Sixteen compounds isolated from Zingiber aromaticum and showing concentration-dependent inhibition with IC₅₀ values less than 100 μM, were analyzed for their possibility of time-, concentration-, and NADPH-dependent inhibition of CYP3A4 and four were analyzed for CYP2D6. All seven kaempferol glycosides and two kaempferol derivatives (4, 5, 8—14) appear to be the mechanism-based inhibitors of CYP3A4 enzyme in which the inhibition is irreversible and driven by the catalytic process. The other compounds showed no NADPH-dependent inhibition or reversible inhibition, and thus do not appear to be mechanism-based inhibitors. Kᵢ values for compounds 4, 5, 8—14 were in the range of 2.21—27.01 μM, whereas the kᵢ₅₀ values were 0.23—0.65 min⁻¹. Kaempferol-3-O-(2,3,4-tri-O-acetyl-α-L-rhamnopyranoside) (5) was found to be the most potent CYP3A4 inactivator with Kᵢ and kᵢ₅₀ values of 2.21 μM and 0.45 min⁻¹, respectively.

Key words Indonesian medicinal plant; mechanism-based inhibition; CYP3A4; CYP2D6; Zingiber aromaticum

MATERIALS AND METHODS

Chemicals [N-Methyl-¹⁴C]erythromycin (55 mCi/mmol, >99% pure) and [O-methyl-¹⁴C]dextromethorphan (55 mCi/mmol, >99% pure) were purchased from American Radiolabeled Chemicals, Inc. (St. Louis, MO, U.S.A.). Human liver microsomes (HLM) were obtained from Xenotech, LLC (Kansas, KS, U.S.A.) and stored at −80°C prior to use. 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). All other chemicals and solvents were of the highest grade available.

Preparation of Test Solutions All the samples used in this experiment were isolated and described in the previous studies of our laboratory. 

Mechanism-Based Inhibition of CYP3A4 by Constituents of Zingiber aromaticum

Tepy USIA, a Tadashi WATABE, a Shigetoshi KADOTA, a and Yasuhiro TEZUKA*, a,b

a Department of Natural Products Chemistry, Institute of Natural Medicine, Toyama Medical and Pharmaceutical University; 2630 Sugitani, Toyama 930-0194, Japan; and b 21st Century COE Program.

Received September 30, 2004; accepted November 24, 2004

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* To whom correspondence should be addressed. e-mail: tezuka@ms.toyama-mpu.ac.jp

The human cytochrome P450 (CYP) superfamily contributes to the metabolism of a variety of xenobiotics including therapeutic drugs, carcinogens, steroids and eicosanoids. Recently, several reports have demonstrated that natural compounds and herbal products may cause a pharmacokinetic interaction with western drugs used clinically when they are simultaneously administered. Herbal constituents may be metabolized by CYP to nontoxic metabolites and excreted, but the formation of toxic metabolites is also possible. In some cases, the formation of a reactive intermediate in a metabolism by CYP may lead to the inactivation of the enzyme. CYP substrates, which are metabolized to reactive intermediates and inactivate the enzyme, are classified as mechanism-based inhibitors and are characterized by time-, concentration-, and NADPH-dependent enzyme inactivations.

