Title: Gemfibrozil and its glucuronide inhibit the OATP2 (OATP1B1: SLC21A6)-mediated hepatic uptake and CYP2C8-mediated metabolism of cerivastatin
- Analysis of the mechanism of the clinically relevant drug-drug interaction between cerivastatin and gemfibrozil -

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Running Title:
The mechanism of the drug-drug interaction between cerivastatin and gemfibrozil.

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A list of nonstandard abbreviations:
CER, cerivastatin; GEM, gemfibrozil; GEM-1-O-glu, gemfibrozil 1-O-β-glucuronide; OATP, organic anion transporting polypeptide; CYP, cytochrome P450; HMG-CoA, 3-hydroxy-3-methylglutaryl coenzyme A; LDL, low density lipoprotein; TG, triglyceride; DDI, drug-drug interaction; AUC, area
under the plasma concentration-time curve; $K_i$, inhibition constant; $IC_{50}$, concentration of inhibitor to produce a 50 % reduction in the metabolism or transport; HPLC, high performance liquid chromatography; MDCK, Madin-Darby canine kidney; HLM, human liver microsome; Ab, antibody; TLC, thin-layer chromatography; $CL_{uptake}$, uptake clearance; $CL_{int, all}$, overall hepatic intrinsic clearance; $CL_{int}$, hepatic intrinsic clearance; $PS_{u,influx}$, membrane permeability clearance of the unbound drug for the influx process; $PS_{u,efflux}$, membrane permeability clearance of the unbound drug for the efflux process

A recommended section assignment to guide the listing in the table of contents:
Absorption, Distribution, Metabolism, & Excretion
Abstract

A serious pharmacokinetic interaction between cerivastatin (CER) and gemfibrozil (GEM) has been reported. In the present study, we examined the inhibitory effects of GEM and its metabolites, M3 and gemfibrozil 1-O-β-glucuronide (GEM-1-O-glu), on the uptake of CER by human organic anion transporting polypeptide 2 (OATP2)-expressing cells, and its metabolism in cytochrome P450 (CYP) expression systems. Uptake studies showed that GEM and GEM-1-O-glu significantly inhibited the OATP2-mediated uptake of CER with IC$_{50}$ values of 72 and 24 µM, respectively. They also inhibited the CYP2C8-mediated metabolism of CER with IC$_{50}$ values of 28 and 4 µM, respectively, while M3 had no effects. GEM and GEM-1-O-glu minimally inhibited the CYP3A4-mediated metabolism of CER. The IC$_{50}$ values of GEM and GEM-1-O-glu for the uptake and the metabolism of CER obtained in the present study were lower than their total, and not unbound, plasma concentrations. However, considering the possibly concentrated high unbound concentrations of GEM-1-O-glu in the liver and its relatively larger plasma unbound fraction compared with GEM itself, the glucuronide inhibition of the CYP2C8-mediated metabolism of CER appears to be the main mechanism for the clinically relevant drug-drug interaction. Previously reported clinical drug interaction studies showing that coadministration of GEM with pravastatin or pitavastatin, both of which are known to be cleared from the plasma by the uptake transporters in the liver, only minimally (less than 2-fold) increased the AUC of these statins also supported our present conclusion.
3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase inhibitors (statins) and fibrates are now well-established treatments for hyperlipidaemia to prevent cardiovascular diseases (Wierzbicki et al., 2003). Statins are principally used to reduce low density lipoprotein (LDL), and also triglycerides (TG) in proportion to their LDL-lowering efficacy and the baseline TG level while fibrates are mainly used for the treatment of hypertriglyceridaemia or as second-line agents in patients with statin intolerance (Moghadasian et al., 2000; Wierbicki et al., 2003). The combination therapy of statins and fibrates is widely used in clinical practice. However, there are reports of rhabdomyolysis by this combination therapy, mainly involving gemfibrozil (GEM) with lovastatin and cerivastatin (CER) (Abdul-Ghaffar et al., 1995; Bruno-Joyce et al., 2001; Roca et al., 2002). This may be partly due to drug-drug interactions (DDI) caused by events at a pharmacokinetic level although an event at a pharmacodynamic level, i.e. a strong direct effect on myocytes by combination therapy, may be also involved (Kyrklund et al., 2001; Backman et al., 2002; Matzno et al., 2003). Indeed, concomitant use of GEM markedly increased the area under the plasma concentration-time curve (AUC) of simvastatin acid, lovastatin acid and CER and produced a small increase in the concentrations of pravastatin and pitavastatin (Backman et al., 2000 and 2002; Kyrklund et al., 2001 and 2003; Mathew et al, 2004). Staffa et al. (2002) reported that 31 patients taking CER died due to rhabdomyolysis and 12 of them were concomitantly taking GEM. Due to this severe side effect, CER was voluntarily withdrawn from the world market in August 2001.

