STRUCTURAL ELUCIDATION OF HUMAN OXIDATIVE METABOLITES OF MURAGLITAZAR: USE OF MICROBIAL BIOREACTORS IN THE BIOSYNTHESIS OF METABOLITE STANDARDS

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ABSTRACT:

Muraglitazar (Pargluva), a dual α/γ peroxisome proliferator-activated receptor activator, is currently in clinical development for treatment of type 2 diabetes. This study describes the structural elucidation of the human oxidative metabolites of muraglitazar through the use of a combination of microbial bioreactors, NMR and accurate mass analyses, and organic synthesis. Plasma, urine, and feces were collected from six healthy subjects following oral administration of [14C]muraglitazar (10 mg, 100 μCi) and pooled samples were analyzed. Approximately 96% of the recovered radioactive dose was found in the feces and 3.5% in the urine. The parent compound represented >85% of the radioactivity in plasma. The fecal radioactivity was distributed among 16 metabolites (M1–M12, M14–M16, and M8α) and the parent drug, of which hydroxylation and O-demethylation metabolites (M5, M10, M11, M14, and M15) represented the prominent human metabolites. The urinary radioactivity was distributed into several peaks including muraglitazar glucuronide (M13) and the parent drug. Low concentrations of metabolites in human samples prevented direct identification of metabolites beyond liquid chromatographic (LC)-mass spectrometric analysis. Microbial strains Cunninghamella elegans and Saccharopolyspora hirsuta produced muraglitazar metabolites that had the same high performance liquid chromatography retention times and the same tandem mass spectrometric (MS/MS) properties as the corresponding human metabolites. The microbial metabolites M9, M10, M11, M14, M15, and M16 were isolated and analyzed by NMR. Based on these LC-MS/MS and NMR analyses, and organic synthesis, the structures of 16 human oxidative metabolites were identified. The oxidative metabolism of muraglitazar was characterized by hydroxylation, O-demethylation, oxazole opening, and O-demethylation/hydroxylation, as well as O-dealkylation and carboxylic acid formation. This study demonstrated the utilization of microbial bioreactors for the identification of metabolites.

Peroxisome proliferator-activated receptors (PPARs) are a set of nuclear hormone receptors (comprising the α, γ, and δ subtypes). The two most intensively investigated subtypes have been PPARα (primarily expressed in the liver and demonstrated to play a critical role in lipid metabolism) and PPARγ (primarily expressed in adipose tissue and implicated in insulin sensitization as well as glucose and fatty acid utilization). PPARα is the target of the fibrate class of hypolipidemic drugs such as fenofibrate (Balfour et al., 1990; Packard, et al., 2002; Despres, 2001) and gemfibrozil (Spencer and Barradell, 1996), whereas PPARγ is the target of the thiazolidinedione (Mudaliar and Henry, 2001) class of antidiabetic drugs such as rosiglitazone (Balfour and Plosker, 1999; Cheng-Lai and Levine, 2000; Goldstein, 2000) and pioglitazone (Gillies and Dunn, 2000). Muraglitazar (N-[(4-methoxyphenoxy)carbonyl]-N-[(4-[2-(5-methyl-2-phenyl-4-oxazolyl)ethoxy] phenyl)methyl]glycine, BMS-298585, Paragluva; Fig. 1), is an oxybenzylglycine analog (nonthiazolidinedione) dual α/γ PPAR activator currently in clinical development for the treatment of type 2 diabetes (Devasthale et al., 2005). This study describes the structural elucidation of oxidative metabolites of muraglitazar in humans following oral administration of [14C]muraglitazar through the use of a combination of microbial bioreactors, NMR, and accurate mass LC/MS.

The advantages of microbial bioreactors as a complementary in vitro system for drug metabolism are the relatively low cost, mild conditions, ease of use, potential for efficient conversion with a high yield of metabolites, scale-up capability, and a potential to reduce the use of animals. Microbial strains including the zygomycete fungus Cunninghamella elegans and the actinomycete Saccharopolyspora hirsuta have been used to study biotransformation and biosynthesis of several drugs and have shown the ability to produce oxidative metabolites.

ABBREVIATIONS: PPAR, peroxisome proliferator-activated receptor; HPLC, high performance liquid chromatography; LC/MS, liquid chromatography/mass spectrometry; P450, cytochrome P450; TFA, trifluoroacetic acid; ESI(+), positive electrospray ionization; Q-TOF, quadrupole time of flight; 1D, one-dimensional; 2-D, two-dimensional; TOCSY, total correlation spectroscopy; THF, tetrahydrofuran; EtOAc, ethyl acetate; TBS, Tris-buffered saline; MeOH, methanol; IBX, 1-hydroxy-1,2-benziodoxole-3(1H)-one-1-oxide; DMSO, dimethyl sulfoxide; ADME, absorption, distribution, metabolism, and excretion.

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olite profiles similar to those found in mammalian species (Ikeda et al., 1985; Zhang et al., 1995, 1996a, 1997). Some of these microbial biotransformation reactions are catalyzed by cytochrome P450 enzymes (Zhang et al., 1995, 1996b; Yang et al., 1997). Therefore, microbial bioreactors should be useful for generation of metabolites in sufficient quantity to aid in structural elucidation of human metabolites by spectroscopic and chromatographic methods.

