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Glucuronidation Converts Gemfibrozil to a Potent, Metabolism-Dependent Inhibitor of CYP2C8: Implications for Drug-Drug Interactions

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Abbreviations used are: CYP, Cytochrome P450; HLM, human liver microsomes; IC_{50}, inhibitor concentration that causes 50% inhibition; LC/MS-MS, liquid chromatography/tandem mass spectrometry; K_I, inhibitor concentration that supports half the maximal rate of inactivation; k_{inact}, maximal rate of enzyme inactivation; OATP, organic anion transporting polypeptide; UGT, UDP-glucuronosyltransferase.

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ABSTRACT

Gemfibrozil more potently inhibits CYP2C9 than CYP2C8 in vitro, and yet the opposite inhibitory potency is observed in the clinic. To investigate this apparent paradox, we evaluated both gemfibrozil and its major metabolite, an acyl-glucuronide (gemfibrozil 1-*O*-β-glucuronide) as direct-acting and metabolism-dependent inhibitors of the major drug-metabolizing cytochrome P450 (CYP) enzymes (CYP1A2, 2B6, 2C8, 2C9, 2C19, 2D6, and 3A4) in human liver microsomes. Gemfibrozil most potently inhibited CYP2C9 (IC_{50} of 30 μM), whereas, gemfibrozil glucuronide most potently inhibited CYP2C8 (IC_{50} of 24 μM). Unexpectedly, gemfibrozil glucuronide, but not gemfibrozil, was found to be a metabolism-dependent inhibitor of CYP2C8 only. The IC_{50} for inhibition of CYP2C8 by gemfibrozil glucuronide decreased from 24 μM to 1.8 μM after a 30-min incubation with human liver microsomes and NADPH. Inactivation of CYP2C8 by gemfibrozil glucuronide required NADPH, and proceeded with a K_I (inhibitor concentration that supports half the maximal rate of enzyme inactivation) of 20-52 μM and a k_{inact} (maximal rate of inactivation) of 0.21 min^{-1}. Potent inhibition of CYP2C8 was also achieved by first incubating gemfibrozil with alamethicin-activated human liver microsomes and UDP-glucuronic acid (to form gemfibrozil glucuronide) followed by a second incubation with NADPH. LC/MS/MS analysis established that human liver microsomes and recombinant CYP2C8 both convert gemfibrozil glucuronide to a hydroxylated metabolite, with oxidative metabolism occurring on the dimethylphenoxy moiety (the group furthest from the glucuronide moiety). The results described have important implications for the mechanism of the clinical interaction reported between gemfibrozil and CYP2C8 substrates such as cerivastatin, repaglinide, rosiglitazone, and pioglitazone.

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INTRODUCTION

There have been several reports of clinical interactions between gemfibrozil (e.g., Lopid, Parke-Davis) and CYP2C8 substrates such as cerivastatin, repaglinide, rosiglitazone, and pioglitazone (Backman et al., 2002; Niemi et al., 2003a; Niemi et al., 2003b; Jaakkola et al., 2005). Reports on the in vitro inhibitory potential of gemfibrozil demonstrated that this lipid-lowering drug is a more potent inhibitor of CYP2C9 than CYP2C8 (Wen et al., 2001; Wang et al., 2002; Fujino et al., 2003). However, in the clinic, gemfibrozil is a more potent inhibitor of CYP2C8 than CYP2C9. Coadministration of gemfibrozil with the CYP2C9 substrate warfarin does not increase the plasma concentrations of either *R*- or *S*-warfarin (in fact, it actually decreases them) (Lilja et al., 2005). An important step in providing a potential explanation for why gemfibrozil is a more potent inhibitor of CYP2C9 than CYP2C8 in vitro but is a more potent inhibitor of CYP2C8 than CYP2C9 in vivo was provided by Shitara et al. (2004), who demonstrated that gemfibrozil 1-*O*-β-glucuronide is a more potent inhibitor than gemfibrozil of CYP2C8. These same authors demonstrated that gemfibrozil 1-*O*-β-glucuronide inhibits in vitro the CYP2C8-mediated metabolism of cerivastatin, as well as the OATP2-mediated uptake of cerivastatin (Shitara et al., 2004). Other recent reports of glucuronide metabolites interacting directly with CYP2C8 have been published, including the CYP2C8-mediated hydroxylation of the acyl glucuronide of diclofenac, and the 17β-*O*-glucuronide of estradiol (Kumar et al., 2002; Delaforge et al., 2005).

This study was designed to compare the in vitro inhibitory potential of gemfibrozil and its 1-*O*-β-glucuronide towards the major human drug-metabolizing CYP enzymes, namely CYP1A2, 2B6, 2C8, 2C9, 2C19, 2D6, and 3A4. In addition to confirming that gemfibrozil glucuronide is a more potent inhibitor of CYP2C8 than is gemfibrozil, we discovered that gemfibrozil glucuronide is a metabolism-dependent inhibitor of CYP2C8, suggesting that the clinical interactions between gemfibrozil and CYP2C8 substrates involves, at least in part, the conversion of gemfibrozil to an acyl glucuronide followed by the NADPH-dependent inactivation of CYP2C8 (Fig. 1).

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The conversion of gemfibrozil to its acyl glucuronide, and the subsequent metabolism-dependent inhibition of CYP2C8 by gemfibrozil glucuronide, could also be demonstrated in vitro by incubating gemfibrozil with alamethicin-activated human liver microsomes and UDP-glucuronic acid (to form the acyl glucuronide) followed by incubation with NADPH. This two-step procedure may serve as a generic method to screen drugs for their ability to be converted to a glucuronide that inhibits CYP enzymes either directly or in a metabolism-dependent manner.

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MATERIALS AND METHODS

Chemicals and Reagents

Gemfibrozil was purchased from Sigma Chemical Co. (St. Louis, MO). Gemfibrozil 1-*O*-β-glucuronide was prepared as described below. Human liver microsomes (pooled from 16 donors) were prepared and characterized at XenoTech LLC (Lenexa, KS), and recombinant CYP2C8 (Bactosomes) was obtained from Cypex (Dundee, Scotland). All other reagents were obtained from commercial sources, most of which have been described elsewhere (Robertson et al., 2000; Madan et al., 2002).