Reports have appeared describing the mechanism of the
pharmacokinetic interaction between CER and GEM (Wen et al., 2001; Wang et al., 2002; Prueksaritanont et al., 2002a and 2002b). In humans, CER is subject to a dual metabolic pathway mediated by cytochrome P450 (CYP) 2C8 and 3A4 (Mück, 2000). Wen et al. (2001) and Wang et al. (2002) reported that GEM inhibits multiple isoforms of CYPs including 2C8 but has no inhibitory effect on 3A4. Therefore, the inhibition of CYP2C8-mediated metabolism may be one mechanism responsible for this clinically relevant DDI. In addition, Prueksaritanont et al. (2002c) have suggested that UGT-mediated glucuronidation of statins is an important metabolic pathway because they are spontaneously converted to lactones following UGT-mediated glucuronidation. Prueksaritanont et al. (2002a and 2002b) have also reported that GEM inhibits this UGT-mediated glucuronidation of CER as well as CYP-mediated oxidation. The inhibition constants (Ki) or the concentrations of inhibitors to produce a 50 % reduction in the metabolism of CER (IC50) in their reports were as high as the total plasma concentration of GEM in the therapeutic range (Wang et al. 2002; Prueksaritanont et al. 2002a and 2002b). However, taking the high protein binding of GEM into consideration, the unbound concentration of GEM is much less than the reported Ki or IC50 values (Todd and Ward, 1988; Wang et al. 2002; Prueksaritanont et al. 2002a).

We previously reported that CER was actively taken up into the liver via transporter(s) including organic anion transporting polypeptide 2 (OATP2 (OATP1B1); SLC21A6) (Shitara et al., 2003). We investigated the inhibitory effects of cyclosporin A on the transporter-mediated uptake of CER in hepatocytes and showed that cyclosporin A inhibited it without any effects on its
metabolism at therapeutic concentrations (Shitara et al., 2003). This partly explained the mechanism responsible for the clinically relevant DDI between CER and cyclosporin A (Shitara et al., 2003). However, until now, there have been no reports of the interaction between GEM and transporters. Gemfibrozil 1-O-β-glucuronide (GEM-1-O-glu), a metabolite of GEM, is taken up and accumulates in isolated perfused rat liver (Sallustio et al., 1996; Sabordo et al., 1999 and 2000). This uptake is inhibited by coadministration of dibromosulfophthalein and clofibric acid while acetoaminophen and its glucuronide produced no inhibition (Sabordo et al., 1999 and 2000). These results suggest an involvement of transporter(s) in the hepatic uptake of GEM-1-O-glu and, therefore, GEM-1-O-glu may affect the transporter-mediated uptake of CER.

GEM is metabolized to M1-4 by CYPs with M3 being the major metabolite (Nakagawa et al., 1991). GEM also undergoes glucuronidation, mainly to GEM-1-O-glu (Nakagawa et al., 1991) and the plasma concentrations of these metabolites are reported to be relatively high (Okerholm et al., 1976; Nakagawa et al., 1991). Therefore, in the present study, we examined the effects of GEM and its major metabolites, M3 and GEM-1-O-glu, on the metabolism and the transporter-mediated uptake of CER in order to analyze the mechanism of the clinically relevant DDI between CER and GEM.
Materials and Methods

Materials

[14C]CER (2.03 GBq/mmol) and unlabeled CER were kindly provided by Bayer AG (Wuppertal, Germany). GEM was purchased from Sigma-Aldrich (St. Louis, MO). A metabolite of GEM, M3 (purity: 99.6 %), was chemically synthesized in KNC Laboratories, Co. Ltd. (Kobe, Japan). GEM-1-O-glu was enzymatically synthesized from GEM. 2 mg/mL GEM was incubated at 37 °C with rat microsomes (3.6 mg protein/mL) and 5 mg/mL UDP glucuronic acid in 100 mM glycine-NaOH buffer (pH 8.5) supplemented with 20 % (w/v) glycerin for 27 h, followed by the addition of 1 N HCl to bring the pH to 4.0. This sample was chromatographed on an ODS column (Cosmosil 75C18-PREP, 300 mL, Nakalai Tesque, Kyoto, Japan) with MeOH:H2O (30:70 – 80:20, stepwise) as the mobile phase and analyzed by high performance liquid chromatography (HPLC) using an ODS column (Inertsil ODS-2, φ4.6 mm x 150 mm, GL Sciences, Inc., Tokyo, Japan) with a mobile phase of 0.05 % trifluoroacetic acid/acetonitrile (53/47) at a flow rate of 1.0 mL/min. The fraction containing GEM-1-O-glu, detected by its absorbance at 254 nm, was collected and evaporated. The fraction was rechromatographed on an ODS column with MeOH:H2O (50:50 – 80:20, stepwise) and purified, followed by evaporation, to obtain GEM-1-O-glu. The purity determined by HPLC detected by the absorbance at 230 nm was 99.4 %. The chemical structures and molecular weights of M3 and GEM-1-O-glu were confirmed by nuclear magnetic resonance (NMR) and mass spectrometry (MS) analyses, respectively. The
chemical structures of GEM and its metabolites used in the present study are shown in Figure 1. All other reagents were of analytical grade.

**Uptake of [\(^{14}\)C]CER in OATP2-expressing cells.**

An uptake study of CER in OATP2-expressing cells was conducted in the presence of GEM and its metabolites, M3 and GEM-1-O-glu. The construction of OATP2-expressing Madin-Darby canine kidney (MDCK) cells has been previously described (Sasaki et al., 2002). The uptake of [\(^{14}\)C]CER was examined by the method described previously (Shitara et al., 2003). The inhibitors, GEM (0-300 µM), M3 (0-1000 µM) or GEM-1-O-glu (0-300 µM), were added at the same time along with [\(^{14}\)C]CER when the uptake reaction was initiated.

