Plasma stability-dependent circulation of acyl glucuronide metabolites in humans. How circulating metabolite profiles of muraglitazar and peliglitazar can lead to misleading risk assessment

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Abbreviations used: ADME, absorption, distribution, metabolism, and excretion; AG, 1-O-β-acyl-glucuronide; CPM, counts per minutes; HLM; human liver microsomes; LC/MS or LC/MS/MS, High performance liquid chromatography tandem mass spectrometry; MIST, metabolites in safety testing; NADPH, β-nicotinamide adenine dinucleotide phosphate sodium (reduced form); NMR, nucleic magnetic resonance; PPAR, peroxisome proliferator-activated receptors; UDPGA, uridine 5'-diphospho-glucuronic acid; UGT, uridine 5'-diphospho-glucuronosyltransferase; UV, ultraviolet, TFA, trifluoroacetic acid.

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ABSTRACT

Muraglitazar and peliglitazar, two structural analogs differing by a methyl group, are dual alpha/gamma peroxisome proliferator-activated receptor activators. Both compounds were extensively metabolized in humans through acyl glucuronidation to form 1-O-β-acyl-glucuronide (AG) metabolites as the major drug-related components in bile, representing at least 15-16% of dose following oral administration. Peliglitazar AG was the major circulating metabolite while muraglitazar AG was a very minor circulating metabolite in humans. Peliglitazar AG circulated at lower concentrations in animal species than in humans. Both compounds had a similar glucuronidation rate in UDPGA-fortified human liver microsomal incubations and a similar metabolism rate in human hepatocytes. Muraglitazar AG and peliglitazar AG were chemically synthesized and found to be similarly oxidized through hydroxylation and O-demethylation in NADPH-fortified human liver microsomal incubations. Peliglitazar AG had a greater stability than muraglitazar AG in incubations in buffer, rat or human plasma (pH 7.4). Incubations of muraglitazar AG or peliglitazar AG in plasma produced more aglycon than acyl migration products compared to incubations in the buffer. These data suggested that the difference in plasma stability, not differences in intrinsic formation, direct excretion, or further oxidation of muraglitazar AG or peliglitazar AG, contributed to the observed difference in the circulation of these AG metabolites in humans. The study demonstrated the difficulty in doing risk assessment based on metabolite exposure in plasma as the more reactive muraglitazar AG would not have triggered a threshold of concern based on recent FDA guidance on Metabolites in Safety Testing (MIST) whereas the more stable peliglitazar AG would have.
Introduction

Acyl glucuronidation is one of the major metabolic clearance pathways for many carboxylic acid containing drugs. 1-O-β-Acy1 glucuronides, which are formed by 5’-diphospho-glucuronosyltransferase (UGT) enzymes as the original isomer, can undergo a number of reactions including hydrolysis, rearrangement via acyl migration (Stachulski et al., 2006; Xue et al., 2008; Zhang et al., 2006), further metabolism (Kumar et al., 2002; Kochansky et al., 2007; Ogilvie et al., 2006), and reaction with nucleophiles (Bailey and Dickinson, 2003; Akira et al., 2002; Sallustio et al., 1995). The chemical reactivity of acyl glucuronides proceeds via two distinct pathways (Bailey and Dickinson, 2003; Stachulski et al., 2006): (1) direct displacement of the acyl residue with a nucleophile to produce an aglycon (hydrolysis product) and an acylated nucleophile, (2) alternatively, migration of the acyl group around the sugar-ring to yield 2-, 3-, and 4- acyl isomers. These isomers may undergo transient ring opening with concurrent formation of a reactive aldehyde group. Either of these pathways could lead to covalent binding to cellular proteins (Bailey and Dickinson, 2003; Akira et al., 2002; Sallustio et al., 1995). These pathways are effectively catalyzed at physiological pH (pH 7.4) and occur more rapidly under basic pH conditions (Xue et al., 2008). Acyl glucuronides are relatively stable under acidic conditions (pH 4-5). Careful acidification and cooling are required to stabilize acyl glucuronides in biofluids such as plasma, urine, and bile (Wang et al., 2007). Muraglitazar and peliglitazar, oxybenzylglycine analogs (non-thiazolidinedione), are novel dual PPAR α/γ activators. The two compounds are structurally very similar with the difference being the addition of a methyl group (Figure 1). Following oral administration to humans, both [14C]muraglitazar and [14C]peliglitazar underwent
extensive conjugation and the major portion of each radioactive dose was excreted in bile as acyl glucuronide metabolites in humans (Wang et al., 2006; Wang et al., 2010). The acyl glucuronide metabolites of muraglitazar and peliglitazar appeared to be stable in \textit{ex vivo} plasma, urine and bile under acidic and low temperature conditions since no significant amounts of acyl migration isomers were formed in plasma, urine, and bile of animals and humans that were acidified and stored at low temperature. In addition, the parent concentrations were not increased over time (Wang et al., 2006; Wang et al., 2010). The major circulating drug-related component (>90%) after oral administration in humans was muraglitazar, however, both peliglitazar and peliglitazar AG were major drug-related components (approximately equal concentrations) in humans following oral administration of peliglitazar. There was no protein covalently bound radioactivity found in the protein pellet after extraction with organic solvents from plasma samples of humans with oral administration of either C-14 labeled compound.

The circulation of a metabolite, in general, would mainly depend on its formation, further metabolism, and direct clearance into urine and bile. For reactive metabolites such as acyl glucuronides, there could also be reactions to form various small metabolites or large molecular adducts. This study investigates the mechanism responsible for the distributional difference between muraglitazar and peliglitazar, two structurally similar analogs by studying formation, stability, excretion and further metabolism of their glucuronide metabolites.
Materials and Methods

Materials. Muraglitazar, peliglitazar, and their C-14 labeled materials were synthesized at Bristol-Myers Squibb. Their structures are shown in Figure 1. The C-14 labeled materials had a radiochemical purity of >99%. Acetonitrile and trifluoroacetic acid was purchased from EM Science (Gibbstown, NJ). Pooled human liver microsomes (n = 22) were purchased from BD Biosciences (Boston, MA). The human hepatocytes were acquired as freshly prepared cell suspensions from Clonetics Co. (Walkersville, MD). All other chemicals used were of reagent grade or better.

Synthesis of peliglitazar AG and muraglitazar AG.

Synthesis of muraglitazar AG and peliglitazar AG basically followed the procedure described by (Perrie et al., 2005).

