Effects of Menthol on the Pharmacokinetics of Triazolam and Phenytoin

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We have previously shown that menthol attenuates the anticoagulant effect of warfarin by increasing the expression levels of CYP3A and CYP2C in the liver. This study evaluated the effects of menthol on the pharmacokinetics of the CYP3A substrate triazolam and the CYP2C substrate phenytoin. Menthol was orally administered to mice for 7 d. Twenty-four hours after the administration of menthol, triazolam was orally administered, and the plasma concentration was measured. In addition, the CYP3A metabolic activity for triazolam and the CYP3A expression level in the liver were determined. The effects of menthol on the pharmacokinetics of phenytoin were assessed in the same manner. In the menthol-treated group, the area under the blood concentration–time curve (AUC) of triazolam was lower and its clearance was higher compared with the control group. The CYP3A metabolic activity and CYP3A expression level in the liver were significantly increased in the menthol-treated group compared with the control group. Similarly, the AUC of phenytoin was lower and the hepatic CYP2C expression level was higher in the menthol-treated group. Thus, menthol lowered the plasma concentrations of triazolam and phenytoin when concurrently administered. These effects may be attributed to an increased metabolic activity for these drugs due to the increased expression of CYP3A and CYP2C in the liver.

Key words  CYP; menthol; warfarin; triazolam; phenytoin

CYP is a drug-metabolizing enzyme that is mainly expressed in the liver. There are various subtypes of CYP, and CYP3A is one of the most important subfamilies because approximately 50% of the drugs used in clinical practice today are metabolized by CYP3A. The expression levels and activity of CYPs have been shown to dynamically vary according to various factors, such as drugs, natural products, and food and beverages. Because variations in the expression and activity of CYPs may affect the pharmacokinetics of drugs that are substrates of the CYPs and impair their therapeutic effects, it is important to identify potential factors affecting the expression and activity of CYPs to ensure the appropriate use of drugs.

Based on reports showing that the intake of menthol attenuates the anticoagulant effect of warfarin when concurrently administered, we performed a pharmacokinetic study and demonstrated that menthol induces the expression of CYP3A and CYP2C, which are hepatic enzymes participating in the metabolism of warfarin, and thereby lowers the plasma concentration of warfarin, resulting in a reduced anticoagulant effect. Menthol is contained in candies, cigarettes, and herbal teas, as well as pharmaceutical products, and its safety has been assured. However, our findings suggest that the concurrent use of menthol with a drug may lower the plasma concentration of the drug and resultantly attenuate its therapeutic effects.

The present study evaluated whether this effect of menthol is exerted only on warfarin or also on other drugs that are substrates of CYP3A or CYP2C using the hypnotic triazolam as a CYP3A substrate and the antiepileptic phenytoin as a CYP2C substrate.

MATERIALS AND METHODS

Materials  Triazolam, phenytoin, and l-menthol were purchased from Wako Pure Chemical Industries, Ltd. (Osaka, Japan). Bovine serum albumin (BSA), TRI reagent, ethylene-diaminetetraacetic acid (EDTA), mouse anti-rat CYP2C6 antibody, and α-hydroxytriazolam were purchased from Sigma-Aldrich Corp. (St. Louis, MO, U.S.A.). 4-Hydroxytriazolam was purchased from BIOMOL (Exeter, U.K.). The reduced nicotinamide adenine dinucleotide phosphate (NADPH) regeneration system was purchased from Becton, Dickinson and Company (Tokyo, Japan). Rabbit anti-rat CYP3A2 antibody was purchased from Daiichi Pure Chemicals (Tokyo, Japan). Mouse anti-rabbit glyceraldehyde-3-phosphate dehydrogenase (GAPDH) antibody was purchased from Chemicon International Inc. (Temecula, CA, U.S.A.). Donkey anti-rabbit immunoglobulin G-horseradish peroxidase (IgG-HRP) antibody and ECL plus Western blotting detection reagents were purchased from GE Healthcare (Chalfont St. Giles, U.K.). A high-capacity cDNA synthesis kit was purchased from Applied Biosystems (Foster City, CA, U.S.A.), and iQ SYBR Green Supermix was purchased from Bio-Rad Laboratories ( Hercules, CA, U.S.A.). Primers were purchased from Invitrogen Corp. (Tokyo, Japan). All other reagents were of the highest commercially available grade.

