

## Studies on Interactions between Functional Foods or Dietary Supplements and Medicines. I. Effects of *Ginkgo biloba* Leaf Extract on the Pharmacokinetics of Diltiazem in Rats

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The effects of *Ginkgo biloba* leaf extract (GBE), one of the most widely used herbal dietary supplements in Japan, on the pharmacokinetics of diltiazem (DTZ), a typical probe of cytochrome P450 (CYP) 3A, were examined in rats. The simultaneous addition of GBE to small intestine and liver microsomes inhibited the formation of *N*-demethyl DTZ (MA), an active metabolite of DTZ produced by CYP3A, in a concentration-dependent manner, with an IC<sub>50</sub> of about 50 and 182 µg/ml, respectively. This inhibition appeared to be caused, at least in part, by a mechanism-based inhibition. Both the rate of formation of MA and total amount of CYP in intestinal or hepatic microsomes after a single oral pretreatment with GBE (20 mg/kg) decreased transiently. The pretreatment significantly decreased the terminal elimination rate constant and increased the mean residence time, after intravenous administration of DTZ (3 mg/kg). Furthermore, it significantly increased the area under the concentration–time curve and absolute bioavailability after oral administration of DTZ (30 mg/kg). These results indicated that the concomitant use of GBE in rats increased the bioavailability of DTZ by inhibiting both intestinal and hepatic metabolism, at least in part, via a mechanism-based inhibition for CYP3A.

**Key words** *Ginkgo biloba* leaf; diltiazem; pharmacokinetic interaction; P450; metabolism; rat

Recently, the demand for functional foods or dietary supplements including herbal remedies, vitamins, minerals, *etc.*, has been steadily increasing with the diffusion of self-medication, as well as alternative and supplemental medicines throughout the world. In such situations, these foods or supplements have frequently been taken with various ethical drugs prescribed for the treatment of many diseases.<sup>1)</sup> In fact, grapefruit juice<sup>2)</sup> and St. John's wort<sup>3)</sup> have been reported to strongly inhibit and promote, respectively, the activity of cytochrome P450 (CYP) 3A4, the most important isoform related to the oxidative biotransformation of numerous medicines because it is the major or an abundant enzyme in critical tissues such as the small intestine and liver,<sup>4,5)</sup> and subsequently to affect the pharmacokinetics of drugs.<sup>6,7)</sup> However, there is far less information about the pharmacokinetic interactions between functional foods or supplements and medicines than that between pairs of drugs.<sup>3)</sup> Accordingly, to prevent adverse reactions dependent on potential interactions or to utilize available interactions clinically, detailed investigations of the interactions between functional foods and medicines are urgently required.

Standardized *Ginkgo biloba* leaf extract (GBE) has been widely used as a botanical dietary supplement in Japan as well as the United State or a phytomedicine in many countries in Europe in recent years, while the seeds are commonly employed in traditional Chinese herbal medicine.<sup>8,9)</sup> The primary active components of GBE include flavonoid glycosides and various unique diterpenes such as ginkgolides which are potent inhibitors for platelet activating factor.<sup>8)</sup> Clinical studies have indicated that GBE exhibits therapeutic effects in a variety of disorders including Alzheimer's dis-

ease, failing memory, age-related dementias, *etc.* Side effects of GBE are very rare and consist of mild gastric upset, headache and allergic skin reactions.<sup>8)</sup> On the other hand, it was reported as clinical cases that GBE given with aspirin, warfarin or trazodone was associated with spontaneous hyphema, intracerebral haemorrhage and acute depression, respectively.<sup>10,11)</sup> Budzinsky *et al.*<sup>12)</sup> and Umegaki *et al.*<sup>13)</sup> reported that commercial GBE significantly inhibited human CYP3A4 and rat CYP3A activities *in vitro*, respectively, whereas the feeding of GBE for 4 weeks markedly induced CYP3A1 and CYP3A2 mRNA expression, weakening the hypotensive effect of nicardipine in rats.<sup>14)</sup> However, the possibility of pharmacokinetic interactions between GBE and drugs metabolized by CYP3A in animals or humans remained unclear.

Therefore, in this study, the inhibitory effects and mechanism of action of GBE against CYP3A activity estimated from the formation of *N*-demethyl diltiazem (MA), an active metabolite,<sup>15)</sup> from diltiazem (DTZ), a typical substrate for CYP3A<sup>16–19)</sup> and flow-limited drug in both rats and humans,<sup>20)</sup> were examined using rat small intestine and liver microsomes. We also examined the effects of single oral pretreatment with GBE on the activity and the total amount of CYP in both microsomes and the pharmacokinetics of DTZ and MA after intravenous and oral administrations of DTZ to rats.