In our previous studies, we examined the inhibitory activity of some Indonesian medicinal plants against CYP3A4 and CYP2D6 and isolated (2R,3S,5R)-2,3-epoxy-6,9-humuladien-5-ol-8-one (1), (2R,3R,5R)-2,3-epoxy-6,9-humuladien-5-ol-8-one (2), (5R)-2,6,9-humulatrien-5-ol-8-one (3), kaempferol-3-O-(2,3-di-O-acetyl-α-L-rhamnopyranoside) (4), kaempferol-3-O-(2,3,4-tri-O-acetyl-α-L-rhamnopyranoside) (5), zerumbone (6), zerumbone epoxide (7), kaempferol-3-O-(2,4-di-O-acetyl-α-L-rhamnopyranoside) (8), kaempferol-3-O-(3,4-di-O-acetyl-α-L-rhamnopyranoside) (9), kaempferol-3-O-(2-O-acetyl-α-L-rhamnopyranoside) (10), kaempferol-3-O-(3-O-acetyl-α-L-rhamnopyranoside) (11), kaempferol-3-O-(4-O-acetyl-α-L-rhamnopyranoside) (12), kaempferol-3-O-methyl ether (13), kaempferol-3,4′-di-O-methyl ether (14), (5S)-6-gingerol (15), and trans-6-shogaol (16) (Chart 1) from Zingiber aromaticum as inhibitors against the CYP3A4-mediated metabolism. In addition, compounds 5, 9, 13, and 14 also showed inhibitory activity to the metabolism by CYP2D6. Z. aromaticum is one of the popular traditional medicines extensively used in Indonesia, and thus it is important to determine the possibility of mechanism-based inactivation on CYP3A4 and CYP2D6 by these compounds. In this report, we have performed kinetic analyses to investigate the mechanism-based inhibition of CYP3A4 and CYP2D6 by these compounds.
paper.\(^6\) The test solutions were prepared by dissolving each compound in MeOH.

**Time- and Concentration-Dependent Inhibition Assay**

Inhibitory activity on the metabolism mediated by CYP3A4 or CYP2D6 in vitro was determined using a radiometric measurement of \(^{14}\)Cformaldehyde formed by the reaction with [N-methyl-\(^{14}\)C]erythromycin (0.1 \(\mu\)Ci/incubation, 1 mM in 5% of MeOH) or [O-methyl-\(^{14}\)C]dextromethorphan (0.1 \(\mu\)Ci/incubation, 100 \(\mu\)M in 5% of MeOH) as a substrate, respectively.\(^6\) For studies of time- and concentration-dependent inhibitions, various concentrations of sample (0—200 \(\mu\)M) according to the previous concentration-dependent assay and IC\(_{50}\) data,\(^6\) which dissolved in MeOH (final concentration of MeOH was 1%) were added to the incubation mixture containing 150 \(\mu\)l of phosphate buffer (0.1 M, pH 7.4), 197.5 \(\mu\)l of ultrapure water, and 50 \(\mu\)l of HLM (4 mg/ml). After 5 min preincubation under shaking at 37 °C, the reaction was initiated by addition of 50 \(\mu\)l of NADPH-generating system (4.20 mg/ml of NADP\(^+\), 100 \(\mu\)M of G-6-P, 100 \(\mu\)M of MgCl\(_2\), and 10 U/ml of G-6-P dehydrogenase). At certain intervals, 50 \(\mu\)l of substrate was added and incubation was continued for 10 min (CYP3A4) or 20 min (CYP2D6) under the same conditions. The reaction was stopped by the addition of 125 \(\mu\)l of 10% trichloroacetic acid and CYP activity was assayed. An equivalent volume of MeOH was treated similarly as control. The inhibitory effect of samples was assessed from difference between the sample and corresponding control.

**Time- and NADPH-Dependent Inhibition Assay**

Samples of a certain dose were preincubated with phosphate buffer (0.1 M, pH 7.4) and HLM (4 mg/ml) in the presence or absence of the NADPH-generating system (as described above). At various times (0, 5, 10, 20 min), 50 \(\mu\)l of substrate and 50 \(\mu\)l of NADPH-generating system were added and incubation was continued for 10 min (CYP3A4) or 20 min (CYP2D6) under the same conditions. The reaction was stopped by the addition of 125 \(\mu\)l of 10% trichloroacetic acid and CYP activity was assayed. Activities were normalized to the activity at 0 min so that the percent decrease in CYP activity reflected activity loss due only to inactivation and not reversible inhibition.