Materials and Methods

Materials. [14C]Muraglitazar, with a radiospecific activity of 10 μCi/mg and a radiochemical purity of 99.4%, was synthesized in multiple steps from [U-14C]phenol in a 20% overall yield at the Pharmaceutical Research Institute, Bristol-Myers Squibb. The structure of muraglitazar and the positions of 14C labels are shown in Fig. 1. PEG-400 was purchased from Aldrich Chemical Co. (Milwaukee, WI). Trifluoroacetic acid (TFA) was purchased from EM Scien
tific (Gibbstown, NJ). Ecolite liquid scintillation cocktail was purchased from PerkinElmer Life and Analytical Sciences (Boston, MA). Deuterated solvents D3O, CD3CN, and CDCl3 (D 99.8%) were obtained from Cambridge Isotope Laboratories, Inc. (Andover, MA). All organic solvents and water were of HPLC grade. Microbial strains were purchased from The American Type Culture Collection (Manassas, VA).

Sample Collection and Preparation. The study in human subjects had Institutional Review Board approval and all subjects were required to give informed and written consent before participation in the study. Six healthy human subjects each received an oral dose of 10 mg (100 μCi) of [14C]muraglitazar in PEG-400. Plasma (1, 4, 12, 24, and 48 h), urine (0–240 h), and feces (0–240 h) were collected. Plasma was collected in EDTA and pooled across the six subjects. The plasma (1 ml) was extracted with acetonitrile (3 volumes) and centrifuged at 2000g for 10 min. The supernatant was saved and the pellet was extracted twice with acetonitrile (3 ml). The radioactivity extraction efficiency was >95%. The combined supernatants were evaporated to dryness by evaporation under a stream of nitrogen. The residue was redissolved in 200 μl of acetonitrile/water (3:7) and centrifuged at 2000g for 10 min before HPLC analysis. Urine and feces were collected for 0 to 240 h at 24-h intervals, and the volume of urine and the weight of feces were determined for mass balance. Fecal paste was prepared and pooled from all samples collected up to 10 days across six subjects. Samples of pooled feces (1 g) were extracted by mixing with ethyl acetate (3 ml) and centrifuging at 2000g for 10 min. The supernatant was saved and the pellet was extracted twice with ethyl acetate (3 ml). The extraction efficiency of radioactivity was 84.5%. The combined supernatants were evaporated to dryness by evaporation under a stream of nitrogen. The residue was redissolved in 200 μl of acetonitrile/water (3:7) and centrifuged at 2000g for 10 min before HPLC analysis. Urine was concentrated directly under a stream of nitrogen. The residues were each reconstituted in 2 ml of acetonitrile/water (3:7, v/v). The extracts were analyzed by accurate mass LC/MS and LC-MS/MS. For metabolite isolation, fractions were collected from five injections (100 μl) onto a 4.6 × 150 mm YMC HPLC column (YMC Co., Ltd., Kyoto, Japan). The microbial isolates were analyzed by LC/MS, LC-MS/MS, and NMR.

Radioactivity Detection. Radioactivity in the biological samples was determined by mixing an aliquot of the sample with 15 ml of Ecolite cocktail and counting for 10 min using a Tri-Carb 2250 liquid scintillation analyzer (PerkinElmer Life and Analytical Sciences). The radioactivity in fecal homog
enates was determined by combusting the samples, followed by liquid scintillat
counting. For HPLC profiling, the HPLC eluent was collected in 96-deep-well Lumaplates and dried in a SpeedVac (Thermo Electron Corpora
tion, Waltham, MA). The plates were counted for 10 min per well using a TopCount scintillation analyzer (PerkinElmer Life and Analytical Sciences).

HPLC. HPLC was performed on a Shimadzu VP system equipped with two pumps (model LC-10AT), an autoinjector (SIL 10AD), and a diode array detector (SPD-M10A) (Shimadzu, Kyoto, Japan). YMC ODS AQ C-18 columns (4.6 or 2.0 × 150 mm, 5 μ) were used for all separations. HPLC effluent fractions were collected into the 96-well plates at 0.26-min intervals for 70 min after sample injection with a Gilson model 202 fraction collector (Gilson Medical Electronics, Middleton, WI). The columns were eluted with solvents A and B. Solvent A was water containing 0.06% TFA. Solvent B was acetonitrile containing 0.06% TFA. The initial condition was 5% solvent B. The B composition was increased linearly to 25% (5 min), 40% (15 min), 53% (40 min), 60% (3 min), and 90% (2 min), and then was held at 90% for 7 min. The flow rates were 1 ml/min for the 4.6 × 150 mm column and 0.3 ml/min for the 2.0 × 150 mm column. Metabolites in plasma, urine, and fecal extracts were monitored by radioactivity detection in collected fractions, and metabolites in microbial incubations were monitored by on-line UV detection at 280 nm.

LC/MS and LC-MS/MS Analyses. A YMC ODS AQ C-18 column (2.0 × 150 mm, 5 μ) was used for LC/MS. A Thermo Electron LCQ deca XP mass spectrometer with an ESI(+) source (Thermo Electron Corporation) was used for initial MS/MS analysis of fungal metabolites. The capillary temperature used for analysis was 230°C. The nitrogen gas flow rate, spray current, and voltages were adjusted to give maximum sensitivity for the parent compound. The collision energy was 20%. Accurate mass analysis was conducted on a Micromass Q-TOF Ultima mass spectrometer that was equipped with a Lock-Spray and an ESI(+) source (Waters, Milford, MA). LC-MS/MS analysis of the human and microbial metabolites was also performed with the Q-TOF with a collision energy of 15 to 25%. The HPLC eluent was directed to the mass spectrometer. The desolvation temperature used for analysis was 300°C. The nitrogen gas flow rate, and the spray and cone voltages were adjusted to give maximum sensitivity for muraglitazar. The m/z 556.2771 of an infused 20 ng/glucine enkephalin solution was used as lock mass. The Q-TOF was tuned to 18000 resolution at half-peak height using an insulin tuning solution (at m/z 956.3), and was calibrated up to 1500 Da using a polyalanine calibration solution. The experimentally obtained masses matched their respective calculated values with an error of less than 5 mDa (<10 ppm).