### MATERIALS AND METHODS

**Chemicals** GBE (Ginkgolon-24; Lot No. 270009030), ginkgolide A, ginkgolide B, ginkgolide C and bilobalide

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powders were kindly provided by Tokiwa Phytochemical Co., Ltd. (Chiba, Japan). GBE was produced in a standard fashion by extraction from the milled leaves with ethanol. The final quality of this extract was assured by maintaining the prescribed range of index components (over 24% flavonoid glycosides and 6% terpene lactones and less than 1 ppm ginkgolic acids), and the yield was about 2%. The GBE contained 1.80% ginkgolide A, 0.85% ginkgolide B, 1.09% ginkgolide C and 2.32% bilobalide. The DTZ hydrochloride and imipramine hydrochloride (an internal standard for HPLC analysis) used were purchased from Wako Pure Chemical Ind., Ltd. (Osaka, Japan). The MA hydrochloride was synthesized from DTZ hydrochloride at the Department of Functional Molecular Chemistry in our university. Ketoconazole, quercetin and kaempferol were purchased from Sigma Chemical Co. (St. Louis, MO, U.S.A.). NADP, glucose-6-phosphate and glucose-6-phosphate-dehydrogenase were purchased from Oriental Yeast Co., Ltd. (Tokyo, Japan). All other chemicals were reagent- or HPLC-grade commercial products.

**Animals** Nine-week-old male Wistar rats (Japan SLC, Inc., Hamamatsu, Japan), weighing 220–280 g, were used throughout this study. All animal experimental protocols described below were approved by the Experimental Animal Research Committee at Kyoto Pharmaceutical University.

**Preparation of Small Intestine and Liver Microsomes** Small intestine and liver microsomes were prepared using normal rats or rats treated with a GBE solution. Briefly, the GBE (20 mg/kg) was administered orally, and then the small intestine and liver were excised 1, 6, 12 and 24 h after the treatment. Small intestine and liver microsomes were prepared by a slight modification of the EDTA-separation of enterocytes and ultracentrifugation procedures reported by Mohri and Uesawa<sup>21</sup>) and the conventional method using differential centrifugation by Omura and Sato,<sup>22</sup>) respectively. Protein concentrations in microsomes were determined by the method of Lowry *et al.*<sup>23</sup>) with bovine serum albumin as the standard.

**Enzyme Activity Assay** The formation rate of MA from DTZ was measured in a similar manner to that reported by Murray and Butler.<sup>16</sup>) Briefly, the reaction mixture contained 30  $\mu$ l of 5 or 10 mg protein/ml microsomes (final concentration; 0.5 or 1.0 mg protein/ml), 10  $\mu$ l of 18 mM NADP (0.6 mM), 10  $\mu$ l of 180 mM glucose-6-phosphate (6 mM), 10  $\mu$ l of 60 units/ml glucose-6-phosphate dehydrogenase (2 units/ml), 150  $\mu$ l of 200 mM phosphate buffer (100 mM, pH 7.4) containing 2 mM EDTA (1 mM) and 30  $\mu$ l of distilled water. In the conventional experiment, the reaction was started by addition of 30  $\mu$ l of 250  $\mu$ M DTZ (25  $\mu$ M) with 30  $\mu$ l of distilled water or with various concentrations of GBE solution after preincubation at 37 °C for 1 min (for 10 min in the examination on the inhibitory mechanism of action). The total volumes of the incubation mixtures were adjusted to 300  $\mu$ l. Tubes including the whole mixtures were incubated in a shaking water bath (100 strokes/min) at 37 °C for 3 or 5 min. The relationship between MA formed and incubation time was linear up to 10 min. The reaction was terminated by adding 500  $\mu$ l of ice-cold ethyl acetate and cooling to 0 °C. The preincubation of small intestine or liver microsomes with an NADPH-generating system alone at 37 °C for at least 10 min did not affect the rate of formation of MA in the sub-

sequent incubation.

**Preincubation Experiments of Liver Microsomes with GBE** The incubation mixture containing liver microsomes (150  $\mu$ g) were preincubated with GBE (200  $\mu$ g/ml), an NADPH-generating system or both GBE and an NADPH-generating system in a shaking water bath (100 strokes/min) at 37 °C for 10 min. Then, subsequent incubations were performed in the incubation mixture containing preincubated microsomes, an NADPH-generating system and DTZ (25  $\mu$ M) without or with GBE at 37 °C for 3 min, under various conditions as shown in Fig. 3. The final volumes of the incubation mixtures were adjusted to 300  $\mu$ l. The reaction was terminated as described above.

