DMD012450

**COMPARATIVE METABOLISM OF RADIOLABELED MURAGLITAZAR IN
ANIMALS AND HUMANS BY QUANTITATIVE AND QUALITATIVE
METABOLITE PROFILING**

DONGLU ZHANG, LIFEI WANG, NIRMALA RAGHAVAN, HAIYING ZHANG,
WENYING LI, PETER T CHENG, MING YAO, LITAO ZHANG, MINGSHE ZHU,
SAMUEL BONACORSI, SURESH YEOLA, JAMES MITROKA, NARAYANAN
HARIHARAN, VINAYAK HOSAGRAHARA, GAMINI CHANDRASENA, WEN
CHYI SHYU, AND W GRIFFITH HUMPHREYS

Pharmaceutical Candidate Optimization (DZ, LW, NR, WL, HZ, MY, MZ, SY, JM, VH,
GC, WS, WGH), Discover Chemistry (PTC and SB), Metabolic Disease Biology (LZ,
NH), Pharmaceutical Research Institute, Bristol-Myers Squibb, Princeton, NJ 08543

DMD012450

Running title: Qualitative and quantitative profiling of muragliatizar metabolites

Address correspondence to:

Donglu Zhang, Ph.D.

Pharmaceutical Candidate Optimization,

Bristol-Myers Squibb, P.O. BOX 4000,

Princeton, NJ 08543.

Phone: 609-252-5582.

Email: Donglu.Zhang@BMS.com

Abbreviations used: HPLC, High-performance Liquid Chromatography; LC/MS, liquid chromatography/mass spectrometry; TFA, trifluoroacetic acid

Text pages: 30

of tables: 8; # of figures: 8

of references: 18

of words in abstract: 245

of words in introduction: 350

of words in discussion: 750

DMD012450

ABSTRACT

Muraglitazar (Pargluva), a dual alpha/gamma PPAR activator, has both glucose and lipid lowering effects in animal models and in patients with diabetes. This study describes the *in vivo* and *in vitro* comparative metabolism of [¹⁴C]muraglitazar in rats, dogs, monkeys, and humans by quantitative and qualitative metabolite profiling. Metabolite identification and quantification methods used in these studies included LC/MS, LC/MS/MS, LC/radiodetection, LC/UV, and a newly described mass defect filtering technique in conjunction with high resolution MS. Following oral administration of [¹⁴C]muraglitazar, absorption was rapid in all species, reaching a concentration peak for parent and total radioactivity in plasma within 1 hour. The most abundant component in plasma at all times in all species was the parent drug, and no metabolite was present in greater than 2.5% of the muraglitazar concentrations at 1 hour post dose in rats, dogs, and humans. All metabolites observed in humans plasma were also present in rats, dogs, or monkeys. Urinary excretion of radioactivity was low (<5% of the dose) in all intact species and the primary route of elimination was via biliary excretion in rats, monkeys, and humans. Based on recovered doses in urine and bile, muraglitazar showed a very good absorption in rats, monkeys, and humans. The major drug-related components in bile of rats, monkeys, and humans were glucuronides of muraglitazar and its oxidative metabolites. The parent compound was a minor component in bile, suggesting extensive metabolism of the drug. In contrast, the parent drug and oxidative metabolites were the major components in feces and no glucuronide conjugates were found, suggesting that glucuronide metabolites were excreted in bile and hydrolyzed in the gastrointestinal tract. The metabolites of muraglitazar resulted from both glucuronidation and oxidation. The metabolites in general had greatly reduced activity as PPAR α/γ activators relative to muraglitazar. In conclusion, muraglitazar was rapidly absorbed, extensively metabolized through glucuronidation and oxidation, and mainly eliminated in the feces via biliary excretion of glucuronide metabolites in all species studied. Disposition and metabolic pathways were qualitatively similar in rats, dogs, monkeys, and humans.