Synthesis of gemfibrozil glucuronide

Gemfibrozil (4 mM) was incubated at 37^{o}C for 5 hours with liver 9000 × *g* supernatant fraction from Aroclor 1254-induced rats (7.2 mg/mL) in a 50-mL reaction mixture containing 100 mM glycine buffer (pH 8.6), 10% glycerol (v/v), 2.2% acetonitrile (v/v) and 8 mM UDP-glucuronic acid. The reaction was quenched with 2 mL of 2N HCl. Gemfibrozil glucuronide was purified by semi-preparative HPLC on a YMC ODS A 30 × 100 mm, S5 column at a flow rate of 10 mL/min. The step-wise linear gradient was 10% to 20% Solvent B over 5 min, and from 20% to 90% Solvent B over 34 min, where Solvent A was 0.1% formic acid in water and Solvent B was 0.1% formic acid in acetonitrile. Fractions containing gemfibrozil glucuronide were pooled and lyophilized. Gemfibrozil glucuronide was obtained as a white solid (44% yield, HPLC purity: >99% at 275 nm). LC/MS (ESI $[M-H]^-$): m/z 425; MS^2: m/z 425→ m/z 249, 175; MS^3: m/z 425 → m/z 249 → m/z 121. High resolution MS: $C_{21}H_{30}O_9$, calculated m/z for $[M+Na^+]$: 449.17875, found 449.1777 (error, -2.4 ppm). ^{1}H NMR (400 MHz, DMSO-D6 + D_2O) δ ppm 1.15 (s, 3 H), 1.15 (s, 3H), 1.64 (s, 2 H), 2.06 (s, 3H), 2.22 (s, 3H), 3.17 (t, *J*=8.56 Hz, 1 H), 3.27 (t, *J*=8.80 Hz, 1 H), 3.33 (t, *J*=9.05 Hz, 1 H), 3.70 (d, *J*=9.29 Hz, 1 H), 3.83 - 3.91 (m, *J*=4.89 Hz, 1 H), 5.34 (d, *J*=8.31 Hz, 1 H), 6.60 (d, *J*=7.34 Hz, 1 H), 6.68 (s, 1H), 6.96 (d, *J*=7.34 Hz, 1 H).

CYP Enzyme Inhibition: IC_{50} determination

Unless indicated otherwise, CYP activities in human liver microsomes were determined according to previously published procedures (Pearce et al., 1996; Robertson et al., 2000; Madan et al., 2002; Toren et al., 2004). In general, incubations were conducted in 400-μL incubation mixtures (pH 7.4) containing high purity water, potassium phosphate buffer (50 mM), $MgCl_2$ (3 mM), EDTA (1 mM), NADP (1 mM), glucose-6-phosphate (5 mM), glucose-6-phosphate dehydrogenase (1 Unit/mL), marker substrate and human liver microsomes (50 – 100 μg protein/mL). The concentration of each marker substrate was based on previously determined Km values as follows: 20 μM phenacetin (for CYP1A2), 50 μM bupropion (for CYP2B6), 15 μM paclitaxel (for CYP2C8), 4 μM diclofenac (for CYP2C9), 35 μM *S*-mephenytoin (for CYP2C19), 5 μM dextromethorphan (for CYP2D6), 100 μM testosterone (for CYP3A4), and 3 μM midazolam (second substrate for CYP3A4). All incubations were conducted at 37°C for 5 min prior to addition of the stop reagent with internal standard, which in most cases was a deuterated analog of the metabolite measured. Positive controls for both direct and metabolism-dependent inhibition were included at a single concentration.

Aliquots of the stock and/or working solutions of either gemfibrozil or its 1-*O*-β-glucuronide were manually added to buffer mixtures containing the components described above, but prior to addition of the NADPH-generating system. Fresh solutions were prepared on the day of the experiment.

To evaluate the potential for metabolism-dependent inhibition, gemfibrozil or its 1-*O*-β-glucuronide (at the same concentrations used to evaluate direct inhibition) were incubated at 37°C with human liver microsomes and an NADPH-generating system for approximately 30 min. Reactions were initiated by the addition of the NADPH-generating system. After the 30-min preincubation, the marker substrate (at a concentration approximately equal to its Km) was added, and the incubation was continued for 5 min to measure residual CYP activity.

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K_I and k_{inact} determination

To determine the K_I and k_{inact} values for the inactivation of CYP2C8, various concentrations of gemfibrozil glucuronide (0.25 to 64 μM) were incubated for 2 to 40 min with pooled human liver microsomes (0.1 and 1.0 mg/mL) at 37ºC . After the preincubation, an aliquot (40 μL) was transferred to another incubation tube (final volume 400 μL) containing 10 μM paclitaxel and an NADPH-generating system in order to measure residual CYP2C8 activity. This procedure diluted the microsomes to 10 or 100 μg/mL and diluted gemfibrozil glucuronide to one tenth its original concentration to minimize any direct inhibitory effects on CYP2C8. The incubations were then continued for 2 min to allow for conversion of paclitaxel to 6α-hydroxypaclitaxel. A short incubation with paclitaxel was used to minimize additional inactivation of CYP2C8 when measuring residual CYP2C8 activity. Reactions were carried out in triplicate.

Metabolism of gemfibrozil glucuronide

Gemfibrozil glucuronide (64 μM) was incubated for zero or 30 min with pooled human liver microsomes (1 mg/mL) or recombinant CYP2C8 (~40 pmol/mL) in potassium phosphate buffer (50 mM, pH 7.4), $MgCl_2$ (3 mM), EDTA (1 mM), NADP (1 mM), glucose-6-phosphate (5 mM) and glucose-6-phosphate dehydrogenase (1 Unit/mL). The samples were stopped with an equal volume of acetonitrile and 4% formic acid, and precipitated protein was removed by centrifugation. Aliquots of these samples were analyzed on an API-4000 Q-trap (see "Analytical methods") using TurboIonSpray source in the negative ionization mode. Data acquisition was conducted using Analyst software (version 1.4) with information-dependent-acquisition (IDA) scan function. Constant neutral loss survey scans at 176 Da were used to trigger product ion data acquisition on the gemfibrozil glucuronide. Separations were performed using a Develosil RP 150 x 2.0 mm analytical column with a linear gradient over 45 minutes.