**In vitro metabolism of CER**

To measure the effect of GEM and its metabolites on the metabolism of [\(^{14}\)C]CER and to estimate the contributions of CYP2C8 and 3A4, its *in vitro* metabolism was examined in CYP2C8- and 3A4-expressing insect cells supplemented with the expression of human CYP reductase and cytochrome b5 (Supersome\(^\text{®}\); BD Gentest, Woborn, MA), and pooled human liver microsomes (HLM: BD Gentest). To estimate the contribution of CYP2C8, HLM was preincubated with a specific inhibitory antibody (Ab) against CYP2C8 (BD Gentest: 0-10 µL/0.1 mg mg protein HLM) at 4 °C for 20 min. To estimate the contribution of CYP3A4, ketoconazole (0-1 µM), a potent CYP3A4 inhibitor, was used. Prior to the metabolism study, human CYP2C8 and 3A4 expression
systems (final 20 nM CYP) or HLM (final 0.2 mg protein/mL) were incubated at 37 °C for 10 min in 100 mM potassium phosphate buffer (pH 7.4) containing 3.3 mM MgCl₂, 3.3 mM glucose-6-phosphate, 0.4 U/mL glucose-6-phosphate dehydrogenase, 1.3 mM NADPH and 0.8 mM NADH. A 500 µL-volume of incubation mixture was transferred to a polyethylene tube and [¹⁴C]CER (0.25 µM) was added to initiate the reaction with GEM, M3 or GEM-1-O-glu (0-300 µM). After incubation for 30 min, the reaction was terminated by the addition of 500 µL ice-cold acetonitrile as this method had been shown to terminate the enzymatic reaction in a pilot study (data not shown), subsequently followed by centrifugation. To measure the metabolic rate of [¹⁴C]CER, the supernatant was collected and concentrated to approximately 20 µL in a centrifugal concentrator (VC-36N, TAITEC, Saitama, Japan), followed by thin-layer chromatography (TLC). The analyte was separated on silica-gel 60F₂₅₄ (Merck KGaA, Darmstadt, Germany) using a suitable mobile phase (toluene / acetone / acetic acid, 70 : 30 : 5, v/v/v). The intensity of the bands for intact [¹⁴C]CER and its metabolites separated by TLC was determined by the BAS 2000 system (Fuji Film, Tokyo, Japan).

**Protein binding of GEM and its metabolites**

To estimate the fraction not bound to human serum protein, 300 µM GEM, M3 and GEM-1-O-glu were added to serum (Nissui Pharmaceuticals, Inc., Tokyo, Japan) buffered with 50 mM potassium phosphate at 37°C and incubated for 2 min. After that, the sample underwent ultrafiltration (Amicon...
Centrifree, Millipore, Billerica, MA) and the GEM and its metabolites in the filtrate were determined by HPLC. To 0.5 mL filtrate, 0.5 mL phosphate buffered saline containing 5 µL 1 mM ibuprofen (internal standard) and 20 µL formic acid were added, followed by vigorous shaking. Subsequently, the sample was extracted with 5 mL ethyl acetate/cyclohexane (20/80), then 4 mL of the organic phase was collected and evaporated. The sample obtained was dissolved in 0.5 mL acetonitrile and separated on an ODS column (Super ODS column, φ4.6 mm x 150 mm, Tosoh, Tokyo, Japan). The mobile phase for GEM was 10 mM acetate buffer (pH 4.7) / acetonitrile (55/45) while that for M3 and GEM-1-O-glu was a mixture of 10 mM acetate buffer (pH4.7) and acetonitrile with a linear gradient from 70 % to 55 % acetate buffer for 30 min, and the flow rate was 1.0 mL/min for all analyses. The retention times for GEM and ibuprofen were 28 and 13 min, respectively, and those for M3, GEM-1-O-glu and ibuprofen were 13.5, 10 and 31 min, respectively. The absorbance was measured at 254 nm and quantitation was carried out by comparison with the absorbance of a standard curve prepared for each compound.

Data analysis

The time-courses of the uptake of [14C]CER into OATP2-expressing cells were expressed as the uptake volume [µL/mg protein] of radioactivity taken up into the cells [dpm/mg protein] divided by the concentration of radioactivity in the incubation buffer [dpm/µL]. The uptake velocity of [14C]CER was calculated using the uptake volume obtained at 2 min and expressed as the
uptake clearance (CL\textsubscript{uptake}: µL/min/mg protein). The metabolic rate of [\textsuperscript{14}C]CER was calculated by the decrease in unchanged [\textsuperscript{14}C]CER or the formation of its metabolites, M1 and M23.

To calculate the IC\textsubscript{50} values of GEM and its metabolites in terms of the OATP2-mediated uptake, the following equation was used:

\[
\Delta \text{CL}_{\text{uptake}} (+\text{inhibitor}) = \frac{\Delta \text{CL}_{\text{uptake}} (\text{control})}{1 + |I|\text{IC}_{50}} \quad \text{...(1)}
\]

where \(\Delta \text{CL}_{\text{uptake}}\) is the CL\textsubscript{uptake} for OATP2-mediated uptake, which is the CL\textsubscript{uptake} of [\textsuperscript{14}C]CER minus that estimated in the presence of excess unlabeled CER, \(\Delta \text{CL}_{\text{uptake}} (+\text{inhibitor})\) and \(\Delta \text{CL}_{\text{uptake}} (\text{control})\) are the \(\Delta \text{CL}_{\text{uptake}}\) values estimated in the presence and absence of inhibitors, respectively, and \(I\) is the inhibitor concentrations.