DMD012450

INTRODUCTION

The peroxisome proliferator-activated receptors (PPARs) are a set of nuclear hormone receptors. The two most intensively investigated subtypes have been PPAR α (expressed primarily in the liver and plays a critical role in lipid metabolism) and PPAR γ (predominantly expressed in adipose tissue and implicated in insulin sensitization as well glucose and fatty acid utilization). PPAR α is the target of the fibrate class of hypolipidemic drugs such as fenofibrate (Balfour et al., 1990; Despres, 2001) and gemfibrozil (Packard et al., 2002), whereas PPAR γ is the target of the thiazolidinedione (Mudaliar and Herry, 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), a novel dual PPAR α/γ activator, is an oxybenzylglycine analog (non-thiazolidinedione) and has both glucose and lipid lowering effects when tested in animal models of diabetes and dyslipidemia and in patients with diabetes (Devasthale et al., 2005; Harrity et al., 2006; Kendall et al., 2006). Seventeen human oxidative metabolites of muraglitazar were identified following oral administration of [^{14}C]muraglitazar (Zhang et al., 2006) and glucuronidation was the major clearance pathway of muraglitazar in humans (Wang et al., 2006).

This study was conducted to determine comparative metabolite profiles quantitatively and qualitatively in plasma, urine, bile and feces from rats, dogs, monkeys, and humans following oral administration of [^{14}C]muraglitazar and in incubations with hepatocytes from mice, rats, monkeys, and humans. Seven identified metabolites were synthesized for verification and tested for their PPAR α/γ activation activity. Qualitative and quantitative profiling provides identities, amounts (or concentrations), and changes versus time of muraglitazar and its metabolites in plasma and in excreta (urine, bile, and feces) of rats, dogs, monkeys, and humans. The recently published biotransformation of [^{14}C]muraglitazar in mice (Li et al., 2006) was included in some sections for species comparison.

DMD012450

MATERIALS AND METHODS

Materials

[¹⁴C]Muraglitazar was prepared at Bristol-Myers Squibb and had a radiochemical purity of 99.5%. Metabolite standards M1, M5, M10, M11, and M15 were prepared as described previously (Zhang et al., 2006; Wang et al., 2006); M9 and M16 were also prepared at Bristol-Myers Squibb. Ecolite[®] liquid scintillation cocktail was purchased from ICN Biomedicals, Inc. (Costa Mesa, CA). Deepwell LumaPlate[™]-96-well plates were purchased from PerkinElmer[™] (Boston, MA). For solid phase extraction, Oasis[®] HLB C-18 cartridge columns (5 mL) were obtained from Waters Co. (Milford, MA). PEG-400 and sodium hydroxide were purchased from Sigma-Aldrich Co. (Milwaukee, WI). Acetonitrile and trifluoroacetic acid was purchased from EM Science (Gibbstown, NJ). All other chemicals used were of reagent grade or better. Cryopreserved hepatocytes from male cynomolgus monkeys, and three human donors (pooled before use) were purchased from In Vitro Technologies (Baltimore, MD). Hepatocytes were stored in liquid nitrogen before use. Fresh hepatocytes from male Sprague-Dawley rats and male ICR/CD-1 mice were prepared in house using a literature method (Berry and Friend, 1969).

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. Dogs and monkeys were non-naive animals. Animals completed at least 4 days of quarantine or conditioning before dose administration. LabChow[™] or LabDiet[™] (Purina Mills Inc) was fed to dogs and monkeys daily. LabDiet[™] was available to rats and mice *ad libitum*. Tap water was provided to animals *ad libitum*. Mice were fasted for 4 h and rats, dogs, monkeys, and humans were fasted overnight before dose administration. Food was returned at 4 h post dosing for rats, dogs, and monkeys. All human subjects were required to give informed and written consent prior to participation of this study. The extrapolated radiation dose equivalent for tissues in humans following a 100 μ Ci oral dose ranged from 0 to 33.2

DMD012450

millirem based on the tissue distribution study in rats (data not shown). This level is >75 times lower than the annual allowable exposure limit. Therefore, the administration of 100 μCi of [^{14}C]muraglitazar to healthy male subjects was considered safe. The dose was prepared by dissolving [^{14}C]muraglitazar in PEG-400. Plasma, urine, bile, and fecal samples were obtained following a single oral administration of [^{14}C]muraglitazar to rats, dogs, monkeys, or humans.

