STUDIES TO FURTHER INVESTIGATE THE INHIBITION OF HUMAN LIVER MICROSOMAL CYTOCHROME P450 2C8 (CYP2C8) BY THE ACYL-β-GLUCURONIDE OF GEMFIBROZIL


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Running Title: Inactivation of CYP2C8 by gemfibrozil acyl-β-glucuronide analogues

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Abbreviations:

ArRS9, liver 9000g supernatant fraction of Aroclor 1254-treated rats; BSA, bovine serum albumin; CH3CN, acetonitrile; CH3OH, methanol; CYP, cytochrome P450; gemfibrozil, 5-(2,5-
dimethylphenoxy)-2,2-dimethyl-pentanoic acid; HLM, human liver microsomes; HPLC, high-performance liquid chromatography; IC<sub>50</sub>, inhibitor concentration that causes 50% inhibition at time = 0 min (IC<sub>50(0)</sub>) and Time = 30 min (IC<sub>50(30)</sub>); K<sub>I</sub>, inhibitor concentration that supports half the maximal rate of inactivation; k<sub>inact</sub>, maximal rate of enzyme inactivation; LC/MS-MS, liquid chromatography/tandem mass spectrometry; m-Me, meta-methyl; MS, mass spectrometry; MS<sup>2</sup>, product ion mass spectrum; MS<sup>n</sup>, sequential product ion mass spectrum; NADPH, reduced nicotinamide adenine dinucleotide phosphate; NH<sub>4</sub>OAc, ammonium acetate; o-Me, ortho-methyl; rhCYP2C8, human recombinant CYP2C8; SAL, saccharic acid lactone; UDPGA, Uridine diphosphate glucuronic acid; UGT, UDP-glucuronosyltransferase.
ABSTRACT

In previous studies, gemfibrozil acyl-β-glucuronide, but not gemfibrozil, was found to be a mechanism-based inhibitor of cytochrome P450 2C8 (CYP2C8). In order to better understand whether this inhibition is specific for gemfibrozil acyl-β-glucuronide, or if other glucuronide conjugates are potential substrates for inhibition of this enzyme, we evaluated several pharmaceutical compounds (as their acyl-glucuronides) as direct-acting and metabolism-dependent inhibitors of CYP2C8 in human liver microsomes. Out of eleven compounds that were evaluated as their acyl-glucuronide conjugates, only gemfibrozil acyl-β-glucuronide exhibited mechanism-based inhibition, indicating that CYP2C8 mechanism-based inhibition is very specific to certain glucuronide conjugates. Structural analogues of gemfibrozil were synthesized and their glucuronide conjugates were prepared in order to further examine the mechanism of inhibition. When the aromatic methyl groups on the gemfibrozil moiety were substituted with trifluoromethyls, the resulting glucuronide conjugate was a weaker inhibitor of CYP2C8 and mechanism-based inhibition was abolished. However, the glucuronide conjugates of mono-methyl gemfibrozil analogues were mechanism-based inhibitors of CYP2C8, although not as potent as gemfibrozil acyl-β-glucuronide itself. The ortho-monomethyl analogue was a more potent inhibitor than the meta-monomethyl analogue, indicating that CYP2C8 favors the ortho position for oxidation and potential inhibition. Molecular modeling of gemfibrozil acyl-β-glucuronide in the CYP2C8 active site is consistent with the ortho-methyl position being the favored site of covalent attachment to the heme. Moreover, hydrogen bonding to four residues (S100, S103, Q214, and N217) is implicated.
INTRODUCTION

There have been several reports of clinical interactions between gemfibrozil (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). Although in vitro studies indicate that gemfibrozil is a more potent inhibitor of CYP2C9 than CYP2C8 (Wen et al., 2001), the results of clinical studies have shown that gemfibrozil is a more significant inhibitor of CYP2C8. This discrepancy was explained by demonstrating that the major metabolite of gemfibrozil, gemfibrozil acyl-β-glucuronide, is a potent inhibitor of CYP2C8 based on in vitro studies. These same authors demonstrated that gemfibrozil acyl-β-glucuronide inhibits the CYP2C8-mediated metabolism of cerivastatin in vitro, as well as the organic anion transporting peptide mediated uptake of cerivastatin (Shitara et al., 2004). In subsequent studies, Ogilvie et al. (2006) found that gemfibrozil acyl-β-glucuronide was a direct-acting and metabolism-dependent inhibitor of CYP2C8 with a $K_i$ of 20 to 52 μM and a $k_{inact}$ of 0.21 min$^{-1}$. When gemfibrozil was coadministered with repaglinide (a substrate for CYP3A4 and CYP2C8) in a clinical study, an increase in the area under the curve (AUC) was observed for repaglinide that persisted for at least 12 hr after the dose, indicating this mechanism-based inhibition occurs in vivo (Tornio et al., 2008). The effect is rapid and is evident 1 hr after gemfibrozil administration (Honkalammi et al. 2011). Recently, Baer et al. (2009) showed that gemfibrozil acyl-β-glucuronide was covalently bound to the heme of CYP2C8 and proposed P450-catalyzed oxidation of either of the benzylic methyl groups to form a benzyl radical intermediate (Fig. 1a).
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). Acyl glucuronides have long been known to be reactive; capable of undergoing reactions such as hydrolysis, rearrangement and covalent binding to proteins, possibly resulting in pharmacological or toxicological effects (Bailey and Dickinson, 2003).

The purpose of this study was to determine if the acyl-β-glucuronide of gemfibrozil is a unique mechanism-based inhibitor of CYP2C8 or if other glucuronide conjugates are capable of inhibiting this enzyme. Several compounds were evaluated as their acyl-glucuronide conjugates in this regard. As follow up to the work of Baer et al. (2009), gemfibrozil analogues and their glucuronide conjugates were synthesized and evaluated as inhibitors of CYP2C8 activity in HLM. Molecular modeling was also conducted to better understand how the gemfibrozil acyl-β-glucuronide and its analogues interact with the CYP2C8 active site.
MATERIALS AND METHODS

Chemicals and Reagents. Amodiaquine, clotrimazole, diclofenac, gemfibrozil, ibuprofen, indomethacin, ketoconazole, ketoprofen, mafenamic acid, phenelzine, SAL and simvastatin were purchased from Sigma Chemical Co. (St. Louis, MO). Atorvastatin, dihydroketoprofen, montelukast and all glucuronide conjugates (except those prepared below) were purchased from Toronto Research Chemicals (North York, Canada). (R)- and (S)-Naproxen were purchased from VWR (Bridgeport, NJ). HLM and P450 microsomes (Supersomes™) derived from baculovirus-infected insect cells were obtained from BD Biosciences (Woburn, MA). ArRS9 was purchased from Moltox Inc. (Boone, NC). Alamethicine, BSA, NADPH and UDPGA were obtained from Sigma-Aldrich (St. Louis, MO). MgCl₂ was purchased from Ambion (Austin, Texas). NH₄OAc was purchased from EM Science (Cherry Hill, NJ). HPLC grade acetonitrile and water were purchased from J. T. Baker (Phillipsburg, NJ). All other reagents were obtained from commercial sources.