Allyl glucuronate: To a solution of D-glucuronic acid (5.0 g, 25.7 mmol) in dimethyl formamide (50 mL) at 20°C was added 1,8 - diazabicyclo[5.4.0]undec-7-ene (DBU) (4.3 mL, 28.2 mmol). The mixture was stirred for 15 min, after which allyl bromide (2.8 mL, 30.8 mmol) was added. The reaction was stirred overnight, after which volatiles were removed in vacuo at 60°C. The crude residue was then purified by flash chromatography twice on a 120-g silica gel column with a continuous gradient from 0-20% methanol (MeOH) in methylene chloride (CH₂Cl₂). The isolated material was then dissolved in MeOH (5 mL) and concentrated in vacuo. CH₂Cl₂ (40 mL) was added and the solution was cooled to 0°C for 0.5 h. The precipitated solid was filtered off and washed with ice cold CH₂Cl₂, then dried in vacuo to give the allyl glucuronate (3.4 g, 57% yield, α/β anomer ratio @ 2:1).
Peliglitazar β-anomer allyl ester: To a -5°C solution of peliglitazar (5.5 g, 10.4 mmol) and PPh₃ (2.72 g, 10.4 mmol) in tetrahydrofuran (THF, 40 mL) and dimethyl formamide (DMF, 5 mL) was added diisopropyl azodicarboxylate (2.0 mL, 10.4 mmol). After 10 min, a solution of the allyl glucuronate (1.44 g, 6.15 mmol) in THF (10 mL) and DMF (2.5 mL) was added slowly over 10 min using a syringe pump. After the addition was complete the reaction solution had turned dark brown. The reaction was stirred at -5°C for 2 h and the reaction was monitored by thin layer chromatography (TLC) until completion. The formation of product(s) was monitored by HPLC-MS analysis on a Luna C18, 50 x 4.6 mm column with a 0-100% B linear gradient in 4 min with Solvent A: 10% MeOH in 10 mM ammonium acetate (NH₄OAc) solution and Solvent B: 90% MeOH in 10 mM NH₄OAc solution at a flow rate of 4 mL/min. Volatiles were removed in vacuo, and the crude residue was purified by flash chromatography twice on a 120-g silica gel column with a continuous gradient of 0-10% ethanol (EtOH) in CH₂Cl₂. The resulting material contained the desired α and β anomers as major products. The α and β anomers with a 2-3:1 ratio had been partially separated by the flash chromatography procedure described above and the mixture was further purified by preparative HPLC. The preparative HPLC conditions used a 250 x 21 mm, 4 μ Synergy Hydro-RP 80A column (Phenomenex, Torrance, CA) at room temperature with a flow rate of 25 mL/min and a mobile phase of 0.05% acetic acid pH 3.3/acetonitrile (50:50, v/v) of a run time of 48 min at 278 nm. A portion of 35 mg was injected on the column each time. After careful removal of acetonitrile (CH₃CN) in vacuo at room temperature, the remaining aqueous solution was extracted with ethyl acetate (EtOAc, 300 mL) and the organic phase was
concentrated and dried \textit{in vacuo} to provide the $\alpha$ and $\beta$ anomer esters as white powders. The $\beta$ anomer was recovered in a 10% yield.

Bigelitazar AG: To a 0°C solution of the $\beta$-anomer allyl ester (370 mg, 0.44 mmol) in THF (2.0 mL) was added (PPh$_3$)$_4$Pd (59.2 mg, 0.051 mmol) followed by pyrrolidine (35.2 $\mu$L, 0.44 mmol). The mixture was stirred at 0°C for 30 min, after which volatiles were immediately removed \textit{in vacuo} to give the crude carboxylic acid. LC/MS using the previously described Luna C18 column method indicated the formation of desired product. The crude product was purified by previously described preparative HPLC on a Synergi, Hydro-RP column. After removal of CH$_3$CN, the remaining solution was lyophilized to give the desired acyl glucuronide $\beta$-anomer as a white powder (98% pure by HPLC, 300 mg, @75% yield). $^1$H NMR (500 MHz, DMSO-$d_6$) $\delta$ ppm: 1.50 (d, $J$=7.15 Hz, 3H), 1.57 (d, $J$=6.60 Hz, 2H), 2.36 (s, 3H) 2.93 (t, $J$=6.60 Hz, 2H), 3.07 - 3.12 (m, 1H), 3.15 (s, 1H), 3.23 (s, 1H), 3.35 (d, $J$=9.90 Hz, 1H), 3.38 (d, $J$=9.90 Hz, 1H), 3.73 (s, 3H), 3.84 (d, $J$=18.70 Hz, 1H), 3.90 (d, $J$=18.15 Hz, 1H), 4.07 (d, $J$=18.50 Hz, 1H), 4.11 (d, $J$=18.15 Hz, 1H), 4.22 (t, $J$=6.60 Hz, 2H), 5.30 (q, $J$=7.70 Hz, 1H), 5.35 (q, $J$=7.70 Hz, 1H), 5.39 (d, $J$=8.25 Hz, 1H), 6.89 (d, $J$=8.80 Hz, 2H), 6.94 (d, $J$=8.80 Hz, 2H), 6.98 (d, $J$=8.80 Hz, 1H), 7.29 (d, $J$=8.25 Hz, 2H), 7.34 (d, $J$=8.25 Hz, 2H), 7.49 (t, $J$=7.88 Hz, 2H), 7.49 (s, 1H), 7.92 (dd, $J$=7.70, 1.10 Hz, 2H). The anomeric proton of the $\beta$-anomer was at $\delta$5.39, with $J$ = 8.25 Hz, which is typical for the beta configuration. The following resonance signals corresponded to the minor rotational conformer: $\delta$1.57 (proton #18); $\delta$3.90 (15); $\delta$5.30 (17); $\delta$5.35 (2); $\delta$6.98 (24, 26); $\delta$7.34 (23, 27). $^{13}$C NMR (126 MHz, DMSO-$d_6$) $\delta$ ppm: 10.44 (s, 1C), 17.42 (s, 1C), 26.16 (s, 1C), 39.61 (s, 1C), 45.31 (s, 1C), 54.11 (s, 1C), 55.90 (s, 1C), 66.74 (s, 1C), 72.47 (s, 1C), 73.03 (s, 1C), 75.08 (s, 1C),
1-O-Acyl-α-glucuronide of peliglitazar: The isolated acyl glucuronide α anomer allyl ester obtained by preparative HPLC was deprotected by the identical procedure as described for the synthesis of the β anomer acid as a white powder (98% purity by HPLC) after lyophilization. $^1$H NMR (500 MHz, DMSO-$d_6$) δ ppm: 1.47 (d, $J$=6.60 Hz, 3H) 1.55 (d, $J$=6.60 Hz, 3H) 2.37 (s, 3H) 2.94 (t, $J$=6.32 Hz, 2H) 3.15 (d, $J$=8.80 Hz, 1H) 3.73 (s, 3H) 4.22 (t, $J$=6.32 Hz, 2H) 4.94 (d, $J$=3.85 Hz, 1H) 5.06 (d, $J$=6.60 Hz, 1H) 5.34 (d, $J$=7.15 Hz, 2H) 5.39 (d, $J$=6.60 Hz, 2H) 5.97 (d, $J$=2.61 Hz, 1H) 6.02 (d, $J$=3.30 Hz, 1H) 6.89 (d, $J$=6.86 Hz, 2H) 6.91 (d, $J$=6.89 Hz, 2H) 6.95 (d, $J$=8.25 Hz, 2H) 7.02 (d, $J$=8.80 Hz, 2H) 7.28 (d, $J$=8.25 Hz, 2H) 7.33 (d, $J$=8.80 Hz, 2H) 7.49 (s, 1H) 7.50 (t, $J$=7.15 Hz, 2H) 7.92 (d, $J$=7.70 Hz, 2H). Some of the signals corresponded to the minor rotational conformer, including δ1.55 doublet (for proton 18) and δ5.97 doublet (for the anomeric proton #2). Since the amount of material was limited for carbon-proton NMR heterocorrelations, the chemical shift (@ 6 ppm) and coupling (<4 Hz) of the anomeric proton were used to determine the molecule to be in an alpha conformation.