Animals  Male ICR mice (8 weeks of age) were purchased from Japan SLC, Inc. (Shizuoka, Japan). The mice were maintained at room temperature (24±1°C) and 55±5% humidity with 12 h of light (artificial illumination; 08:00–20:00). The present study was conducted in accordance with the Guiding Principles for the Care and Use of Laboratory Animals, as adopted by the Committee on Animal Research at Hoshi University.
Triazolam/Phenytoin Pharmacokinetics

An aqueous solution of 1% dimethyl sulfoxide (DMSO) or menthol (10 mg/kg) was orally administered to the mice for 7 d. Twenty-four hours after menthol administration, the mice were orally administered 5 mg/kg triazolam or 30 mg/kg phenytoin (administered to 25 mice in each group). After administration, approximately 1 mL of blood was collected from the abdominal vena cava using a heparinized syringe (five individuals/time point). Plasma fractions were separated by centrifugation of the blood samples.

The plasma triazolam concentration was measured as described previously.6 Briefly, after adding isopropanol to the plasma, the sample was mixed, centrifuged (15000×g for 10 min at 25°C), and deproteinated. Borate buffer (50 mM; pH 11) and chloroform were added to the supernatant, and the sample was subsequently mixed and centrifuged (10000×g for 5 min at 25°C). The lower layer was collected and dried with nitrogen gas. The residue was dissolved in the mobile phase, and triazolam was assayed by HPLC-UV.

The plasma phenytoin concentration was measured as described previously.6 Briefly, after adding acetonitrile to the plasma, the sample was mixed, centrifuged (5000×g for 5 min at 4°C), and deproteinated. Chloroform was added to the supernatant, and the sample was subsequently mixed and centrifuged (3000×g for 10 min at 4°C). The lower layer was collected and dried with nitrogen gas. The residue was dissolved in the mobile phase, and phenytoin was assayed by HPLC-UV.

HPLC-UV

The HPLC apparatus consisted of a Waters 2695 Separation Module (Waters, Tokyo, Japan) and a Waters 2489 UV/Visible Detector (Waters), and the measured data were recorded and analyzed using the Empower analysis software (Waters). We used an Inertsil C18 ODS-3 column (mean particle size: 5 µm, 4.6×250 mm, GL Sciences Inc., Tokyo, Japan). For the triazolam measurements, the mobile phase was acetonitrile–methanol–10 mM potassium phosphate buffer (3:6:11, pH 7.4). The flow rate was 1.0 mL/min, the temperature was 40°C, and the detection wavelength was 220 nm.8 For the phenytoin measurements, the mobile phase was methanol–water–glacial acetic acid (55:44:1), the flow rate was 1.0 mL/min, the temperature was 30°C, and the detection wavelength was 230 nm.9

Data Analysis10 The area under the plasma concentration–time curve extrapolated to infinity (AUCinf) was calculated using the average concentrations from five mice at each time point with the linear trapezoidal rule. The elimination rate constant (k0) and elimination half-life (t1/2=0.693/k0) were calculated from the concentration profiles of the elimination phase. The maximum plasma concentration (Cmax) and the time-to-maximum plasma concentration (tmax) were obtained from actual measurements. The body clearance (CL/F, where F is the bioavailability) was calculated using the following formula:

\[ CL/F (\text{mL/min}) = \text{dose (µg)}/AUC_{\text{inf}} (\text{µg} \cdot \text{min/mL}) \]

F was treated as a constant because the same formulation of triazolam or phenytoin was used, and we assumed that the bioavailability did not change among different mice.

Treatments Menthol (10 mg/kg) was orally administered to mice for 7 d. An aqueous solution of 1% DMSO was orally administered to the control group. The liver and small intestine were removed after the final administration and stored at −80°C.

Microsome Preparation11 The liver and small intestine were homogenized using dissecting buffer (0.3 M sucrose, 25 mM imidazole, 1 mM EDTA, 8.5 µM leupeptin, and 1 µM phenylmethylsulfonyl fluoride; pH 7.2). The resulting suspension was centrifuged (9000×g for 15 min at 4°C), and the supernatant was centrifuged (105000×g for 1 h at 4°C). Dissecting buffer was added to the precipitate and homogenized using an ultrasonic homogenizer to yield the microsomal fraction.