Synthesis of gemfibrozil analogs and isolation of glucuronide conjugates (Supplement 1).

Time-Dependent Inhibition of CYP2C8 in HLM. Test compounds and positive controls (60 nL, in DMSO) at 10 concentrations were incubated with 15 μL of HLM (0.1 mg/mL HLM, 1 mM NADPH, 100 mM potassium phosphate buffer, pH 7.4, 2 mM MgCl₂) for 30 min (or as specified) at 37°C. This mixture was then diluted with an equal volume (15 μL) of substrate mixture (4 μM amodiaquine, 1 mM NADPH, 100 mM potassium phosphate buffer, pH
7.4, 2 mM MgCl₂), and the reaction continued for 10 min at 37°C. To determine the IC₅₀ value of the test compounds at the 0 minute time point, test compounds were first mixed with 15 μL of the substrate mixture, then immediately diluted with an equal volume (15 μL) of HLM mixture and incubated for 10 min at 37°C. At the end of 10-minute incubation, these reactions were terminated by the addition of 30 μl of quench buffer (94% water: 5% acetonitrile: 1% formic acid) containing internal standard (0.15 μM [²H₅]-N-desethylamodiaquine).

In one experiment, the test compounds were first preincubated (30 min at 37°C) with alamethicin-treated (25 μg/mL) HLM (0.1 mg/mL) with and without 2 mM UDPGA, followed by addition of NADPH (1 mM final) and further incubation for 0, 15 and 30 min at 37°C. At the end of the incubation time with NADPH, the reactions were continued by addition of the probe substrate mixture, containing 4 μM amodiaquine, 1 mM NADPH, 100 mM phosphate buffer (pH7.4) and 2 mM MgCl₂. After 10-min of incubation at 37°C, the reaction was terminated by addition of the quench buffer containing internal standard (described above).

Immediately prior to sample analysis, the denatured protein was precipitated by centrifugation for 10 min at 2500g and the supernatant was used to perform sample analysis. The amount of N-desethylamodiaquine produced in each reaction was determined by RapidFire™ - Mass Spectrometry (BIOCIUS Life Sciences, Wakefield, MA) using on-line solid phase extraction (SPE) with tandem mass spectrometry (MS/MS). Four compounds, known CYP2C8 inhibitors, were tested as positive controls in each experiment. They included three reversible CYP2C8 inhibitors (montelukast, clotrimazole and ketoconazole) and one time-dependent inhibitor (phenelzine).
**Metabolic Profiling.** Gemfibrozil, gemfibrozil glucuronide, BTFM gemfibrozil, and BTFM gemfibrozil glucuronide (50 µM) were individually incubated at 37°C with pooled HLM (1 mg/mL) or recombinant CYP2C8 (~50 pmol/mL) in phosphate buffer (100 mM, pH 7.4). Reactions were started by adding NADPH (3 mM) in phosphate buffer. Sample aliquots were taken at various times: 0, 30, and 60 min. Reactions were quenched by adding equal volumes of acetonitrile containing 4% formic acid and the precipitated proteins were removed by centrifugation (1000 × g for 5 min). The supernatant was analyzed by direct injection onto the LC/UV/MS^n system for metabolic profiling.

**Analytical.** *Synthesis of gemfibrozil analogues.* Microwave reactions were performed using a CEM Discover microwave (Ramp: 1 min, Stirring: On, Power Max: On). Column chromatography was performed using a Biotage flash chromatography system (Biotage, LLC, Charlotte, NC). HPLC analysis was performed using a Varian ProStar DAD detector (model 335) with Varian ProStar pumps (model 215) with 25 mL pump heads (Column: Waters Sunfire C18, 3.5 µm, 4.6x150 mm). HRMS analysis was performed using a Thermo Fisher Scientific, Exactive (Fourier Transform Orbitrap mass spectrometer) in negative ionization electrospray mode using Xcalibur software (Xcalibur, Inc., Reston, VA). Proton NMR spectra were recorded on 400 MHz Bruker DPX400A spectrometer.

*Isolation of glucuronides.* In the course of isolation, chromatographic fractions were screened by LC/MS for glucuronide products with a Thermo-Finnigan Deca LCQ LC/MS system; Waters Sunfire C-18 column, 5 µm, 4.6x150 mm. Mobile phase was 10 mM NH₄OAc with CH₃CN 95:5 (solvent A) and 10 mM NH₄OAc with CH₃CN 5:95 (solvent B). Conditions:
start 85:15 (A:B) to 0:100 (A:B) over 25 min, held at 100% solvent B for 5 min, then back to initial conditions over 2 min (32 min total); Flow rate: 1.2 mL/min, Wavelength: 254 nm, ESI-MS (Negative ion mode). NMR data were recorded in CD$_3$OD (5 mm tube), using a Bruker Avance III 500 MHz spectrometer.

Metabolite identification. HPLC analyses were carried out using an Agilent 1100 Series Separation Module (Palo Alto, CA) and a Phenomenex, Synergy, Hydro-RP (2.0 x 150 mm, 4µ) column maintained at room temperature. The mobile phase consisted of 98/2 mixture of 10 mM NH$_4$OAc and CH$_3$CN (Solvent A) and 70/30 mixture of CH$_3$CN and CH$_3$OH (Solvent B). Separations of components present in the incubation mixture was achieved under the following gradient conditions: initially solvents were held isocratically at 0% solvent B for 1 min, followed by a linear gradient to 100% solvent B over 30 min. Solvent B was then held isocratic at 100% for 3 min. Thereafter, solvents were immediately brought back to initial conditions, 0% solvent B in 0.1 min and re-equilibrated column for 4.9 min with a total chromatographic run time of 38 min.

The eluent from the HPLC column was routed in-line to an Agilent1100 series photodiode-array detector (scanning 200-600 nm at 5 Hz) and then to a Thermo Fisher LCQ Deca Xp Plus ion trap mass spectrometer. Electro-spray ion source was operated in negative ionization mode with data-dependent product-ion scanning. The product ion MS$^n$ spectra were obtained by collision induced dissociation (CID) using normalized energy of 35% and an isolation width of 3 Da.

