Note

Green Tea Extract Affects the Cytochrome P450 3A Activity and Pharmacokinetics of Simvastatin in Rats

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Summary: Effects of green tea extract (GTE) on the activity of cytochrome P450 (CYP) enzymes and pharmacokinetics of simvastatin (SIM) were investigated in rats. Inhibitory effects of GTE on CYP3A activity were investigated in rat hepatic microsomes (RHM) using midazolam (MDZ) 1’-hydroxylation as a probe reaction. SD female rats received a single oral dose of GTE (400 mg/kg) or troleandomycin (TAO, a CYP3A selective inhibitor, 500 mg/kg), followed 30 min later by SIM (20 mg/kg). Plasma concentrations of SIM and its active metabolite, simvastatin acid, were determined up to 6 h after the SIM administration using LC/MS/MS. In RHM, GTE inhibited MDZ 1’-hydroxylation with IC50 and Kapp values of 12.5 and 18.8 µg/mL, respectively, in a noncompetitive manner. Area under plasma concentration-time curves for SIM in the GTE and TAO groups were increased by 3.4- and 10.2-fold, respectively, compared with the control. The maximum concentrations of SIM were higher in the GTE (3.3-fold) and TAO (9.5-fold) groups. GTE alters the pharmacokinetics of SIM, probably by inhibiting intestinal CYP3A.

Keywords: CYP3A; green tea extract; midazolam; pharmacokinetics; rat; simvastatin

Introduction

Over recent decades, consumption of green tea (Camellia sinensis) has been growing worldwide in accordance with an increasing number of publications reporting beneficial effects such as chemoprevention, anti-oxidation and anti-infection.1–3 Catechins, the major polyphenol constituents of freshly brewed green tea, are thought to exert those effects.4 Naturally occurring catechins in green tea comprise (−)-epigallocatechin-3-gallate (EGCG), (−)-epigallocatechin (EGC), (−)-epicatechin (EC) and (−)-epicatechin-3-gallate (ECG). Previously, Muto et al. demonstrated that green tea catechins, especially galloylated catechins, inhibited drug oxidation catalyzed by cytochrome P450 (CYP) enzymes in vitro.5 In addition, green tea catechins, especially EGCG, have been reported to exhibit pharmacokinetic interactions with various drugs in animal experiments.6–10 For instance in rats, repeated oral administration of green tea extract (GTE) for one week significantly increased the maximum plasma concentration (Cmax) and area under the time-concentration curve (AUC) of midazolam, which is metabolized extensively by CYP3A, suggesting that catechins in GTE inhibited CYP3A activity in the intestine.11 Recently, our group reported that the consumption of green tea containing large amounts of catechins increased the bioavailability of simvastatin in a hypercholesterolemia patient.12 The effect on CYP3A activity of drinking green tea may contribute to the alteration of simvastatin pharmacokinetics; however, to the best of our knowledge, there are no reports concerning the interaction of simvastatin with green tea catechins in rodents.

Simvastatin is clinically used as a lactone prodrug and is hydrolyzed by esterase in the liver to yield an active metabolite,
Both simvastatin and simvastatin acid are further metabolized to several inactive metabolites including 6′-hydroxy or 3′,5′-dihydrodiol forms by CYP3A in rats and humans. A previous study demonstrated that the metabolism of simvastatin in female Sprague-Dawley (SD) rats is more similar than in male rats to that observed in humans, suggesting that female rats could represent a proper model to clarify the underlying mechanism of herb-drug interaction reported in our previous human case. As an example, Ishigami et al. showed that the co-administration of simvastatin and itraconazole resulted in significant increases in the C_{min} and AUC of simvastatin due to the inhibition of CYP3A in female rats. Concerning the pharmacokinetic interaction between simvastatin and dietary components, Butterweck et al. reported that repeated intake of grapefruit juice elevated the plasma concentrations of simvastatin and simvastatin acid in rats. Given that green tea catechins actually inhibit CYP3A activity in vivo, plasma concentrations of simvastatin and simvastatin acid may be altered.

