyme. In the latter case inhibition may be reversible, quasi-irreversible, or irreversible (mechanism-based) (*20*). *trans*-Anethole, the major constituent isolated from this plant, was reported to be metabolized in rat and mouse (*21*). In our investigation, we observed weak inhibitory activity on erythromycin N-demethylation activity mediated by CYP3A4 in human liver microsomes. Interestingly, the metabolites of *trans*-anethole, such as benzoic acid derivatives (**2**–**4**) and anethole diols (**11**, **12**) were also isolated from the MeOH extract of this plant. Weak inhibitory activity of CYP3A4 by these compounds (IC_{50} > 100 μM) indicated that these compounds are not the active metabolite of *trans*-anethole (metabolism-independent inhibition). However, less effect dependent on preincubation time by *trans*-anethole should suggest its direct inhibition (**Figure 2A**).

With the mechanism-based inactivation, time is required for metabolism and activation of the inhibitor, which is indicated by time-dependent inhibition (*6*). Thus, the time dependency of the inhibition by the isolated compounds was examined with preincubation times of 0 and 20 min. Troleandomycin, a known mechanism-based inactivator (*22*), showed a decrease of the residual CYP3A4 activity as the preincubation time was prolonged, whereas ketoconazole, a reversible inactivator (*20*), showed an increase of the residual CYP3A4 activity (**Figure 2A**). Of the isolated compounds, only 5-MOP showed a 40% decrease of the residual CYP3A4 activity (preincubation of 0

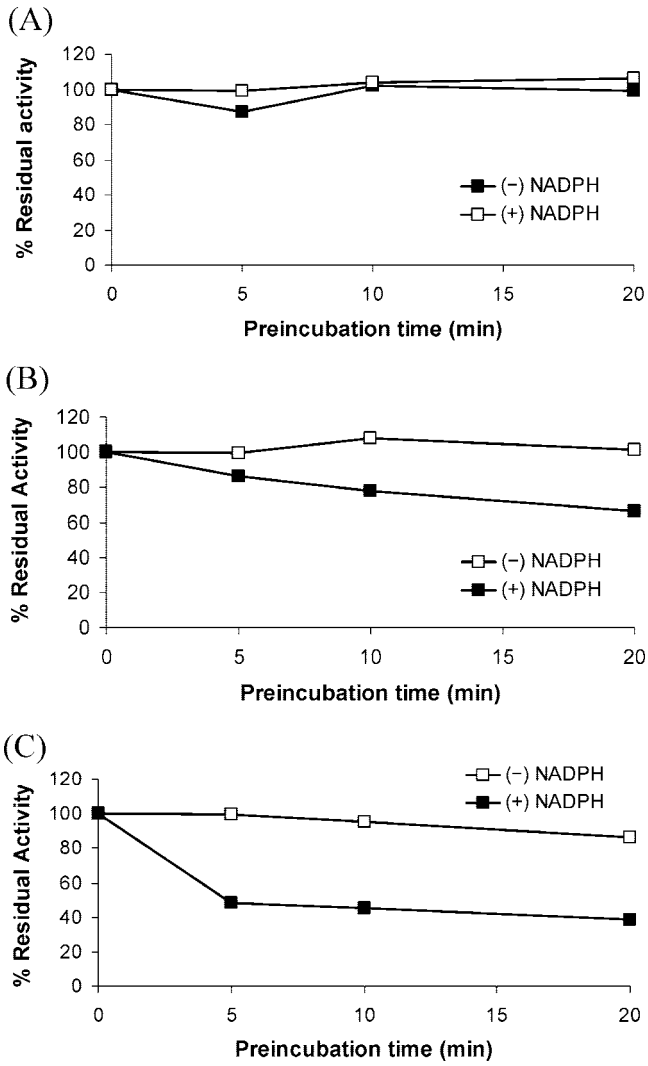

Figure 3. Inhibition of erythromycin N-demethylation activity in human liver microsomes by ketoconazole (**A**), troleandomycin (**B**), and 5-MOP (**C**) in the absence or presence of a NADPH-generating system at preincubation times of 0, 5, 10, and 20 min. Each symbol represents the means of duplicate experiments.

min, 58.9%; 20 min, 23.8%) as the prolongation of the preincubation time. Furthermore, the preincubation time dependency was confirmed with the preincubation times of 0, 10, 20, and 30 min (**Figure 2B**). The IC_{50} value was 4-fold decreased by increasing the preincubation time from 0 to 20 min.