Quantification of N-desethylamodiaquine (CYP2C8 inhibition). The amount of substrate probe metabolite (N-desethylamodiaquine) was measured using solid phase extraction with
tandem mass spectrometry (SPE-MS/MS) analysis on a RapidFire™-MS/MS system, which consisted of RapidFire™ 200 HT System (BIOCIUS Life Sciences, Wakefield, MA) and ABSciex 4000 QTRAP® hybrid triple quadrupole linear ion trap mass spectrometer using a Turbo V™ source with an ESI probe. The samples (20 μL) were loaded onto the BIOCIUS extraction SPE C4 column with mobile phase A (water with 0.01% TFA, 0.09% FA) at 1.5 mL/min for 3.2 seconds and were eluted with mobile phase B (CH₃CN with 0.01% TFA, 0.09% FA) at 1.25 mL/min for 3 seconds, followed by a re-equilibration of the SPE cartridge with mobile phase A at 1.5 mL/min for 0.5 seconds. The total cycle time was ~10 seconds per injection. The SPE eluent was then introduced to an ABSciex 4000 QTRAP® mass spectrometer.

Selected reaction monitoring (SRM) was used for analysis of metabolite and internal standard. The SRM instrument parameters were: ESI (positive ion mode), m/z 328.2 → 282.8 transition for N-desethylamodiaquine, m/z 331.2 → 282.8 transition for [²H₅]-N-desethylamodiaquine, 55V declustering potential, 26 eV collision energy, 80 ms dwell time. The acquired data was processed with RapidFire™ peak integration software, and the results were exported as an Excel file for IC₅₀ calculation.

**Data Analysis.** The signal intensity of the N-desethylamodiaquine (from MS/MS analysis) was normalized to the signal of internal standard, [²H₅]-N-desethylamodiaquine; thus signal intensity in each reaction was expressed as signal ratio. The sample signal ratios were then normalized to the average signal ratio of the reactions performed in the absence of the test substance (solvent control, 0% inhibition) and in the absence of enzyme (background, 100%
inhibition). Results were expressed as percent inhibition of the enzyme activity with solvent control, calculated as:

\[
\% \text{ Inhibition} = \left[1 - \frac{(S - B)}{(T - B)}\right] * 100
\]

where S = Sample, T = Average Solvent Control, B = Average Background signals.

The results were then imported into in-house curve fitting software (CurveMaster), which utilizes MathIQ (ID Business Solutions, Ltd., UK) to determine the IC\textsubscript{50} value for each test compound. The IC\textsubscript{50} is defined as the concentration corresponding to 50% inhibition of the enzyme activity observed with solvent control, derived from the fitted 10-point curve as determined using a 4-parameter logistic regression model:

\[
Y = A + \left[\frac{B - A}{1 + (C / X)^D}\right]
\]

where: A = minimum inhibition; B = maximum inhibition; X = inhibitor concentration; C = X, at which Y = A+ (B-A)/2, (ie concentration of inhibitor at which half of the maximal inhibition is observed); D=Hill coefficient (slope). IC\textsubscript{50} values (where % Inhibition = 50) for each compound at each time point were determined. IC\textsubscript{50} values and percent inhibition at the highest concentration are reported for each tested time point.

**Molecular Modeling.** All compounds were sketched as using ChemDraw and converted to three dimensional structures using Omega v2.2 (OpenEye Scientific Software, LLC, Santa Fe, NM). The structures were minimized using Batchmin (Schrödinger, Inc, New York, NY) using the OPLS2005 forcefield with implicit solvation. The CYP2C8 X-ray crystal structure was...
retrieved from the RCSB (Research Collaboratory for Structural Bioinformatics) (code: 2NNI). The protein was prepared using the Protein Prep Wizard within Maestro v8.5 (Schrödinger, Inc, New York, NY) using the standard defaults for metal treatment, amino acid deletions, etc. Docking was performed using Glide (Schrödinger, Inc, New York, NY) using flexible conformations for the ligand. Grids were prepared using the Glide Grid generation tool by selecting montelukast to specify the binding site. No constraints were applied for docking. Poses were evaluated using the Glide score and by visual inspection. Poses were preferred that included an interaction with the heme group.
**RESULTS**

**Effect of Various Glucuronide Conjugates on CYP2C8 Activity.** The acyl glucuronide conjugates of 11 compounds were evaluated as direct-acting and time/mechanism-dependent inhibitors of CYP2C8 activity in HLM. Activities were assessed with a marker substrate (amodiaquine) at 2 µM, a concentration close to its reported K_m of 1.86 µM (Walsky 2004). The results are summarized in Table 1. Of the compounds tested, only gemfibrozil acyl-β-glucuronide elicited a time/mechanism-dependent effect, with a 16-fold shift in IC_{50} (IC_{50(0)} = 21 µM, IC_{50(30)} = 1.4 µM). These results are similar to that reported by Ogilvie et al. (2006), where IC_{50(0)} = 24±5 µM and IC_{50(30)} = 1.8±0.5 µM. No time/mechanism-dependent effect was observed when gemfibrozil itself was tested in this assay (IC_{50(0)}/IC_{50(30)} ratio ~ 1.0) also similar to that reported by Ogilvie et al. Therefore, gemfibrozil itself is not a metabolism-dependent inhibitor of CYP2C8. Controls for the CYP2C8 inhibition experiments included three reversible inhibitors - montelukast (IC_{50} = 0.03 µM), clotrimazole (IC_{50} = 0.7 µM) and ketoconazole (IC_{50} = 2.8 µM) and one time-dependent inhibitor - phenelzine (IC_{50(0)} = > 200 µM, IC_{50(30)} = 60 µM). Control values were in agreement with published results (Walsky et al., 2005; Polasek et al., 2004) and were included for all CYP2C8 inhibition experiments.

Although all other compounds were not time-dependent inhibitors of CYP2C8 as their acyl glucuronides, three could be considered ‘strong’ inhibitors (simvastatin-glucuronide > mafenamic- glucuronide > diclofenac- glucuronide) with IC_{50}s less than 20 µM and greater than 90% inhibition. Three compounds were ‘weak’ inhibitors (ketoprofen- glucuronide > indomethacin- glucuronide > atorvastatin- glucuronide) with IC_{50}s between 25 and 50 µM and
greater than 65% inhibition. Four compounds did not exhibit CYP2C8 inhibition (ibuprofen-glucuronide, (R)- and (S)-naproxen- glucuronide and dihydroketoprofen- glucuronide) (Table 1).

The aglycone (parent) compounds were also tested as inhibitors of CYP2C8 (Table 2). Simvastatin, mefenamic acid and atorvastatin were moderate to weak inhibitors of CYP2C8, with IC₅₀s similar to that of their glucuronide conjugates. Ibuprofen, (R)- and (S)-naproxen and dihydroketoprofen did not show CYP2C8 inhibition, also similar to that of their glucuronide conjugates. However, the glucuronide conjugates of diclofenac and indomethacin were approximately 2-fold more potent as CYP2C8 inhibitors compared with the corresponding aglycones. The glucuronide conjugate of ketoprofen showed the greatest inhibition (versus its aglycone), with a greater than 5-fold lower IC₅₀. No time-dependent CYP2C8 inhibition was observed for the parent aglycones.