**CYP and Cytochrome b<sub>5</sub> Assays** The amounts of CYP or cytochrome b<sub>5</sub> in intestinal and hepatic microsomes were estimated by the conventional method of Omura and Sato.<sup>22</sup>)

**Pharmacokinetic Experiments** The left carotid artery of each rat was cannulated with polyethylene tubing (PE-50; Clay Adams, Dickinson & Co., Parsippany, NJ, U.S.A.) under anesthesia the day before the pharmacokinetic experiment. The animals were fasted but allowed free access to water for 18 h before the administration of drugs. A 2% (w/v) arabic gum solution (vehicle) or GBE suspended in the vehicle (20 mg/kg) was administered orally *via* gastric intubation to unanesthetized rats, and then DTZ dissolved in water was administered at bolus doses of 3 or 30 mg/kg intravenously or orally, respectively, 1 h after the pretreatment. Considering the difference in metabolic clearance between rats and humans,<sup>20</sup>) the oral doses of GBE and DTZ in this study were designed to be about 10-fold of those per day to humans. Blood samples (0.25 ml) were collected through the cannula in heparinized plastic microcentrifuge tubes (1.5 ml) before and at 0.05, 0.083, 0.167, 0.333, 0.5, 0.75, 1, 1.5 and 2 h (*i.v.*) or 0.083, 0.167, 0.25, 0.5, 1, 1.5 and 2 h (*p.o.*) after drug administration. The samples were centrifuged at 13000 rpm for 3 min at room temperature in a Centrifuge 5415C (Eppendorf GmbH, Germany), and the plasma fraction was frozen at –80 °C until the assay. The assays were performed within 1 week of collection.

**Assays of DTZ and MA** The assay of DTZ and MA was conducted by means of the HPLC-UV method reported by Murray and Butler<sup>16</sup>) with a slight modification as follows. The sample (800  $\mu$ l) in which the reaction was stopped with ethyl acetate as previously indicated in the enzyme activity assay or the plasma (100  $\mu$ l) pretreated with 500  $\mu$ l of ethyl acetate immediately before the assay was placed in a glass tube (10 ml), and then 200  $\mu$ l or 100  $\mu$ l of imipramine solution (internal standard; 15 or 100  $\mu$ M for microsomes or plasma, respectively), 200  $\mu$ l of distilled water, 1 g of solid NaHCO<sub>3</sub> (for microsomes) or 200  $\mu$ l of a 7.5 w/v% NaHCO<sub>3</sub>-distilled water solution (for plasma), and ethyl acetate (5 ml) were added. After vortex mixing for 30 s, the mixture was centrifuged at 3000 rpm for 15 min. Then, the organic phase (4.5 ml) was also extracted with 200  $\mu$ l of 10 mM HCl, and the water phase (150  $\mu$ l) was transferred to another tube and evaporated to dryness at 40 °C under a stream of nitrogen. The residue was completely reconstituted with 100  $\mu$ l of 2 mM HCl, and then 20 or 40  $\mu$ l of the sample was injected into an HPLC apparatus (LC-6A; Shimadzu, Kyoto, Japan) equipped with a column oven (CTO-6A; Shimadzu) and an ultraviolet detector (SPD-6A; Shimadzu). The

conditions for analysis were as follows: column size, 25 cm×4.0 mm i.d.; packing, STR ODS-II (Shinwa Chemical Industries, Ltd., Kyoto, Japan); mobile phase, 50 mM KH<sub>2</sub>PO<sub>4</sub>-acetonitrile-triethylamine (70:28.8:0.2, pH 5.9); column temperature, 40 °C; flow rate, 1.0 ml/min; wavelength, 238 nm; and sensitivity, 0.00125 a.u.f.s. The retention times for DTZ, MA and imipramine were *ca.* 16, 11 and 21 min, respectively. The calibration curves for DTZ (0.05–4.0 µg/ml) and MA (0.2–5.0 µM or 0.05–3.0 µg/ml) showed good linearity ( $R^2 > 0.999$ ).

**Pharmacokinetic Analysis** The peak plasma concentration ( $C_{\max}$ ) and the time to reach  $C_{\max}$  ( $T_{\max}$ ) of DTZ and MA were determined from the actual data obtained after intravenous or oral administration. The terminal elimination rate constant ( $\lambda$ ) was calculated by fitting individual data for three terminal points of the plasma concentration profile with a log-linear regression equation using the least-squares method. The corresponding elimination half-life ( $t_{1/2(\lambda)}$ ) was calculated by dividing  $\ln 2$  by  $\lambda$ . The areas under the plasma concentration–time curves from zero to infinity ( $AUC_{0-\infty}$ ) for DTZ and MA were calculated by means of the trapezoidal rule with extrapolation to infinity with  $\lambda$ . The mean residence time from zero to infinity ( $MRT$ ) for DTZ and MA was estimated by moment analysis.<sup>24</sup> The absolute bioavailability of DTZ after oral administration ( $F$ ) was estimated as follows:  $(AUC_{p.o.} \times D_{i.v.}) / (AUC_{i.v.} (DTZ \text{ alone}) \times D_{p.o.}) \times 100$ .