NMR. The synthetic materials and microbial isolates were analyzed on a JEOL ECL-500 MHz spectrometer (JEOL, Tokyo, Japan) or a Bruker Avance 600 MHz system (Bruker, Newark, DE) equipped with a 5-mm Z-gradient probe, or a 3-mm Nalorac probe. 1D 1H, 2D correlation spectroscopy, 2D TOCSY (total correlation spectroscopy), heteronuclear multiple quantum co
erherence spectroscopy (one-bond carbon-proton correlation), heteronuclear multiple-bond correlation spectroscopy (long-range carbon-proton correla
tion), and edited distortionless enhancement by polarization transfer experi
ts were performed. All chemical shifts are reported in ppm relative to
tetramethylsilane in CD$_3$CN. For reference, a complete proton and carbon peak assignment of the NMR spectra was made on the parent drug.

Synthesis of M5. The proposed structure for M5 based on mass spectral analysis had a hydroxyl group on carbon-12 (Table 1). To confirm this structure, 12-hydroxy muraglitazar was synthesized in several steps (Fig. 2A), as described below.

Intermediate 1. A solution of 4-hydroxybenzaldehyde (1.0 g, 8.2 mmol), tetrabutylsilyl chloride (1.48 g, 9.8 mmol), and imidazole (660 mg, 9.8 mmol) in N,N-dimethylformamide (10 ml) was stirred at room temperature for 3 h. The reaction mixture was then partitioned between ethyl acetate (EtOAc) and brine (saturated NaCl solution). The organic phase was washed with 10% aqueous lithium chloride, dried over anhydrous magnesium sulfate (MgSO$_4$), and concentrated under vacuum to give the TBS ether, which was used in the next step without further purification. A solution of the TBS ether, glycine methyl ester hydrochloride (1.23 g, 9.8 mmol), and triethylamine (Et$_3$N, 0.4 ml, 9.8 mmol) in methanol (MeOH, 20 ml) was stirred at room temperature for 18 h, and then sodium borohydride (NaBH$_4$, 370 mg, 9.8 mmol) was cautiously added portion-wise. The reaction was stirred for 1 h at room temperature, followed by addition of saturated aqueous sodium bicarbonate (NaHCO$_3$) and removal of volatiles under vacuum. The residue was partitioned between EtOAc and saturated aqueous NaHCO$_3$. The organic phase was dried (MgSO$_4$) and concentrated under vacuum to give the amino ester intermediate 1 (1.3 g, 51%) as an oil. LC/MS [M + H]$^+$ = 310. The oil was used in the next reaction without further purification.

Intermediate 2. A solution of amino-ester intermediate 1 (500 mg, 1.6 mmol), 4-benzyloxyphenyl chloroformate (510 mg, 1.9 mmol), and Et$_3$N (0.27 ml, 1.9 mmol) in methylene chloride (CH$_2$Cl$_2$, 10 ml) was stirred at room temperature for 2 h. The reaction mixture was then partitioned between CH$_2$Cl$_2$ and saturated aqueous NaHCO$_3$. The organic phase was dried (MgSO$_4$) and concentrated under vacuum to give the desired carbamate ester, LC/MS [M + H]$^+$ = 536, which was used in the next step without further purification. To a solution of the TBS ether carbamate ester in tetrahydrofuran (THF, 10 ml) was added tetrabutylammonium fluoride (Bu$_4$NF, 1.9 ml of a 1 M solution in THF, 1.9 mmol) at room temperature. The reaction was stirred for 1 h at room temperature and then was concentrated under vacuum. The residue was chromatographed on a silica gel column (stepwise gradient from 85:15 to 55:45 hexane/EtOAc) to give the phenol carbamate ester intermediate 2 (300 mg, 44%) as a clear oil. LC/MS [M + H]$^+$ = 422.
Intermediate 3. A mixture of the phenol intermediate 2 (120 mg, 0.28 mmol), the mesylate intermediate 4 (which was prepared at Bristol-Myers Squibb; 100 mg, 0.24 mmol; Devasthale et al., 2005), and potassium carbonate (K₂CO₃, 67 mg, 0.48 mmol) in acetonitrile (10 ml) was stirred at 90°C for 36 h. The reaction then was cooled to room temperature and stirred for 36 h. Volatile materials were removed under vacuum and the residue was partitioned between EtOAc and brine. The organic phase was dried (MgSO₄) and concentrated under vacuum to give the desired alkylated phenol as an oil, which was used in the next step without further purification.