Muraglitazar AG: This compound was synthesized with similar procedures to peliglitazar AG at an overall 3.3% yield and the purity of 98%. $^1$H NMR (500 MHz, DMSO-$d_6$) δ ppm: 2.36 (s, 3H), 2.93 (t, $J$=6.60 Hz, 2H), 3.07 - 3.16 (m, 2H), 3.20 - 3.27 (m, 1H), 3.35 (t, $J$=9.62 Hz, 1H), 3.73 (s, 3H), 4.09 - 4.25 (m, 4H), 4.45 (dd, 2H), 4.58
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(dd, 1H), 5.38 (d, J=8.25 Hz, 0H), 5.42 (d, J=7.70 Hz, 1H), 6.94 (dd, 4H), 7.01 (d, J=8.80 Hz, 1H), 7.08 (d, J=8.80 Hz, 1H), 7.26 (d, J=8.25 Hz, 1H), 7.31 (d, J=8.25 Hz, 1H), 7.43 - 7.55 (m, 3H), 7.91 (d, J=7.15 Hz, 2H). The anomeric proton of the β-anomer was at 5.38 ppm (J=8.25 Hz) for the minor rotational conformer, and at 5.42 ppm (J=7.70 Hz) for the major conformer. The downfield chemical shift and large coupling observed are typical for the beta configuration. The following resonance signals corresponded to the minor rotational conformer: 4.58 (dd, 1H), 5.38 ppm (J=8.25 Hz), 7.31 (d, J=8.25 Hz, 2H), 7.01 (d, J=8.80 Hz, 1H). 13C NMR (126 MHz, DMSO-d6) δ ppm: 10.95 (s, 1C), 26.69 (s, 2C), 49.50 (s, 1C), 51.88 (br s, 1C), 56.47 (s, 1C), 67.29 (s, 1C), 72.98 (s, 1C), 73.52 (br s, 1C), 75.69 (s, 1C), 77.36 (s, 1C), 96.15 (br s, 1C), 115.10 - 115.47 (m, 2C), 115.65 (br s, 2C), 123.30 - 124.14 (m, 2C), 126.53 (s, 2C), 128.22 (s, 1C), 130.03 (s, 1C), 130.14 (s, 1C), 130.39 (s, 1C), 131.14 (s, 2C), 133.78 (s, 1C), 145.51 (s, 1C), 146.20 (s, 1C), 155.71 (s, 1C), 157.65 (br s, 1C), 158.86 (s, 1C), 159.47 (s, 1C), 169.30 (s), 169.62 (s, 1C), 172.61 (br s, 1C). The carbonyl signal at 169.30 corresponded to the minor rotational conformer.

Liver microsomal and hepatocyte incubations. Incubations (250 μL) contained muraglitazar or peliglitazar (10 μM, 2.5 mM stock solution in 50:50, v/v, acetonitrile:Tris buffer), HLM (1 mg/mL protein), UDPGA (2 mM), magnesium chloride (10 mM), and Tris buffer (100 mM, pH 7.4). Incubations were initiated by addition of a substrate at 37°C and quenched after 15 min by addition of one volume of acetonitrile to the incubation mixture. After centrifugation to remove the precipitated microsomal proteins, the clear supernatant (100 μL) was analyzed by LC/MS. The HPLC system I consisted of a Waters 600 pump, 717 Autosampler, and a 996
Photodiode Array detector. The column used was a C₁₈ YMC ODS-AQ reverse phase column (4.6 x 150 mm, 3 μ) maintained at room temperature with a flow rate of 0.7 mL/min. The mobile phase A consisted of 5% acetonitrile in water containing 0.1% TFA and the mobile phase B consisted of 95% acetonitrile containing 0.1% TFA. A linear gradient was 10 to 100% B in 20 min and then held at 100% B for 10 min. The retention times were 12.1, 7.3, 15.4, and 8.6 min for muraglitazar, muraglitazar AG, peliglitazar, and peliglitazar AG, respectively. The HPLC was interfaced to a LCQ mass spectrometer (ThermoFisher, San Jose, CA) operated in the positive ionization mode to acquire full scan LC/MS data with a mass scan range of 200-1000 Da. The percent metabolism was calculated based on the ratio of peak areas of the metabolite versus metabolite plus the parent (metabolite/metabolite + parent) from the UV chromatogram (at 278 nm).

Hepatocyte incubations were performed with cells in suspension in 24-well tissue culture plates shaken at 90 rpm on an orbital shaker. The incubations were in Krebs-Henseleit buffer in a 5% CO₂/95% air atmosphere at 37°C with muraglitazar or peliglitazar (5 μM, 0.5 mM stock in 50:50 acetonitrile/potasium phosphate, v/v) and 1 x 10⁶ hepatocytes/mL. Incubation time was 1 hour. The samples were quenched by addition of an equal volume of acetonitrile. The quenched samples were treated and analyzed by LC/MS with the HPLC system I as described for the microsomal incubations.

Muraglitazar AG or peliglitazar AG at 20 μM was separately incubated for 15 min with human liver microsomes (2 mg/mL protein) in 1 mL of 50 mM sodium phosphate buffer with and without 1 mM NADPH. The samples were separated by HPLC system II using a Shimadzu LC-10AT system using the analytical column used for the microsomal
incubation samples. The mobile phase consisted of two solvents: A) 0.06% TFA in water and B) 0.06% TFA in acetonitrile. The gradient consisted of the following steps: Solvent B started at 5%, then linearly increased to 25% at 5 min, to 40% at 20 min, to 53% at 60 min, to 60% at 63 min, to 90% at 65 min, held at 90% for 7 min, and then decreased to 5% at 75 min. HPLC effluent was 1 mL/min. The quantities of muraglatzar and peligitazar AGs were estimated by HPLC separation with UV detection at 278 nm. The metabolites were identified by LC/MS as described in the supplemental section.

**Study Subjects, Dosing, and Sample Collection**

All animal housing and care conformed to the standards recommended by the Guide for the Care and Use of Laboratory Animals. Animal rooms were maintained on a 12-hour light and dark cycle. The human study was performed in accordance with the principles of the Declaration of Helsinki and its amendments, and the study protocol was approved by the Institutional Review Board and Radiation Safety Committee at the investigational site. All subjects were in good health and gave written, informed consent to participate in the study.

Male CD-1 mice (n=5, 25-35 g), male Sprague-Dawley rats (n=3, 250-280 g), and male Cynomolgus monkeys (n=3, 3-5 kg) were fasted for approximately 8 hours before dosing. Each animal received an oral gavage dose of 30 mg/kg (300 μCi/kg, 5 mL/kg) for mouse, 15 mg/kg (150 μCi/kg, 5 mL/kg) for rat, 3 mg/kg (30 μCi/kg, 1 mL/kg) for monkey of [14C]peliglitazar dissolved in PEG-400. Human studies were conducted as described previously (Wang et al., 2006). Four healthy male subjects, aged 18 to 45 years, each received a single dose of [14C]muraglitazar (20 mg) or [14C]peliglitazar (10 mg)
containing approximately 100 μCi of radioactivity as an oral solution in PEG-400 after at least an 8 h overnight fast.