Rat. Following oral administration of [^{14}C]muraglitazar to male Sprague-Dawley rats (11 mg/kg, 8.4 $\mu\text{Ci}/\text{mg}$), blood (1, 4, and 12 h) was obtained from rats (three rats per collection time) by terminal bleeding via cardiac puncture following carbon dioxide anesthesia. Urine (0-12, 12-24, and 24 h-interval thereafter for 0-168 h) and feces (24 h intervals for 0-168 h) were collected from three rats on dry-ice. In a separate biotransformation study, bile, urine, and feces (0-24 h) were collected from three bile duct-cannulated (BDC) male rats for 0-24 h following oral administration of [^{14}C]muraglitazar (10 mg/kg, 5 $\mu\text{Ci}/\text{mg}$). For bile collection, a bile salt solution (18 mg/mL cholic acid and 1.1 mg/mL sodium bicarbonate in saline, pH 7.2) was infused via the duodenal cannula at 1 mL/h for BDC rats. For the pharmacokinetic study, blood (300 μL) was collected from three rats via a jugular vein cannula at 0, 0.25, 0.5, 1, 2, 4, 6, 8, 12, 24, 48, 72, 96, 120, 144, and 168 h post dose.

Mouse. Bile, urine, and feces were collected (0-24 h) from 5 bile-duct cannulated male CD-1 mice following an oral dose of [^{14}C]muraglitazar (1 and 40 mg/kg, 11.2 $\mu\text{Ci}/\text{mg}$). For bile collection, a solution of taurocholic acid (2.3 mg/mL in 0.9% saline) was infused via the duodenal cannula at 0.3 mL/h for BDC mice (Li et al., 2006).

Dog. Blood (10 mL at 1, 4, and 12 h) was collected from the indwelling venous catheter from three male beagle dogs following oral doses of [^{14}C]muraglitazar (1.9 mg/kg, 2.8 $\mu\text{Ci}/\text{mg}$). Urine (0-12, 12-24, and 24 h-intervals thereafter for 0-168 h) and feces (24 h intervals for 0-168 h) were also collected on dry-ice. In addition, another series of blood samples (2 mL) were collected at 0,

DMD012450

0.25, 0.5, 1, 2, 4, 6, 8, 12, 24, 48, 72, 96, 120, 144, and 168 h post dose for pharmacokinetic analysis.

Monkey. Blood (5 mL at 1, 4, and 12 h) was collected from the indwelling venous catheter from three male cynomolgus monkeys following oral doses of [^{14}C]muraglitazar (2 mg/kg, 6.6 $\mu\text{Ci}/\text{mg}$). Urine (0-12, 12-24 h, and 24 h-interval thereafter for 0-168 h) and feces (24 h intervals for 0-168 h) were also collected on dry-ice. In a separate study, bile, urine, and feces were collected (0-8, 8-24, 24-48 h for 0-48 h) from three bile-duct cannulated male cynomolgus monkeys following an oral dose of [^{14}C]muraglitazar (5 mg/kg, 6 $\mu\text{Ci}/\text{mg}$). For bile collection, a bile salt solution (18 mg/mL cholic acid and 1.1 mg/mL sodium bicarbonate in saline, pH 7.2) was administered via a distal (flushing) catheter at 1 mL/kg/h for BDC monkeys. In addition, another series of blood samples (2 mL) were collected at 0, 0.25, 0.5, 1, 2, 4, 6, 8, 12, 24, 48, 72, 96, 120, 144, and 168 h post dose for pharmacokinetic analysis.

Human. Blood (10 mL at 1, 4, and 12 h) was obtained by venipuncture from six healthy male subjects following a single 10-mg oral dose of 100 μCi . Urine (0-8, 8-24 h, and 24 h-interval thereafter for 0-240 h) and feces (24 h intervals for 0-240 h) were obtained. In addition, another series of blood samples (2 mL) were collected at 0, 0.25, 0.5, 1, 2, 4, 6, 8, 12, 24, 48, 72, 96, 120, 144, and 168 h post dose for pharmacokinetic analysis. Bile was collected over 3-8 h post dose from 4 subjects following an oral 20 mg (100 μCi) dose of [^{14}C]muraglitazar (Wang et al., 2006). An intravenous dose of cholecystokinin was used to stimulate gallbladder contraction at 7 h post dose in humans.