A solution of the alkylated phenol in THF (5 ml) and Bu₄NF (0.50 ml of a 1 M solution in THF, 0.50 mmol) was stirred at room temperature for 15 min to give a solution of the crude alcohol intermediate 3, LC/MS [M + H]⁺ = 623. Methanol (10 ml) and aqueous NaOH (2 ml of a 1 N solution) were added to the reaction mixture and the reaction was stirred at room temperature for 16 h. The solution was acidified with 1 N aqueous HCl and then was concentrated under vacuum. The residue was partitioned between EtOAc and 1 N aqueous HCl. The organic phase was dried (MgSO₄) and concentrated under vacuum. The residue was purified by preparative HPLC on a Phenomenex (Torrance, CA) Luna C18 column (5 μ, 21.2 × 100 mm; detection at 220 nm; flow rate = 25 ml/min; continuous gradient from 90% solvent A/10% solvent B to 100% solvent B over 8 min, where solvent A = 90:10:0.1 H₂O/MeOH/TFA and solvent B = 90:10:0.1 MeOH/H₂O/TFA) to give the

Fig. 2. Synthetic scheme of metabolite M5 (12-hydroxy O-demethyl muraglitazar) and M12 (muraglitazar 12-carboxylic acid).
O-benzylphenyl carbamate acid 3 (69 mg, 40%) as a white solid. LC/MS [M + H]+ = 609.

Metabolite M5. To a solution of the O-benzylphenyl carbamate acid 3 in MeOH (10 ml) was added 10% palladium on carbon catalyst (50 mg), and the reaction was stirred under an atmosphere of hydrogen at room temperature for 30 min. After the catalyst was filtered off, the solution was concentrated under vacuum and the residue was purified as described for purification of the intermediate 3 to give metabolite M5 (25 mg; 43%) as a white powder. LC/MS [M + H]+ = 519. HNMR (400 MHz, CD-Od): 7.91 (2H), 7.40 (2H), 7.15 (2H), 6.82 (2H), 6.67 (2H), 4.58 (s, 2H), 4.47 (2s, 2H), 4.18 (t, J = 6.5 Hz, 2H), 3.89 (2s, 2H), 2.98 (t, J = 6.5 Hz, 2H).

Synthesis of M12. The proposed structure for M12 based on mass spectral analysis was oxidation of the methyl-12 to a carboxylic acid. To confirm this, 281.0 mg, 1.1 mmol), dimethyl sulfoxide (DMSO; 4.0 ml), and a magnetic stir bar. The vial was capped and placed in an oil bath heated at 110°C for 24 h. The reaction mixture was diluted with 4.0 ml of acetonitrile and subjected to preparative HPLC isolation using a Phenomenex Luna C18, column, 21.2 × 150 mm, 5 μ, with solvent A (water with 0.05% trifluoroacetic acid) and solvent B (acetonitrile with 0.05% TFA). The linear gradient used was 50% B to 90% B in 10 min at a flow rate of 21.0 ml/min (monitored at 280 nm). Fractions containing the product were pooled, concentrated via rotary evaporation, and freeze-dried to afford a white powder (ca. 1 mg). $^1$H NMR (600 MHz, DMSO-d$_6$) 7.96 (d, 8.0 Hz, 2H), 7.63 (m, 1H), 7.58 (m, 2H), 7.26 (m, 2H), 6.97 (m, 2H), 6.90 (m, 4H), 4.53, 4.39 (2s, 2H), 4.33 (m, 2H), 3.89 and 3.63 (s, 2H, rotamers), 3.78 (s, 2H), 3.29 (2s, 2H).

Results

Biotransformation Profiles in Human Plasma, Urine, and Feces and in Microbial Incubations. After oral administration of [1$^{13}$C]muraglitazar (10 mg) to humans, urinary excretion was minimal (3.3% of the dose) and the majority of radioactivity was excreted in feces (approximately 96% of the recovered radioactive dose). The overall radioactivity recovery was low (64%) from this study, possibly because of difficulties in accurately measuring the radioactivity in a fecal paste preparation. An additional human ADME study showed recovery >93% of the radioactive dose in which feces was homogenized in a water and ethanol mixture. The fecal metabolite profile was similar in both studies (data not shown). The parent compound represented >90% of the radioactivity in human plasma at early time points and >85% at 48 h. No metabolites accounted for >5% of the radioactivity in plasma at any time point. Human metabolites identified in plasma included M18, M5, M10, M11, M13, and M15. Figure 3 shows metabolite profiles of human feces (by radioactivity) and microbial incubations (by UV at 280 nm). Four metabolite peaks (M5, M10, M11, and M15) and the parent compound (P) were the major radioactive components in human feces and, together, accounted for >70% of the radioactivity in the fecal sample. Human urine contained several radioactive peaks including M13 and P, with M13 accounting for approximately 1% of the dose. Figure 4 shows the selected ion chromatographic profiles of muraglitazar metabolites in human feces. There were four hydroxy metabolites (M8a, M10, M11, and M14), four hydroxy O-demethyl metabolites (M2, M5, M6, and M7), one O-demethyl metabolite (M15), three dihydroxy metabolites (M3, M4, and M8), one dioxygenation with dehydrogenation metabolite (M12), and two oxazole ring-opening metabolites (M9 and M16). Because human feces, as the major excretion route, contained low concentrations of metabolites of muraglitazar at a 10-mg dose, detailed structural identification of metabolites through isolation was not practical. Microbial bioreactors were thus used to generate sufficient quantities for isolation and identification of the human metabolites. Microbes S. hirsuta and C. elegans produced high yields of metabolites that had the same retention times as human fecal metabolites M9, M10, M11, M14, M15, and M16 (Fig. 3). In addition to these major metabolites, the microbial strains also produced other oxidative metabolites that had the same retention times and fragmentation patterns as those found in human feces (partial data are shown in Table 2). Comparative HPLC/accurate MS chromatographic profiles were very similar between human feces and microbial incubations for selected muraglitazar metabolites as demonstrated in Fig. 5. In addition, the mass spectral fragmentation patterns and molecular formula calculated with a 5-ppm accuracy of these microbial metabolites matched the corresponding human metabolites (Table 2). Based on these comparisons, the microbial metabolites were judged to be identical to the human metabolites.