The objective of the present study was to assess the effect of GTE on the metabolism of simvastatin in rats. Using rat hepatic microsomes, the in vitro inhibitory effect of GTE on CYP3A activity was tested with midazolam as a specific probe substrate of CYP3A. To investigate the pharmacokinetic interaction, rats were given a single dose of simvastatin 30 min after receiving GTE or troleandomycin, a CYP3A selective inhibitor, by oral gavage, and plasma concentrations of simvastatin and simvastatin acid were measured.

**Methods**

**Reagents:** Simvastatin, simvastatin acid, lovastatin, lovastatin acid and 1′-hydroxy midazolam were purchased from Toronto Research Chemicals (North York, ON, Canada). Midazolam and troleandomycin were purchased from Wako Pure Chemical Industries (Osaka, Japan). Diazepam, carboxymethylcellulose (CMC) and β-nicotinamide adenine dinucleotide phosphate, reduced form (NADPH), were purchased from Sigma Aldrich (St. Louis, MO). As a GTE, Sunphenon BG3™ was provided by Taiyo Kagaku (Yokkaichi, Japan), containing EGCG, EGC, EC and ECG at 43.4, 24.8, 9.7 and 1.7% with a total catechin content of 86.5% (w/w).

**Animal experiments:** Female SD rats (11 weeks, 250–300 g) (Japan SLC, Hamamatsu, Japan) were maintained at 24 ± 2°C with free access to food and water in a 12-h light-dark cycle. After overnight fasting, rats received single oral doses of 400 mg/kg GTE, 500 mg/kg troleandomycin or saline (control) by gavage. GTE was dissolved in distilled water, and troleandomycin was suspended in saline (pH 4.0). 30 minutes later, simvastatin suspended in 0.5% CMC was administered by gavage at a dose of 20 mg/kg. Blood samples were taken periodically from a tail vein up to 6 h after simvastatin administration. Plasma samples were stored at −80°C prior to analysis. The experimental procedure was approved by the Animal Ethical Committee of the University of Shizuoka.

**Assay of midazolam 1′-hydroxylation in rat hepatic microsomes:** Rat hepatic microsomes were prepared according to a previous report. Protein concentrations of microsomal samples were determined by Lowry’s method, and samples were stored at −80°C before use. The midazolam 1′-hydroxylation assay was performed according to a previous study with some modifications. The incubation mixture contained 100 mM potassium phosphate buffer (pH 7.4), rat hepatic microsomes (0.5 mg/mL protein) and midazolam (1, 5, 10, 25 and 50 µM) in a final volume of 100 µL. The reaction was initiated by the addition of 1 mM NADPH. Incubations were carried out for 5 min at 37°C with increasingly higher concentrations of GTE (from 0 to 100 µM), and terminated by adding 100 µL of 10 µM diazepam, an internal standard, in ice-cold methanol. The samples were then centrifuged at 10,000×g for 10 min at 4°C, and filtered through 0.2 µm membrane filters (Millex-LG, Millipore, Bedford, MA).

**Determination of simvastatin and simvastatin acid concentrations:** Simvastatin and simvastatin acid were extracted from plasma using a solid phase extraction system (Oasis HLB, Waters, Milford, MA), and their concentrations were quantified using a high-performance liquid chromatography (HPLC)/electrospray ionization (ESI) tandem mass spectrometry (MS/MS) system (Finnigan TSQ4000, Thermo Scientific, Waltham, MA). Lovastatin and lovastatin acid were used as internal standards for simvastatin and simvastatin acid, respectively. Chromatographic separation was carried out with the Agilent 1100 HPLC system (Agilent Technologies, Santa Clara, CA) using an Inertsil ODS-3 C18 analytical column (2.1 × 50 mm i.d., 2.0 µm; GL Science Inc., Tokyo, Japan) at 40°C. The mobile phases were 5 mM ammonium acetate with pH 4.3 (A) and acetonitrile (B) with a flow rate of 0.3 mL/min. The gradient of the mobile phase was 0–5.5 min, 58% (B); 5.5–6.0 min, 55–95% (B); and 6.0–8.5 min, 95% (B), followed by 4 min of column equilibration. The mass spectrometer was operated in the positive ionization mode for the detection of lactone compounds, and in the negative ionization mode for the detection of acid compounds. The monitored fragmentation reactions were from 441 m/z to 325 m/z for simvastatin, from 427 m/z to 325 m/z for lovastatin, from 435 m/z to 319 m/z for simvastatin acid, and from 421 m/z to 101 m/z for lovastatin acid. The lower limits of quantification were 0.1 ng/mL for simvastatin and 0.5 ng/mL for simvastatin acid. The interday coefficients of variation (CV) for simvastatin and simvastatin acid were 7% and 4%, respectively, at plasma concentrations of 10 ng/mL.