To stabilize acylglucuronide metabolites in bile samples, acetic acid was added to 2 or 5% (v/v) after bile sample collection prior to sample freezing at -20°C . The blood was collected in tubes containing K3EDTA and centrifuged within 30 min of collection to harvest plasma (10 min, 1300xg at 4°C). Animal fecal homogenate was prepared by mixing water:ethanol (50:50, v/v) and feces (5:1, w/w) followed by homogenization. A paste of human feces was prepared as

DMD012450

homogenate before analysis for radioactivity and sample pooling. Urine and fecal homogenate were pooled by combining urine and feces obtained during each collection interval (1% for feces and 2% for urine). A pooled plasma sample was prepared by combining equal volumes of plasma (1-2 mL) from all subjects for each collection time point. Pooled plasma, urine, and feces were analyzed for radioactivity distribution and metabolite identification by LC/MS/MS. A description of the studies and recovery of radioactive doses in animals and humans are shown in Table 1. The dose recovery data from humans with bile collection were presented previously and are included here for comparison (Wang et al., 2006).

Incubations of [^{14}C]Muraglitazar in Hepatocytes

Cryopreserved hepatocytes from monkeys and humans were thawed as recommended by the manufacturer. [^{14}C]Muraglitazar (25.6 μg in 10 μL of methanol) was added to 1 mL portions (final drug concentration = 25.6 $\mu\text{g/mL}$) of cell suspensions in Krebs-Henseleit buffer in open 22 mL glass vials. The cell concentrations were 3, 3, 2 and 2 million cells/mL for hepatocytes from mice, rats, monkeys and humans, respectively. The cell viability before incubation was 65, 92, 78, and 82% for mouse, rat, monkey, and human hepatocytes, respectively. The reaction vials were incubated with shaking (120 rpm) for 4 h in a 37°C chamber. The chamber was maintained under an oxygen/carbon dioxide (95/5) atmosphere. A 4-h incubation of [^{14}C]muraglitazar (25.6 $\mu\text{g/mL}$) in buffer served as a negative control. At the end of the incubations, acetonitrile (2 mL) was added to each sample, and the mixtures were sonicated for 5 min in a Branson 5210 sonicator. The samples were centrifuged at 3000xg for 10 min. The supernatants were removed and aliquots were taken for radioactivity counting. The radioactivity recoveries were 95.6, 96.5, 99.2, and 98.7%, respectively, from mouse, rat, monkey, and human hepatocyte incubations. The remainder of supernatant was evaporated to near dryness under a stream of nitrogen. The residues were reconstituted in 0.2 mL of water/acetonitrile (2:1, v/v) for HPLC/UV, LC/MS/MS, and LC/radiodetection.

DMD012450

Radioactivity Analysis and High-Performance Liquid Chromatography (HPLC)

The radioactivity detection and HPLC analysis were performed as described previously (Zhang et al., 2006). Under these conditions, the synthetic metabolite standards, M1, M5, M9, M10, M11, M15, M16, each had the same retention times (monitored by UV at 275 nm) as the corresponding metabolites in the human fecal extract (monitored by radioactivity detection). These metabolite standards also had the same MS fragmentation patterns as the *in vivo* metabolites.

Preparation of Biological Samples for Metabolite Profiling

Plasma. Pooled plasma was extracted by addition of 2 mL of acetonitrile to 1 mL of plasma, vortexed for 1 min, and centrifuged at 2000xg for 10 min (Eppendorf 5810 R centrifuge, Eppendorf Company, Hamburg, Germany). The supernatant was removed and saved. The pellet was treated with additional three extractions with 2 mL of acetonitrile/water (2:1, v/v) and all supernatants were combined. Aliquots of 100-300 μ L of this solution were mixed with Ecolite cocktail and counted for radioactivity. After the solvent was evaporated to dryness under nitrogen, the residue was re-dissolved in 0.20 mL of water/acetonitrile (2:1, v/v) and centrifuged at 2000xg for 10 min before injecting onto the HPLC column.

Urine. Pooled urine (5 mL) was concentrated either by passing through a 5 mL C18 cartridge (OasisTM, Waters) or by direct evaporation under a stream of nitrogen. The cartridge was eluted with methanol after washing with a half-column volume of water and the eluate was concentrated under a stream of nitrogen. The residue from direct evaporation of the urine or from the evaporation of the methanol eluate was suspended in 0.20 mL of water/acetonitrile (2:1, v/v) and centrifuged at 2000xg for 10 min, and the supernatant (50 μ L) was subjected to HPLC analysis.