For the inhibitory effects of GEM and its metabolites on the metabolism of [\textsuperscript{14}C]CER in CYP2C8 and 3A4 expression systems, the IC\textsubscript{50} values were calculated from the following equation:

\[
v(+\text{inhibitor}) = \frac{v(\text{control})}{1 + |I|\text{IC}_{50}} \quad \text{...(2)}
\]

where \(v(+\text{inhibitor})\) and \(v(\text{control})\) are the metabolic rates of CER in the presence and absence of inhibitors, respectively.

These equations were fitted to the data obtained in the present study using a computerized version of the non-linear least squares method, WinNonlin\textsuperscript{®} (Pharsight, Mountain View, CA), to obtain the IC\textsubscript{50} values with computer calculated S.D. values.

For the inhibition study using pooled HLM, the observed values of the
metabolic rates were compared with the simulated values using the following equation:

$$v(+\text{inhibitor}) = v(\text{control}) \times \left( \frac{R_{\text{CYP2C8}}}{1 + \frac{1}{IC_{50,\text{CYP2C8}}}} + \frac{R_{\text{CYP3A4}}}{1 + \frac{1}{IC_{50,\text{CYP3A4}}}} \right) \ldots (3)$$

where, \(R_{\text{CYP2C8}}\) and \(R_{\text{CYP3A4}}\) are the contributions of CYP2C8 and 3A4 to the metabolism of CER (total metabolism and the formations of M1 and M23), respectively, and \(IC_{50,\text{CYP2C8}}\) and \(IC_{50,\text{CYP3A4}}\) are the IC\(_{50}\) values for CYP2C8- and 3A4-mediated metabolism of CER, respectively. For this simulation, the contributions of CYP2C8 and 3A4 to the formation of M1 and M23 in HLM were calculated based on the contributions of these enzymes to the total metabolism of CER in HLM and the ratio of the initial formation rate of each metabolite in CYP expression systems to that in HLM.
Results

Inhibitory effects of GEM and its metabolites on OATP2-mediated uptake of [14C]CER.

The effects of GEM, M3 and its glucuronide on the OATP2-mediated uptake of [14C]CER were examined (Figure 2). GEM and GEM-1-O-glu significantly inhibited OATP2-mediated uptake of [14C]CER without any effects on the uptake in vector-transfected cells while M3 did not show a statistically significant inhibition up to a concentration of 1000 µM (Figure 2). The IC₅₀ values of GEM and GEM-1-O-glu for the OATP2-mediated uptake of [14C]CER were 72.4 ± 28.4 and 24.3 ± 19.8 µM, respectively (mean ± S.D.).

Inhibitory effects of GEM and its metabolites on the in vitro metabolism of [14C]CER in CYP2C8 and 3A4 expression systems.

The in vitro metabolism of [14C]CER was examined in CYP2C8 and 3A4 expression systems. In the TLC analysis, one band for parent [14C]CER and two other bands for metabolites were detected in the CYP2C8 expression system while one band for [14C]CER and only one band for a metabolite were detected in the CYP3A4 expression system. The band for one of the metabolites produced by CYP2C8 and CYP3A4 matched, suggesting that these 2 enzymes produced the same metabolite. This metabolite was identified as M1 and the other was M23. The Rf values for [14C]CER, M1 and M23 were 0.13, 0.09 and 0.055, respectively. In Figure 3, the metabolism of [14C]CER in the CYP2C8 and 3A4 expression systems in the presence or absence of GEM
and its metabolites are shown. GEM and GEM-1-O-glu significantly inhibited the metabolism of [14C]CER both in CYP2C8 and 3A4 expression systems while M3 had no effects (Figure 3). GEM and GEM-1-O-glu preferentially inhibited CYP2C8-mediated metabolism compared with CYP3A4-mediated metabolism (Figure 3). The IC50 values of GEM and GEM-1-O-glu for the CYP2C8-mediated metabolism were 28.0 ± 4.3 and 4.07 ± 1.23 µM (mean ± S.D.), respectively, and the corresponding values for the CYP3A4-mediated metabolism were 372 ± 100 and 243 ± 59 µM (mean ± S.D.), respectively. In Figures 4 and 5, the CYP2C8-mediated M1 and M23 formation rates and the CYP3A4-mediated M1 formation rate in the presence or absence of GEM and GEM-1-O-glu are shown. GEM and GEM-1-O-glu inhibited CYP2C8-mediated M1 formation with IC50 values of 36.8 ± 5.3 and 5.38 ± 1.29 µM (mean ± S.D.), respectively, and M23 formation with IC50 values of 29.7 ± 4.4 and 4.30 ± 1.48 µM (mean ± S.D.), respectively (Figure 4). They slightly inhibited CYP3A4-mediated M1 formation with IC50 values of 406 ± 106 and 267 ± 62 µM (mean ± S.D.), respectively (Figure 5). The IC50 values of GEM and GEM-1-O-glu for the OATP2-mediated uptake and the CYP2C8- and 3A4-mediated metabolism of [14C]CER are summarized in Table 1.