NADPH is required in the mechanism-based inactivation to activate the inhibitor. In the reconstructed experiment, thus, the residual activity of CYP3A4 was decreased by increasing the preincubation time in the presence of NADPH, whereas almost no effect was observed in the absence of NADPH (**Figure 3**). The mechanism-based inactivator is not released from the active site of the enzyme after metabolite activation (*6*). This characteristic inhibition is not observed in other types of metabolite inhibitor. The experimental method can be constructed by addition of a nucleophile on inhibition of the mechanism-based inactivator. Failure of the protection by the nucleophile indicates that the metabolite is not released from the binding site of the enzyme. Lack of the protecting effect of glutathione and *N*-acetylcysteine on inhibition of CYP3A4 by 5-MOP should indicate that the metabolite cannot be released from the active site of the enzyme. This was similar to characteristic inhibition of the positive control, troleandomycin (**Figure 4**). The presence

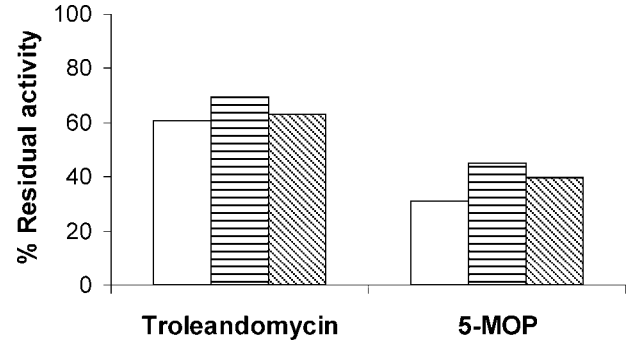

Figure 4. Effect of the presences of water (white bars), glutathione (striped bars), or *N*-acetylcysteine (slashed bars) on inhibition of CYP3A4 by troleandomycin and 5-MOP. Percent residual activity was obtained from the percentage of CYP3A4 activity after 20 min of preincubation, which was normalized by the activity observed at 0 min.

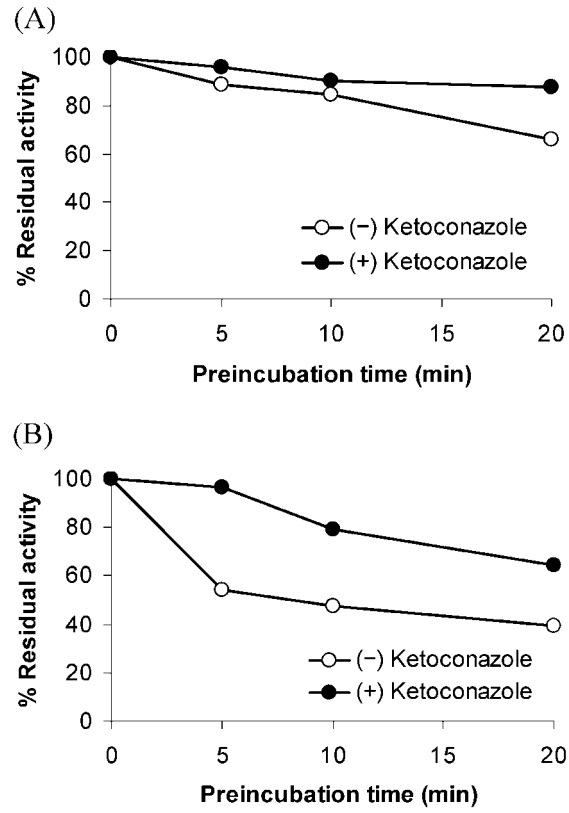

Figure 5. Effect of competitive inhibitor 0.5 μM ketoconazole on inhibition of CYP3A4 by 10 μM troleandomycin (**A**) and 25 μM 5-MOP (**B**). CYP activity was normalized by the activity observed at 0 min, which was arbitrarily set as 100%. The data points refer to duplicate experiments.