**Determination of 1′-hydroxy midazolam concentrations:** The concentrations of 1′-hydroxy midazolam in microsomal samples were quantified by ultra-performance liquid chromatography (UPLC)/ESI-MS using the Waters ACQUITY UPLC system. Chromatography was performed on an ACQUITY BEH C18 column (2.1 × 50 mm i.d., 1.7 µm; Waters) with a column oven temperature of 40°C. The mobile phases were 5 mM ammonium acetate (A) and acetonitrile (B) with a flow rate of 0.25 mL/min. The gradient of the mobile phase was 0–2.0 min, 40–60% (B), followed by 1.5 min of column equilibration. Selective ion monitoring was employed to detect [M+H]⁺ ions for 1′-hydroxy midazolam and diazepam, and the monitored m/z of 1′-hydroxy midazolam and diazepam was 342 and 326, respectively. The lower limit of quantification for 1′-hydroxy midazolam was 10 ng/mL with an interday CV of less than 15%.

**Data analysis:** Data are presented as the mean ± standard error mean (SEM). Enzyme kinetic analyses were performed using SigmaPlot 12.0 (Systat Software, San Jose, CA). IC₅₀ was defined as the concentration of inhibitor showing a 50% reduction in metabolite formation rate, and the value was estimated through nonlinear regression of relative reaction velocities at a single substrate concentration in the presence of varying inhibitor concentrations. The pharmacokinetic parameters of simvastatin and simvastatin acid were calculated using a non-compartmental analysis by WinNonlin (Pharsight, Mountain View, CA).
analyses were performed with a one-way ANOVA and Dunett’s test as a post-hoc test using Prism (GraphPad Software, San Diego, CA) with a criterion for significance of p < 0.05.

Results and Discussion

Our group previously reported that the consumption of green tea increased the bioavailability of simvastatin in a hypercholesterolemic patient, which poses the intriguing question about the mechanics that explain the pharmacokinetic interaction between green tea and simvastatin.12) Since simvastatin is extensively metabolized by the CYP3A subfamily, the present study was aimed to investigate the effects of an extract of green tea (Sunphenon BG3) on CYP3A activity in vitro and on simvastatin pharmacokinetics in vivo in rats.

The inhibitory effect of GTE on CYP3A activity was investigated in rat hepatic microsomes using midazolam as a CYP3A probe. The $K_{in}$ and $V_{max}$ values for midazolam 1′-hydroxylation were $1.3 \pm 0.1 \mu M$ and $4.4 \pm 0.1 \text{pmol/mg protein/min}$, and the intrinsic clearance was $3.4 \mu L/min/mg$ protein. When GTE was added to the incubation mixture at concentrations ranging from 1 to 100 $\mu g/mL$, midazolam 1′-hydroxylation was dose-dependently inhibited with an IC$_{50}$ of $12.5 \pm 1.4 \mu g/mL$ (Fig. 1A). This value is in good agreement with a previous study showing the IC$_{50}$ of GTE of $12.6 \mu g/mL$ for CYP3A-mediated testosterone 6β-hydroxylation in human hepatic microsomes, although the contents of catechins in the GTE were different.11) GTE also had an apparent $K_i$ of $18.8 \pm 2.7 \mu g/mL$ in a noncompetitive manner (Fig. 1B). Xia et al. suggested that if the IC$_{50}$ value of an inhibitor for CYP3A activity was less than 4 $\mu g/mL$, the inhibition would be moderate in rat hepatic microsomes.20) Accordingly, GTE used in this study was found to be a weak to moderate inhibitor for CYP3A.