Measurement of CYP3A Metabolic Activity12 The triazolam solution (final concentrations ranging from 0 to 750 µM), hepatic microsomal suspension (final concentration was 0.27 mg/mL), and nicotine adenine dinucleotide phosphate (NADPH) regenerating system solution were mixed and incubated for 30 min at 37°C. The reaction was stopped by the addition of acetonitrile. The sample was centrifuged (16 000×g for 15 min at 4°C), and the supernatant was evaporated under a flow of nitrogen. The residue was dissolved in the HPLC mobile phase [acetonitrile–methanol–10 mM potassium phosphate buffer (3:6:11, pH 7.4)], and HPLC-UV was used to quantify 4-hydroxytriazolam and α-hydroxytriazolam. The initial linear rate conditions were confirmed with respect to the microsomal protein concentration (up to 1 mg/mL) and incubation time (up to 40 min). The relationship between the triazolam concentration and metabolic rate was fitted to the Michaelis–Menten equation (4-hydroxylation) or the Michaelis–Menten equation with substrate inhibition (α-hydroxylation) using the nonlinear least-squares regression program (MULTI)13 to calculate the kinetic parameters (maximum velocity: Vmax, Michaelis constant: Km, and substrate inhibition constant: Ki). Furthermore, we calculated the intrinsic clearance (CLint=Vmax/Km) for each metabolic pathway.

Electrophoresis and Immunoblotting The protein concentrations were measured by the bicinchoninic acid (BCA) method14 using BSA as a standard. The proteins were diluted using loading buffer. The samples were boiled prior to loading on a polyacrylamide gel. After electrophoresis, the separated proteins were transferred onto a polyvinylidene difluoride membrane, which was incubated in skim milk blocking buffer. After blocking, the membrane was incubated with primary antibodies for 1 h. The primary antibodies used were as follows: mouse anti-rat CYP2C6 (1/1000), rabbit anti-rat CYP3A2 (1/10000), and mouse anti-rabbit GAPDH (1/2000). The membrane was incubated with secondary antibodies for 1 h. The membrane was incubated with ECL plus detection reagent and visualized with an LAS-3000 Mini Lumino image analyzer (FUJIFILM, Tokyo, Japan).

RNA Preparation from Tissue Samples The RNA from the liver and small intestine was extracted using TRI reagent. The resulting solution was diluted using Tris/EDTA buffer (TE buffer), and the purity and concentration of RNA were calculated by measuring the absorbance at 260 and 280 nm using a NanoDrop Lite Spectrophotometer (Thermo Fisher Scientific, Waltham, MA, U.S.A.).

Real-Time Polymerase Chain Reaction (PCR) A high-capacity cDNA synthesis kit was used to synthesize cDNA from 1 µg of RNA. The target gene expression was analyzed by real-time RT-PCR using the primers listed in Table 1. The iQ SYBR Green Supermix, target gene forward/reverse prim-
Menthol-treated group was significantly decreased 120 min ally decreased. The plasma triazolam concentration in the administration and the plasma triazolam concentration gradu-
kinetic parameters were analyzed (Fig. 1, Table 2).

Beers, cDNA TE buffer solution, and RNase-free water were added to each well of a PCR plate. Real-time RT-PCR was conducted at a denaturation temperature of 95°C for 15 s, an annealing temperature of 56°C for 30 s, and an elongation temperature of 72°C for 30 s. The amplification fluorescence intensity was monitored using the MyiQ™ single-color real-time RT-PCR detection system (Bio-Rad Laboratories). The mRNA gene expression levels were normalized to the GAPDH gene expression level.

Statistical Analyses The numerical data are expressed as the means±standard deviation. The significance of the differences was examined using Student’s t-test for pairs of values. Differences of $p<0.05$ were considered to be statistically significant.

**RESULTS**

**Effect of Menthol on Pharmacokinetics of Triazolam** It is known that triazolam is metabolized by CYP3A in humans and by its ortholog in mice to produce $\alpha$-hydroxytriazolam and 4-hydroxytriazolam.15) Many of the CYP3A substrates also act as substrates for P-glycoprotein (P-gp), a transporter that mediates drug excretion.16,17) However, triazolam is not a P-gp substrate.18) Therefore, we examined the effect of menthol on the pharmacokinetics of a CYP3A substrate using triazolam. Briefly, the plasma concentration of triazolam was measured after the oral administration of triazolam 24 h after the oral administration of menthol for 7 d, and the pharmacokinetic parameters were analyzed (Fig. 1, Table 2).

The $C_{\text{max}}$ of triazolam reached 30 min after triazolam administration and the plasma triazolam concentration gradually decreased. The plasma triazolam concentration in the menthol-treated group was significantly decreased 120 min and 180 min after triazolam administration compared with that in the control group (Fig. 1). In the menthol-treated group, the triazolam $AUC$ decreased to approximately 60% of that in the control group, and the $CL/F$ increased to approximately 1.7-fold that in the control group (Table 2).

