Mechanism-Based Inhibition of CYP3A4 by Constituents of *Zingiber aromaticum*

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Sixteen compounds isolated from *Zingiber aromaticum* and showing concentration-dependent inhibition with IC_{50} 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. K_I values for compounds 4, 5, 8—14 were in the range of 2.21—27.01 μM, whereas the k_{inact} 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 K_I and k_{inact} values of 2.21 μM and 0.45 min^{-1}, respectively.

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

The human cytochrome P450 (CYP) superfamily contributes to the metabolism of a variety of xenobiotics including therapeutic drugs, carcinogens, steroids and eicosanoids.[1)] 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.[2—4)] 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.[1)]

In our previous studies, we examined the inhibitory activity of some Indonesian medicinal plants against CYP3A4 and CYP2D6[5)] and isolated (2*R*,3*S*,5*R*)-2,3-epoxy-6,9-humuladien-5-ol-8-one (**1**), (2*R*,3*R*,5*R*)-2,3-epoxy-6,9-humuladien-5-ol-8-one (**2**), (5*R*)-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**), (*S*)-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.[6)] *Z. aromaticum* is one of the popular traditional medicines extensively used in Indonesia,[7,8)] 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.

MATERIALS AND METHODS

Chemicals [*N*-Methyl-^{14}C]erythromycin (55 mCi/mmol, >99% pure) and [*O*-methyl-^{14}C]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 as described in the previous

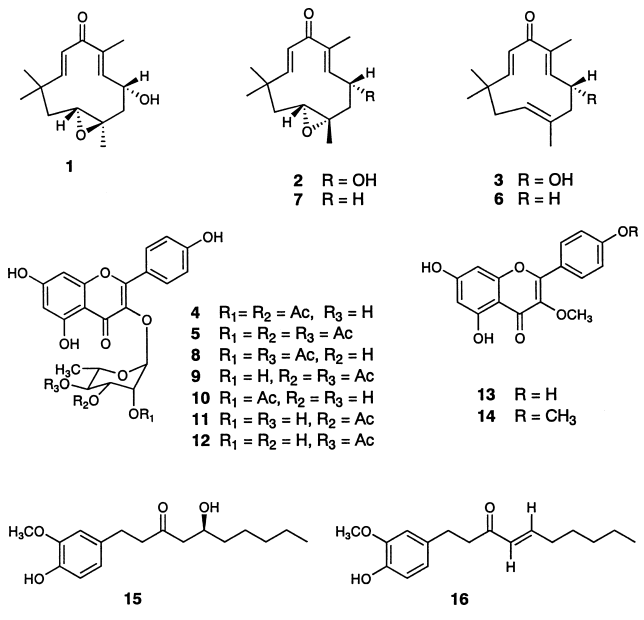

Chart 1. Structures of Compounds

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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}C]formaldehyde formed by the reaction with [*N*-methyl-^{14}C]erythromycin (0.1 μCi/incubation, 1 mM in 5% of MeOH) or [*O*-methyl-^{14}C]dextromethorphan (0.1 μCi/incubation, 100 μM in 5% of MeOH) as a substrate, respectively.[6] For studies of time- and concentration-dependent inhibitions, various concentrations of sample (0—200 μM) 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 μl of phosphate buffer (0.1 M, pH 7.4), 197.5 μl of ultrapure water, and 50 μl of HLM (4 mg/ml). After 5 min preincubation under shaking at 37 °C, the reaction was initiated by addition of 50 μl of NADPH-generating system (4.20 mg/ml of $NADP^+$, 100 mM of G-6-P, 100 mM of $MgCl_2$, and 10 U/ml of G-6-P dehydrogenase). At certain intervals, 50 μl 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 μl 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 μl of substrate and 50 μl 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 μl 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_{app}) were determined by multiplication of the resulting slopes by 2.303. The inactivation rate constant at an infinite concentration of inhibitor (k_{inact}) and the inhibitor concentration required for a half-maximal rate of inactivation (K_I) values were calculated from the double reciprocal plots of k_{app} *versus* sample concentration by linear regression analyses using the software product WinNonlin Ver. 3.1 (Pharsight Corp., Mountain View, CA, U.S.A.). The *y*-intercept is known to equal $1/k_{inact}$ while the *x*-intercept equals $-1/K_I$.