To clarify the effects of GTE on the pharmacokinetics of simvastatin and simvastatin acid, a single oral dose of GTE (400 mg/kg) was administered to rats 30 min before the oral administration of simvastatin. Plasma concentration-time profiles and pharmacokinetic parameters of simvastatin and simvastatin acid are shown in Figure 2 and Table 1. Compared with the control, pretreatment with GTE resulted in a significant increase in the AUC$_{0-6}$ values of simvastatin by 3.4-fold. The $C_{max}$ of simvastatin was also increased, by 3.3-fold, with little change in the time at the maximum plasma concentration ($t_{max}$). The terminal half-life ($t_{1/2}$) of simvastatin was not changed by GTE, suggesting that GTE did not affect the elimination of simvastatin from blood. For simvastatin acid, the GTE increased the $C_{max}$ and AUC$_{0-6}$ by 2.8- and 2.0-fold, respectively. However, these values did not reach...
statistical significance due to large individual differences. Single pretreatment with troleandomycin, a specific CYP3A inhibitor, markedly elevated the \( C_{\text{max}} \) and AUC\(_{0.6}\) of simvastatin by 9.5- and 10.2-fold, respectively, as compared to the control. Pretreatment with troleandomycin also led to increases in the \( C_{\text{max}} \) and AUC\(_{0.6}\) of simvastatin acid by 3.3- and 3.0-fold, respectively, although we did not observe significant differences between the control and troleandomycin groups. Previous studies in rats showed that the coadministration of EGCG or GTE significantly increased the plasma concentrations of CYP3A substrate drugs including, diltiazem, midazolam, nicardipine, and tamoxifen.8-11) In accordance with these reports, our results indicate that GTE elevates the plasma concentration of simvastatin. We have recently demonstrated that after a single oral administration of GTE (400 mg/kg) to rats, \( C_{\text{max}} \) values of EC, ECG, EGC, and EGCG were 755, 110, 1,399 and 1,217 ng/mL, respectively.23) The total concentration of these four catechins was approximately 3.5 µg/mL, lower than the \( K_{\text{ppp}} \) value estimated in this study. This could support the hypothesis that the inhibition of hepatic CYP3A activity was weak in the case of a single oral administration of GTE. On the other hand, intestinal concentrations of catechins are considered to be higher than blood concentrations after the oral administration of GTE. Therefore, the results suggested that a single administration of GTE mainly inhibited simvastatin metabolism in the intestine, and might not affect the elimination of simvastatin.

Previous studies suggested that both simvastatin and simvastatin acid are substrates of several drug transporters such as P-glycoprotein (P-gp) and organic anion transporting polypeptide (OATP) 1B1.22) There is also a report showing an increased distribution of simvastatin to the brain and liver in multidrug-resistance gene (mdr1a/b) knockout mice as compared with wild-type mice.23) Jodoin et al. demonstrated that EGCG can modulate the bioavailability of a P-gp substrate.24) Thus, to elucidate whether GTE influences the activities of drug transporters in vivo, it is necessary to examine its effects on the tissue distribution of simvastatin by measuring the concentration of simvastatin in the tissue. In addition, it may also be necessary to investigate whether chronic treatment with GTE or increasing doses of GTE further alter the pharmacokinetics of simvastatin as well as the measurement of plasma concentration profiles of green tea catechins. Those studies will reveal more detailed mechanisms underlying green tea–simvastatin interaction. There is known to be a significant interspecies difference between humans and rats in the metabolism of simvastatin.15,25) Furthermore, our group recently reported that GTE and EGCG also inhibited CYP3A-mediated midazolam 1'-hydroxylation in human liver and intestinal microsomes.26) Therefore, further clinical studies are warranted to investigate the pharmacokinetic interaction between simvastatin and green tea.

The present study showed that GTE weakly to moderately inhibited CYP3A activity in a noncompetitive manner as evaluated by midazolam 1'-hydroxylation in rat hepatic microsomes. A single oral dose of GTE 30 min before simvastatin administration resulted in a significant elevation in plasma concentrations of simvastatin.

In conclusion, GTE may affect the pharmacokinetics of simvastatin and simvastatin acid in rats, possibly by inhibiting CYP3A in the intestine.

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References