of the competitive inhibitor in the enzyme inactivation by a mechanism-based inactivator decreases the rate of inactivation. Preincubation of 5-MOP with ketoconazole, a known competitive inhibitor of CYP3A4 (*23*), showed a decrease of the inactivation rate (**Figure 5**). This result should indicate competition of 5-MOP with the competitive inhibitor, ketoconazole, at the binding site of CYP3A4 to decrease the inactivation rate of 5-MOP. The Lineweaver–Burk plot of 5-MOP on the inhibition of erythromycin N-demethylation activity in human liver microsome showed a mixed type of inhibition (**Figure 6**).

Furanocoumarins isolated from grapefruit juice were reported for their mechanism-based inactivation of CYP3A4 (*24*, *25*). The double bond in the furan ring rather than in the coumarin ring was reported to be the important part for bioactivation to form a reactive intermediate. 4-Ipomeanol [1-(3-furyl)-4-hy-

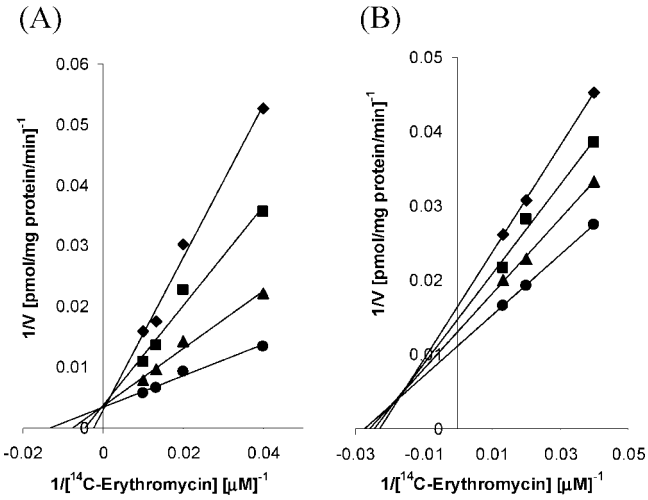

Figure 6. Lineweaver–Burk plots of ketoconazole (0.125, 0.25, 0.5, or 1 μM) and 5-MOP (12.5, 25, 50, or 100 μM) on erythromycin N-demethylation activity in human liver microsomes when the concentration of the substrate was 25, 50, 75, or 100 μM. Each point represents the means of duplicate experiments.

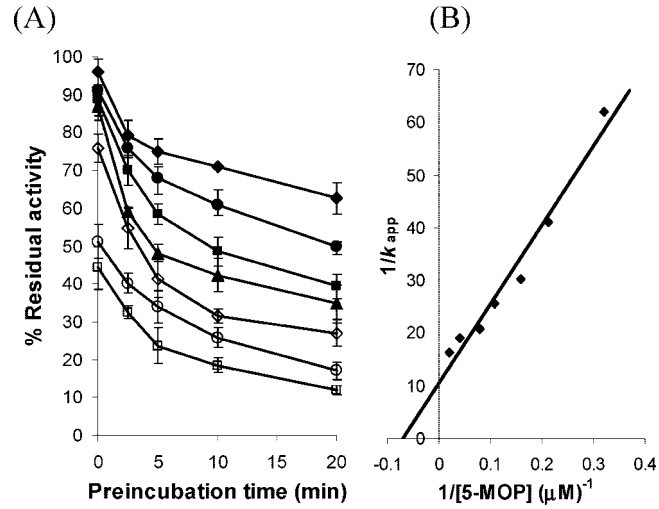

Figure 7. Kinetic analysis on inhibition of CYP3A4 by 5-MOP: (**A**) percentage of the residual activity versus preincubation time (0, 2.5, 5, 10, and 20 min) at a concentration of 3.1 μM (◆), 4.5 μM (●), 6.3 μM (■), 10.2 μM (▲), 12.5 μM (◇), 25 μM (○), and 50 μM (□); (**B**) reciprocal of the k_{app} value obtained from the slope of the line plotted against the reciprocal of 5-MOP concentrations as described under Materials and Methods. The line was produced by linear regression analysis of each point. Each value represents the mean ± SD of three determinations.