Identification of Metabolites. Typical fragmentation patterns of muraglitazar in a full-scan MS analysis showed cleavage at the benzyl C–N bond adjacent to carbamate (C3–N) to give a fragment at m/z 292 and cleavage at the ether bond (C8–O) to give a fragment at m/z 186 (as shown in Fig. 1). Muraglitazar had a molecular ion [M + H]$^+$ at m/z 517 (Table 2). The key $^1$H NMR data (Table 3) for metabolite identification are the chemical shifts (ppm) at 2.35 for the methyl group at position 12, 2.92 for the methylene at position 9, 3.77 for the methoxy group at position 17, and 7.47 or 7.48 for the aromatic protons at positions 16 and 17.

Similar fragmentation patterns were observed with muraglitazar metabolites M2 to M16. All monohydroxylation metabolites (M2, M5, M6, M7, M8a, M10, M11, and M14) showed a fragmentation ion at m/z 202 (186 + 16) or its dehydrated form at m/z 184 (202 – H$_2$O), indicating that oxygen addition occurred on the oxazole-ring side of the molecules. Monohydroxylation metabolites, M2, M5, M7, M8a, M10, and M14 (but not M6 and M11, which were hydroxylated at position 17), also showed a fragmentation ion at m/z 187.0756, which had a formula of C$_{12}$H$_{11}$O$_2$. Nitrogen was lost in the oxidation process, giving the nitrogen mass spectrometer after ether cleavage of the oxygenated molecules (187 = 186 – NH + oxygen). The mechanism for this gas-phase reaction is not currently understood. NMR data of metabolites were analyzed by comparison to the spectra of the parent muraglitazar. Metabolites M9, M10, M11, M14, M15, and M16 were isolated from incubations with C. elegans and S. hirsuta and identified by NMR and LC-MS/MS analyses. The structures of other human metabolites were
identified by comparing their MS/MS spectra with those metabolites identified by NMR. M5 and M12 were synthesized as reference standards following preliminary identification by LC-MS/MS analysis.

Metabolites M8a, M10, M11, and M14 (Monohydroxylated Metabolites). These four metabolites had the same molecular ion \([M + H]^+\) at \(m/z\) 533. They all had fragmentation patterns similar to each other and to the parent compound, cleaving at the C8–O and C3–N bonds (Fig. 6A). Their MS/MS spectra suggested that the hydroxylation was on the oxazole-ring side of the molecules.

Metabolite M11 showed fragments at \(m/z\) 308 (cleavage at the C3–N bond) and 202 (cleavage at the C8–O bond) (Fig. 6A). The proton 1D NMR spectrum (Fig. 7A) of the microbial isolate showed that this metabolite had two characteristic changes with respect to the parent drug. These changes were: 1) absence of a signal for the proton at position 17 and 2) a significant upfield shift of the protons at position 16, from 7.48 ppm to 6.92 ppm. The signals for the protons at position 15 also showed a small shift from 7.94 ppm to 7.80 ppm. These results indicated that the hydroxylation was at the para-position 17, and metabolite M11 was thus assigned as 17-hydroxy muraglitazar.

Metabolite M10 showed fragment ions at \(m/z\) 308 (cleavage at the C3–N bond), 290 (290 = 308 – H₂O), 202 (cleavage at the C8–O bond), and 184 (184 = 202 – H₂O) (Fig. 5A). The fragment ion at \(m/z\) 278 (278 = 292 + 16 – 30) was consistent with the loss of formaldehyde from cleavage at the C3–N bond. The proton 1D NMR spectrum (Fig. 7A) of the microbial isolate showed the absence of the methyl signal at 2.35 ppm and the appearance of a new signal at 4.6 ppm (a singlet integrating for two protons, characteristic of a CH₂OH group). All other protons could be accounted for. These results indicated that the hydroxylation was at position 12 (oxazole-methyl group), and metabolite M10 was thus assigned as 12-hydroxy muraglitazar.

Metabolite M14 showed fragment ions at \(m/z\) 290 (cleavage at the C3–N bond, 290 = 308 – H₂O) and 202 (cleavage at the C8–O bond). The proton 1D NMR spectrum (Fig. 7A) showed that the proton 9 resonance of this metabolite had a dramatic downfield shift from 2.92 ppm to 5.04 ppm. This signal was still a triplet and coupled...
FIG. 4. Accurate LC/MS ion chromatographic profiles of [14C]muraglitazar metabolites in human feces: oxazole ring-opening metabolite M9 (A); dioxygenation and dehydrogenation metabolite M12 (B); N-acetyl oxazole ring-opening metabolite M16 (C); the parent drug (D); dihydroxylation metabolites M3, M4, and M8 (E); O-demethylation metabolite M15 (F); hydroxylation and O-demethylation metabolites M2, M5, M6, and M7 (G); hydroxylation metabolites M8a, M10, M11, and M14 (H); and radiochromatogram of human feces for comparison purpose (I). Ion chromatograms ([M + Na]+ for M9 and M16 and [M + H]+ for other metabolites) were extracted with narrow mass ranges (0.1700–0.2200 Da) of metabolites and parent drug from accurate mass analyses. XIC, extracted ion chromatograms.
(in the 2D TOCSY spectrum) to the signal at 4.27 ppm of proton 8. Even so, there was no significant shift on the resonance frequency of proton 8 relative to the parent drug. Proton 8 was no longer a triplet but, rather, a broad doublet, supporting the idea that it was coupling to only one other proton. These results indicated that the hydroxylation was on position 9, and metabolite M14 was thus assigned as 9-hydroxy muraglitazar. The absolute stereochemistry of M14 is not known.