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Two-step activation of gemfibrozil to a CYP2C8 inhibitor

Having obtained evidence that gemfibrozil glucuronide is a metabolism-dependent inhibitor of CYP2C8, we evaluated whether such inhibition could be achieved starting with gemfibrozil in a two-step process. Accordingly, gemfibrozil was incubated with human liver microsomes in the presence of UDP-glucuronic acid (to support the formation of gemfibrozil glucuronide) and then in the presence of NADPH (to support the metabolism-dependent inactivation of CYP2C8 by gemfibrozil glucuronide). Pooled human liver microsomes (1.1 mg) were preincubated with 27.5 μg alamethicin (a pore-forming peptide that increases microsomal UGT activity without compromising CYP activity (Fisher et al., 2000)) on ice in a total volume of 2 mL. Aliquots (250 μL) of the alamethicin-activated microsomes were transferred to a second set of tubes containing 2.0 mL of potassium phosphate buffer (55 mM, pH 7.4), $MgCl_2$ (3.3 mM), EDTA (1.1 mM), saccharic acid 1,4-lactone (100 μM) and gemfibrozil (30 or 100 μM) for a final microsomal protein concentration of 50 μg/mL. Glucuronidation reactions were started by the addition of 250 μL of a 40 mM solution of UDP-glucuronic acid (final concentration = 4 mM). After a 0 or 60-min incubation at 37°C, the samples were placed on ice. The samples were then divided into 0- and 30-min NADPH-preincubation samples. After a 0- or 30- min preincubation with NADPH, an aliquot (40 μL) was transferred to another incubation tube (final volume 400 μL) containing paclitaxel (10 μM) and an NADPH-generating system in order to measure residual CYP2C8 activity. This procedure diluted the microsomal protein to 5 μg/mL and diluted gemfibrozil and its glucuronide to one tenth their original concentration to minimize any direct inhibitory effects on CYP2C8. The incubations were then continued for 5 min to allow for conversion of paclitaxel to 6α-hydroxypaclitaxel.

Analytical methods

All analyses were performed with an Applied Biosystems / Sciex API2000, API3000, or API-4000 Q-trap HPLC-MS/MS systems equipped with an electrospray (Turbo IonSpray) ionization source (Applied Biosystems, Foster City, CA), two LC-10ADvp pumps with an SCL-10Advp controller, SIL-HTA autosampler and DGU-14 solvent degasser (Shimadzu, Columbia, MD). The HPLC column used was a

Phenomenex Develosil RPAqueous, 5 µm, 50 × 2.0 mm column (Torrance, CA), which was preceded by a direct connection guard column with a C8, 4.0 mm × 2.0 mm cartridge (Phenomenex, Torrance, CA).

Statistical Analyses

Data for the IC_{50} determinations were processed with a validated customized add-in (DI IC_{50} LCMS Template version 1.1) for the computer program Microsoft Excel, (Office 2000; Microsoft Inc., Redmond, WA). When inhibition of CYP enzymes was observed, the data were processed to determine IC_{50} values by nonlinear regression with XLFit (Version 3.0, IDBS, Limited, Surrey, UK).

Data for the K_I and k_{inact} investigation were analyzed to determine the rate of enzyme inactivation at each inhibitor concentration tested (Kitz and Wilson, 1962). Based on this method, the natural log of the ratio of the residual activity (E_i) to the control activity (E_t) (where the residual activity is the rate after a defined incubation period with the inhibitor) was plotted against incubation time for each concentration of inhibitor. Linear regression (GraFit, Version 4.021, Erithacus Software, Ltd., Surrey, UK) was then performed such that (-1)× the value of the slope represents the observed initial rate of enzyme inactivation at each concentration of inhibitor. The rates of inactivation were then plotted against the inhibitor concentration and the data fitted by non-linear regression (GraFit, Version 4.021, Erithacus Software, Ltd., Surrey, UK) according to the following equation (Jones et al., 1999):

$$\lambda = \frac{k_{\text{inact}} \cdot [\text{I}]}{(K_{\text{I}} + [\text{I}])} \qquad (1)$$

This equation is analogous to the Michaelis-Menten equation where λ represents the rate constant for inactivation at each inhibitor concentration, [I] is the inhibitor concentration, K_I is the inhibitor concentration that produces half the maximal rate of inactivation (analogous to Km) and k_{inact} represents the maximal rate of inactivation (analogous to Vmax). This equation assumes there is negligible change in inhibitor concentration during incubation and that the loss of enzyme activity is due to inactivation by a first-order process.

RESULTS

Effect of gemfibrozil and gemfibrozil 1-*O*-β-glucuronide on selected CYP enzymes: IC_{50} determinations. Gemfibrozil and gemfibrozil glucuronide were evaluated as direct-acting and metabolism-dependent inhibitors of the major CYP enzymes in human liver microsomes, namely CYP1A2, 2B6, 2C8, 2C9, 2C19, 2D6 and 3A4, the activities of which were assessed with marker substrates at concentrations equal to Km. The results are summarized in Table 1. Gemfibrozil was a more potent inhibitor of CYP2C9 than CYP2C8 (IC_{50} value of 30 μM versus 120 μM, respectively), whereas the converse was true of gemfibrozil glucuronide (the IC_{50} value was 24 μM for CYP2C8 and > 300 μM for CYP2C9).

Gemfibrozil inhibited CYP1A2 (IC_{50} 99 μM) and CYP2C19 (IC_{50} 100 μM) with slightly greater potency than it inhibited CYP2C8 (IC_{50} 120 μM), but it did not inhibit CYP2B6 or CYP2D6 (IC_{50} > 300 μM), nor did it inhibit CYP3A4 regardless of whether testosterone or midazolam was used as marker substrate. As summarized in Table 1, preincubation of gemfibrozil with NADPH-fortified human liver microsomes for 30 min did not increase its inhibitory effect, suggesting that gemfibrozil is not a metabolism-dependent inhibitor of the CYP enzymes examined. This is further illustrated in Fig. 2A, which shows that preincubation had no effect on the potency with which gemfibrozil inhibited CYP2C8 activity toward paclitaxel.

With the notable exception of CYP2C8, gemfibrozil glucuronide was less potent than gemfibrozil at inhibiting all of the CYP enzymes examined. Consequently, gemfibrozil glucuronide was a more selective inhibitor than gemfibrozil in that gemfibrozil glucuronide was at least an order of magnitude more potent as an inhibitor of CYP2C8 than the other CYP enzymes examined. Furthermore, the potency with which gemfibrozil glucuronide inhibited CYP2C8 increased by a further order of magnitude

following a 30-min preincubation with NADPH-fortified human liver microsomes, as shown in Fig. 2B. No such increase in inhibitory potency was apparent with any of the other CYP enzymes examined.