RESULTS AND DISCUSSION

Metabolism by CYP represents the rate-limiting step in the metabolism of a large number of drugs; hence, inhibition of CYP is recognized as an important cause of drug interactions by the United States Food and Drug Administration (FDA) and other regulatory agencies.[9] Inhibitory drug interactions can cause symptoms of drug overdose, including an exaggerated pharmacological response and/or drug toxicity. These interactions generally fall into two categories. The first involves "direct" inhibition of the metabolism of one drug by the other. Direct inhibition may exhibit Michaelis–Menten kinetics characteristic of competitive, noncompetitive, uncompetitive, or mixed (competitive and noncompetitive) inhibition. The second type of drug interaction results from "irreversible" (or "quasi-irreversible") inhibition of CYP and often involves metabolism-dependent inhibition or suicide inactivation of CYP. This type of inhibition can completely block the metabolism of other drugs.[9]

In a previous paper, we reported 16 compounds to be CYP3A4 inhibitory constituents of *Z. aromaticum*, which showed concentration-dependent CYP3A4 inhibition with an IC_{50} value of 102 μg/ml.[5] Thus, we have examined the possibility of their mechanism-based inhibition. All compounds except for zerumbone (**6**) decreased the rate of metabolism mediated by CYP3A4 in both concentration- and time-dependent manners (Fig. 1). Furthermore, in the NADPH-dependent assay of metabolism mediated by CYP3A4, compounds **1**, **3**, and **7** showed only 4.8, 6.3, and 0.9% decrease by incubation with NADPH for 20 min, while other compounds decreased by 26—82% (Fig. 2). Thus, compounds **2**, **4**, **5**, **8**—**16** showed NADPH-dependent inhibition, but others did not.

Pseudo-first-order kinetics was observed on the inactivation of the enzyme activity. The inactivation rate constant at an infinite concentration of inactivator (k_{inact}) and the concentration of inactivator required for a half-maximal rate of inactivation (K_I) were determined from double-reciprocal plots of k_{app} values and inactivator concentrations.[10] Based on time-, concentration-, and NADPH-dependent inactivation 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 μM, whereas they had k_{inact} values of 0.23—0.65 min^{-1} (Table 1). Kaempferol-3-*O*-(2,3,4-tri-*O*-acetyl-α-L-rhamnopyranoside) (**5**), the most potent CYP3A4 inhibitor (IC_{50}, 14.4 μM),[6] was found to be the most potent CYP3A4 inactivator with K_I and k_{inact} values of 2.21 μM 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 μM). 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-