Metabolite M8a showed fragment ions at \( m/z \) 308 (cleavage at the C3–N bond) and 187 (cleavage at the C8–O bond). The absence of the other cleavage fragment at \( m/z \) 202 observed with other hydroxy metabolites indicated the instability of this fragment in the mass
spectrometer. The result suggested that the hydroxylation was probably at position 8 (methylene group). This hemiacetal structure was expected to have some degree of instability in aqueous environments, which was consistent with the low concentration of this metabolite in human feces and the subsequent formation of the O-dealkyl metabolite M1. Although the position for the hydroxyl group in M8a was not determined, available data supported a tentative assignment for M8a as 8-hydroxy muraglitazar.

**Metabolite M15.** M15 showed a molecular ion [M + H]⁺ at m/z 503 and major fragment ions at m/z 186 and 292 in the LC/MS analysis, consistent with O-demethylation. ¹H and ¹³C NMR analyses showed that M15 lacked the OCH₂ group (at 3.8 ppm for ¹H in Fig. 7B and 55 ppm for ¹³C) observed for the parent muraglitazar. The only change was the disappearance of the methoxy resonance of position 23. All other signals were in the same range as in the parent molecule, including the ortho- and meta-positions in the corresponding aromatic ring (Table 3; Fig. 7B). This metabolite was assigned as O-demethyl muraglitazar.

**Metabolites M9 and M16.** These two metabolites had mass spectral characteristics distinct from those of muraglitazar and other metabolites. They showed a strong Na adduct of the molecular ions in LC/MS analyses. Both of these metabolites did show typical fragmentation patterns, cleaving at the C₈–O and C₃–N bonds.

Metabolite M9 had a molecular ion [M + H]⁺ at m/z 507 (loss of 10 Da from the parent compound) and fragment ions at m/z 282 (cleavage at the C₈–O bond, 282 = 292 – 10) and 176 (cleavage at the C₈–O bond, 176 = 186 – 10). Accurate mass analysis and formula calculation indicated that the 10-Da loss was due to elemental composition changes with an addition of oxygen and loss of ethylene (O + C₂H₂). The UV spectral changes (from a strong absorption peak around 250–315 mm and a weak absorption peak around 210–240 mm to a strong absorption peak around 200–255 mm and a weak absorption peak around 265–285 mm) suggested the removal of the extended aromatic system, consistent with an oxazole-ring opening.

**Proton 1D NMR spectrum (Fig. 7B)** showed that the methyl resonance at position 12 was lost and there was no new signal that could account for these protons, suggesting that this position was not simply hydroxylated. These results indicated that the metabolite had an oxazole ring-opening structure. Metabolite M9 was assigned as the oxazole ring-opening imide derivative of muraglitazar.

Metabolite M16 showed a molecular ion [M + H]⁺ at m/z 549 (plus 32 Da from the parent compound) and fragment ions at m/z 324 (cleavage at the C₈–N bond, 324 = 328 – 42, an acetyl group), 282 (cleavage at the C₈–N bond, 282 = 292 – 10) and 176 (cleavage at the C₈–O bond, 176 = 186 – 10). This fragmentation pattern suggested that M16 was a derivative of M9 with the addition of an acetyl group. The proton 1D NMR spectrum showed a new signal in the aliphatic region at 2.28 ppm that might be attributed to an acetyl group (not shown). A theoretical prediction of the NMR spectrum of this proposed structure gave a value of 2.24 ppm for the acetyl group, in good agreement with the experimental value. These results supported the idea that this metabolite was an N-acetyl derivative of M9.
FIG. 6. Q-TOF MS/MS spectra of human fecal metabolites of muraglitazar: (A) hydroxylation metabolites M14, M11, M10, and M8a, (B) hydroxylation/O-demethylation metabolites M7, M6, M5, and M2, and (C) dihydroxylation metabolites M3, M4, and M8 and 12-carboxylic acid metabolite M12.
Fig. 7. Proton 1D NMR spectra of microbial isolates of muraglitazar metabolites: A, M10, M11, and M14; B, M15 and M9.
and metabolite M16 was thus assigned as the oxazole ring-opening
N-acytelyimide derivative of muraglitazar.

Metabolites M2, M5, M6, and M7 (O-Demethylated and Hydroxy-
lated Metabolites). These four metabolites had the same molecular ion
[M + H]⁺ at m/z 519 (519 = 517 + 16 – 14), consistent with
hydroxylation and O-demethylation. They had typical fragmentation
patterns of cleavage at either the C8–O or the C3–N bond (Fig. 6B).
Their MS/MS spectra also suggested that the hydroxyations were on
the oxazole-ring side of the molecules. M2, M5, M6, and M7 had the
same fragmentation patterns as monohydroxy metabolites M8a, M10,
M11, and M14, respectively. Therefore, these compounds were
assigned as the O-demethylation metabolites corresponding to the hy-
droxy metabolites M8a, M10, M11, and M14. M2 was tentatively
assigned as 8-hydroxy O-demethyl muraglitazar. Similar to the as-
signment for M8a, available data supported the hydroxyl group at C8,
although the final structure for M2 was not determined. M5 was
assigned as 12-hydroxy O-demethyl muraglitazar. M6 was assigned as
17-hydroxy O-demethyl muraglitazar. M7 was assigned as 9-hydroxy
O-demethyl muraglitazar.