The marked increase in CYP2C8 inhibition caused by preincubating gemfibrozil glucuronide with human liver microsomes for 30 min was dependent on the presence of NADPH. In the presence of NADPH, the 30-min preincubation resulted in more than a 10-fold shift in IC_{50} (from 24 to 1.8 μM), whereas in its absence the preincubation actually resulted in a slight decrease in inhibitory potency (the IC_{50} value was 48 μM [results not shown]).

Inactivation of CYP2C8 by gemfibrozil 1-*O*-β-glucuronide: K_I and k_{inact} determination. The results in Fig. 2b suggest that gemfibrozil glucuronide is a metabolism-dependent inhibitor of CYP2C8. Accordingly, experiments were performed to determine K_I (the concentration of gemfibrozil glucuronide supporting half maximal rate of CYP2C8 inactivation) and k_{inact} (the first order rate constant for CYP2C8 inactivation). The results are summarized in Fig. 3. The inactivation of CYP2C8 was dependent on the concentration of gemfibrozil glucuronide (over the range examined, which was 1.25 to 64 μM) and the time course conformed to a first order process (as indicated by the linearity of plots of the log of the residual enzyme activity against time). The K_I values differed slightly depending on whether gemfibrozil glucuronide was preincubated with human liver microsomes at 0.1 mg/mL (and subsequently diluted 10 fold to determine CYP2C8 activity) or 1.0 mg/mL (and subsequently diluted 10 fold to determine CYP2C8 activity). The K_I value was 20 μM in the former case, and 52 μM in the latter. In both cases, the k_{inact} value was 0.21 min^{-1}, meaning that, in the presence of saturating concentrations of gemfibrozil glucuronide, 21% of CYP2C8 was inactivated every minute. As a metabolism-dependent inhibitor, gemfibrozil glucuronide inhibited CYP2C8 with roughly the same potency regardless of whether the concentration of human liver microsomes was 0.1 or 1.0 mg/mL. If the mechanism-based inhibition of CYP2C8 involved product inhibition (i.e., reversible inhibition of CYP2C8 by a metabolite of

gemfibrozil glucuronide), then gemfibrozil glucuronide would have been expected to cause more inhibition when incubated with a 10-fold higher concentration of human liver microsomes, which was not the case. This in turn suggests, but does not prove that gemfibrozil glucuronide is an irreversible or quasi-irreversible inactivator of CYP2C8.

Metabolism of gemfibrozil glucuronide

In the presence of NADPH, human liver microsomes and recombinant CYP2C8 both converted gemfibrozil glucuronide to a hydroxylated metabolite, as determined by LC/MS/MS. The mass fragmentation pattern of this metabolite is shown in Fig. 4. The presence of the ion at m/z 265 is due to the loss of a 176-Da fragment from the hydroxy-gemfibrozil glucuronide molecular ion of 441 Da, which is characteristic of collision-induced dissociation of the glucuronic acid moiety. The presence of the ion at m/z 137 indicates that hydroxylation of gemfibrozil glucuronide occurred at the dimethylphenoxy moiety.

Preincubation of gemfibrozil with UDPGA and alamethicin

As shown in Fig. 5, incubating gemfibrozil with alamethicin-activated human liver microsomes and UDP-glucuronic acid increased its ability to inhibit CYP2C8, presumably due to the formation of gemfibrozil glucuronide. Incubating these same samples with NADPH caused a further loss of CYP2C8 activity, presumably due to the metabolism-dependent inhibition of CYP2C8 by gemfibrozil glucuronide (Fig. 5).

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DISCUSSION

For the purpose of prioritizing clinical drug interaction studies, the FDA frequently evaluates in vitro CYP inhibition data on the basis of rank order, which tacitly assumes that the CYP enzyme inhibited most potently in vitro will be the same enzyme most potently inhibited in vivo (Bjornsson et al., 2003; Huang, 2004). This approach has proven reliable on numerous occasions, especially when a drug candidate has been evaluated as both a direct-acting and a metabolism-dependent inhibitor of the major drug-metabolizing CYP enzymes in human liver microsomes. Gemfibrozil represents an exception to the rule, because it inhibits CYP2C9 more potently than it inhibits CYP2C8 in vitro, and yet the opposite is observed in vivo, where gemfibrozil does not inhibit the CYP2C9-dependent metabolism of warfarin, and has only minimal effect on the metabolism of the CYP2C9-substrate nateglinide (Lilja et al., 2005; Niemi et al., 2005) but does inhibit the metabolism of several CYP2C8 substrates, including cerivastatin, repaglinide, rosiglitazone, and pioglitazone (Backman et al., 2002; Niemi et al., 2003a; Niemi et al., 2003b; Jaakkola et al., 2005). In the present study, we have confirmed the previous finding of Shitara et al. (2004) that gemfibrozil 1-*O*-β-glucuronide is a direct inhibitor of CYP2C8, and moreover, that the potency with which gemfibrozil inhibits CYP enzymes is shifted from CYP2C9 to CYP2C8 by glucuronidation (Table 1). Furthermore, we have shown that gemfibrozil glucuronide is an NADPH-dependent, mechanism-based inhibitor of CYP2C8 (Fig. 2b, Fig. 3). We propose that this mechanism-based inhibition of CYP2C8 by gemfibrozil glucuronide explains, at least in part, the observed clinical interactions between multiple-dose gemfibrozil and the aforementioned CYP2C8 substrates (Backman et al., 2002; Niemi et al., 2003a; Niemi et al., 2003b; Jaakkola et al., 2005).

The mechanism by which gemfibrozil glucuronide inactivates CYP2C8 remains to be determined, but some mechanistic information is apparent from the results of the present study. Gemfibrozil glucuronide is an acyl glucuronide, and some acyl glucuronides can rapidly rearrange to form reactive aldehydes that form Schiff's bases (covalent adducts) with lysine residues on proteins (Li and Benet, 2003). Accordingly, we evaluated gemfibrozil glucuronide as both a time-dependent and metabolism-dependent

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inhibitor of CYP2C8 (by incubating human liver microsomes with gemfibrozil glucuronide in the absence and presence of NADPH, respectively). This experiment established that gemfibrozil glucuronide is a metabolism-dependent inhibitor, not simply a time-dependent inhibitor, of CYP2C8. In addition, normal Michaelis-Menten type kinetics were observed in the experiment to determine k_{inact} (Fig. 3b), making the spontaneous formation of reactive metabolites a less likely explanation for the increase in the inhibition of CYP2C8. Furthermore, gemfibrozil glucuronide has an apparent half-life of 44 hours in aqueous buffer (pH 7.4, 37°C), making it one of the least reactive acyl glucuronides (Ebner et al., 1999). Finally, incubating gemfibrozil glucuronide with ten times the concentration of human liver microsomes (1.0 vs. 0.1 mg/mL) did not increase its inhibitory effect, suggesting that the mechanism-based inactivation of CYP2C8 is not due to product inhibition but is due to irreversible or quasi-irreversible inactivation of CYP2C8.