Blood samples (terminal for mouse and rat, and via the cephalic vein for monkey, direct venipuncture using vacutainers for human) were collected at 1, 4, 12, and 24 hours using K$_2$EDTA as the anticoagulant. Plasma was prepared by centrifugation at approximately 1000xg for 15 min at 4°C. Acetic acid was added to plasma to a final concentration of 5% (v/v, 0.83 M) immediately following processing. All plasma samples were frozen and stored at -20°C.

**Sample preparations and analysis.** Pooled plasma samples were prepared separately by mixing an equal volume (0.2-0.5 mL) of plasma sample from each subject. Portions (0.5-1 mL) of the pooled plasma samples were extracted by addition of a mixture of one volume of methanol and 3 volume of acetonitrile and mixed on a Vortex mixer. The mixtures were centrifuged at 2000xg at 10°C for 30 min, and then the supernatants were transferred into a polypropylene centrifuge tube. The extraction was repeated two more times and all supernatants were combined. The recovery of radioactivity in the supernatant after extraction was quantitative. The plasma protein pellets were digested in 1 M NaOH for 12 h before neutralized with 1 M HCl and counted for radioactivity in 15 mL of Ecolite™ scintillation fluid. The combined supernatants were concentrated under a stream of nitrogen, the residues were then reconstituted in 0.2-0.5 mL of a solution of 70% of HPLC mobile phase A (0.06% TFA in water) and 30% mobile phase B (0.06% TFA in acetonitrile), vortexed, centrifuged at 2000xg for 10 min, and 100 μL of the supernatant was used for the HPLC analysis. Metabolites in plasma were analyzed by HPLC system II using a Shimadzu LC-10AT system as described in last section. HPLC
effluent (1 mL/min) was collected into Deepwell LumaPlate™-96 (PerkinElmer life sciences, Meriden, CT) at 0.26 min. The plates were dried with a Speed-Vac (Savant) and counted for 10 min per well with a TopCount analyzer (PerkinElmer life sciences, Meriden, CT) to quantify radioactivity. Biotransformation profiles were prepared by plotting the resulting net CPM values vs time-after-injection. Radiochromatograms were reconstructed from the Topcount data using Microsoft® Excel software.

**Stability studies in incubations in buffer and plasma.** A portion of 0.75 mL of 1 M phosphate buffer at pH 7.4 and 14.1 mL of human or rat plasma were mixed, and 0.15 mL of 5 mg/mL 1-O-β-acyl glucuronide stock solution in acetonitrile and water (50:50, v/v) was spiked to that solution to give a final concentration of 50 µg/mL of the 1-O-β-acyl glucuronide. Each spiked plasma solution was aliquoted into a 0.5 mL portion of 27 tubes. Twenty four of them were placed in the water bath at 37°C, and three of them were quenched with 3 volumes of 1.35% acetic acid in acetonitrile/methanol (1:1, v/v). The remaining samples were quenched at 0.25, 0.5, 1, 2, 4, 6, 24, and 48 h (3 tubes/time point) following the same quenching procedure. The samples were mixed well and immediately frozen at -80°C until analysis. The entire experiment was done on ice water bath. Before analysis samples were taken out and thawed in ice water bath. Similarly, for incubations in phosphate buffer, 0.75 mL of 1 M phosphate buffer at pH 7.4 and 14.1 mL water were mixed and 0.15 mL of 5 mg/mL 1-O-β-acyl glucuronide stock solution. The remaining steps were same as the incubations for buffered plasma.

1-O-β-Acyl glucuronides and their isomers as well as aglycons were separated isocratically by HPLC system III using a mobile phase comprised of 65:35 (v/v) acetonitrile/water containing 0.05% formic acid at a flow rate of 0.3 mL/min. The
separation was performed using a Phenomenex Luna C18 (2) analytical column (3 x 150 mm, 3 μm, Torrance, CA) operated at 25°C. The injection volume was 5 μL, and the run time was 12.0 min. Under these conditions, the retention time was 6.17, 7.04, 7.55, 8.13, 8.35, 8.82, and 15.08 min, respectively, for Isomer 1, 2, muraglitazar AG, Isomer 3, 4, 5, and muraglitazar. The retention time was 7.51, 8.62, 9.55, 10.25, 11.05, and 14.80 min, respectively, for Isomer 1, 2, peliglitazar AG, Isomer 3, 4, and peliglitazar. The HPLC effluent was monitored at 278 nm and analyzed by a LTQ mass spectrometer (ThermoFisher, San Jose, CA).

**Results**

Table 1 and Figure 2 show the exposures of peliglitazar and peliglitazar AG in the plasma of mouse, rat, monkey, and human. The exposure multiples of Cmax and AUC values for the parent compound in mouse, rat, and monkey were >21. The exposure multiples of Cmax and AUC values for the major glucuronide metabolite, peliglitazar AG, were 3.8-6.4 in the mouse, 1-2 in monkey, and <0.5 in rat from the ADME studies. The projected exposure multiples of Cmax and AUC values for the metabolite were higher (all were >1) in mouse, rat, even monkey with linearly scaled doses for the term-toxicological studies. However, the exposure multiples of Cmax and AUC values for the metabolite were still <1 with the projected carcinogenicity testing doses. Overall, peliglitazar AG exposure multiples of Cmax and AUC in toxicological species were marginal even at the projected high toxicological doses.

Figure 1 showed that peliglitazar AG circulated in humans as a major metabolite, while muraglitazar AG had little circulation in humans in the 1-h plasma samples. Radioactivity profiles of human plasma at 4, 12, and 24 h also showed that peliglitazar AG but not
Muraglitazar AG was a significant circulating metabolite in humans. There was minimal to no acyl isomers detected in the plasma samples from subjects after administration of C-14 peliglitazar or muraglitazar. In addition, the plasma protein pellets after extraction with the organic solvent contained no detectable levels of radioactivity. Together, the low level of isomerization and protein covalent binding suggest ex vivo stabilization of the plasma samples by addition of acetic acid, and low levels of total plasma radioactivity. Overall, muraglitazar AG represented approximately 4% of the muraglitazar AUC$_{0-24h}$ while peliglitazar AG represented 240% of the peliglitazar AUC$_{0-24h}$. With concentrations of peliglitazar AG of 102, 48.8, and 21.3 ng/mL at 1, 4, and 12 h postdose, the estimated half life of the peliglitazar AG was approximately 3 h based on the limited data points (Wang et al., 2010). Estimation of half life for muraglitazar AG was not possible. Although both muraglitazar and peliglitazar underwent extensive conjugation metabolism to form AGs as the major metabolite, only peliglitazar AG was the major circulating component following oral administration of the C-14 labeled drug. Similar distributional difference was observed in monkeys as well as in rats following oral administration of C-14 labeled muraglitazar or peliglitazar (data not shown).