**Metabolism-Dependent Inhibition of CYP2C8 by Gemfibrozil and BTFM Analogue.**

Gemfibrozil, BTFM gemfibrozil and gemfibrozil acyl-β-glucuronide were preincubated with HLM in the presence and absence of UDPGA and NADPH (0, 15 and 30 min) prior to addition of amodiaquine to monitor CYP2C8 activity (data not shown). BTFM gemfibrozil was a CYP2C8 inhibitor (IC₅₀ 15 to 26 μM), but the inhibition was not dependent on UDPGA or NADPH, indicative of direct (reversible) inhibition of CYP2C8 by this analogue. In the presence of UDPGA and NADPH, gemfibrozil was a time / mechanism-dependent inhibitor of CYP2C8 after 30 min of incubation (IC₅₀(30) = 25 μM), indicating that glucuronidation of gemfibrozil is necessary for inhibition of CYP2C8. In the absence of NADPH at zero min, gemfibrozil acyl-β-glucuronide did not demonstrate CYP2C8 inhibition, but showed increasing
inhibition after a longer preincubation time in the presence of NADPH (IC$_{50(30)}$ = 4.0 μM), indicating the dependence on metabolism for inhibition. Although the metabolism of gemfibrozil to gemfibrozil acyl-β-glucuronide was not monitored in this experiment, it appears that incomplete formation of the glucuronide conjugate occurred, as indicated by the discrepancy in IC$_{50}$ in the two experiments. These results indicate that the glucuronide conjugate has to be prepared prior to its incubation with HLM and that insufficient glucuronide would be formed in a reaction mixture containing the aglycone and cofactor-fortified HLM. For this reason, glucuronides of gemfibrozil analogues were biosynthesized from their respective aglycones, either by incubation with HLM (BTFM gemfibrozil) or rat liver ArRS9 (o-Me gemfibrozil and m-Me gemfibrozil) in the presence of UDPGA.

**Effect of Gemfibrozil and Analogue Glucuronide Conjugates on CYP2C8 Activity.**

The IC$_{50}$ of the gemfibrozil analogues as well as their glucuronide conjugates were evaluated for time / mechanism-dependent inhibition as shown in Table 3. As observed previously, gemfibrozil acyl-β-glucuronide behaved as a time- and metabolism-dependent inhibitor of CYP2C8. By comparison, BTFM gemfibrozil was an inhibitor of CYP2C8 (IC$_{50}$ of 13 to 17 μM), but its acyl-β-glucuronide was not a time-dependent inhibitor after 30-min preincubation with NADPH. The o-Me and m-Me gemfibrozil analogues exhibited almost no CYP2C8 inhibition and their corresponding acyl-β-glucuronides demonstrated weak inhibition without preincubation with NADPH (Fig. 3). However, after 30-min preincubation, the o-Me and m-Me gemfibrozil acyl-β-glucuronides trended toward time-dependent inhibition, with the o-Me
gemfibrozil glucuronide more potent an inhibitor compared to m-Me gemfibrozil glucuronide (Table 3).

**Metabolism of Gemfibrozil Acyl-β-Glucuronide and BTFM Gemfibrozil Glucuronide in the Presence of HLM and Human Recombinant CYP2C8.** Gemfibrozil acyl-β-glucuronide was stable to metabolism in the presence of both NADPH-fortified HLM and recombinant CYP2C8 (≥98% remaining after 60 min of incubation). Only three minor (<1%) metabolites M1, M2 and M3 were detected by HPLC/UV/MS in both incubations; representative chromatograms (HLM) are shown (Fig. 4C). The structures of the metabolites were assigned based on their MS² product ion mass spectra compared to that of gemfibrozil acyl-β-glucuronide (Fig. 4). Gemfibrozil acyl-β-glucuronide gave a protonated molecular ion at \( m/z \) 425. Subsequent MS² fragmentation gave a daughter ion at \( m/z \) 249 indicating loss of glucuronide moiety (176 Da). Further fragmentation (MS³) of the ion at \( m/z \) 249 gave rise to an ion at \( m/z \) 121, which corresponded to the dimethyl-phenoxy part of the molecule. The three metabolites, M1-M3, produced similar full scan mass spectra with a deprotonated molecular ion at \( m/z \) 441, a mass 16 Da higher than gemfibrozil acyl-β-glucuronide, denoting that a single oxidation had occurred. MS² spectrum of M1, M2 and M3 produced an abundant ion at \( m/z \) 265 (loss of glucuronide moiety). MS³ fragmentation of \( m/z \) 265 produced an ion at \( m/z \) 137, implying that oxidation occurred on the dimethyl-phenoxy moiety for all three metabolites. An attempt was made to further characterize these metabolites, but efforts were unsuccessful due to the very low amounts in the incubates. Under conditions where gemfibrozil acyl-β-glucuronide metabolites...
M1-M3 were detected, no metabolites of BTFM gemfibrozil glucuronide were detectable (data not shown).

**Computational Docking Studies.** Computational docking studies were performed to investigate the orientation of the gemfibrozil acyl-β-glucuronide in the active site of CYP2C8 (Fig 6). Docking poses with the o-Me position oriented toward the gamma-meso position of the heme were superior to those with the m-Me oriented towards the heme. Both orientations include numerous hydrogen bonds between the glucuronide and the CYP2C8 active site. The carboxylate of the glucuronide forms hydrogen bonds to sidechains of N217 and S100 and to the backbone NH of S103. Additional hydrogen bonds are formed to Q214 and S100. The pose orienting the o-Me towards the heme (Fig. 5a) forms numerous hydrophobic contacts to the site, including T301, I113, V366, S114, and V477. In addition, the 5-position methyl fits into small hydrophobic recess between V477 and F205, contributing to the orientation of the o-Me position toward the heme.