The results of the present study suggest that the clinically significant interactions observed between gemfibrozil and CYP2C8 substrates are due, at least in part, to the NADPH-dependent inactivation of CYP2C8 by gemfibrozil 1-*O*-β-glucuronide. A method to predict in vivo inhibition from quasi-irreversible or irreversible inhibition was reported by Lu and colleagues (equation 1 in Lu et al., 2003) as follows:

$$\frac{AUC_{po(i)}}{AUC_{po(c)}} = \frac{1}{\left(\frac{f_m \bullet f_{m,P450}}{1+\left[k_{inact} / K_I \bullet [I]_u / k_e\right]}\right) + \left[1-\left(f_m \bullet f_{m,P450}\right)\right]}$$

where [*I*] is the inactivator concentration, and k_{inact} and K_I are parameters estimable by nonlinear regression, ($f_m \cdot f_{m,P450}$) represents the fraction of a hypothetical concomitantly administered drug metabolized by a given CYP enzyme, and k_e is the rate constant for enzyme degradation (which has not been determined experimentally, but which was assumed to be 0.0008 min^{-1} which in turn assumes a half-life of 21 h (Mayhew et al., 2000)). This relationship relies on certain assumptions including (1) the conditions of the well-stirred pharmacokinetic model are met, (2) the substrate exhibits linear

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pharmacokinetics and is metabolized only in the liver, and (3) complete absorption from the gastrointestinal tract occurs (Mayhew et al., 2000; Lu et al., 2003). As illustrated in Fig. 6, the in vitro k_{inact} (0.21 min^{-1}) and K_I (51 μM) data were used to simulate the effects of gemfibrozil glucuronide on the pharmacokinetics of concomitantly-administered CYP2C8 substrates ($AUC_{po(i)}$-to-$AUC_{po(c)}$ ratio). For these simulations, we used various published values of the in vivo concentrations of gemfibrozil glucuronide: (1) 89 μM, which is the average unbound (free) C_{max} in liver, (2) 20 μM, which is the total (free + bound) in plasma, and (3) 2.3 μM, which is the unbound (free) C_{max} in plasma (Shitara et al. 2004). We did not base these simulations on an estimate of the concentration of free gemfibrozil glucuronide in the intra-hepatic blood, as has been proposed by others (Kanamitsu et al., 2000; Ito et al., 2004), because this approach was developed for orally administered drugs, whereas gemfibrozil glucuronide is presumably formed in the liver. Based on these simulations, for a drug whose clearance was 95% dependent on metabolism by CYP2C8 (i.e., $f_m \cdot f_{m,CYP2C8} = 0.95$), gemfibrozil glucuronide would be predicted to cause an 8-fold to a 19-fold increase in AUC depending on which concentration of gemfibrozil glucuronide is used. The fold-increase in AUC of a victim drug would be less for drugs whose clearance was less dependent on metabolism by CYP2C8 as shown in Fig. 6. In agreement, the AUC ratio for repaglinide, cerivastatin, pioglitazone and rosiglitazone is ~8.0 (range 5.5 to 15), 4.4 (range 1.1 to 8.0), 3.2 (range 2.3 to 6.5) and 2.3 (range 1.5 to 2.8), respectively (Backman et al., 2002; Niemi et al., 2003a, 2003b; Jaakola et al., 2005). Conversely, the fold-increase in AUC of a victim drug would be greater if the half-life of CYP2C8 were longer than 21 h (i.e., if k_e were smaller), or if the lower estimate of K_I (20 μM instead of 51 μM) were used in the simulations. This predicted effect when using either the average unbound (free) C_{max} concentration in liver, or the total (free + bound) plasma concentration of gemfibrozil glucuronide is larger than that observed clinically, while the predicted effect using the unbound (free) C_{max} in plasma is similar to the observed clinical effect.

The results of the present study also provide a likely explanation for the discordant results reported by Prueksaritanont et al. (2005), who recently evaluated gemfibrozil as a CYP enzyme inducer in primary

cultures of human hepatocytes. These investigators observed that treatment of human hepatocytes with 250 μM gemfibrozil caused an increase in the levels of mRNA encoding CYP3A4 (3.8 fold) and CYP2C8 (4.9 fold). Gemfibrozil induced CYP3A4 activity (testosterone 6β-hydroxylation) to the same extent it induced CYP3A4 mRNA. In contrast, rather than inducing CYP2C8 activity (amodiaquine *N*-dealkylation) to the same extent that it induced CYP2C8 mRNA, gemfibrozil caused a marked decrease (>60%) in CYP2C8 activity. The discrepancy between the gemfibrozil-induced increase in CYP2C8 mRNA and the decrease in CYP2C8 activity can be rationalized on the basis that gemfibrozil up-regulated the transcription of CYP2C8 (and CYP3A4) but was converted by human hepatocytes to its acyl glucuronide, which subsequently inactivated CYP2C8 (but not CYP3A4).

The observation that gemfibrozil is an enzyme inducer may help explain the effects of gemfibrozil on warfarin metabolism. Gemfibrozil (600 mg b.i.d. for 8 days) was evaluated for its ability to inhibit the CYP2C9-dependent metabolism of warfarin (because CYP2C9 is the enzyme most potently inhibited by gemfibrozil), but, unexpectedly, gemfibrozil was found to increase, not decrease, the metabolic clearance of warfarin (Lilja et al., 2005). Although Prueksaritanont et al. (2005) did not assess the effects of gemfibrozil on CYP2C9, this enzyme is typically up-regulated together with CYP3A4 and CYP2C8, and it seems reasonable to speculate that gemfibrozil increases the metabolic clearance of warfarin by inducing CYP2C9.