droxypentanone] isolated from *Ipomea batatas* having been infected with *Fusarium solani* was also reported as a mechanism-based inactivator of CYP3A4 (*26*). The furan ring is assumed to be oxidized or epoxidized to produce an active metabolite causing the inactivation. 8-Methoxypsoralen and other furanocoumarins were reported for their inhibition of CYP2A6, CYP2B1, and CYPs in mouse liver microsomes (*25*, *27*). In these papers, the furan ring was reported as an important part for bioactivation to form a reactive intermediate, and their irreversible inactivation is caused by modification of the apoprotein. Thus, the furan ring of 5-MOP might be an important part on its inhibition of erythromycin N-demethylation activity mediated by CYP3A4 in human liver microsomes.

The inhibition of CYP3A4 by 5-MOP was time-, concentration-, and NADPH-dependent, and the inactivation was unaffected by the presence of nucleophiles and competitive inhibitor. From these results, 5-MOP was concluded to be a mechanism-based inactivator of CYP3A4. The kinetic parameter for the mechanism-based inactivation of 5-MOP showed K_I of 15.0 μM (inactivator concentration required for half-maximal inactivation) and $k_{inact}$was 0.098 min^{-1} (maximal rate constant of inactivation) (**Figure 7**). The efficiency of enzyme inactivation determined from the ratio of k_{inact} to K_I of 5-MOP was 5.8 min^{-1} nM^{-1}. This value was not as high as those reported for GF-I-1 (161.29) and GF-I-4 (384.62), the strong mechanism-based inhibitor reported from grapefruit juice on testosterone 6β-hydroxylation mediated by human liver microsomes (*8*). However, this value is comparable to those reported for erythromycin (5.13), amiodarone (4.48), rutaecarpine (3.59) (the major component of Evodia fruit extract), and bergamottin (2.0) (a constituent of grapefruit juice). Thus, 5-MOP, a constituent isolated from fennel, would be the constituent responsible for the mechanism-based inactivation of inhibition of CYP3A4.

5-MOP, the strongest inhibitor in this extract, showed weaker inhibitory potency with an IC_{50} value of 18.3 μM compared to the potent inhibitor GF-I-4 isolated from grapefruit juice, which showed IC_{50} values of 3 nM on nifedipine oxidation, 13 nM on omeprazole sulfoxidation, and 12 nM on omeprazole 3-hydroxylation. The clinical use of 5-MOP for treatment of psoriasis was reported to reach a maximum plasma concentration of <1.3 μM (*28*), and another study with human volunteers showed that consumption of 300 g of the celery roots led to a plasma concentration below than 10 nM (*29*). These plasma concentrations are less than the IC_{50} value of 5-MOP. Usually in vivo drug−drug interaction would be observed when the in vitro inhibitory potency was <1 μM. However, the possibility of drug interaction was suggested even in the case of inhibitory potency of >10 μM (*30*). On inhibition by 5-MOP, the IC_{50} value was decreased 4-fold by increasing the preincubation time to 20 min (IC_{50} = 4.6 μM). As the mechanism-based inactivator, the inhibitory potency is increased by increasing the preincubation time, resulting in the increase of the potential interactions with concomitant drug metabolized by the same CYPs. Further studies are required to clarify the absorption of 5-MOP and its metabolic stability in the small intestine and liver to estimate the possibilities of drug interaction in clinical situations.

ABBREVIATIONS USED

CYP, cytochrome P450; CYP3A4, cytochrome P450 3A4; 5-MOP, 5-methoxypsoralen; HLM, human liver microsomes; K_I, inhibitor concentration required for a half-maximal inactivation; k_{inact}, maximal rate constant of the inactivation.

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