Estimation of the contributions of CYP2C8 and 3A4 to the metabolism of [14C]CER in pooled HLM.

To estimate the contributions of CYP2C8 and 3A4, we examined the effect of a specific inhibitory Ab for CYP2C8 and ketoconazole, a potent inhibitor of
CYP3A4, on the metabolism of \([^{14}C]\text{CER}\) in the pooled HLM (Figure 6). Incubation with HLM produced three different metabolites detected by TLC, and one of them (Rf = 0.04) was identified as M24, a metabolite spontaneously produced from M1 and M23. In the present analysis, only the formation of M1 and M23 was analyzed. The inhibitory Ab for CYP2C8 inhibited the microsomal metabolism of \([^{14}C]\text{CER}\) in a concentration-dependent manner at low concentrations and maximum inhibition was obtained at 5 \(\mu\text{L}/100 \ \mu\text{g}\) microsomes (Figure 6a). At maximum inhibition, the microsomal metabolism of \([^{14}C]\text{CER}\) decreased to 38.9 ± 2.7 % (mean ± S.E.) of the control (Figure 6a) and, therefore, the contribution of CYP2C8 was estimated to be 61 %. In the presence of inhibitory Ab for CYP2C8, M23 formation was completely inhibited while M1 formation fell only to 61.2 ± 2.9 % (mean ± S.E.) of the control (Figures 6b and 6c). Ketoconazole also reduced the microsomal metabolism of \([^{14}C]\text{CER}\) in a concentration-dependent manner (Figure 6d). However, the inhibition studies using CYP2C8 and 3A4 expression systems showed that it inhibited not only CYP3A4-mediated metabolism but also that mediated by CYP2C8 (Figure 6d). At 0.1 \(\mu\text{M}\), most of the CYP3A4-mediated metabolism of \([^{14}C]\text{CER}\) was inhibited with only a minimal effect on that mediated by CYP2C8 (Figure 6d) and, therefore, 0.1 \(\mu\text{M}\) ketoconazole was used to estimate the contribution of CYP3A4. It was found that 0.1 \(\mu\text{M}\) ketoconazole reduced the metabolism of \([^{14}C]\text{CER}\) to 63.4 ± 7.2 % (mean ± S.E.) of the control (Figure 6d), suggesting that the contribution of CYP3A4 was, at most, 37 %. In addition, 0.1 \(\mu\text{M}\) ketoconazole reduced M1 formation to 62.6 ± 5.6 % (mean ± S.E.) of
the control and slightly, but not significantly, reduced M23 formation to 77.6 ± 11.3 % (mean ± S.E.) of the control (Figures 6e and 6f).

Inhibitory effects of GEM and its metabolites on the \textit{in vitro} metabolism of [\textsuperscript{14}C]CER in pooled HLM.

We examined the inhibitory effects of GEM and GEM-1-O-glu on the metabolism of [\textsuperscript{14}C]CER in pooled HLM (Figure 7). GEM and GEM-1-O-glu inhibited the metabolism of [\textsuperscript{14}C]CER in pooled HLM in a concentration-dependent manner (Figure 7) while M3 had no effects (data not shown). Figure 7 also shows simulation curves for the inhibitory effects of GEM and GEM-1-O-glu in pooled HLM based on equation (3).

Human serum protein binding of GEM and its metabolites.

We examined the protein binding of GEM and its metabolites in 50 mM phosphate-buffered human serum (pH7.4). The unbound fractions (f\textsubscript{u}) of GEM, M3 and GEM-1-O-glu were 0.648 ± 0.037, 1.23 ± 0.00, 11.5 ± 2.3 % (mean ± S.E.), respectively.
Discussion

It has already been reported that GEM is an inhibitor of CYP- and UGT-mediated metabolism of CER (Wang et al., 2002; Prueksaritanont et al. 2002a and 2002b). In the present study, we showed that GEM inhibited the OATP2-mediated uptake of CER as well as its metabolism (Figure 2). The IC\textsubscript{50} value of GEM for OATP2-mediated uptake of [\textsuperscript{14}C]CER (72 \text{ \textmu M}) was similar to or lower than the reported IC\textsubscript{50} values for metabolism (Wang et al., 2002; Prueksaritanont et al. 2002a and 2002b). We also found that a metabolite of GEM, GEM-1-O-glu, was a potent inhibitor of OATP2-mediated hepatic uptake of CER with a lower IC\textsubscript{50} value (24 \text{ \textmu M}) than that of GEM itself (Figure 2). This finding was matched by the fact that many glucuronides are recognized by OATP family transporters as substrates and/or inhibitors with a high affinity (König et al., 2000a and 2000b, Cui et al., 2001).