In addition to the glucuronide, gemfibrozil itself was also docked into CYP2C8. The docking pose (Fig. 5c) for gemfibrozil (shown with slate blue carbons) is substantially different compared to glucuronide metabolite. The pose of gemfibrozil acyl-β-glucuronide is shown for reference with cyan carbons. The only contact residue in common for the poses is S103. The dimethylphenyl ring of gemfibrozil is placed in a largely hydrophobic pocket formed by I106, F201, V237, and A292. As gemfibrozil is not a particularly potent inhibitor of CYP2C8 (Table 2 and 3), it is likely that multiple weak binding poses are probable.
Inhibition of CYP2C8 (IC$_{50}$ ~ 10 µM) was observed with mefenamic acyl-β-glucuronide and diclofenac acyl-β-glucuronide, so the docking studies described herein were expanded to include these two glucuronides. In both cases, poses were generated forming similar H-bonds to the glucuronide as for the gemfibrozil acyl-β-glucuronide. In addition, good hydrophobic contacts are formed with hydrophobic residues proximal to the heme. In neither case, however, were any functional groups oriented toward the heme that would imply likely mechanism-based inhibition through covalent attachment. The methyl substituents of mefenamic acyl-β-glucuronide were oriented away from the heme in the favored binding pose (Fig. 6). A similar result was obtained with diclofenac acyl-β-glucuronide (data not shown).
DISCUSSION

Although it is known that gemfibrozil acyl-β-glucuronide is a mechanism-based inhibitor of CYP2C8 (Ogilvie 2008), it is not known if gemfibrozil acyl-β-glucuronide is unique in its ability to inhibit CYP2C8, or if other acyl glucuronides are able to inhibit this enzyme, either reversibly or mechanistically. Of the acyl glucuronide conjugates tested in this study, seven out of eleven showed some degree of reversible CYP2C8 inhibition. Although some of these compounds show significant inhibition, the impact on CYP2C8 in vivo is not known. For example, Hatorp et al (2003) have assessed the impact of simvastatin on the PK of repaglinide. Unfortunately, given the role of CYP2C8 and 3A4 in the clearance of repaglinide the results are difficult to interpret. Additional clinical data are needed in order to place the results described herein in context. It is worth noting that when the aglycone (parent) compounds were tested for CYP2C8 inhibition, the IC₅₀s were similar to the corresponding glucuronide conjugates, indicating that glucuronide conjugates may fit into the CYP2C8 pocket just as well as the parent molecule. Also noteworthy is that ketoprofen acyl glucuronide was even more potent than ketoprofen itself. Ketoprofen acyl glucuronide is present in human plasma and excreted in the urine (Upton 1980), although CYP2C8 inhibition in a clinical setting has not been reported. Only gemfibrozil acyl-β-glucuronide was a time-dependent inhibitor of CYP2C8, indicating that there is a particular metabolic liability for this glucuronide conjugate; gemfibrozil itself was not a time-dependent inhibitor of CYP2C8 (Table 1).

This study confirms the previous finding that gemfibrozil causes time-dependent inhibition of CYP2C8 when incubated in the presence of UDPGA and NADPH. However, generation of the glucuronide in the incubation resulted in less potent inhibition of CYP2C8
compared to inhibition observed upon incubation with the gemfibrozil acyl-β-glucuronide (IC\textsubscript{50} after 30 min of incubation is 25 vs. 4.0 μM). It appears that although generation of the glucuronide conjugate in the incubation mixture indicates the presence of time-dependent inhibition, incubation with the glucuronide conjugate itself is necessary to accurately determine the time-dependent shift in IC\textsubscript{50}. Therefore, further studies with gemfibrozil analogues were also conducted with their respective glucuronide conjugates.

NADPH was also required in order to observe time-dependent inhibition of CYP2C8 in the presence of gemfibrozil acyl-β-glucuronide, indicating that the gemfibrozil glucuronide is likely oxidized by P450. Such a hypothesis is supported by the fact that incubation of gemfibrozil acyl-β-glucuronide with NADPH-fortified HLM produced three minor hydroxylation products (Fig. 4). Unfortunately, turnover was low and the oxidative metabolites could not be further characterized (metabolism on the benzylic moiety of gemfibrozil is implicated). CYP2C8-catalyzed oxidation of glucuronide is not a novel finding, because it has been known for some time that the enzyme is capable of hydroxylating estradiol-17β-glucuronide (Delaforge et al., 2005). Following a thorough docking of the molecule within the CYP2C8 crystal structure, the same authors concluded that the active site is large enough to accommodate a glucuronide conjugate. Likewise, Kumar et. al. (2002) have described CYP2C8-mediated glucuronide metabolism; oxidation of diclofenac acyl glucuronide to 4'-hydroxy diclofenac acyl glucuronide. Based on the results presented herein, such metabolism does not render pronounced time-dependent inhibition of CYP2C8 in HLM (Table 1), in contrast to what is observed for gemfibrozil acyl-β-glucuronide.
Recently, Baer et al. (2009) have shown that following incubation with recombinant CYP2C8, the gemfibrozil acyl-β-glucuronide is covalently bound to the heme moiety, based on mass spectrometry and deuterium isotope effects. Oxidation of the aromatic o-Me or p-Me group to form a benzyl radical was proposed as the reactive intermediate that binds to the γ-meso position of the heme. The authors concluded that because of the rotameric flexibility of the alkyl chain of gemfibrozil acyl-β-glucuronide, there was no indication that either the o-Me or the m-Me would have a favored orientation. In order to further probe this hypothesis, three gemfibrozil analogues and their corresponding glucuronides were evaluated in the CYP2C8 inhibition assay. These three analogues were BTFM gemfibrozil, o-Me gemfibrozil and m-Me gemfibrozil.

When the gemfibrozil aromatic methyl groups were substituted with trifluoromethyls to give BTFM gemfibrozil, this compound was a competitive CYP2C8 inhibitor; however time-dependent inhibition of CYP2C8 was not observed either for the analogue or the corresponding glucuronide conjugate of this analogue. This is to be expected, based on the hypothesis that oxidation of the methyl groups is necessary for time-dependent inhibition of CYP2C8 and that such metabolism can be blocked by substitution with trifluoromethyls. In agreement, turnover of BTFM gemfibrozil and its acyl-β-glucuronide was not detectable after incubation with NADPH-fortified HLM (Fig. 5).

Time-dependent inhibition of CYP2C8 was not observed with the mono-methyl gemfibrozil analogues. However, the glucuronide conjugate of o-Me gemfibrozil was a time-dependent inhibitor of CYP2C8. The o-Me gemfibrozil glucuronide analogue appears to be a more potent CYP2C8 inhibitor compared to the m-Me gemfibrozil glucuronide analogue, but less potent than gemfibrozil acyl-β-glucuronide itself (Table 3).
In contrast to what Baer et al. had observed in their computational docking studies, the orientation of the o-Me position of the gemfibrozil acyl-β-glucuronide appears to fit better in the CYP2C8 binding pocket compared to the m-Me position, based on our modeling studies (Fig. 6). The pose of orientation of the o-Me position with the methyl group oriented towards the heme appears to fit better in the CYP2C8 binding pocket compared to the m-Me being oriented towards the heme position. It appears that more hydrophobic interactions are present in the o-Me analogue oriented pose compared to the m-Me oriented pose. The loss of potency of the o-Me glucuronide analogue compared to gemfibrozil acyl-β-glucuronide may be due to additional rotational freedom of the ortho-phenyl position in the active site. It appears that the additional methyl in the meta position allows the molecule to fit into a shallow pocket of the CYP2C8 molecule and appears to limit the rotation of the phenyl ring. Thus, the proposed reaction pathway for gemfibrozil acyl-β-glucuronide more likely involves oxidation of the o-Me position leading to covalent binding and inactivation of the CYP2C8 (Fig. 1b), rather than a lack of a favored oxidation position as indicated by Baer et al. (Fig. 1a). It is also possible that a dimethide analogue could also be formed as a reactive intermediate, which would help explain the loss of potency in the mono-methyl analogues, but there is no current evidence to indicate this is occurring.