The synthesis of the metabolite M5 is outlined in Fig. 2A. 4-Hy-
droxybenzaldehyde was protected as the tert-butyldimethylsilyl ether
and then was subjected to reductive amination with glycine methyl
ester hydrochloride to give the secondary amine 1. Acylation of amine
1 with 4-benzyloxyphenyl chloroformate, followed by deprotection of
the tert-butyldimethylsilyl ether, furnished the phenol carbamate ester
2. Alkylation of phenol 2 with the hydroxylated phenyloxazole me-
sylate 4, followed by deprotection of the TBS ether, provided the
hydroxymethyl oxazole carbamate ester 3. Deprotection of 3 (hydrog-
enolysis of the O-benzyl ether followed by ester hydrolysis) provided
the metabolite M5. Synthetic M5 had the same HPLC retention time
and MS/MS fragmentation patterns as the corresponding metabolites
from human feces (data not shown).

Metabolites M3, M4, M8, and M12. Metabolites M3, M4, and M8 had
the same molecular ion [M + H]⁺ at m/z 549 (549 = 517 + 32),
which was consistent with dihydroxylation. Metabolite M12 had a
molecular ion [M + H]⁺ at m/z 547 (549 = 517 + 32 – 2), which
was consistent with dioxygenation and dehydrogenation. All of these
metabolites showed a typical fragmentation pattern, with cleavage at
the C8–O and C3–N bonds (Table 2; Fig. 6C). The MS/MS spectra
for all the metabolites suggested that both hydroxylations were on
the oxazole-ring side of the molecules.

Metabolite M4 showed fragment ions at m/z 531 (549 – H₂O), 324
(cleavage at the C3–N bond, 324 = 292 + 16 + 16), 306 (306 =
324 – H₂O), 218 (cleavage at the C8–O bond, 218 = 186 + 16 + 16)
and 200 (200 = 218 – H₂O) (Fig. 6C). This fragmentation
pattern was similar to that of the 12-monohydroxylation metabolite
M10 (Table 2; Fig. 6A). In addition, M4 had a characteristic fragment
at m/z 294, loss of formaldehyde (from cleavage at the C3–N bond,
294 = 324 – CH₂O); this fragmentation was also observed in the
12-hydroxy metabolites M5 and M10 (at m/z 278). M4 also showed a
characteristic fragment at m/z 192.0687 (C₁₀H₈(NO₃), which could
be formed from the cleavage between the C-9 and C-10 bond followed
by a hydration. These results suggested dihydroxylation at the 9- and
12-positions. Metabolite M4 was tentatively assigned as 9,12-
dihydroxy muraglitazar.

Metabolite M8 showed fragment ions at m/z 324 (cleavage at the
C3–N bond, 324 = 292 + 16 + 16) and 218 (cleavage at the C8–O
bond, 218 = 186 + 16 + 16) (Fig. 6C). The fragmentation pattern
was similar to that of 17-hydroxy muraglitazar except for the addi-
tional 16-mass unit increase for each fragment. M8 was tentatively
assigned as 12,17-dihydroxy muraglitazar. Although another possibil-
ity could be that the second hydroxylation occurs on the same phenyl
ring (16,17-dihydroxy muraglitazar), a trace level of a fragment at m/z
306 (306 = 324 – H₂O) in the MS/MS of M8 suggested that M8 was
more likely to be 12,17-dihydroxy muraglitazar.

Metabolite M3 showed fragment ions at m/z 324 (cleavage at the
C3–N bond, 324 = 292 + 16 + 16), 187 (cleavage at the C8–O bond,
C₁₂H₁₀O₂), and 192 (C₁₀H₈NO₃) (Fig. 6C). Detection of the frag-
ment ion at m/z 187 ruled out the possibility of 17-hydroxylation. No
other major fragment ions were observed with this metabolite. The
result suggested dihydroxylation at positions 8 and 12. M3 was
tentatively assigned as 8,12-dihydroxy muraglitazar.

Metabolite M12 showed fragments at m/z 322 (cleavage at the
C3–N bond, 324 = 292 + 16 + 16 – 2) and 218 (cleavage at the
C8–O bond, 218 = 186 + 16 + 16 – 2) (Fig. 6C). One structure that
was consistent with these observations was muraglitazar 12-car-
boxylic acid. Other structures including 12-oxa-17-hydroxy mura-
glitazar, 9-oxa-17-hydroxy muraglitazar, and 12-oxa-9-hydroxy mu-
raglitazar could not be ruled out. To confirm the structure of M12,
muraglitazar 12-carboxylic acid was synthesized with two oxidation
steps from muraglitazar. Literature procedures for the oxidation of a
methyl group that is directly attached from an oxazole ring to an
aldehyde, and from an aldehyde to a carboxylic acid were used for the
preparation (Nicolaou et al., 2001; Mazitschek et al., 2002). Synthetic
M12 had the same HPLC retention time and MS/MS fragmentation
patterns as the corresponding metabolites from human feces (data not
shown).