We also examined the inhibitory effect of GEM and its metabolites on the CYP2C8- and 3A4-mediated metabolism of [\textsuperscript{14}C]CER. In the present TLC analysis, two clear bands for metabolites were detected in the experiment using the CYP2C8 expression system while only one clear band for the metabolite was detected in the CYP3A4 experiment. As Wang et al. (2002) reported that CYP2C8 and 3A4 equally catalyzed the formation of M1 while the formation rate of M23 was 14-fold lower in CYP3A4 than in CYP2C8, we identified these two bands as M1 and M23, respectively. The results of the inhibition studies should be discussed in relation to previous reports (Wang et al., 2002; Prueksaritanont et al., 2002a). Wang et al. (2002) reported that GEM inhibited CYP2C8-mediated metabolism of CER to M1 and M23 with IC\textsubscript{50} values of 78
and 68 μM, respectively. The corresponding values in the present analysis were 37 and 30 μM, respectively (Figure 4), and these are comparable with the values reported by Wang et al. (2002). Prueksaritanont et al. (2002a) reported that the IC₅₀ values of GEM for M1 and M23 formations in HLM were 220 and 87 μM, respectively, and Wang et al. (2002) reported the corresponding values to be >250 and 95 μM, respectively. As shown in Figure 7, we also observed a concentration-dependent reduction in M1 and M23 formation in HLM. The apparent IC₅₀ values of GEM for M1 and M23 formation in HLM was calculated to be 234 and 26 μM, respectively (Figure 7), and these are also similar to results in previous reports (Wang et al., 2002; Prueksaritanont et al., 2002a).

Atypical effects of inhibitors were found in the present study, i.e. M3 apparently activated the CYP2C8-mediated metabolism of [¹⁴C]CER at low concentrations, and GEM-1-O-glu activated the CYP3A4-mediated metabolism and M1 formation at low concentrations while it inhibited them at higher concentrations (Figures 3 and 5), though these activations were not statistically significant with a few exceptions. These atypical effects of inhibitors may be explained by a multisite kinetic analysis involving a mixed effect of GEM and its metabolites as inhibitors and activators of enzymatic reactions (Galetin et al., 2002 and 2003). However, in the present study, the enhancing effect was, at most, 1.5-fold, and this would have only a minimal effect on drug disposition in clinical situations, if any. Therefore, we analyzed the effects of GEM and its metabolites by a simple equation (2).

In the present study, we showed that GEM and GEM-1-O-glu inhibited
both OATP2-mediated hepatic uptake and metabolism of $^{14}$C]CER. Hence, the coadministration of GEM may lead to a DDI due to the inhibition of hepatic uptake and/or metabolism of CER. The possibility of a clinically relevant DDI should be discussed taking the therapeutic concentration of GEM and its metabolites into consideration because the intrinsic hepatic clearance will fall to $1/(1+\frac{I}{IC_{50}})$ of control, where $I$ is the inhibitor concentration (Ueda et al., 2001).

In the report by Backman et al. (2002), the mean maximum concentration of GEM after repeated oral administration of 600 mg twice daily was 150 µM. Okerhelm et al. (1976) measured the plasma concentrations of free GEM, its glucuronide conjugates and other metabolites after a single oral administration of 600 mg $[^3]$H]GEM in normal human subjects receiving 600 mg unlabeled GEM twice daily for 6 days and reported that the maximum concentration of glucuronide conjugates, mainly GEM-1-O-glu, was approximately 20 µM while that of total GEM (GEM + glucuronide conjugate) was approximately 100 µM. Hengy and Kölle (1985) also reported that 10 - 15 % of GEM in plasma was present as glucuronide conjugates. The reported values of the total concentrations of GEM and GEM-1-O-glu were similar or higher than the $IC_{50}$ values for the metabolism and hepatic uptake of CER in the present study. However, because of the high plasma protein binding, the unbound concentrations of GEM and GEM-1-O-glu were at most 0.97 and 2.3 µM, respectively, i.e. less than the $IC_{50}$ values obtained in the present study. Because only unbound drugs interact with transporters, this result suggests that it is unlikely to cause the reported serious DDI between CER and GEM.
However, it is possible that GEM or its metabolites inhibit the metabolism of CER in the liver if they are actively transported to the liver and accumulate there. Indeed, Sallustio et al. (1996) have reported that GEM-1-O-glu is actively taken up by perfused rat liver and the liver/perfusate concentration ratio is 35 – 42. Assuming that it also accumulates in human liver, its unbound concentration there would be higher than the IC\textsubscript{50} value for the microsomal metabolism, which gives a \[1 + \frac{1}{\text{IC}_{50}}\] value of 3.1 - 3.2, i.e. more than a 3-fold reduction in the intrinsic hepatic clearance, suggesting that it may cause a serious DDI. In Table 2, the therapeutic total and unbound concentrations in the blood, the estimated unbound concentration in the liver and the effects on the intrinsic hepatic clearances are summarized.

In the present study, GEM and GEM-1-O-glu preferentially inhibited CYP2C8-mediated metabolism of CER compared with CYP3A4-mediated metabolism (Figures 3-5). These results support the findings by Backman et al. (2002) who reported that the AUC of M23 was markedly reduced to 17 % of the control while that of the open acid form of CER, the lactone form of CER and M1 were 4.4, 3.5 and 3.5 times higher than the control. As M23 formation is predominantly mediated by CYP2C8, and not by 3A4, the inhibition of CYP2C8 satisfactorily explains this DDI. In the report by Backman et al. (2002), all the AUC ratios for each of the metabolites to the open acid form of CER fell, following coadministration of GEM, to 82, 8.8 and 80 % of the control for M1, M23 and the lactone form of CER, respectively. The slight reduction in the AUC of M1 and the lactone form may be partly due to GEM and GEM-1-O-glu
inhibition of the hepatic uptake of CER, followed by M1 and M23 formation and lactonization in the liver.