**Statistical Analysis** Data are expressed as the mean ± standard error (S.E.). Comparisons between two groups and among more than three groups were performed using the unpaired Student's *t*-test and repeated or non-repeated measures analysis of variance (ANOVA) followed by Fischer's PLSD test, respectively, with StatView J5.0 for Macintosh (Abacus Concepts Inc., Berkeley, CA, U.S.A.), and differences were considered statistically significant when  $p < 0.05$ .

## RESULTS

**Inhibitory Effects of GBE on CYP3A Activity in Microsomes** GBE inhibited the *in vitro* formation of MA from DTZ by small intestine and liver microsomes in a concentration-dependent manner (Fig. 1). The 50% inhibitory concentration ( $IC_{50}$ ) for small intestine was estimated to be approximately  $50 \pm 16$  µg/ml. In the case of liver, the  $IC_{50}$  was about  $182 \pm 13$  µg/ml. These two values were significantly different. Also, ketoconazole (25 µM), a typical inhibitor of CYP3A, strongly inhibited the formation of MA in both microsomes, as well as at 1000 µg/ml of GBE (data not shown). The intact rates of the control in intestine and liver microsomes were 0.125 and 0.918 nmol/min/mg protein, respectively.

**Inhibitory Mechanism of Action against CYP3A Activity by GBE** The inhibitory mechanism of action against DTZ *N*-demethylase when GBE was simultaneously added was examined using hepatic microsomes. As shown in Fig. 2, based on Lineweaver–Burk plots, the mode of inhibition for CYP3A was estimated to be essentially competitive at 100 and 200 µg/ml GBE, concentrations near the  $IC_{50}$ . In contrast, the inhibition observed at 500 µg/ml GBE was determined to be mixed with both competitive and non-competitive modes.

To elucidate whether these inhibitory actions were based

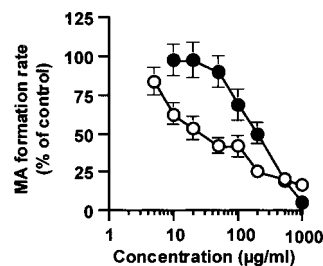


Fig. 1. Inhibitory Effects of GBE on the Formation of MA from DTZ by Rat Small Intestine and Liver Microsomes

Each point represents the mean ± S.E. of 3 or 6 rats. The reactions were performed in an NADPH-generating system containing microsomes (150 µg) and DTZ (25 µM) with or without various concentrations of GBE at 37 °C for 3 min. ○, small intestine; ●, liver.

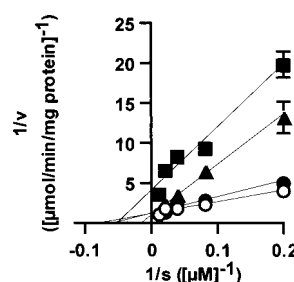


Fig. 2. Lineweaver–Burk Plots Showing the Inhibitory Effects of GBE on the Formation of MA from DTZ by Rat Liver Microsomes

Each point represents the mean ± S.E. of 6 rats. The reactions were performed in an NADPH-generating system containing rat liver microsomes (150 µg) and DTZ (5, 12.5, 25, 50 or 100 µM) with or without three concentrations of GBE at 37 °C for 3 min. ○, control; ●, with 100 µg/ml GBE; ▲, 200 µg/ml GBE; ■, 500 µg/ml GBE.

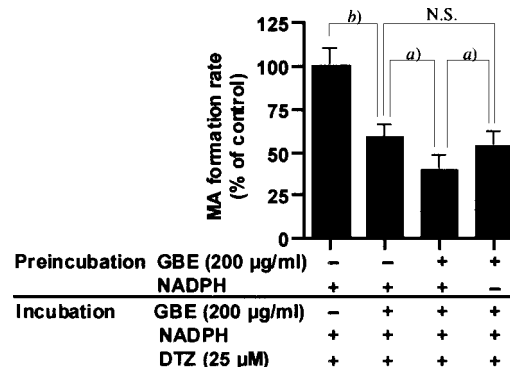


Fig. 3. Effects of the Preincubation under Various Conditions on the Formation of MA from DTZ by Rat Liver Microsomes

Each column represents the mean ± S.E. of 3 rats. *a)*  $p < 0.05$  and *b)*  $p < 0.01$ , respectively (repeated measures ANOVA and Fisher's PLSD test). After preincubation for 10 min, the incubations were performed in an NADPH-generating system containing microsomes (150 µg) and DTZ (25 µM) without or with GBE (200 µg/ml) at 37 °C for 3 min.

on an irreversible and mechanism-based inhibition, we examine the effects of preincubation under various conditions on the formation of MA by liver microsomes. As shown in Fig. 3, the rate of formation of MA by hepatic microsomes preincubated for 10 min in an NADPH-generating system with GBE (200 µg/ml) was significantly less than that without GBE. This significant decrease disappeared when the preincubation was performed with no NADPH-generating system.