Metabolites M13 and M18. A very small trace level of M13 was
detected in human fecal extract by LC-MS/MS; however, this was a
relatively major metabolite in human urine. This metabolite had a
molecular ion [M + H]⁺ at m/z 693 and fragment ions at m/z 517,
292, and 186 (the same fragment ions as muraglitazar), consistent
with an acyl glucuronide of the parent compound. M13 was tenta-
atively assigned as an acyl glucuronide of muraglitazar. M18 was a
minor metabolite detected in plasma and urine. M18 had a
molecular ion [M + H]⁺ at m/z 709 and fragment ions of 533, 308,
and 202, consistent with a glucuronide of hydroxy muraglitazar.
M18 was assigned as the glucuronide of an isomer of hydroxy
muraglitazar, although the position for the glucuronic acid was not
determined.

Metabolite M1. This peak was a minor metabolite in human feces
(Fig. 3). The radioactive peak had the same retention time as a
metabolite in microbial incubations. Based on LC/MS and LC-
MS/MS analyses, M1 was tentatively assigned as O-dealkyl muragli-
tazar. The structures of muraglitazar metabolites are shown in Fig. 1.

Discussion

After oral administration of [¹⁴C]muraglitazar to healthy sub-
jects, the parent drug was the major radioactive component in the
circulation. No single metabolite accounted for >5% of the total
plasma radioactivity. Muraglitazar acyl glucuronide, M13, ac-
counted for approximately 1% of the dose in urine. Fecal excretion
represented >96% of the recovered radioactivity. Major fecal
metabolites included M5, M10, M11, and M15. The parent drug
accounted for approximately 15% of the recovered radioactive
dose in feces, whereas oxidative metabolites accounted for the
remaining dose. Altogether, 16 oxidative and 2 glucuronide me-
tabolites were identified from plasma, urine, and feces. Muragli-
tazar and structurally characterized metabolites represented >95% of
the radioactivity in excreta. The oxidative metabolites of
[¹⁴C]muraglitazar in human feces were probably generated by
intestinal and liver P450 enzymes followed by biliary excretion.
An additional mechanism could include intestinal excretion of
metabolites. It is unlikely that the fecal metabolites were generated
by the enzymes in the intestinal microflora since these enzymes are generally reductive in nature. Consistent with these findings, incubation of $[^{14}C]$muraglitazar in human liver microsomes in the presence of NADPH generated M1, M9, M10, M11, M12, M14, M15, and M16 as prominent oxidative metabolites (data not shown). Although P450 enzymes are expected to catalyze formation of the initial oxidation product at C-12 of muraglitazar (M10) and other oxidative metabolites, the additional oxidation steps of the hydroxyl group in M10 to a carboxylic acid group in M12 could be catalyzed by an alcohol dehydrogenase and an aldehyde oxidase.

During the development of new chemical entities, there are two general questions regarding metabolite identification that need to be addressed. The first concern is which metabolites (above what concentration or what percentage of the dose) should be structurally characterized, and the second concerns the degree of characterization that is necessary for the metabolites. At the compound selection and optimization stage, only major metabolites and potentially toxic and/or reactive metabolite(s) need to be identified to avoid compounds that may have unacceptably high clearance values or have the potential to cause toxicity through reactive metabolite generation. However, radioactive materials are often not available at this time, and quantitation is often limited to estimation based on LC/UV detection. Minor metabolites are often missed by UV detection at this stage. Once in the development stage, ADME data in animals and humans with radiolabeled materials will reveal the major circulating metabolite(s) and major clearance pathways. The ADME data will also define relative concentrations of each metabolite in plasma, urine, bile, and feces. Since one of the main objectives for metabolism studies is to support drug safety evaluation studies, the Metabolite In Safety Testing (MIST) committee suggested that any circulating metabolites and any significant metabolites in excreta of humans should be characterized to understand the formation pathways of metabolites (Baillie et al., 2002). Identification of the major metabolites in human excreta aids not only in defining the major clearance pathways but also in the design of drug-drug interaction studies. Major metabolites in animals, which may not be as important as human metabolites, also should be identified since they may explain the species-specific toxicities observed in animals. Thus, there is a need to identify quantitatively and qualitatively important radioactive metabolites in studies with radiolabeled materials.

The structural identification of metabolites can be carried out at multiple levels. The goal of initial identification of metabolites is often accomplished through LC/UV, LC/MS, and LC/MS/MS analyses to determine the biotransformation pathways involved in the clearance of the compound. Determination of the nature of the metabolites, such as oxygenation (hydroxylation or oxidation of a heteroatom), dioxygenation, dealkylation, and conjugation (glucuronide, sulfate, glutathione, etc.), is often sufficient. The identification of metabolites from biological matrices beyond this level is often challenging. Due to low concentrations and partial identification of the microbial metabolite, which directed organic synthesis of the metabolite. The synthetic compound matched the human metabolite both by HPLC retention time and LC/MS and LC/MS/MS analyses.

In summary, the oxidative biotransformation pathways of muraglitazar in humans include hydroxylation, O-demethylation, hydroxylation/O-demethylation, oxazole-ring opening, O-dealkylation, and carboxylic acid formation. A combination of microbial bioreactors, NMR and LC/accurate mass analyses, and organic synthesis helped to successfully identify human fecal metabolites following oral administration of $[^{14}C]$muraglitazar. Microbial bioreactors proved to be extremely useful as a source for oxidative metabolites of muraglitazar and greatly aided in metabolite identification. This methodology should be broadly applicable to the determination of metabolite structures for other new chemical entities.

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References


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