Other statins, including simvastatin, lovastatin, pravastatin and pitavastatin, are also affected by the coadministration of GEM (Backman et al., 2000 and 2002; Kyrklund et al., 2001 and 2003; Mathew et al., 2004). GEM increases the AUC of the open acid form of these statins (Backman et al., 2000 and 2002; Kyrklund et al., 2001 and 2003; Mathew et al., 2004). However, it does not affect the AUC of the lactone form of simvastatin and lovastatin, and reduces that of pitavastatin (Backman et al., 2000; Kyrklund et al., 2001; Mathew et al., 2004), and it has no effect at all on the plasma concentration of fluvastatin (Spence et al., 1995). The limited effect of GEM only on the plasma concentrations of the open acid forms of simvastatin and lovastatin can be explained by inhibition of lactone formation followed by UGT-mediated glucuronidation (Prueksaritanont et al., 2002a). The reduced AUC of the lactone form of pitavastatin may also be explained by the same mechanism (Fujino et al., 2003). On the other hand, the increase in the AUC of the open acid forms of pravastatin and pitavastatin may be partly explained by minor inhibition of their OATP2-mediated uptake (Table 2), as these statins are substrates of OATP2 (Hsiang et al., 1999; Nakai et al., 2001; Hirano et al., 2004). GEM increases the AUC of pravastatin and pitavastatin only by 2.0- and 1.5-fold, respectively, while it increases that of CER 4.4-fold (Backman et al., 2002; Kyrklund et al., 2003; Mathew et al., 2004). The increase in the AUC of pravastatin can be partly explained by its reduced renal excretion and, therefore, the effect of GEM on its elimination in the liver is weaker (Kyrklund et
al., 2003). On the other hand, cyclosporin A, an inhibitor of OATP2, markedly increases the AUC of pravastatin and pitavastatin as well as CER (Regazzi et al., 1993; Hasunuma et al., 2003). The variety of effects on different statins may be due to the fact that GEM and its metabolites inhibit both the uptake and CYP2C8-mediated metabolism of CER in the liver while they minimally inhibit only the hepatic uptake of pravastatin and pitavastatin (Table 2) and, on the other hand, cyclosporin A inhibits their hepatic uptake at therapeutic concentrations (Shitara et al., 2003).

In conclusion, we have shown that GEM moderately inhibits while GEM-1-O-glu potently inhibits the CYP2C8-mediated metabolism and both moderately inhibit the OATP2-mediated hepatic uptake of drugs. Their inhibition of the CYP2C8-mediated metabolism of CER (mainly by GEM-1-O-glu) is a major mechanism which governs the clinically relevant DDI between CER and GEM, while their inhibition of the OATP2-mediated hepatic uptake of CER may also contribute to the DDI, but to a lesser extent.
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References


(OATP1B1) and OATP8 (OATP1B3) to the hepatic uptake of pitavastatin in humans. *J Pharmacol Exp Ther* accepted.


Footnotes

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Figure Legends

Figure 1

**Chemical structures of GEM and its metabolites.**

(a) GEM, (b) M3, (c) GEM-1-O-glu

Figure 2

**Effect of GEM and its metabolites on the OATP2-mediated uptake of \[^{14}C\]CER.**

The inhibitory effects of GEM (a), M3 (b) and GEM-1-O-glu (c) on the OATP2-mediated uptake of \[^{14}C\]CER were examined. Uptake of \[^{14}C\]CER in OATP2-expressing (■) and vector-transfected cells (□) in the presence of GEM and its metabolites is shown. Uptake of \[^{14}C\]CER in the presence of excess unlabeled CER (30 μM) was also examined (●). Each symbol represents the mean value of three independent experiments ± S.E. and solid lines represent the fitted lines. The asterisk represents a statistically significant difference shown by Dunnet’s test (∗…p < 0.05, ∗∗…p < 0.01).

Figure 3

**Effect of GEM and its metabolites on the CYP2C8- and 3A4-mediated metabolism of \[^{14}C\]CER.**

Inhibitory effects of GEM (a, d), M3 (b, e) and GEM-1-O-glu (c, f) on the metabolism of \[^{14}C\]-CER in CYP2C8- (a, b, c) and 3A4- (d, e, f) expression
systems were examined. Each symbol represents the mean value of three independent experiments ± S.E. and solid lines represent the fitted lines. Asterisk represents statistically significant difference by Dunnet’s test (*...p < 0.05, **...p < 0.01).

Figure 4
Effect of GEM and GEM-1-O-glu on the CYP2C8-mediated M1 and M23 formation of [14C]CER.

Inhibitory effects of GEM (a, c) and GEM-1-O-glu (b, d) on the CYP2C8-mediated M1 formation (a, b) and M23 formation (c, d) were examined. Each symbol represents the mean value of three independent experiments ± S.E. and solid lines represent the fitted lines. The asterisk represents a statistically significant difference shown by Dunnet’s test (*...p < 0.05, **...p < 0.01).

Figure 5
Effect of GEM and GEM-1-O-glu on the CYP3A4-mediated M1 and M23 formation of [14C]CER.