**CYP3A Activities and Levels of Cytochromes in Microsomes after Single Oral Pretreatment with GBE** The ef-

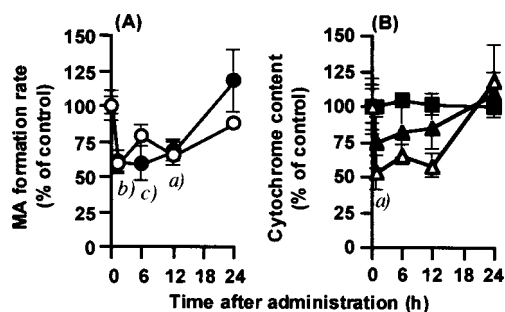


Fig. 4. Effects of Single Oral Pretreatment with GBE on the Formation of MA from DTZ (A) and Cytochrome Content (B) in Rat Small Intestine and Liver Microsomes

Each point represents the mean  $\pm$  S.E. of 3–6 rats. *a*) and *b*)  $p < 0.05$  and  $p < 0.01$  vs. each point at 0 h for small intestine, respectively, and *c*)  $p < 0.05$  vs. the point at 0 h for liver (non-repeated measures ANOVA and Fisher's PLSD test). The GBE suspension (20 mg/kg) was administered orally to unanesthetized rats, and then the small intestine and liver were excised at indicated time points. (A) The reactions were performed in an NADPH-generating system containing small intestine or liver microsomes (300 or 150  $\mu$ g) and DTZ (25  $\mu$ M) at 37  $^{\circ}$ C for 5 or 3 min, respectively.  $\circ$ , small intestine (MA formation);  $\bullet$ , liver (MA formation);  $\triangle$ , small intestine (CYP content);  $\blacktriangle$ , liver (CYP content);  $\blacksquare$ , liver (cytochrome  $b_5$  content).

ffects of a single oral pretreatment with GBE (20 mg/kg) on the formation of MA and the amounts of CYP or cytochrome  $b_5$  in small intestine and liver microsomes were examined (Figs. 4A, B). The MA formation rates in small intestine microsomes significantly decreased to about 60 and 66% at 1 and 12 h, respectively, after the treatment with GBE. Also, in the case of liver microsomes, they showed a significant decrease to approximately 59% at 6 h. However, these decreases in CYP3A activities in small intestine and liver microsomes were transient and disappeared at 24 h, and the respective values at 24 h (% of 0 h) were estimated to be about 89 and 118% (Fig. 4A).

In addition, the total CYP content of small intestine microsomes significantly decreased to 53% 1 h after the treatment and recovered after 24 h. Similar behavior was observed in liver microsomes although there were no significant differences. On the other hand, the cytochrome  $b_5$  content of the liver was not affected at all by the pretreatment with GBE (Fig. 4B). The CYP content of small intestine microsomes at 0 h was  $0.026 \pm 0.005$  nmol/mg protein. In the case of liver microsomes, the levels of CYP and cytochrome  $b_5$  were  $0.256 \pm 0.041$  and  $0.316 \pm 0.036$  nmol/mg protein, respectively, and were almost the same as those reported by Ohnishi *et al.*<sup>25)</sup>

**Effects of Single Oral Pretreatment with GBE on DTZ Pharmacokinetics** The plasma DTZ and MA concentration–time curves after intravenous administration of DTZ (3 mg/kg) with or without single oral GBE pretreatment (20 mg/kg) are shown in Fig. 5, and Table 1 summarizes the pharmacokinetic parameters. Pretreatment 1 h before with GBE significantly decreased the  $\lambda$  value and increased the MRT value for DTZ. The  $C_{\max}$  value for MA in the GBE group was markedly lower than that in the control group ( $p < 0.05$ ). There were no significant differences in other values between the two groups. The  $\lambda$  and  $t_{1/2(\lambda)}$  values for DTZ in the non-treatment group were comparable with those shown in previous reports.<sup>26)</sup>

Furthermore, the effects of a single oral pretreatment with GBE (20 mg/kg) on the plasma concentration of DTZ and MA after oral administration of DTZ (30 mg/kg) were exam-