The biotransformation of drugs and other xenobiotics frequently occurs in two sequential phases. During Phase I metabolism, enzymes that catalyze oxidative, reductive or hydrolytic reactions introduce or expose a functional group that is subsequently conjugated, often with a highly water-soluble moiety such as glucuronic or sulfonic acid, during Phase II metabolism (Parkinson, 2001). Although Phase I metabolism generally precedes Phase II, there are reports of phase II metabolites undergoing oxidation by CYP enzymes. Examples include the oxidation of sulfate conjugates of testosterone,

dehydroepiandrosterone (DHEA) and estrogens (Ingelman-Sundberg et al., 1975; Kitada et al., 1987; Ohmori et al., 1998). In one case, cytochrome P450 (namely CYP2C12, which is expressed in female but not male rats) catalyzes the oxidation of a steroid *di*-sulfate (namely 5α-androstane-3α,17β-diol-3,17-disulfate) (Ryan et al., 1984). More recently, CYP2C8 has been shown to catalyze the oxidation of two glucuronides, namely the acyl glucuronide of diclofenac and the 17β-glucuronide of estradiol (Kumar et al., 2002; Delaforge et al., 2005). In both cases, hydroxylation occurs well away from the glucuronide moiety (4´-hydroxylation in the case of diclofenac glucuronide and 2-hydroxylation in the case of estradiol-17β-glucuroinide). In the case of the aglycones (i.e., prior to glucuronidation), these same reactions are catalyzed by CYP2C9 (Leemann et al., 1993; Lee et al., 2003) and even CYP2C8, albeit at a much slower rate (Lee et al., 2003; Melet et al., 2004). It remains to be determined whether, as a general rule, substrates for CYP2C9 become substrates for CYP2C8 once they have been glucuronidated. The active site of CYP2C8 has been mapped by X-ray crystallography and determined to be considerably larger than that of CYP2C9 (based on the rigid crystal structures of the enzymes, which does not take into account the potential flexibility of their active sites). As such, the former is capable of accommodating large substrates, including glucuronides, in its active site, with metabolism predicted to occur distal to the glucuronide moiety (Melet et al., 2004; Schoch et al., 2004).

In the presence of NADPH, human liver microsomes and recombinant CYP2C8 both converted gemfibrozil glucuronide to a hydroxylated metabolite. Hydroxylation occurred well away from the glucuronide moiety on the dimethylphenoxy ring of gemfibrozil. This distal site of hydroxylation is analogous to that reported for the glucuronides of diclofenac and estradiol, and is consistent with the proposed orientation of large acidic substrates in the active site of CYP2C8 (Melet et al., 2004). From the mass fragmentation pattern, we cannot discern whether CYP2C8 oxidizes one of the methyl groups (aliphatic hydroxylation) or the phenol ring (aromatic hydroxylation). The latter reaction could produce a substituted hydroquinone, which is a candidate for the putative reactive metabolite that ostensibly

inactivates CYP2C8. Further experiments will be required to ascertain whether formation of the hydroxylated metabolite or its subsequent metabolism leads to the inactivation of CYP2C8. It also remains to be determined whether inactivation of CYP2C8 by gemfibrozil glucuronide involves covalent modification of the protein and, if so, which amino acid residue or residues are modified.

Finally, because it was considered that formation of glucuronide metabolites that can cause clinically significant inhibition of CYP enzymes might be a more widespread phenomenon than was heretofore appreciated, we investigated an experimental design that might be applied generally during pre-clinical CYP inhibition testing. We have shown that, in a manner analogous to preincubating test drugs with human liver microsomes and NADPH (a method commonly used to identify metabolism-dependent inhibitors), a test drug (gemfibrozil in this case) can be preincubated with human liver microsomes first with UDP-glucuronic acid (in the presence of alamethicin to stimulate UGT activity) and then with NADPH prior to measuring CYP activity (CYP2C8 activity in this case). Along similar lines, Kajosaari et al. (2005) used the first step of this approach to show that glucuronidation of gemfibrozil by alamethicin-activated human liver microsomes increased the degree of inhibition of CYP2C8. Our results show that the ability of gemfibrozil to inhibit CYP2C8 increased after the "glucuronidation step" and increased even further after the subsequent "cytochrome P450 step" (Fig. 5). This two-step procedure might prove useful in screening drug candidates for their potential to be converted to glucuronides that can function as direct-acting or metabolism-dependent inhibitors of CYP2C8 (or any of the other drug-metabolizing CYP enzymes in human liver microsomes).

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Footnotes:

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FIGURE LEGENDS

Fig. 1: Metabolism of gemfibrozil

Fig. 2: Evaluation of gemfibrozil and gemfibrozil glucuronide as direct-acting and metabolism-dependent inhibitors of CYP2C8. (a) Gemfibrozil inhibited CYP2C8 in pooled human liver microsomes (0.05 mg/mL) with IC_{50} values of 120 μM (without preincubation) and 150 μM (with a 30-min preincubation with NADPH); (b) Gemfibrozil glucuronide inhibited CYP2C8 in pool human liver microsomes (0.05 mg/mL) with an IC_{50} values of 24 μM (without preincubation) and 1.8 μM with a 30-min preincubation with NADPH. Each symbol represents the average of duplicate determinations.

Fig. 3. Determination of K_I and k_{inact} for the metabolism-dependent inhibition of CYP2C8 by gemfibrozil glucuronide. (a) Gemfibrozil glucuronide (GFG) was preincubated (at concentrations indicated) with NADPH and pooled human liver microsomes (1 mg/mL) for the time points indicated at 37°C. After the preincubation, aliquots (40 μL) were removed and residual CYP2C8 activity determined as described in *Materials and Methods*. Individual points represent the average of triplicate determinations ± standard deviation. The graph in (b) represents the direct plot of the initial rates of inactivation of CYP2C8 from two experiments, the first with 0.1 mg/mL pooled human liver microsomes, and the second with 1 mg/mL (the latter is shown in graph (a). Values are the slopes of the initial rates of inactivation (λ) at each concentration of gemfibrozil glucuronide, shown ± standard error.

Fig. 4. LC/MS/MS analysis of the metabolite formed from gemfibrozil glucuronide by human liver microsomes or recombinant CYP2C8. The full scan of MS spectra of putative hydroxylated gemfibrozil glucuronide is shown, along with the proposed fragmentation of the molecule. The samples were incubated with human liver microsomes or recombinant CYP2C8, NADPH and gemfibrozil glucuronide (64 μM) for 60 minutes.