Although most glucuronide conjugates are eliminated without inhibiting major metabolic pathways, some have the potential to bring about drug-drug interactions (Faed 1984). Consequently, the 2008 FDA safety guidelines state that if a drug conjugate is reactive (e.g., acyl glucuronide), then additional safety assessment may be needed (US 2008). Although P450 inhibition screening could be part of such an assessment, such studies are not conducted...
routinely (Bode 2010). It has been suggested that glucuronides may be generated by incubation of a test compound with alamethicin-treated (UDPGA-fortified) HLM to form the glucuronide, followed by incubation with NADPH and a probe substrate(s) to assess inhibition of one or more P450s (Ogilvie et al., 2006; Bode, 2010). However, a more accurate determination of time-dependent inhibition would need to be conducted with the isolated glucuronide once mechanism-based inhibition was suspected. In any case, it appears from this study that if the parent compound is an inhibitor of CYP2C8, the corresponding acyl glucuronide may also contribute to the inhibition. Although gemfibrozil acyl-β-glucuronide has a relatively unique configuration that renders mechanism-based inhibition and this mechanism is not applicable to all acyl glucuronides, P450 inhibition (including time-dependent inhibition) should be evaluated in the presence (and absence) of UDPGA for all compounds that produce acyl glucuronide conjugates. If inhibition is observed in the presence of UDPGA, the corresponding acyl glucuronide should be generated and tested for P450 inhibition. Although mechanism-based inhibition of additional P450s (beyond CYP2C8) by glucuronides has not been reported to date, in vitro testing of multiple human P450s is advisable.
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Authorship Contribution

Participated in research design: Jenkins, Zvyaga, Burrell, Turley, Rodrigues.

Conducted experiments: Zvyaga, Hurley, Turley, Wagner, Leet, Philip.

Contributed new reagents or analytic tools: Burrell, Turley, Leet.

Performed data analysis: Jenkins, Zvyaga, Johnson, Hurley, Wagner, Philip.

Wrote or contributed to the writing of the manuscript: Jenkins, Zvyaga, Johnson, Turley, Leet, Philip, Rodrigues.
REFERENCES


clinically relevant drug-drug interaction between cerivastatin and gemfibrozil. J Pharmacol Exp Ther 311:228-236.


Figure Legends

Figure 1. A) Reaction pathway for time-dependent inhibition of CYP2C8 by gemfibrozil acyl-ß-glucuronide proposed by Baer et al. 2009 B) Reaction pathway for time-dependent inhibition as proposed in this paper, indicating o-Me is the predominant configuration for oxidation to a reactive metabolite.

Figure 2. Chemical preparation of gemfibrozil analogues. DMF, dimethylformamide.

Figure 3. IC₅₀ curves for inhibition of CYP2C8-mediated amodiaquine N-deethylation by gemfibrozil and analogue compounds, and their respective acyl-ß-D-glucuronides, following incubation with NADPH-fortified HLM for 0 min (open circles) and 30 min (solid diamonds).

Figure 4. Negative LC/MS/MS analysis of (A) gemfibrozil acyl-ß-glucuronide (B) oxidative metabolite after incubation with NADPH-fortified HLM for 60 min (Gluc = glucuronide) and (C) UV chromatogram (272 nm) of gemfibrozil acyl-ß-glucuronide oxidative metabolites after incubation with HLM and NADPH for 60 min. Metabolites were also observed using recombinant CYP2C8 (results not shown).

Figure 5. Gemfibrozil acyl-ß-glucuronide orientation in CYP2C8 (A) orientation of o-Me toward heme; and (B) orientation of m-Me toward heme. Key contact residues are highlighted in the figure. (C) pose of gemfibrozil (slate blue carbons) in CYP2C8. The pose of gemfibrozil
acyl-β-glucuronide from (A) is shown for spatial reference (cyan carbons). Note that the only contact residue in common is a hydrogen bond to S103.

**Figure 6.** Mefenamic acyl-β-glucuronide docked into the CYP2C8 active site. The molecule has similar binding between the protein and the glucuronide as for the gemfibrozil acyl-β-glucuronide. The methyl substituents are oriented away from the heme making covalent attachment unlikely. No poses were identified that would orient the methyl groups toward the heme.
Table 1: IC_{50} values for inhibition of CYP2C8-catalyzed amodiaquine N-deethylation by various glucuronide conjugates following incubation with NADPH-fortified HLM for 0 and 30 minutes.

<table>
<thead>
<tr>
<th>Structure</th>
<th>Substance</th>
<th>Average IC_{50} (μM)(^a)</th>
<th>Ratio</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>IC_{50(0)}</td>
<td>IC_{50(30)}</td>
</tr>
<tr>
<td><img src="image1.png" alt="Structure" /></td>
<td>Gemfibrozil</td>
<td>120 ± 10</td>
<td>110 ± 10</td>
</tr>
<tr>
<td><img src="image2.png" alt="Structure" /></td>
<td>Gemfibrozil Acyl-β-D-Glucuronide</td>
<td>21 ± 1.3</td>
<td>1.4 ± 0.03</td>
</tr>
<tr>
<td><img src="image3.png" alt="Structure" /></td>
<td>Simvastatin Acyl-β-D-Glucuronide</td>
<td>3.8 ± 0.5</td>
<td>3.5 ± 0.4</td>
</tr>
<tr>
<td><img src="image4.png" alt="Structure" /></td>
<td>Mefenamic Acyl-β-D-Glucuronide</td>
<td>8.5 ± 0.9</td>
<td>8.4 ± 0.8</td>
</tr>
<tr>
<td><img src="image5.png" alt="Structure" /></td>
<td>Diclofenac Acyl-β-D-Glucuronide</td>
<td>14 ± 0.7</td>
<td>9.6 ± 0.5</td>
</tr>
<tr>
<td><img src="image6.png" alt="Structure" /></td>
<td>rac-Ketoprofen Acyl-β-D-Glucuronide</td>
<td>26 ± 0.7</td>
<td>22 ± 0.9</td>
</tr>
<tr>
<td><img src="image7.png" alt="Structure" /></td>
<td>Indomethacin Acyl-β-D-Glucuronide</td>
<td>26 ± 2.2</td>
<td>26 ± 2.3</td>
</tr>
<tr>
<td><img src="image8.png" alt="Structure" /></td>
<td>Atorvastatin Acyl-β-D-Glucuronide</td>
<td>45 ± 3.7</td>
<td>35 ± 3.1</td>
</tr>
<tr>
<td><img src="image9.png" alt="Structure" /></td>
<td>Ibuprofen Acyl-β-D-Glucuronide</td>
<td>&gt; 100 (39 ± 4)(^\dagger)</td>
<td>&gt; 100 (47 ± 4)(^\dagger)</td>
</tr>
<tr>
<td><img src="image10.png" alt="Structure" /></td>
<td>(R)-Naproxen Acyl-β-D-Glucuronide</td>
<td>&gt; 100 (27 ± 6)(^\dagger)</td>
<td>&gt; 100 (37 ± 4)(^\dagger)</td>
</tr>
<tr>
<td><img src="image11.png" alt="Structure" /></td>
<td>(S)-Naproxen Acyl-β-D-Glucuronide</td>
<td>&gt; 100 (17 ± 6)(^\dagger)</td>
<td>&gt; 100 (16 ± 7)(^\dagger)</td>
</tr>
<tr>
<td><img src="image12.png" alt="Structure" /></td>
<td>Dihydro-Ketoprofen Acyl-β-D-Glucuronide</td>
<td>&gt; 100 (1 ± 5)(^\dagger)</td>
<td>&gt; 100 (5 ± 6)(^\dagger)</td>
</tr>
</tbody>
</table>