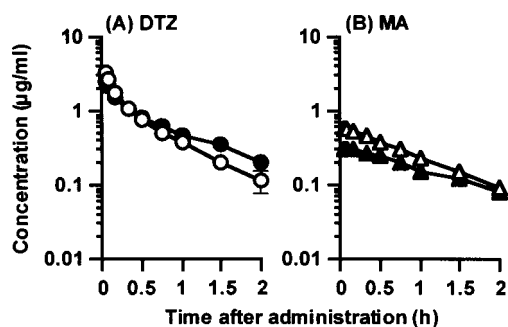


Fig. 5. Effects of Single Oral Pretreatment with GBE on the Plasma DTZ and MA Concentrations after Intravenous Administration of DTZ to Rats

Each point represents the mean  $\pm$  S.E. of 7 or 8 rats. The vehicle (control) or GBE suspension (20 mg/kg) was administered orally to unanesthetized rats, and then the DTZ solution (3 mg/kg) was administered intravenously 1 h later.  $\circ$ , control (DTZ);  $\bullet$ , GBE (DTZ);  $\triangle$ , control (MA);  $\blacktriangle$ , GBE (MA).

Table 1. Effects of Single Oral Pretreatment with GBE on the Pharmacokinetic Parameters of DTZ and MA after Intravenous Administration of DTZ to Rats

Parameter	DTZ		MA	
	Control	GBE	Control	GBE
$C_{\max}$ ( $\mu$ g/ml)	—	—	$0.62 \pm 0.09$	$0.36 \pm 0.05^a$
$T_{\max}$ (h)	—	—	$0.09 \pm 0.02$	$0.16 \pm 0.06$
$\lambda$ (/h)	$1.43 \pm 0.19$	$0.95 \pm 0.11^a$	$1.02 \pm 0.11$	$0.80 \pm 0.11$
$t_{1/2(\lambda)}$ (h)	$0.56 \pm 0.08$	$0.78 \pm 0.07$	$0.74 \pm 0.07$	$0.94 \pm 0.09$
$AUC_{0-\infty}$ ( $\mu$ g $\cdot$ h/ml)	$1.47 \pm 0.20$	$1.64 \pm 0.21$	$0.65 \pm 0.06$	$0.48 \pm 0.06$
MRT (h)	$0.63 \pm 0.08$	$0.96 \pm 0.10^a$	$1.10 \pm 0.09$	$1.40 \pm 0.15$

Each value represents the mean  $\pm$  S.E. of 7 or 8 rats. *a*)  $p < 0.05$  vs. each control value (unpaired Student's *t*-test). The vehicle (control) or GBE suspension (20 mg/kg) was administered orally to unanesthetized rats, and then the DTZ solution (3 mg/kg) was administered intravenously 1 h later.

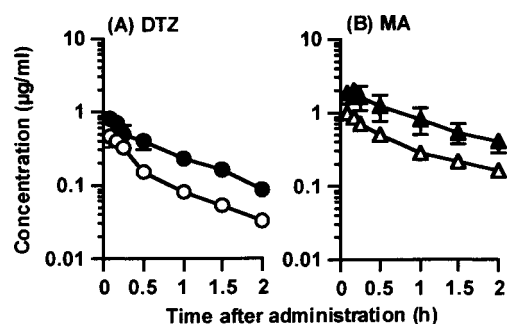


Fig. 6. Effects of Single Oral Pretreatment with GBE on the Plasma DTZ and MA Concentrations after Oral Administration of DTZ to Rats

Each point represents the mean  $\pm$  S.E. of 5 or 6 rats. The vehicle (control) or GBE suspension (20 mg/kg) was administered orally to unanesthetized rats, and then the DTZ solution (30 mg/kg) was administered orally 1 h later.  $\circ$ , control (DTZ);  $\bullet$ , GBE (DTZ);  $\triangle$ , control (MA);  $\blacktriangle$ , GBE (MA).

ined (Fig. 6). The plasma concentrations of DTZ or MA in the GBE group were higher than those in the control group. The pharmacokinetic parameters are summarized in Table 2. The  $AUC_{0-\infty}$  and  $F$  for DTZ were significantly increased by the pretreatment with GBE, while other parameters of DTZ or MA were not significantly different between the two groups.

Table 2. Effects of Single Oral Pretreatment with GBE on the Pharmacokinetic Parameters of DTZ and MA after Oral Administration of DTZ to Rats

Parameter	DTZ		MA	
	Control	GBE	Control	GBE
$C_{\max}$ ( $\mu\text{g/ml}$ )	0.55 $\pm$ 0.06	0.92 $\pm$ 0.19	1.11 $\pm$ 0.21	2.24 $\pm$ 0.66
$T_{\max}$ (h)	0.12 $\pm$ 0.02	0.13 $\pm$ 0.02	0.12 $\pm$ 0.02	0.14 $\pm$ 0.02
$\lambda$ (h)	0.96 $\pm$ 0.08	1.11 $\pm$ 0.09	0.61 $\pm$ 0.06	0.68 $\pm$ 0.10
$t_{1/2(\lambda)}$ (h)	0.75 $\pm$ 0.06	0.65 $\pm$ 0.05	1.20 $\pm$ 0.15	1.28 $\pm$ 0.35
$AUC_{0-\infty}$ ( $\mu\text{g}\cdot\text{h/ml}$ )	0.30 $\pm$ 0.04	0.67 $\pm$ 0.14 <sup>a)</sup>	1.03 $\pm$ 0.18	2.63 $\pm$ 0.80
MRT (h)	0.90 $\pm$ 0.07	1.02 $\pm$ 0.13	1.57 $\pm$ 0.16	1.77 $\pm$ 0.48
F (%)	2.0 $\pm$ 0.3	4.6 $\pm$ 0.9 <sup>a)</sup>	—	—