Table 2 shows the glucuronidation rates of muraglitazar and peliglitazar in human liver microsomes and hepatocytes and biliary/urinary excretion of acylglucuronide metabolites in humans following oral administration of C-14 labeled compounds. At clinically relevant concentrations, 5 μM in hepatocytes and 10 μM in human liver microsomes (Zhang et al., 2006; Wang et al., 2010), muraglitazar and peliglitazar showed similar glucuronidation rates in incubations with both UDPGA-fortified human liver microsomes and human hepatocytes. In humans, muraglitazar and peliglitazar AGs showed similar...
elimination profiles with at least 15-16% of dose excreted in the bile over the 3-8 h postdose collections and 0.2-0.8% of the dose in the urine over 0-168 h collections following oral administration of C-14 labeled compounds. These data suggest that muraglitazar AG was formed in the liver and excreted into the urine and bile in a similar manner to peliglitazar AG.

Table 3 shows the stability of muraglitazar AG and peliglitazar AG in incubation in buffer or human plasma. Figure 3 shows distinct concentration-time profiles of muraglitazar AG and peliglitazar AG degradation in the buffer and rat or human plasma samples. Muraglitazar AG degraded in a phosphate buffer or human plasma at approximately 7-8 times faster than peliglitazar AG by hydrolysis to aglycons and to a lesser extent by acyl migration to positional isomers. Muraglitazar and peliglitazar AGs degraded approximately 3-5 times faster in human plasma than in the buffer (Table 3). Both glucuronides had degradation that was faster in rat plasma than in buffer but was different from that in human plasma (Figure 3). Good separation of the positional isomers of muraglitazar AG and peliglitazar AG and their degradation products was achieved by simple reversed-phase HPLC using an isocratic elution as described by Xue et al. (2008). The hydrolysis rates for formation of muraglitazar and peliglitazar were 5-10 times faster in plasma than in buffer (Figures 4 and 5). In the incubation with muraglitazar AG in human plasma, the acyl migration isomers 3 and 4 were formed and then quickly hydrolyzed to form the aglycon (Figure 4B); however, both the isomers and the aglycon were formed slowly in the buffer; in addition, the acyl isomers degraded to aglycon at a slower rate than that in plasma (Figure 4A). Similar degradation profiles were observed with peliglitazar AG but at a much slower rate than muraglitazar AG (Figure 5).
Oxidative metabolism of muraglitazar AG and peliglitazar AG was investigated in human liver microsomes in the presence of NADPH. Multiple hydroxylated metabolites and O-demethylated metabolite of muraglitazar AG were observed in the incubation starting with muraglitazar and peliglitazar AGs and metabolite identification was presented in the supplemental section. The glucronide oxidation rates were estimated to be 22 and 24 pmol/min/mg, respectively, for muraglitazar AG and peliglitazar AG.

**Discussion**

Figure 6 illustrates the potential factors that would be expected to affect the circulating levels of an acyl glucuronide metabolite include formation rate, biliary and renal excretion, further metabolism, tissue distribution, and reactions with small and large molecules. The disproportional exposures to peliglitazar AG across species and the striking differences in exposures to AGs of peliglitazar and muraglitazar in humans lead us to evaluate factors that may influence AG exposures for these two compounds. Intrinsic formation in vitro and direct excretion for acyl glucuronide metabolites in vivo were found similar between muraglitazar and peliglitazar. Incubations of muraglitazar and peliglitazar in UDPGA-fortified human liver microsomes or in hepatocytes generated the acyl glucuronides as the major metabolite at similar formation rates. Examination of acyl glucuronide excretion profile revealed that the acyl glucuronide was excreted as the major metabolite in the bile of humans and to the same extent. Urinary excretion was a very minor clearance pathway for both compounds. There was no tissue accumulation or other major differences in tissue distribution patterns in rats following oral administration of C-14 muraglitazar or peliglitazar (data not shown). These observations do not explain the *in vivo* data, which showed that muraglitazar AG had a much lower exposure than
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peliglitazar AG. Thus, the difference in chemical stability or further metabolism of their glucuronide metabolites may have contributed to their very different circulation profiles in humans. Further metabolism of glucuronide metabolites were demonstrated in the in vitro incubations using NADPH-fortified human liver microsomes. The HLM incubations of muraglitazar AG or peliglitazar AG in the presence of NADPH produced hydroxylated and O-demethylated glucuronide metabolites. However, the oxidation of both muraglitazar AG and peliglitazar AG was similarly slow and can not be used to explain their differences in the circulation.

In incubations in pH 7.4 buffer at 37°C, muraglitazar AG degraded >7-8 times faster than peliglitazar AG (Figure 3, Table 3). In incubations of muraglitazar and peliglitazar AGs in human plasma, migration isomers were formed to lesser amounts and these incubations generated aglycons as the dominate products (Figures 4B and 5B). In addition, the acyl migration isomers quickly degraded to the aglycon in the plasma incubations. For comparison to incubations in buffer (Figures 4A and 5A), the aglycon was formed 3-5 times more slowly than in human plasma. In addition, more acyl isomers were formed and then slowly degraded to the aglycon in the buffer. The greater formation of aglycon in plasma may be due to hydrolysis catalyzed by β-glucuronidases and esterases or an unknown hydrolase. The differences in the plasma stability of muraglitazar and peliglitazar AGs could be governed by the steric hindrance of nearby groups to the sugar-aglycone C-O-C=O bond for hydrolysis or migration. The additional methyl group that is near the sugar-aglycon bond in peliglitazar compared to muraglitazar might provide the steric hindrance to prevent hydrolysis of peliglitazar AG. This hypothesis of steric effect on hydrolysis may be tested with model compounds in future. Differences in the chemical
stability of structurally related acyl glucuronides have been well documented (Spahn-Langguth et al., 1989; Bailey and Dickinson, 2003). Even stereo isomers (R- vs S-) of acyl glucuronides have been shown to have two-fold difference in reactivity (Fenselau, 1994; Mortensen et al., 2001; Akira et al., 2000; Hasegwa et al., 2001). Compared to literature first order degradation half-lives (listed in hours) in a pH 7.4 aqueous buffer of AGs of tolmetin (0.26), probenecid (0.4), diclofenac acid (0.51), R-naproxen (0.92), salicylic acid (1.3), S-naproxen (1.8), ibuprofen (3.3), bilirubin (4.4), mefenamic acid (16.5), gemfibrozil (44), and valproic acid (79) (Ebner et al., 1999; Shipkova et al., 2003), dabigatran (1) (Ebner et al., 2010), muraglitazar AG (1.23 h) and peliglitazar AG (10.1) showed moderate aqueous buffer stability. However, reactivity evaluation in buffer was not sufficient since both muraglitazar and peliglitazar AGs showed approximately 4-fold additional instability in plasma compared to buffer. This additional degradation of muraglitazar AG and peliglitazar AG is consistent with a catalytic hydrolysis by an unknown hydrolase for acyl glucuronides in plasma samples. This could be the reason for the inconsistent correlation between buffer degradation stability and plasma exposures of various acylglucuronides (Ebner et al., 2010). Although the reactivity in plasma may not completely correlate with chemical stability either as demonstrated in this study, the real exposure to an acyl glucuronide metabolite in animals or humans is a dynamic process and difficult to assess especially when dealing with a metabolite that had a different degradation rate in plasma samples of different species. This study demonstrates an excellent example that muraglitazar glucuronidation as a major metabolic pathway resulted in a minor circulating metabolite due to chemical degradation in humans.
Another potential difference in disposition of AGs that could lead to different circulating levels is further metabolism. Multiple oxidation metabolites of muraglitazar and peliglitazar AGs were identified (as described in the Supplemental section). The extent of oxidative metabolism was similar for both muraglitazar AG and peliglitazar AG. Therefore, the elimination of muraglitazar and peliglitazar AGs may partially depend on oxidation of the AGs themselves but that does not appear to explain the differential exposure seen in humans. Therefore, while other factors such as differential transport of AGs out of the hepatocytes into blood may also play a role, the chemical stability differences certainly would be expected to contribute the observed exposure differences between muraglitazar AG and peliglitazar AG.