\(^a\)Data are reported as mean ± SD of n = 4 determinations. IC_{50(0)} and IC_{50(30)} represent the IC_{50} for the 0 and 30 minute incubation periods, respectively.
determined after 0 and 30 min of incubation with NADPH-fortified HLM.
† % Inhibition observed at 100 μM ± SD
Table 2: IC<sub>50</sub> values for inhibition of CYP2C8-catalyzed amodiaquine N-deethylation by various aglycones following incubation with NADPH-fortified HLM for 0 and 30 minutes.

<table>
<thead>
<tr>
<th>Structure</th>
<th>Substance</th>
<th>Average IC&lt;sub&gt;50&lt;/sub&gt; (μM)&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Ratio</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>IC&lt;sub&gt;50(0)&lt;/sub&gt;</td>
<td>IC&lt;sub&gt;50(30)&lt;/sub&gt;</td>
</tr>
<tr>
<td><img src="image" alt="Gemfibrozil Structure" /></td>
<td>Gemfibrozil</td>
<td>83 ± 7.1</td>
<td>102 ± 4.0</td>
</tr>
<tr>
<td><img src="image" alt="Simvastatin Structure" /></td>
<td>Simvastatin</td>
<td>8.3 ± 1.1</td>
<td>10.2 ± 1.4</td>
</tr>
<tr>
<td><img src="image" alt="Mefenamic Acid Structure" /></td>
<td>Mefenamic Acid</td>
<td>14.9 ± 3.0</td>
<td>22.0 ± 1.8</td>
</tr>
<tr>
<td><img src="image" alt="Atorvastatin Structure" /></td>
<td>Atorvastatin</td>
<td>21.9 ± 1.3</td>
<td>22.5 ± 1.8</td>
</tr>
<tr>
<td><img src="image" alt="Diclofenac Structure" /></td>
<td>Diclofenac</td>
<td>54 ± 11</td>
<td>57 ± 8</td>
</tr>
<tr>
<td><img src="image" alt="Indomethacin Structure" /></td>
<td>Indomethacin</td>
<td>88 ± 16</td>
<td>&gt; 200 (46 ± 6)&lt;sup&gt;†&lt;/sup&gt;</td>
</tr>
<tr>
<td><img src="image" alt="Dihydro-Ketoprofen Structure" /></td>
<td>Dihydro-Ketoprofen</td>
<td>&gt; 200 (41 ± 4)&lt;sup&gt;†&lt;/sup&gt;</td>
<td>&gt; 200 (44 ± 3)&lt;sup&gt;†&lt;/sup&gt;</td>
</tr>
<tr>
<td><img src="image" alt="Ibuprofen Structure" /></td>
<td>Ibuprofen</td>
<td>&gt; 200 (23 ± 3)&lt;sup&gt;†&lt;/sup&gt;</td>
<td>&gt; 200 (23 ± 1)&lt;sup&gt;†&lt;/sup&gt;</td>
</tr>
<tr>
<td><img src="image" alt="Ketoprofen Structure" /></td>
<td>Ketoprofen</td>
<td>&gt; 200 (14 ± 3)&lt;sup&gt;†&lt;/sup&gt;</td>
<td>&gt; 200 (6 ± 5)&lt;sup&gt;†&lt;/sup&gt;</td>
</tr>
<tr>
<td><img src="image" alt="R-Naproxen Structure" /></td>
<td>(R)-Naproxen</td>
<td>&gt; 200 (17 ± 5)&lt;sup&gt;†&lt;/sup&gt;</td>
<td>&gt; 200 (4 ± 4)&lt;sup&gt;†&lt;/sup&gt;</td>
</tr>
<tr>
<td><img src="image" alt="S-Naproxen Structure" /></td>
<td>(S)-Naproxen</td>
<td>&gt; 200 (9 ± 3)&lt;sup&gt;†&lt;/sup&gt;</td>
<td>&gt; 200 (9 ± 3)&lt;sup&gt;†&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

<sup>a</sup>Data are reported as mean ± SD of n = 4 determinations. IC<sub>50(0)</sub> and IC<sub>50(30)</sub> represent the IC<sub>50</sub> values for initial and incubated conditions, respectively.
determined after 0 and 30 min of incubation with NADPH-fortified HLM.
† % Inhibition observed at 200 μM ± SD
Table 3: IC_{50} values for inhibition of CYP2C8-catalyzed amodiaquine N-deethylation by gemfibrozil analogues and their glucuronide conjugates following incubation with NADPH-fortified HLM for 0 and 30 minutes.