These results confirm that menthol enhanced the elimination of triazolam and resulted in a decreased plasma triazolam concentration.

**Effect of Menthol on CYP3A Metabolic Activity in the Liver** We examined whether the increase in triazolam clearance observed after menthol administration could be attributed to an increase in the intrinsic hepatic clearance of the drug (Fig. 2). Hepatic microsomal fractions were prepared from mice, and the amounts of $\alpha$-hydroxytriazolam and 4-hydroxytriazolam produced by the metabolism of triazolam were measured to evaluate the metabolic activity of CYP3A.

The $V_{\text{max}}$ of $\alpha$-hydroxylation in the menthol-treated group was significantly higher than that observed in the control group. The $CL_{\text{int}}$ in the menthol-treated group was significantly higher (approximately 1.6-fold higher) than that observed in the control group. However, the $K_m$ value did not differ significantly between the control group and the menthol-treated group (Fig. 2A).

The $V_{\text{max}}$ and $CL_{\text{int}}$ of 4-hydroxylation activity were significantly higher in the menthol-treated group than in the control group. However, no significant differences in the $K_m$ value were observed between the control group and the menthol-treated group (Fig. 2B).

These results indicated that the increase in the clearance of triazolam after the administration of menthol can be attributed to an increase in the intrinsic hepatic clearance of the drug.

**Effect of Menthol on CYP3A Expression Level in the Liver** We examined whether the increase in intrinsic hepatic clearance observed after menthol administration could be attributed to an increase in the hepatic CYP3A expression level (Fig. 3).

The expression level of CYP3A11 mRNA in the liver 24 h
after the administration of menthol was significantly higher (approximately 2-fold higher) than that observed in the control group (Fig. 3A).

CYP3A was identified as two bands that appeared at approximately 52 kDa. Genes encoding at least eight different mouse CYP3A isozymes have been identified.\(^\text{19}\) Therefore, the identification of CYP3A as two bands on the immunoblots suggests the expression of more than one mouse CYP3A isozyme. In this study, the sum of these band intensities was used to evaluate the expression of CYP3A protein. In the menthol-treated group, the protein expression of CYP3A in the hepatic microsomal fraction was significantly increased compared with that observed in the control group (Fig. 3B).

These results indicated that the increase in the intrinsic hepatic clearance of triazolam after the administration of menthol can be attributed to an increase in the CYP3A expression level.

Effect of Menthol on the CYP3A Expression Level in the Small Intestine  CYP3A is the CYP family member that is most frequently expressed in the small intestine.\(^\text{11}\) Therefore,
the CYP3A expression level in the small intestine of menthol-treated mice was also determined in this study (Fig. 4).

No differences in the CYP3A11 mRNA expression levels in the small intestine were observed between the control group and the menthol-treated group (Fig. 4A). In addition, no changes in the CYP3A protein expression levels in the microsomal fraction of the small intestine that could be attributed to menthol administration were observed (Fig. 4B).

These results indicate that menthol increases the CYP3A expression level specifically in the liver without affecting the expression of CYP3A in the small intestine.

**Effect of Menthol on the Pharmacokinetics of Phenytoin**

Phenytoin is metabolized by CYP2C in the liver primarily to 5-(p-hydroxyphenyl)-5-phenylhydantoin (HPPH), which has no pharmacological effects. In this study, the effect of menthol on the pharmacokinetics of phenytoin was assessed (Fig. 5, Table 3). The $C_{max}$ of phenytoin reached 8 h after phenytoin administration and the plasma phenytoin concentration gradually decreased. The plasma phenytoin concentration in the menthol-treated group was significantly decreased at 12 h and 24 h after phenytoin administration compared with that in the control group (Fig. 5). In the menthol-treated group, the phenytoin $AUC$ decreased to 78% of that in the control group, and the $CL/F$ was increased 1.3-fold compared with that in the control group (Table 3). The hepatic CYP2C29 mRNA expression level in the menthol-treated group was significantly higher (approximately 2-fold higher) than that observed in the control group (Fig. 6A). Furthermore, in the menthol-treated group, the CYP2C protein expression in the hepatic microsomal fraction was significantly increased compared with that observed in the control group (Fig. 6B).

These results indicate that menthol increases the CYP2C expression level in the liver and thus accelerates the metabolism of phenytoin, leading to a decreased plasma concentration of the drug.