The recent US Food and Drug Administration guidance for pharmaceutical industry on metabolites in safety testing (FDA Guidance, 2008; Baillie et al., 2002; Humphreys and Unger, 2006) defines a rigorous set of experiments to ensure that disproportionally exposed humans metabolites are adequately evaluated in toxicology animal species. The exposure multiples found for peliglitazar AG (AUC) were marginal when compared to the doses used in long-term toxicology studies to those seen in humans after a dose thought to be in the appropriate range. The marginal multiples did not precipitate any toxicology studies with peliglitazar AG as the administered agent, but they did prompt for significant analysis works for plasma AG levels (Xue et al., 2008). It is possible that the Company (BMS) could have been asked to consider such a toxicology study by a regulatory agency. In either case, further examination of the situation with these two compounds leads to the conclusion that plasma monitoring of reactive metabolites may provide misleading information. Peliglitazar AG is obviously less reactive which in part
leads to greater circulating concentrations in humans, and could have triggered additional toxicology studies. On the other hand, muraglitazar AG is more reactive which leads to lower plasma concentrations and thus no further consideration of follow-up toxicology studies. This type of scenario could lead to a sponsor de-risking a relatively stable AG with a set of studies while not following up on the more reactive AG product. Overall, this does not seem to provide the best development path for either compound.

LC/MS-based quantitation methods generally apply to chemically stable metabolites and the exposure to a reactive metabolite could only be assessed when the reactive species reacts with an endogenous component to form a stable form such as albumin adduct (Zhang et al., 2005; Zhang et al., 2009). Chemical instability adds complication for quantification of those instable metabolites such as acyl glucuronides (Xue et al., 2008). Although the ex vivo chemical stability of an acyl glucuronide can be improved through sample acidification and storage at low temperature, the chemical instability in vivo is in general difficult to measure directly. In this study, the chemical stability of muraglitazar AG or peliglitazar AG was assessed by in vitro incubations under a neutral condition followed by HPLC separation and quantification of degradation components in plasma or buffer-fortified with a synthetic glucuronide. In vivo, the hydrolysis of muraglitazar AG or peliglitazar AG catalyzed by an unknown hydrolase formed the parent compounds, which also complicated risk assessment of parents, especially when these acylglucuronides showed species dependent degradations (Figure 3). In addition, conversion of glucuronide metabolites to parent compounds should lead to a higher apparent bioavailability for muraglitazar than peliglitazar assumed all other dispositional properties are similar between two compounds. Unfortunately, bioavailability was not
determined for either compound to make that comparison. Furthermore, there were limited data points for peliglitazar AG and no data points for muraglitazar AG to do any detailed pharmacokinetic analysis for these metabolites.

In summary, a significant reason for different circulating levels of AGs of muraglitazar and peliglitazar was shown to result from the difference in chemical stability in human plasma, not their production, direct excretion, or further metabolism. The focus on the disproportionate peliglitazar AG in human plasma would meet the requirement set out in the MIST, however, it is questionable whether studies of this AG would add anything for the risk assessment for the compound. The more meaningful risk assessment in this case should not be studies with the more stable peliglitazar AG observed in plasma, but rather the more reactive muraglitazar AG not observed in plasma due to inherent reactivity.
Acknowledgements. We would like to thank Dr. Wen-Chyi Shyu for critical review of the manuscript.
References


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receptor agonist highlighted by oxidative metabolism of its acyl glucuronide. *Drug Metab Dispos* **33**: 1894-1904.


Legends of Figures

Figure 1. Metabolite profiles in 1 h human plasma samples after single oral administration of C-14 labeled muraglitazar or peliglitazar. The samples were analyzed by HPLC system II as described in Materials and Methods.

Figure 2. Radioactivity profiles in 1 h plasma of mice (30 mg/kg), rats (15 mg/kg), monkeys (3 mg/kg), and humans (10 mg/subject) following a single oral doses of [14C]peliglitazar. Arrows point to the position of peliglitazar AG elution.

Figure 3. Concentration-time degradation profiles of muraglitazar AG and peliglitazar AG at 50 μg/mL in incubations at 37°C in the buffer, rat and human plasma at pH 7.4. The samples were analyzed by HPLC system III as described in Materials and Methods.

Figures 4. Hydrolysis and isomerization profiles of muraglitazar AG (50 μg/mL) in incubations in a buffer or buffered human plasma (pH 7.4) at 37°C. A, buffer; B, human plasma. The samples were analyzed by HPLC system III as described in Materials and Methods.

Figure 5. Hydrolysis and isomerization profiles of peliglitazar AG (50 μg/mL) in incubations in a buffer or buffered human plasma (pH 7.4) at 37°C. A, buffer; B, human plasma. The samples were analyzed by HPLC system III as described in Materials and Methods.

Figure 6. The factors affecting circulation of an acyl glucuronide metabolite.
Table 1. Estimated exposures of peliglitazar and its acyl glucuronide in mice, rats, monkeys, and humans following a single oral dose of peliglitazar