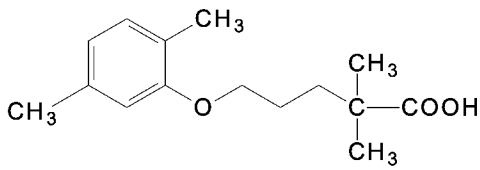
Fig. 5. Two-stage activation of gemfibrozil by human liver microsomes in the presence of UDP-glucuronic acid and alamethicin (the glucuronidation step) and then NADPH (the cytochrome P450 step). Gemfibrozil (30 or 100 μM) was pre-incubated for 0 or 60 min with pooled human liver microsomes, UDP-glucuronic acid and alamethicin, and an additional 0 or 30 min with NADPH prior to measuring residual CYP2C8 activity, as described in *Materials and Methods*. It should be noted that the preincubated samples were diluted 10 fold prior to measuring CYP2C8 activity, hence, the final concentration of gemfibrozil was 10 μM or less. Samples without pre-incubation served as controls. Values represent the average of duplicate determinations.

Fig. 6. Simulation of the effect of gemfibrozil 1-*O*-β-glucuronide on the pharmacokinetics of CYP2C8 substrates. The effect of gemfibrozil glucuronide on CYP2C8 substrates ($AUC_{po(i)}$-to-$AUC_{po(c)}$ ratio) was simulated based on equation 1 from Lu et al. (2003), as described in the text. The y-axis represents the fold increase in AUC of the victim drug (i.e., the drug whose clearance is inhibited by gemfibrozil glucuronide). The x-axis represents the fraction of clearance determined by CYP2C8 (e.g., 0.9 means 90% of the victim drug's clearance is determined by CYP2C8). The three simulations (A, B, C) are based on three estimates of the in vivo concentration of gemfibrozil glucuronide as described in the text.

Table 1. Inhibition of the major CYP enzymes in human liver microsomes by gemfibrozil and its 1-*O*-β-glucuronide

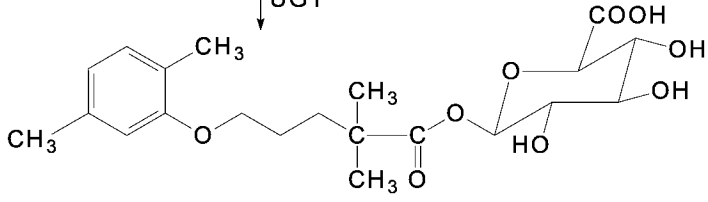
Enzyme	CYP reaction	IC_{50} (μM)[a]			
		Gemfibrozil		Gemfibrozil glucuronide	
		No preincubation	30 min preincubation	No preincubation	30 min preincubation
CYP1A2	Phenacetin *O*-deethylation	99 ± 12	84 ± 6	250 ± 40	> 300
CYP2B6	Bupropion hydroxylation	> 300	> 300	> 300	> 300
CYP2C8	Paclitaxel 6α-hydroxylation	120 ± 20	150 ± 20	24 ± 5	1.8 ± 0.5
CYP2C9	Diclofenac 4´-hydroxylation	30 ± 2	30 ± 3	> 300	> 300
CYP2C19	*S*-Mephenytoin 4´-hydroxylation	100 ± 10	82 ± 18	> 300	> 300
CYP2D6	Dextromethorphan *O*-demethylation	>300	>300	> 300	> 300
CYP3A4/5	Testosterone 6β-hydroxylation	>300	>300	> 300	> 300
CYP3A4/5	Midazolam 1´-hydroxylation	>300	>300	> 300	> 300

[a] Values are displayed to two significant figures, ± standard error



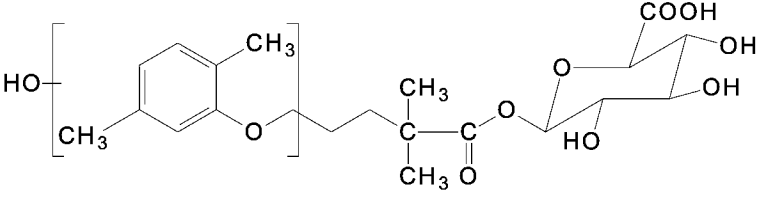
Gemfibrozil

UGT



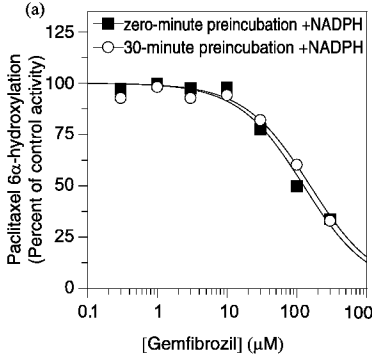
Gemfibrozil 1-*O*-β-glucuronide

CYP



Hydroxy- Metabolite

Fig 1


(a)
zero-minute preincubation +NADPH
30-minute preincubation +NADPH
Paclitaxel 6α-hydroxylation
(Percent of control activity)
125
100
75
50
25
0
0.1
1
10
100
1000
[Gemfibrozil] (μM)

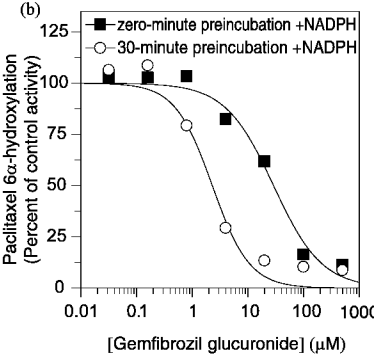

(b)
zero-minute preincubation +NADPH
30-minute preincubation +NADPH
Paclitaxel 6α-hydroxylation
(Percent of control activity)
125
100
75
50
25
0
0.01
0.1
1
10
100
1000
[Gemfibrozil glucuronide] (μM)

Fig 2

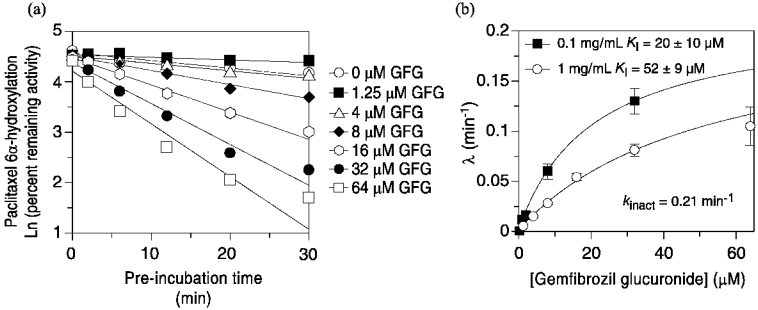

(a)
Paclitaxel 6α-hydroxylation
Ln (percent remaining activity)
5
4
3
2
1
0
10
20
30
Pre-incubation time
(min)
0 μM GFG
1.25 μM GFG
4 μM GFG
8 μM GFG
16 μM GFG
32 μM GFG
64 μM GFG
(b)
0.2
0.15
0.1
0.05
0
λ (min-1)
0.1 mg/mL KI = 20 ± 10 μM
1 mg/mL KI = 52 ± 9 μM
kinact = 0.21 min-1
0
20
40
60
[Gemfibrozil glucuronide] (μM)