**Analysis of Results**

The logarithm of the remaining activities were plotted against incubation times and the slopes of these lines were obtained from linear regression analysis. The inactivation constants (\(k\)\(_{\text{app}}\)) were determined by multiplication of the resulting slopes by 2.303. The inactivation rate constant at an infinite concentration of inactivator (\(k\)\(_{\text{inact}}\)) and the concentration of inactivator required for a half-maximal rate of inactivation (\(K\)\(_{i}\)) were determined from double-reciprocal plots of \(k\)\(_{\text{app}}\) values and inactivator concentrations.\(^{10}\) Based on time-, concentration-, and NADPH-dependent inhibition kinetics, all seven kaempferol glycosides and two kaempferol derivatives (4, 5, 8—14) appear to be the mechanism-based inhibitors for CYP3A4. \(K\)\(_{i}\) values for these compounds were in the range of 2.21—27.01 \(\mu\)M, whereas they had \(k\)\(_{\text{inact}}\) values of 0.23—0.65 min\(^{-1}\) (Table 1). Kaempferol-3-O-(2,3,4-tri-O-acetyl-\(\alpha\)-L-rhamnopyranoside) (5), the most potent CYP3A4 inhibitor (IC\(_{50}\) 14.4 \(\mu\)M),\(^9\) was found to be the most potent CYP3A4 inactivator with \(K\)\(_{i}\) and \(k\)\(_{\text{inact}}\) values of 2.21 \(\mu\)M and 0.45 min\(^{-1}\), respectively.

On the humulene-type sesquiterpenes (1—3, 6, 7), only 2 showed time-, concentration- and NADPH-dependent inactivation of CYP3A4. However, in the NADPH-dependent assay of 2 (Fig. 2, Table 1), a significant fraction of CYP3A4 activity (approximately 66%) remained after a 20 min preincubation with 2 (at 50 \(\mu\)M). Since in the previous concentration-dependent inhibition assay only 51% of CYP3A4 activity remained after incubation with 2 in the same dose, most of the inhibition by 2 may be reversible.\(^{11}\) Similarly, in the NADPH-dependent assay, compounds 15 and 16 showed remaining CYP3A4 activity of 70 and 49% after 20 min preincubation, respectively (Table 1), while in the concentration-dependent inhibition assay, they showed 60 and 42% of CYP3A4 activity remaining, respectively, suggesting that most inhibition of 15 and 16 may also be reversible.

For the CYP2D6 inhibition, only kaempferol-3-O-methyl ether (13) and kaempferol-3,4′-di-O-methyl ether (14) showed time-, concentration-, and NADPH-dependent inhibi-
tion. By comparing the remaining activities in the NADPH-dependent assay (13, 71%; 14, 79%) and those in concentration-dependent studies (13, 66%; 14, 76%), however, most of their inhibition seemed to be reversible. These data should suggest that interactions between the CYP3A4 substrates and 2, 15, or 16 and between the CYP2D6 substrates and 13 or 14 likely require the presence of both the substrates and inactivator. Thus, inhibition of these compounds may be of importance only when drugs and these compounds are administered concomitantly.

Previously, mechanism-based inhibition of some plant constituents were reported. For example, Tassaneeyakul et al. reported bergamottin, 6',7'-dihydroxybergamottin (DHB), GF-I-1, and GF-I-4, four furanocoumarins isolated from grapefruit juice were mechanism-based inhibitors of CYP3A4. Resveratrol, a red wine constituent,
and (−)-hydrastine, an alkaloid isolated from *Hydrastis canadensis*, also showed mechanism-based inhibition against CYP3A4.\textsuperscript{12,13} However, this is the first report about mechanism-based inhibition of kaempferol glycosides or kaempferol derivatives.

In conclusion, these results have demonstrated that all kaempferol glycosides with acetyl group in their structures and two kaempferol derivatives isolated from *Z. aromaticum*...
can cause mechanism-based inhibition of CYP3A4.

**Acknowledgements** Parts of this work were supported by a Grant-in-Aid for the 21st Century COE Program from the Ministry of Education, Culture, Sports, Science and Technology, Japan and by the Uehara Memorial Foundation.