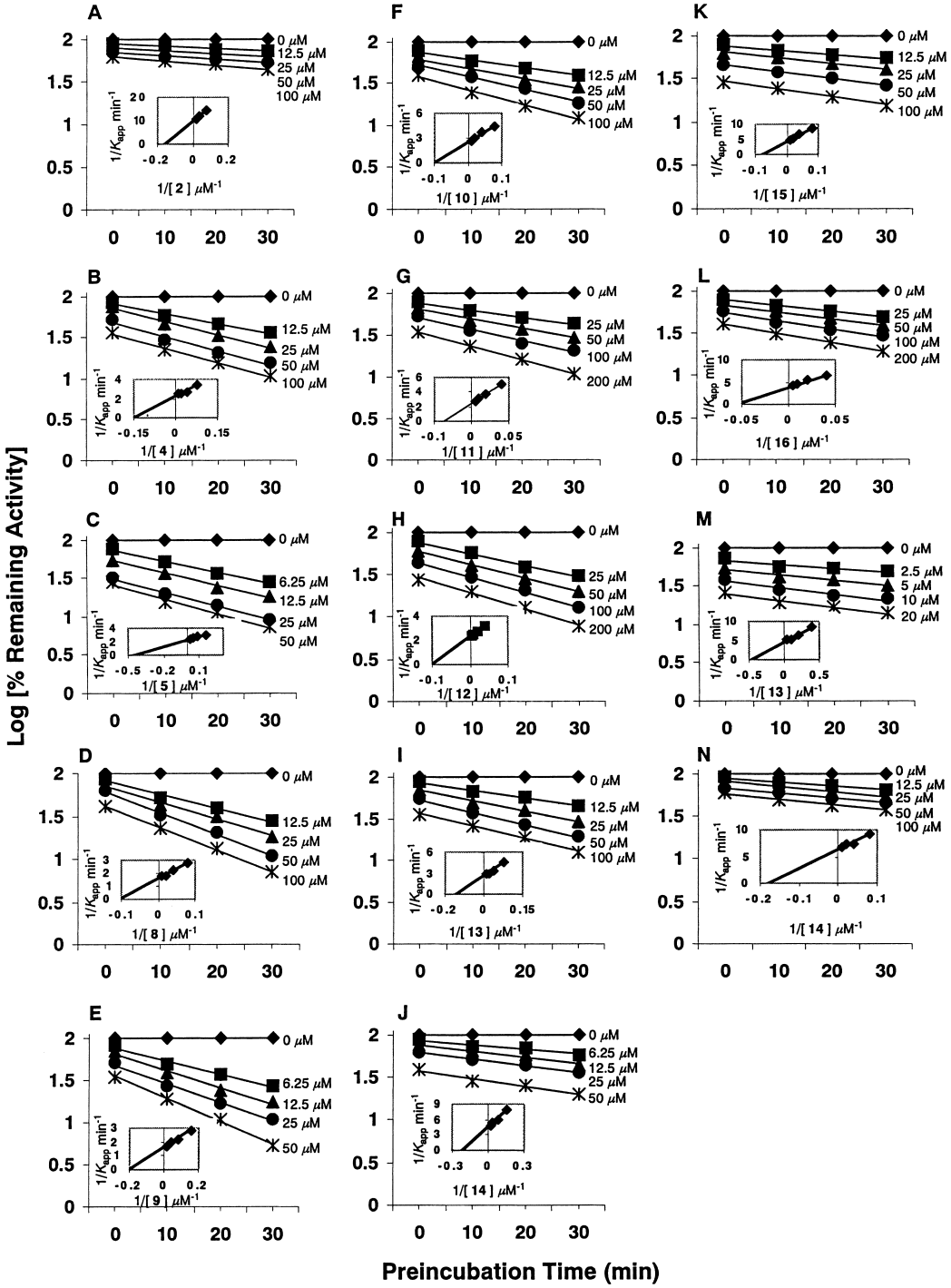

Fig. 1. Time- and Concentration-Dependent Inhibition of CYP3A4 by Compounds **2** (A), **4** (B), **5** (C), **8** (D), **9** (E), **10** (F), **11** (G), **12** (H), **13** (I), **14** (J), **15** (K), and **16** (L); and Time- and Concentration-Dependent Inhibition of CYP2D6 by Compounds **13** (M) and **14** (N). (Insets) Double-Reciprocal Plots of the Relationships between Inactivation Rate Constants (k_{app}) and Compound Concentrations

Each point represents the mean of duplicate determinations. Compound concentrations are shown on the right of the plots. Other experimental details are shown under Materials and Methods.

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.[10,12—15] 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.[10] Resveratrol, a red wine constituent,