<table>
<thead>
<tr>
<th>Study Type</th>
<th>Analyte</th>
<th>Species</th>
<th>Doses (mg/kg or mg/subject*)</th>
<th>Conc. at 1 h (μM)</th>
<th>Conc. Exposure Multiples</th>
<th>AUC&lt;sub&gt;0 to t&lt;/sub&gt; (μM•h)</th>
<th>AUC Exposure Multiples</th>
</tr>
</thead>
<tbody>
<tr>
<td>Radiolabeled ADME&lt;sup&gt;a&lt;/sup&gt;</td>
<td>Peliglitazar</td>
<td>Mouse</td>
<td>30</td>
<td>59.58</td>
<td>397</td>
<td>247.4</td>
<td>333</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Rat</td>
<td>15</td>
<td>4.51</td>
<td>30</td>
<td>17.6</td>
<td>24</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Monkey</td>
<td>3</td>
<td>3.64</td>
<td>24</td>
<td>15.5</td>
<td>21</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Human</td>
<td>10*</td>
<td>0.15</td>
<td>1</td>
<td>0.74</td>
<td>1</td>
</tr>
<tr>
<td>Radiolabeled ADME&lt;sup&gt;a&lt;/sup&gt;</td>
<td>Peliglitazar AG</td>
<td>Mouse</td>
<td>30</td>
<td>1.18</td>
<td>6.4</td>
<td>4.72</td>
<td>3.84</td>
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<td></td>
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<td>Rat</td>
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<td>0.07</td>
<td>0.4</td>
<td>0.21</td>
<td>0.17</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Monkey</td>
<td>3</td>
<td>0.34</td>
<td>1.8</td>
<td>1.45</td>
<td>1.18</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Human</td>
<td>10*</td>
<td>0.18</td>
<td>1</td>
<td>1.23</td>
<td>1</td>
</tr>
<tr>
<td>Long-Term Tox Studies&lt;sup&gt;b,c&lt;/sup&gt;</td>
<td>Peliglitazar AG</td>
<td>Mouse</td>
<td>250</td>
<td>9.8</td>
<td>54</td>
<td>39.3</td>
<td>31.9</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Rat</td>
<td>300</td>
<td>1.4</td>
<td>7.8</td>
<td>4.2</td>
<td>3.41</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Monkey</td>
<td>2.5</td>
<td>0.28</td>
<td>1.6</td>
<td>1.21</td>
<td>1.0</td>
</tr>
<tr>
<td>Carc Studies&lt;sup&gt;c,d&lt;/sup&gt;</td>
<td>Peliglitazar AG</td>
<td>Mouse</td>
<td>10</td>
<td>0.39</td>
<td>2.2</td>
<td>1.57</td>
<td>1.28</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Rat</td>
<td>30</td>
<td>0.14</td>
<td>0.7</td>
<td>0.42</td>
<td>0.34</td>
</tr>
</tbody>
</table>

<sup>a</sup> Concentration is estimated from the relative distribution of the parent or M14 in the plasma, the total concentration of radioactivity and the specific activity of the administered drug; AUC<sub>0 to t</sub> is estimated from the concentration in plasma at limited time points (1, 4, 12 h) using the trapezoidal rule.

<sup>b</sup> Doses are the highest dose from the 6-month rat, 3-month mouse, and 1-year monkey toxicity studies.

<sup>c</sup> Concentration and AUC values are scaled linearly with regard to dose values in ADME studies.

<sup>d</sup> Highest projected doses for the carcinogenicity studies.

<sup>e</sup> Exposure multiples were calculated through diving Cmax or AUC values in animals by that in humans.
Table 2. The glucuronidation of muraglitazar and peliglitazar in human liver microsomes and hepatocytes and excretion of acylglucuronide metabolites in humans following oral administration of C-14 labeled compounds

<table>
<thead>
<tr>
<th></th>
<th>In Vitro Glucuronidation Rates $^a$</th>
<th>Glucuronide Excretion (% of Dose) $^b$</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>HLM (pmol/min/mg proteins)</td>
<td>Hepatocytes (pmol/min/10$^6$ cells)</td>
</tr>
<tr>
<td>Muraglitazar</td>
<td>33</td>
<td>60</td>
</tr>
<tr>
<td>Peliglitazar</td>
<td>32</td>
<td>49</td>
</tr>
</tbody>
</table>

$^a$ The substrate concentration was 5 and 10 $\mu$M, respectively, for hepatocyte and microsomal incubations.

$^b$ Excretion of muraglitazar AG and peliglitazar AG in humans (% of dose) following oral administration of C-14 labeled compounds (20 mg/subject, 100 $\mu$Ci). Urinary elimination represented 2.13 and 1.24 % of dose, biliary elimination represented 39.9 and 24.43 % of dose, respectively, for muraglitazar and peliglitazar.
Table 3. The stability of acyl glucuronides of muraglitazar and peliglitazar in buffer and human plasma incubations at 50 μg/mL and 37°C.

<table>
<thead>
<tr>
<th>Compounds</th>
<th>Products</th>
<th>Buffer</th>
<th>Human plasma</th>
</tr>
</thead>
<tbody>
<tr>
<td>Disappearance of glucuronide (T1/2, hr)\textsuperscript{a}</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Muraglitazar AG</td>
<td>N/A</td>
<td>1.23</td>
<td>0.33</td>
</tr>
<tr>
<td>Peliglitazar AG</td>
<td>N/A</td>
<td>10.1</td>
<td>2.3</td>
</tr>
<tr>
<td>Aglycon formation rates (μg/mL/hr)\textsuperscript{b}</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Muraglitazar AG</td>
<td>Muraglitazar</td>
<td>3.5</td>
<td>16.7</td>
</tr>
<tr>
<td>Peliglitazar AG</td>
<td>Peliglitazar</td>
<td>0.5</td>
<td>5.9</td>
</tr>
</tbody>
</table>

The buffer was 50 mM sodium phosphate, pH 7.4. Plasma samples were buffered with 50 mM phosphate, pH 7.4.

\textsuperscript{a} T1/2 was calculated from initial rates of glucuronide disappearance (T1/2 = 0.693/slope).

\textsuperscript{b} The value was the average of formation rates within the linear ranges (<3 hr).
Fig 1

Human plasma 1 h

Muraglitazar glucuronide

* Indicates C-14 label

Human plasma 1 h

Pielgiltazar glucuronide

* Indicates C-14 label
Fig 2

Mouse 1 h plasma

Rat 1 h plasma

Monkey 1 h plasma

Human 1 h plasma

Peliglitazar

Peliglitazar glucuronide

Time (minutes)
Fig 3

- Buffer muraglitazar-AG
- Human plasma muraglitazar-AG
- Rat plasma muraglitazar-AG
- Buffer peliglitazar-AG
- Human plasma peliglitazar-AG
- Rat plasma peliglitazar-AG
Fig 5

A

B

% of Total (280 nm)

Time (h)

Isomer 1

Isomer 2

Peliglitazar-AG

Isomer 3

Isomer 4

Peliglitazar
Fig 6

- **Bile**
  - *Biliary Excretion*
  - **Formation**
    - Drug → Acyl-gluc
  - **Further metabolism**
    - Acyl-gluc → Metabolite(s)
  - **Liver**
- **Distribution**
  - **Chemical degradation**
    - Acyl-gluc → Drug
  - **Blood**
- **Other tissues / organs**
- **Urine**
  - *Urinary excretion*
Plasma stability-dependent circulation of acyl glucuronide metabolites in humans. How circulating metabolite profiles of muraglitazar and peliglitazar can lead to misleading risk assessment
Donglu Zhang, Nirmala Raghavan, Lifei Wang, Yongjun Xue, Mary Obermeier, Stephanie Chen, Shiwei Tao, Hao Zhang, Peter T Cheng, Wenying Li, Ragu Ramanathan, Zheng Yang, W Griffith Humphreys
Pharmaceutical Candidate Optimization (DZ, NR, LW, YX, MO, WL, RR, ZY, WGH), Discovery Chemistry (SC, ST, HZ, PTC). Bristol-Myers Squibb Research and Development, Princeton, NJ 08543

Supplemental data:
Oxidation of glucuronide metabolites of muraglitazar and peliglitazar in human liver microsomes