**DISCUSSION**

We have previously shown that menthol decreases the plasma concentration of warfarin by inducing the expression of CYP3A and CYP2C and resultanty attenuates the anticoagulant effect of warfarin. This study evaluated the effects of
menthol on the pharmacokinetics of drugs that are known to be metabolized by CYP3A or CYP2C.

Triazolam is a benzodiazepine hypnotic used for the treatment of insomnia.22) Because fluctuations in the plasma concentration of triazolam are associated with impairments in the QOL of patients, the control of the plasma concentration is extremely important. Triazolam is a typical drug that is metabolized by CYP3A, and its sleep-inducing effect may be significantly affected by drugs that alter the activity of CYP3A.23) In this study, triazolam was chosen as a CYP3A substrate, and the effect of menthol on the pharmacokinetics of the drug was evaluated. The results of this study demonstrate that the intake of menthol decreased the AUC of triazolam by approximately 40% and increased the CL/F approximately 1.7-fold in mice (Fig. 1, Table 2). Triazolam is a drug with a relatively low hepatic extraction rate,24) and elimination from the body depends on metabolism in the liver. The protein expression of CYP3A in the hepatic microsomal fraction and the CYP3A metabolic activity increased approximately 1.6-fold and 1.7-fold, respectively, in the menthol-treated group as compared with the control group (Figs. 2, 3). These increases had a good correlation with the increase in the CL/F. In addition, no changes in the CYP3A expression level were observed in the small intestine of menthol-treated mice (Fig. 4). Based on the above, we suspect that the decrease in the AUC of triazolam following menthol administration was attributed to the increase in the expression of CYP3A in the liver. It has been reported that the decreased AUC of triazolam is associated with a markedly attenuated sleep-induced effect.25) The dose of triazolam used in this study is a dose which has a sleep-induced effect on mice.25) Thus, the intake of menthol is expected to impair the QOL of patients who are being treated with triazolam.

So far we have discussed the changes in the expression of CYP3A in the liver due to menthol treatment; however, we also found that the expression levels of CYP3A in the small intestine were not changed (Figs. 3, 4). The reason for this difference, although it is still a matter of speculation, is considered to be as follows. The hepatic expression of CYP3A is mainly mediated by pregnane X receptor (PXR), whereas the vitamin D receptor (VDR) is mainly involved in the intestinal expression of CYP3A.26) The expression level of CYP3A in the small intestine, therefore, depends on the intestinal expression level of VDR. We showed that the nuclear expression of PXR in the liver was increased in the menthol-treated mice.

This study also evaluated the effects of menthol on the pharmacokinetics of a CYP2C substrate. CYP2C is the second most abundantly expressed CYP isoform in the liver. Phenytoin, an antiepileptic, is a CYP2C substrate, and its pharmacokinetics with no proportional relationship between the dose and plasma concentration needs to be strictly controlled in order to prevent seizures. Because phenytoin follows nonlinear pharmacokinetics, it has been confirmed that phenytoin does not show nonlinear pharmacokinetics with the use of inducers and inhibitors of CYPs.28,29) The results of the present study revealed that the administration of menthol lowers the AUC of phenytoin by approximately 20% and increased the CL/F approximately 1.3-fold in mice (Fig. 5, Table 3). Phenytoin is a drug with a low hepatic extraction rate and a capacity-limited hepatic clearance.30) The protein expression of CYP2C in the liver was increased approximately 1.7-fold by the administration of phenytoin following menthol administration was attributed to the increased expression of CYP2C in the liver. The dose of phenytoin used in this study is a dose which has an antiepileptic effect on mice.31) Though phenytoin is a drug which demonstrates nonlinear pharmacokinetics, it has been confirmed that phenytoin does not show nonlinear pharmacokinetics in mice at the dose used in this study.31) Thus, the intake of menthol may impair the therapeutic effect of phenytoin when it is concurrently administered.

In conclusion, in this study, menthol was administrated to mice at a dose of 10mg/kg. At this dose, the clinical reports5,6) showing that menthol attenuated an effect of warfarin were reproducible on mice.7) When calculated based on the amount of menthol contained in foods (cough drop, menthol content:
1–10 mg/drop) and drugs (antiperistaltic drug, menthol content: 160 mg), the dose used in this study is equivalent to 5- to 100-fold the dose for humans. It is not clear if the findings of this study can also occur in humans. We suggested that it is necessary to examine in detail, in clinical practice, whether the findings of this study can also occur in humans.

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Conflict of Interest The authors declare no conflict of interest.

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