Each value represents the mean $\pm$ S.E. of 5 or 6 rats. a)  $p < 0.05$  vs. each control value (unpaired Student's *t*-test). The vehicle (control) or GBE suspension (20 mg/kg) was administered orally to unanesthetized rats, and then the DTZ solution (30 mg/kg) was administered intravenously 1 h later.

## DISCUSSION

We have been studying the possibility of pharmacokinetic interactions between functional foods and drugs for several years. Here, we reported the findings of basic research on the pharmacokinetic interactions between GBE, one of the most popular dietary supplements in Japan, and DTZ as a representative substrate for CYP3A as well as a highly extracted drug in rats.

First, the effects of GBE on CYP3A activity were examined *in vitro* using small intestine and liver microsomes. GBE inhibited CYP3A activities dose-dependently (Fig. 1), and the  $IC_{50}$ s in small intestine and liver microsomes were about 50 and 182  $\mu\text{g/ml}$ , respectively. These results were similar to those obtained using rat liver microsomes reported by Umegaki *et al.*,<sup>13</sup> and indicated that GBE contains a substance(s) that inhibits CYP3A activity. It is already known that GBE contains many kinds of flavonoids such as quercetin and kaempferol,<sup>8,9</sup> which are very popular, and these compounds inhibit CYP3A activity *in vitro*.<sup>27</sup> But quercetin (2.4  $\mu\text{g/ml}$ )<sup>9</sup> and kaempferol (6.0  $\mu\text{g/ml}$ )<sup>9,28</sup> estimated to be contained in GBE at a concentration of 1000  $\mu\text{g/ml}$  did not inhibit the activity of CYP3A in hepatic microsomes (unpublished data). This may be due to much lower concentrations in GBE than the actual inhibitory concentration. In addition, several specific compounds such as ginkgolide A (18.0  $\mu\text{g/ml}$ ), ginkgolide B (8.5  $\mu\text{g/ml}$ ), ginkgolide C (10.9  $\mu\text{g/ml}$ ) and bilobalide (23.2  $\mu\text{g/ml}$ ) also have no inhibitory action (unpublished data). Accordingly, further investigation is required to identify the potential inhibitory compound(s). On the other hand, there is a 3.6-fold difference in the  $IC_{50}$ s for CYP3A between small intestine and liver microsomes, showing a stronger inhibition in small intestine microsomes. The fact that the CYP content per microsomal protein in the small intestine is much lower than that in the liver may be, at least in part, responsible for this result. Furthermore, this may be attributed to the differences in CYP3A isoforms and the ratio of content between two tissues.

Next, to clarify the inhibitory mechanism of action for CYP3A by GBE, we performed a kinetic analysis of inhibition using hepatic microsomes. From the results of Lineweaver–Burk plots, it was suggested that CYP3A activ-

ity was inhibited competitively at low concentrations of GBE below the  $IC_{50}$  but both competitively and non-competitively at a concentration above the  $IC_{50}$  (Fig. 2). Accordingly, the inhibitory mechanism of action was shown to be non-specific inhibition. It also seemed possible that the apparent non-competitive inhibitory effect of GBE reflects an unusually or irreversibly high affinity for CYP3A such as the CYP isoform-nonspecific coordinated binding of some intact inhibitor to the heme moiety of CYP (cimetidine, *etc.*)<sup>29,30</sup> and/or a mechanism-based inhibition by a reactive metabolite of inhibitor (grapefruit juice, *etc.*).<sup>2,30</sup> Therefore, to determine whether these inhibitory actions were associated with the irreversibly mechanism-based inhibition, the effects of preincubation with or without an NADPH-generating system on the CYP3A activity were examined using liver microsomes (Fig. 3). It was found that the inhibition by GBE was closely related to an NADPH-generating system, indicating the existence of mechanism-based inhibition. This result supports that of the *in vitro* kinetic analysis indicated above.