Muraglitazar AG or peliglitazar AG at 20 μM was separately incubated for 15 min with human liver microsomes (2 mg/mL protein) in 1 mL of 50 mM sodium phosphate buffer with and without 1 mM NADPH. The reaction was quenched by adding one volume of ice-cold acetonitrile containing 2% acetic acid following by centrifugation at 2000xg for 10 min. An aliquot of 100 μL was injected to LC/UV/MS/MS analysis. The samples were analyzed by HPLC system II using a Shimadzu LC-10AT system (Shimadzu Scientific Instruments, Kyoto, Japan) and LC/MS analyses were performed using the analytical column used for the microsomal incubation samples. The mobile phase consisted of two solvents: A) 0.06% TFA in water and B) 0.06% TFA in acetonitrile. The gradient consisted of the following steps: Solvent B started at 5%, then linearly increased to 25% at 5 min, to 40% at 20 min, to 53% at 60 min, to 60% at 63 min, to 90% at 65 min, held at 90% for 7 min, and then decreased to 5% at 75 min. HPLC effluent (1 mL/min) was monitored at 278 nm. The HPLC eluent was partially diverted to a LTQ mass spectrometer (ThermoFisher, San Jose, CA). Full LC/MS (Scan range of 200-1000 Da) and MS/MS spectra were collected.
Figures 1S and 2S show oxidative metabolite profiles of incubations of muraglitazar AG and peliglitazar AG in human liver microsomes in the presence of NADPH. Multiple hydroxylated metabolites and \(O\)-demethylated metabolite of muraglitazar and peliglitazar AGs were observed in the incubation starting with muraglitazar AG or peliglitazar AG. After incubations in human liver microsomes, the major components were still the starting materials (muraglitazar and peliglitazar AGs) and the incubations also led to hydrolysis of muraglitazar and peliglitazar AGs (Figures 1S and 2S). Table 1S shows mass spectrometric characterization of these oxidative metabolites. Metabolites M1, M2, and M3 had a molecular ion of \(m/z\) 709 from muraglitazar AG and \(m/z\) 723 from peliglitazar AG, and respective fragmentation ions at \(m/z\) 533 and 547 (loss of 176), which are consistent with hydroxylation products of muraglitazar AG and peliglitazar AG. Although these metabolites could be acyl migration isomers of one metabolite, more likely they are different metabolites with the hydroxyl group at different sites based retention times. Metabolite M4 had a molecular ion of \(m/z\) 679 from muraglitazar AG and \(m/z\) 693 from peliglitazar AG, and respective fragmentation ions at \(m/z\) 503 and 517 (loss of 176), which are consistent with demethylation products of muraglitazar AG and peliglitazar AG. Metabolites M5, M6, and M7 had a molecular ion of \(m/z\) 533 from muraglitazar AG and \(m/z\) 547 from peliglitazar AG, which are consistent with hydroxylated products of muraglitazar and peliglitazar. Metabolite M8 had a molecular ion of \(m/z\) 503 from muraglitazar AG and \(m/z\) 517 from peliglitazar AG, which are consistent with demethylated products of muraglitazar and peliglitazar. Therefore, in addition to these oxidative metabolites of glucuronide, multiple hydroxylated metabolites and an \(O\)-demethylated metabolite of muraglitazar were also observed in the incubation.
Without NADPH, no oxidative metabolites of muraglitazar, peliglitazar, muraglitazar AG, or peliglitazar AG were observed. In the incubations without NADPH, glucuronide isomers of muraglitazar or peliglitazar (e.g. the peak after the starting material peaks in Figures 1S and 2S) were observed. The muraglitazar or peliglitazar oxidative metabolites could be formed from muraglitazar or peliglitazar that resulted from hydrolysis of muraglitazar AG or peliglitazar AG or from hydrolysis of oxidized muraglitazar AG or peliglitazar AG. Very similar degradation profiles were observed for the incubation of muraglitazar AG or peliglitazar AG in the human liver microsomes (Figures 1S and 2S).

Figure 3S shows oxidation pathways as well as hydrolysis and isomerization of muraglitazar AG or peliglitazar AG in the *in vitro* incubations. In the incubations in the presence of NADPH, the oxidized glucuronides could have only been formed by oxidation of the acyl glucuronides since UDPGA was not present in the incubations. There are several reports in the literature on the oxidative metabolism of AGs including diclofenac (Kumar et al., 2002), MRL-C (Kochansky et al., 2007), bilirubin (Crawford et al., 1992), valproic acid (Tang et al., 1996), and gemfibrozil (Ogilvie et al., 2006). In humans, in addition to muraglitazar AG and peliglitazar AG, oxidized (hydroxylated and O-demethylated) acyl glucuronides were also metabolites of muraglitazar and peliglitazar following oral administration (Wang et al., 2006; Wang et al., 2010).

References:


**Supplement Figures**

**Figure 1S.** UV and ion chromatograms of oxidative metabolites of muraglitazar AG formed in HLM incubations in the presence of NADPH.

**Figure 2S.** UV and ion chromatograms of oxidative metabolites of peliglitazar AG formed in HLM incubations in the presence of NADPH.

**Figure 3S.** Hydrolysis, isomerization and metabolic pathways of muraglitazar AG and peliglitazar AG.
Table 1S. LC/MS/MS characterization of oxidative metabolites of muraglitazar AG and peliglitazar AG in human liver microsomes in the presence of NADPH

<table>
<thead>
<tr>
<th>Metabolite</th>
<th>Muraglitazar AG</th>
<th>Peliglitazar AG</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>TR (min)</td>
<td>MH⁺ m/z</td>
</tr>
<tr>
<td>M1</td>
<td>25.25</td>
<td>709</td>
</tr>
<tr>
<td>M2</td>
<td>27.15</td>
<td>709</td>
</tr>
<tr>
<td>M3</td>
<td>29.12</td>
<td>709</td>
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<tr>
<td>M4</td>
<td>29.86</td>
<td>679</td>
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<td>M5</td>
<td>34.42</td>
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<td>Aglycone</td>
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<td>Starting material</td>
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<td>693</td>
</tr>
</tbody>
</table>
Fig 1S

Channel A
UV:278
Mura_glu
HLM_NADPH

M1
M2
M3
M4
M5
M6
M7
M8

Muraglitazar glucuronide
Mur-aglu isomer

Hydroxylated muraglitazar glucuronides
O-Demethylated muraglitazar glucuronide

NL: 7.96E2
m/z = 532.50-533.50
ITMS + c ESI Full ms2
709.00@20.00
Mura_glu_HLM_NADPH

NL: 4.24E3
m/z = 502.50-503.50
ITMS + c ESI Full ms2
679.00@20.00
Mura_glu_HLM_NADPH
Hydroxylated peliglitazar glucuronides

O-Demethylated peliglitazar glucuronide
Fig 3S

2,3,4-O-glucuronide

Acyl migration

1-O-β-glucuronide

UGT/UDPGA Hydrolysis
muraglitazar or peliglitazar

P450

M1, M2, M3 (hydroxy glucuronide)

Hydrolysis
M5, M6, M7 (hydroxy metabolite)

M4 (O-demethyl glucuronide)

Hydrolysis
M8 (O-demethyl metabolite)

$R = H$ for muraglitazar and $R = CH_3$ for peliglitazar