Feces. Pooled fecal homogenate was extracted by addition of 4 mL of acetonitrile to about 1 g of fecal homogenate, vortexed for 5 min, sonicated for 5 min, and centrifuged at 2000xg for 30 min. The supernatant was removed and saved. The extraction was repeated twice with 2 mL of

DMD012450

acetonitrile/water (2:1, v/v) and the supernatants were combined. Aliquots of 0.5 mL of the combined supernatant was mixed with Ecolite[®] cocktail and counted for radioactivity. The combined supernatant was evaporated to dryness under a stream of nitrogen. The residue was suspended in 0.20 mL of water/acetonitrile (2:1, v/v) and centrifuged at 2000xg for 10 min before subjecting 50 μ L aliquots to HPLC analysis.

Bile. Pooled bile samples were mixed with one volume of acetonitrile and centrifuged at 2000xg for 10 min. The supernatant (10-30 μ L) was analyzed directly.

LC/MS for Quantification of Muraglitazar in Plasma

The method utilized trazodone as the internal standard (IS). After the addition of 0.2 mL of acetonitrile containing 100 ng/mL of IS to 0.1 mL of plasma sample, methylene chloride (0.1 mL) was added. The samples were vortexed for 2 min and then centrifuged at 14,000 rpm for 3 min. The organic phase was removed and evaporated to dryness under nitrogen at 40°C. The residue was reconstituted with 0.2 mL of HPLC mobile phase A and 20 μ L of the sample was injected onto the LC/MS. Chromatographic separation was achieved on a Waters 2690 HPLC system using a BDS hypersil C18 analytical column (2 x 50 mm, 3 μ m) at a flow rate of 0.3 mL/min. An isocratic system was employed using 90% mobile phase A (acetonitrile/0.1% formic acid, 50:50, v/v) and 10% mobile phase B (acetonitrile/0.1% formic acid, 95:5, v/v). Analysis (m/z 517.1 for muraglitazar and m/z 372.0 for IS) was accomplished by positive turbo ion spray on a Sciex API 100 mass spectrometer (Applied Biosystems, Beverly, MA).

Pharmacokinetic Analysis

The plasma concentration vs time data for radioactivity and unchanged muraglitazar were analyzed by a noncompartmental method (Gibaldi and Perrier, 1982). The peak plasma concentration, $C_{\max}$, and the time to reach peak concentration as the first occurrence, $T_{\max}$, were recorded directly from experimental observations. The area under the plasma concentration vs time curve (AUC) was calculated by a combination of the trapezoidal methods. The AUC was

DMD012450

calculated from time zero to the time, T , of last measurable concentration [AUC(0- T)]. The first-order rate constant of decline of radioactivity concentrations and unchanged muraglitazar, expressed as equivalents of muraglitazar, in the terminal phase of each plasma concentration vs time profile, K , was estimated by log-linear regression (using no weighting factor) of at least three data points which yielded a minimum mean square error. The absolute value of K was used to estimate the apparent terminal elimination half-life, $T_{1/2}$.

LC/MS and LC/MS/MS for Identification of Metabolites

A YMC ODS AQ C18 column (2.0x 150 mm, 5 μ m) was used for LC/MS analysis. The flow rate was 0.28 or 0.3 mL/min. LC/MS analysis was performed on a LCQ, LTQ (ThermoFinnigan, San Jose, CA) or Q-TOF Ultima mass spectrometer (Micromass, Beverly, MA) with an ESI source. Samples were analyzed in the positive ion mode. The capillary temperature used for the LCQ and LTQ was set at 230°C. The desolvation temperature used on the Q-TOF was 300°C. The nitrogen gas flow rate, spray and cone voltages were adjusted to give maximum sensitivity for muraglitazar. The Q-TOF was tuned to 18,000 resolution at half peak height using an insulin tuning solution, and was calibrated up to 1500 Da using a polyalanine calibration solution. For accurate mass measurement, the m/z 556.2771 of an infused 20 ng/ μ L leucine enkephalin solution were used as Lock Mass. The experimentally obtained masses were within 5 mDa compared to their respective calculated values.