<table>
<thead>
<tr>
<th>Structure</th>
<th>Substance</th>
<th>Average IC_{50} (μM)^a</th>
<th>Ratio</th>
</tr>
</thead>
<tbody>
<tr>
<td><img src="image" alt="Gemfibrozil structure" /></td>
<td>Gemfibrozil</td>
<td>72 ± 7.2</td>
<td>1.0</td>
</tr>
<tr>
<td><img src="image" alt="Gemfibrozil Acyl-β-D-Glucuronide structure" /></td>
<td>Gemfibrozil Acyl-β-D-Glucuronide</td>
<td>13 ± 2.7</td>
<td>13</td>
</tr>
<tr>
<td><img src="image" alt="BTFM Gemfibrozil structure" /></td>
<td>BTFM Gemfibrozil</td>
<td>13 ± 2.1</td>
<td>0.7</td>
</tr>
<tr>
<td><img src="image" alt="BTFM Gemfibrozil Acyl-β-D-Glucuronide structure" /></td>
<td>BTFM Gemfibrozil Acyl-β-D-Glucuronide</td>
<td>37 ± 3.2</td>
<td>&lt; 1.0</td>
</tr>
<tr>
<td><img src="image" alt="o-Me Gemfibrozil structure" /></td>
<td>o-Me Gemfibrozil</td>
<td>&gt; 80 (15 ± 7)^†</td>
<td>1.0</td>
</tr>
<tr>
<td><img src="image" alt="o-Me Gemfibrozil Acyl-β-D-glucuronide structure" /></td>
<td>o-Me Gemfibrozil Acyl-β-D-glucuronide</td>
<td>55 ± 6</td>
<td>3.2</td>
</tr>
<tr>
<td><img src="image" alt="m-Me Gemfibrozil structure" /></td>
<td>m-Me Gemfibrozil</td>
<td>&gt; 80 (10 ± 6)^†</td>
<td>1.0</td>
</tr>
<tr>
<td><img src="image" alt="m-Me Gemfibrozil Acyl-β-D-glucuronide structure" /></td>
<td>m-Me Gemfibrozil Acyl-β-D-glucuronide</td>
<td>&gt; 120 (29 ± 6)^‡</td>
<td>&gt; 1.0</td>
</tr>
</tbody>
</table>
Data are reported as mean ± SD of n = 4 determinations. IC₅₀(0) and IC₅₀(30) represent the IC₅₀ determined after 0 and 30 min of incubation with NADPH-fortified HLM.

† %Inhibition observed at 80 μM ± SD
‡ %Inhibition observed at 120 μM ± SD
Figure 1

A

\[
\begin{align*}
\text{CH}_3 & \quad \text{O} \quad \text{CH}_3 \\
\text{CH}_2 & \quad \text{O} \quad \text{CH}_3
\end{align*}
\]

\[
\text{UDPGA} \quad \text{Glucuronidation} \quad \text{[O]} \quad \text{CYP2C8}
\]

Either configuration

B

\[
\begin{align*}
\text{CH}_3 & \quad \text{O} \quad \text{CH}_3 \\
\text{CH}_2 & \quad \text{O} \quad \text{CH}_3
\end{align*}
\]

\[
\text{UDPGA} \quad \text{Glucuronidation} \quad \text{[O]} \quad \text{CYP2C8}
\]

Covalent binding
Figure 2

1a: $R_1 = CF_3$, $R_2 = CF_3$

1b: $R_1 = H$, $R_2 = CH_3$

1c: $R_1 = CH_3$, $R_2 = H$

2

3a: $R_1 = CF_3$, $R_2 = CF_3$

3b: $R_1 = H$, $R_2 = CH_3$

3c: $R_1 = CH_3$, $R_2 = H$

4a: $R_1 = CF_3$, $R_2 = CF_3$

4b: $R_1 = H$, $R_2 = CH_3$

4c: $R_1 = CH_3$, $R_2 = H$
Figure 4

A

\[
\text{CH}_3 \text{-O-} \text{CH} = \text{C-CH}_3 \text{-O-Gluc}
\]

\[\text{M-H}^- = 425\]

\[
\begin{align*}
\text{MS} & \quad \text{Intensity} \\
0 & \quad 121.10 \\
121.28 & \quad 249.11 \\
120.59 & \quad 122.09 \\
121.22 & \quad 121.10 \\
175.98 & \quad 249.99 \\
249.11 & \quad 851.11 \\
425.19 & \\
507.57 & \quad 774.87 \\
774.87 & \quad 873.20 \\
873.20 & \quad 1298.90 \\
851.11 & \\
1000.02 & \quad 118.29 \\
118.29 & \quad 409.25 \\
409.25 & \quad 980.39 \\
700.76 & \quad 1100.02 \\
1000.02 & \\
200000000 & \quad 137.17 \\
137.17 & \quad 265.12 \\
265.12 & \quad 372.95 \\
372.95 & \quad 400.64 \\
313.74 & \\
15000000 & \quad 138.13 \\
138.13 & \\
5000000 & \\
5000000 & \\
15000000 & \\
15000000 & \\
600000000 & \\
600000000 & \\
400000000 & \\
400000000 & \\
200000000 & \\
200000000 & \\
100000000 & \\
100000000 & \\
50000000 & \\
50000000 &
\end{align*}
\]

B

\[
\text{CH}_3 \text{-O-} \text{CH} = \text{C-CH}_3 \text{-O-Gluc}
\]

\[\text{M-H}^- = 441\]

\[
\begin{align*}
\text{MS} & \quad \text{Intensity} \\
0 & \quad 121.10 \\
121.28 & \quad 249.11 \\
120.59 & \quad 122.09 \\
121.22 & \quad 121.10 \\
175.98 & \quad 249.99 \\
249.11 & \quad 851.11 \\
425.19 & \\
507.57 & \quad 774.87 \\
774.87 & \quad 873.20 \\
873.20 & \quad 1298.90 \\
851.11 & \\
1000.02 & \quad 118.29 \\
118.29 & \quad 409.25 \\
409.25 & \quad 980.39 \\
700.76 & \quad 1100.02 \\
1000.02 & \\
200000000 & \quad 137.17 \\
137.17 & \quad 265.12 \\
265.12 & \quad 372.95 \\
372.95 & \quad 400.64 \\
313.74 & \\
15000000 & \quad 138.13 \\
138.13 & \\
5000000 & \\
5000000 & \\
15000000 & \\
15000000 & \\
600000000 & \\
600000000 & \\
400000000 & \\
400000000 & \\
200000000 & \\
200000000 & \\
100000000 & \\
100000000 & \\
50000000 & \\
50000000 &
\end{align*}
\]

C

\[
\begin{align*}
\text{M1} & \quad \text{Time (min)} \\
10.26 & \\
10.95 & \\
14.94 & \\
15.23 & \\
15.52 & \\
19.34 & \\
18.32 & \\
18.91 & \\
20.21 & \\
\end{align*}
\]

\[
\begin{align*}
\text{M2} & \quad \text{Intensity} \\
10.26 & \\
10.95 & \\
14.94 & \\
15.23 & \\
15.52 & \\
19.34 & \\
18.32 & \\
18.91 & \\
20.21 & \\
\end{align*}
\]

\[
\begin{align*}
\text{M3} & \quad \text{Time (min)} \\
10.26 & \\
10.95 & \\
14.94 & \\
15.23 & \\
15.52 & \\
19.34 & \\
18.32 & \\
18.91 & \\
20.21 & \\
\end{align*}
\]

\[
\text{Gemfibrozil acyl-β-glucuronide}
\]