Fig 3

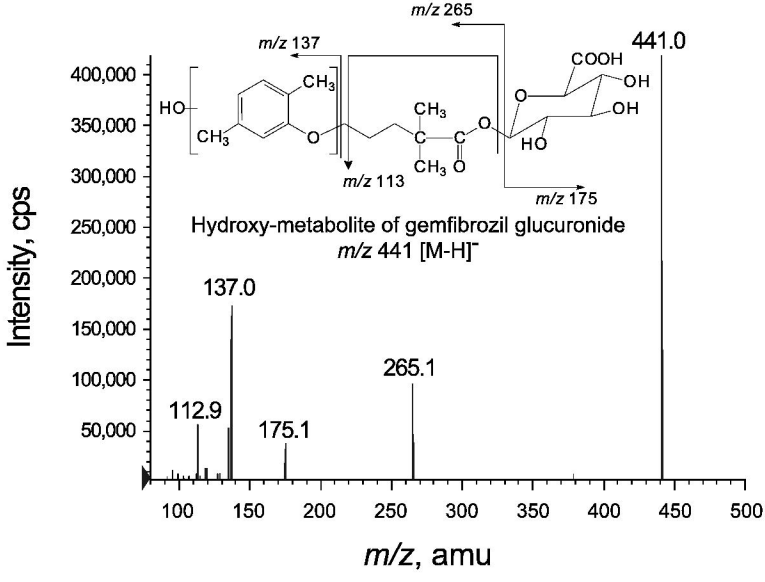

m/z 265
m/z 137
441.0
COOH
OH
OH
HO
CH3
CH3
O
CH3
C
C
O
HO
CH3
O
m/z 113
m/z 175
Hydroxy-metabolite of gemfibrozil glucuronide
m/z 441 [M-H]-
137.0
265.1
112.9
175.1
Intensity, cps
400,000
350,000
300,000
250,000
200,000
150,000
100,000
50,000
100
150
200
250
300
350
400
450
500
m/z, amu

Fig 4

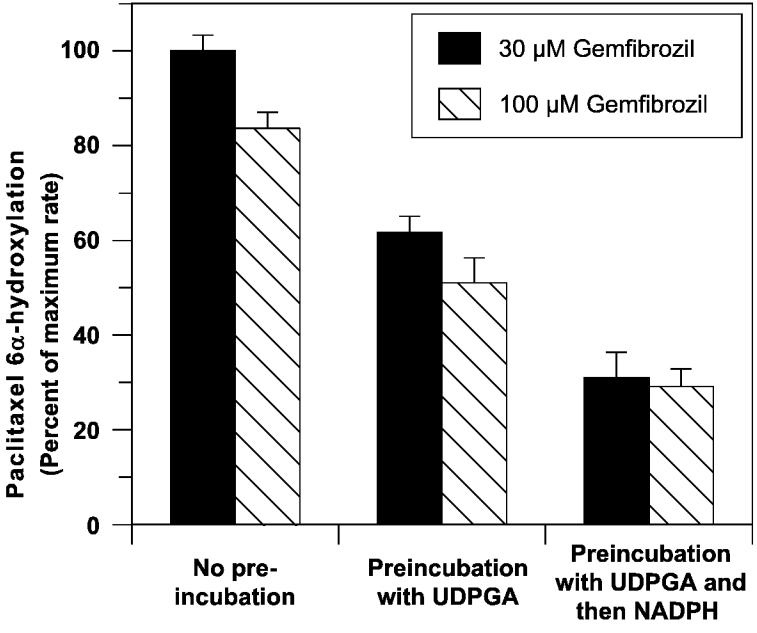

30 μM Gemfibrozil
100 μM Gemfibrozil
Paclitaxel 6α-hydroxylation
(Percent of maximum rate)
100
80
60
40
20
0
No pre-incubation
Preincubation with UDPGA
Preincubation with UDPGA and then NADPH

Fig 5

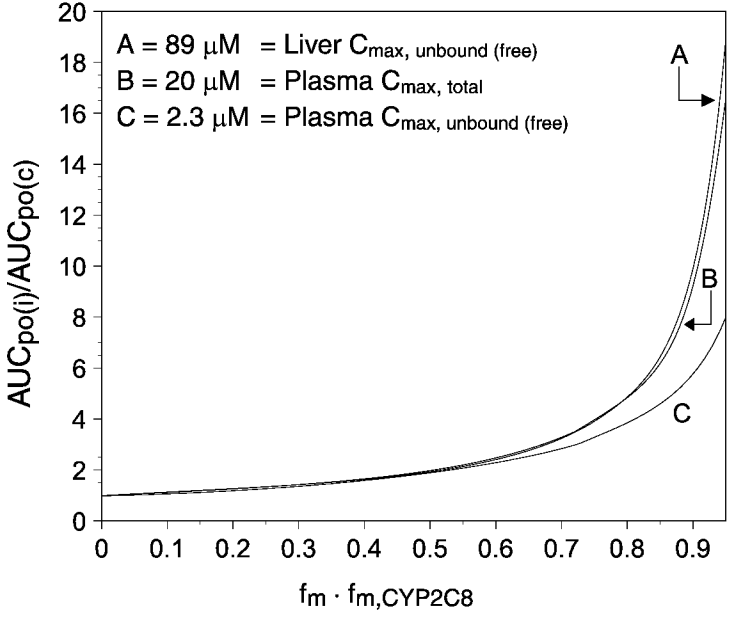

A = 89 μM = Liver Cmax, unbound (free)
B = 20 μM = Plasma Cmax, total
C = 2.3 μM = Plasma Cmax, unbound (free)
A
B
C
AUCpo(i)/AUCpo(c)
fm · fm,CYP2C8
0
2
4
6
8
10
12
14
16
18
20
0.1
0.2
0.3
0.4
0.5
0.6
0.7
0.8
0.9

Fig 6