Furthermore, we performed detailed experiments *ex vivo* to confirm the potential of the irreversible inhibitory effect of GBE on CYP3A found *in vitro*. The CYP3A activities in both small intestine and liver microsomes were transiently (1–12 h) decreased by a single oral administration of GBE (Fig. 4A), corresponding to the decrease in total CYP content (Fig. 4B). These findings indicate that GBE has a significant inhibitory effect on CYP3A even *ex vivo*, and strongly support our conclusion that CYP3A is inhibited by GBE in part via a mechanism-based inhibition where the content of CYP3A seems to be apparently decreased, resulting in an apparent decrease in total CYP content. The inhibitory effects of GBE on CYP3A activity in both small intestine and liver microsomes disappeared 24 h after pretreatment with GBE (Fig. 4A). This recovery seems to result from the regeneration of CYP3A in each tissue as the total CYP content recovered in parallel.

Finally, the effects of a single oral pretreatment with GBE on the pharmacokinetics after intravenous administration of DTZ were examined in rats. The  $\lambda$  and MRT values for DTZ were decreased and increased, respectively, and the  $C_{\max}$  value for its metabolite was lowered by the pretreatment with GBE ( $p < 0.05$ ; Fig. 5 and Table 1), indicating that the elimination of DTZ was slightly but significantly delayed. Furthermore, we examined whether single oral pretreatment with GBE affected the pharmacokinetics after oral administration of DTZ to rats. The  $AUC_{0-\infty}$  and  $F$  values of DTZ were shown to be increased approximately 2-fold without changes in other pharmacokinetic parameters ( $p < 0.05$ ; Fig. 6 and Table 2), suggesting that GBE reduced presystemic metabolism of DTZ. These results *in vivo* corresponded to those *in vitro* and *ex vivo*. Besides, the ratio of  $AUC_{0-\infty}$  for DTZ in the GBE-pretreated group relative to the control group was larger following the oral than intravenous administration of DTZ. Lee *et al.*<sup>26</sup> reported that the extraction ratios for DTZ in the small intestine and liver after oral administration to rats were about 85 and 63%, respectively, confirming that DTZ is highly extracted in the small intestine as well as the liver. Based on our findings and these facts, it was suggested that orally administered GBE suppressed in part the presystemic first-pass metabolism of DTZ not only in the liver but also in the small intestine, by inhibiting CYP3A activity in

both tissues. Furthermore, it is known that DTZ is metabolized to deacetyl DTZ (M1), another major metabolite, by esterases in the intestine and liver.<sup>20</sup> So, GBE might inhibit the enzymes in addition to CYP3A. On the other hand, DTZ and M1 have been reported to be substrates for the multidrug transporter P-glycoprotein (P-gp).<sup>31,32</sup> The inhibition of intestinal P-gp by GBE might be also responsible for the significantly increased absorption of DTZ after oral coadministration with GBE although there is no evidence of this. Unexpectedly, plasma MA concentrations after oral administration of DTZ tended to be higher in the GBE-pretreated group than that the control group, unlike in the case of intravenous administration of DTZ. This phenomenon can be accounted for by the following reasons. First, GBE might inhibit CYP3A activities in the intestine rather than the liver, resulting in an increased intestinal absorption rate of DTZ with a little change in the formation rate of MA in the liver. Second, GBE might suppress the subsequent deacetylation of MA in the intestine. Third, MA might be also a substrate of P-gp and GBE primarily inhibits intestinal P-gp. Further investigation should be undertaken to clarify these points.

The overall rate of metabolization of DTZ is different between rats and humans, as in the case of many drugs; the oral clearance of DTZ in rats is 60-fold that in humans.<sup>20</sup> However, DTZ is classified as a flow-limited or highly extracted drug in both species although the absolute bioavailability in rats (2%; see Table 2) is different from that in humans (30–44%).<sup>33</sup> The metabolic pathways in rats are qualitatively similar to those in humans,<sup>20,34</sup> and the transformation from DTZ to MA is mainly dependent on several CYP3A isoforms such as CYP3A1, 3A2 and CYP3A4 in the rat and human tissues, respectively.<sup>16–19</sup> In addition, we confirmed that the IC<sub>50</sub> of GBE for CYP3A activity in rat liver microsomes (182 ± 13 µg/ml; Fig. 1) was almost consistent with that in commercial human liver microsomes (159 ± 7 µg/ml; unpublished data), similar to the finding reported by Umegaki *et al.*<sup>13</sup> These facts and results suggest the possibility that GBE is likely to inhibit the first-pass metabolism of DTZ in humans.

In conclusion, it was confirmed that the oral coadministration of GBE and DTZ to rats increased the bioavailability of DTZ by inhibiting both intestinal and hepatic metabolism, at least in part, by a mechanism-based inhibition for CYP3A. The findings appear to provide useful information for future investigations in humans on potential pharmacokinetic interactions between GBE and drugs such as DTZ and other flow-limited drugs that are substrates for CYP3A4.

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