Inhibitory effects of GEM (a) and GEM-1-O-glu (b) on the CYP3A4-mediated M1 formation were examined. Each symbol represents the mean value of three independent experiments ± S.E. and solid lines represent the fitted lines. The asterisk represents a statistically significant difference shown by Dunnet’s test (*...p < 0.05, **...p < 0.01).
Figure 6

**Effect of specific inhibitory antibody against CYP2C8 (anti-CYP2C8 Ab) and ketoconazole on the metabolism of CER in HLM.**

The inhibitory effects of anti-CYP2C8 Ab (a) and ketoconazole, a potent CYP3A4 inhibitor, (d) on the metabolism of $[^{14}C]CER$ in pooled HLM (■) were examined. Their effects on M1 formation (b, e) and M23 formation (c, f) are also represented. The effect of ketoconazole on the metabolism of $[^{14}C]$-CER in CYP2C8- (□) and CYP3A4- (○) expression systems was also examined. Each symbol represents the mean value of three independent experiments ± S.E. and solid lines represent the fitted lines. Symbols (*, + and #) represent a statistically significant difference from the control values shown by Dunnet’s test (*…p < 0.05, **…p < 0.01 for the metabolism in HLM, +…p < 0.05, ++…p < 0.01 for CYP2C8-mediated metabolism and #…p < 0.05, ##…p < 0.01 for CYP3A4-mediated metabolism).

Figure 7

**Effect of GEM and its metabolites on the metabolism of $[^{14}C]$-CER in HLM.**

The inhibitory effects of GEM (a, c, e) and GEM-1-O-glu (b, d, f) on the metabolism of $[^{14}C]CER$ (a, b) and the formation of M1 (c, d) and M23 (e, f) in pooled HLM were examined. Each symbol represents the mean value of three independent experiments ± S.E. Solid lines represent simulated lines based on equation (5). The asterisk represents a statistically significant difference shown by Dunnet’s test (*…p < 0.05, **…p < 0.01).
Table 1

IC₅₀ values of GEM and GEM-1-O-glu on the OATP2-mediated uptake and metabolism of [¹⁴C]CER a).

<table>
<thead>
<tr>
<th></th>
<th>GEM [µM]</th>
<th>GEM-1-O-glu [µM]</th>
</tr>
</thead>
<tbody>
<tr>
<td>OATP2-mediated uptake</td>
<td>72.4 ± 28.4</td>
<td>24.3 ± 19.8</td>
</tr>
<tr>
<td>CYP2C8-mediated metabolism</td>
<td>28.0 ± 4.3</td>
<td>4.07 ± 1.23</td>
</tr>
<tr>
<td>CYP3A4-mediated metabolism</td>
<td>372 ± 100</td>
<td>243 ± 59</td>
</tr>
</tbody>
</table>

a) All data are represented as the mean ± computer-calculated S.D.
Table 2

Plasma and liver concentrations of GEM and its metabolites, and their estimated inhibitory effects on the elimination of CER in the liver in clinical situations a).

<table>
<thead>
<tr>
<th></th>
<th>GEM</th>
<th>GEM-1-O-glu</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>C&lt;sub&gt;max&lt;/sub&gt;</strong> b)</td>
<td>[µM]</td>
<td></td>
</tr>
<tr>
<td>1+I/IC&lt;sub&gt;50_OATP2&lt;/sub&gt;</td>
<td>2.4 - 3.1</td>
<td>1.8</td>
</tr>
<tr>
<td>1+I/IC&lt;sub&gt;50_metabolism&lt;/sub&gt;</td>
<td>2.2 - 2.6</td>
<td>2.2</td>
</tr>
<tr>
<td><strong>C&lt;sub&gt;max,u&lt;/sub&gt;</strong> c)</td>
<td>[µM]</td>
<td></td>
</tr>
<tr>
<td>1+I/IC&lt;sub&gt;50_OATP2&lt;/sub&gt;</td>
<td>1.0</td>
<td>1.1</td>
</tr>
<tr>
<td>1+I/IC&lt;sub&gt;50_metabolism&lt;/sub&gt;</td>
<td>1.0</td>
<td>1.3</td>
</tr>
<tr>
<td><strong>C&lt;sub&gt;max,u,liver&lt;/sub&gt;</strong> d)</td>
<td>[µM]</td>
<td></td>
</tr>
<tr>
<td>1+I/IC&lt;sub&gt;50_metabolism&lt;/sub&gt;</td>
<td>-</td>
<td>81 - 97</td>
</tr>
</tbody>
</table>

a) Inhibitory effects of GEM and GEM-1-O-glu are represented by 1+inhibitor concentration (I)/IC<sub>50</sub>.

b) Plasma concentrations of GEM and GEM-1-O-glu are reported by Backman et al. (2002) and Okerhelm et al. (1976).

c) Plasma unbound concentrations.

d) Estimated unbound concentrations in the liver.
Cl\textsubscript{uptake} of [\textsuperscript{14}C]CER [\mu L/min/mg protein]

Concentration of GEM [\mu M]

Concentration of M3 [\mu M]

Concentration of GEM-1-O-glu [\mu M]

(a) (b) (c)
CYP2C8-mediated M1 formation [nmol/min/nmol CYP] vs. Concentration of GEM [µM]

CYP2C8-mediated M23 formation [nmol/min/nmol CYP] vs. Concentration of GEM-1-O-glu [µM]

(a) (b) (c) (d)

** Indicates significance at the 0.05 level.

*** Indicates significance at the 0.01 level.

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CYP3A4-mediated M1 formation [nmol/min/nmol CYP]

Concentration of GEM [µM]

Concentration of GEM-1-O-glu [µM]