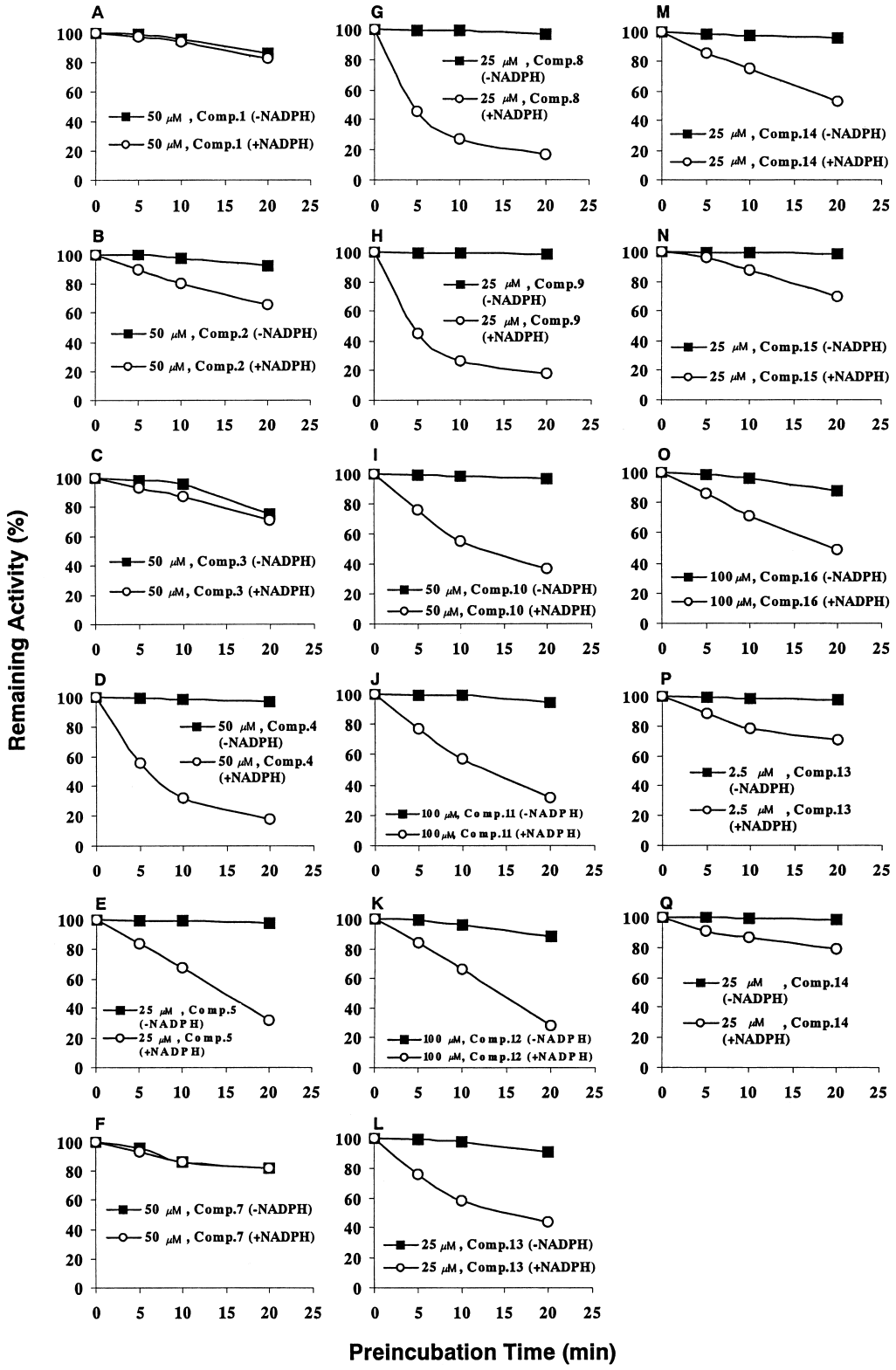

Fig. 2. Time- and NADPH-Dependent Inhibition of CYP3A4 by Compounds **1** (A), **2** (B), **3** (C), 4 (D), **5** (E), **7** (F), **8** (G), **9** (H), **10** (I), **11** (J), **12** (K), **13** (L), **14** (M), **15** (N), and **16** (O); and Time- and NADPH-Dependent Inhibition of CYP2D6 by Compounds **13** (P) and **14** (Q)

Preincubation with CYP3A4 or CYP2D6 was done at 37 °C, +/− NADPH for 0, 5, 10, and 20 min. In order to determine the extent of inactivation, CYP3A4 or CYP2D6 activity was normalized by the activity observed at 0 min, which was arbitrarily set as 100%. Each point represents the mean of duplicate determinations.

and (−)-hydrastine, an alkaloid isolated from *Hydrastis canadensis*, also showed mechanism-based inhibition against CYP3A4.[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*

Table 1. Kinetic Data (k_{inact} and K_I values) and Remaining Activity (%) during NADPH-Dependent Studies of Isolated Compounds from *Z. aromaticum*

Compound	k_{inact} (min^{-1})	K_I (μM)	Remaining activity (%)[a]
On CYP3A4			
2	0.11	6.56	66
4	0.45	6.82	18
5	0.45	2.21	32
8	0.65	9.67	17
9	0.64	5.19	17
10	0.40	9.95	37
11	0.42	27.01	32
12	0.45	10.02	28
13	0.40	9.78	44
14	0.23	4.75	53
15	0.23	12.79	70
16	0.26	17.73	49
On CYP2D6			
13	0.23	2.22	71
14	0.16	5.58	79

a) Remaining activity (%) after 20 min preincubation with NADPH.

can cause mechanism-based inhibition of CYP3A4.

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