Total Ion Chromatograms After Mass Defect Filtering Analysis

The data files from the Q-TOF high resolution mass analysis of fecal extracts of humans, monkeys, and rats were processed using the mass defect filtering method (Zhang et al., 2003). The centroid raw data was processed using a mass defect filter designed to retain ion species with mass defects between 0.006 and 0.106 Da in a mass range of 250-850 Da. The filter was based on the apparent mass of muraglitazar (517.1975 Da) in the uncorrected centroid raw data file.

DMD012450

Radioactivity Profiles and Quantification of Metabolites

The average CPM value from a baseline section (2-3 min) of the chromatogram was subtracted from the CPM value of each fraction. Biotransformation profiles were prepared by plotting the CPM values against time-after-injection using Microsoft® Excel software. Radioactive peaks in the biotransformation profiles were reported as a percentage of the total radioactivity collected during the entire HPLC run. The relative distributions of radioactive metabolites in urine, bile, and feces were calculated from the percent of dose excreted in the matrix (based on DPM by scintillation counting) multiplied by the percent of distribution of metabolites in chromatograms of the matrix (based on CPM counting by Top Count).

Determination of PPAR α / γ Binding and Transactivation Activities of Muraglitazar Metabolites

The *in vitro* PPAR biological activity of the seven synthetic muraglitazar metabolites were evaluated and PPAR α / γ binding and transactivation activity values were determined as described previously (Devasthale et al., 2005).

DMD012450

RESULTS

Excretion of Radioactive Dose

The recovery values of radioactive dose in urine, bile, and feces of rats, mice, dogs, monkeys, and humans are shown in Table 1. Urinary excretion of radioactivity was low (<3.7% of dose) in all intact species and the primary route of excretion was feces (87.7, 89.0, 80.4 and 61.6% of dose for intact rats, dogs, monkeys, and humans, respectively). The bile contained the majority of radioactivity from rats, mice, monkeys, and humans during the given collection periods, representing the major elimination pathway of [¹⁴C]muraglitazar in these four species. Based on recovered dose in urine and bile, the absorption of muraglitazar represented at least 66, 82, 41, and 44% in rats, mice, monkeys, and humans, respectively, following oral administration. The overall recovery of radioactivity in the human study of 10 mg dose was low (64.2%) in which fecal paste was prepared and the reason for the low recovery was not known. An additional study was conducted to address the radioactivity recovery issue by preparing fecal homogenate as slurry in 50:50 (v/v) ethanol/water containing 2% acetic acid following an oral 20 mg dose of [¹⁴C]muraglitazar. The radioactive dose recovery from that study was 3.7% in urine, 50.7% in the feces, and 39.9% of dose in the 3-8 h bile collection (Wang et al., 2006). The fecal metabolite profiles were qualitatively similar between the two human C-14 studies. It was, therefore, concluded that the metabolite profiling results from the samples of the 10-mg dose study were valid despite the lower than an anticipated recovery. Radioactivity excretion profiles of [¹⁴C]muraglitazar in mice following oral administration were similar to other species (Li et al., 2006).

Figure 1 shows the proportion of the doses excreted in urine and feces in each of the collection periods in rats, dogs, monkeys, and humans. The significant portions of the excretion of radioactivity in urine occurred during the first 8 to 12 hours after the dose administration. The concentrations of muraglitazar equivalents (parent drug plus metabolites, calculated using the

DMD012450

actual volumes of urine collected) in rat, monkey, and human urine during this period of urinary excretion are shown in Table 2A. The concentrations of drug-related radioactivity in rat, dog, and monkey urine were approximately 9, 1, and 3 times of that in human urine. The rate of fecal excretion in rats and dogs was rapid, with approximately 85% recovered within 24 h and nearly 95% within 48 h post dose (Figure 1B). In contrast, the rate of fecal excretion in monkeys was slower, with approximately 30% recovered within 48 h and 75% within 96 h post dose. The rate of fecal excretion in humans was similar to that in monkeys, with 25% within 48 h and 80% within 96 h post dose.

The majority of the excretion of radioactivity in bile probably occurred during the first 24 to 48 hours after dose administration. The average radioactive dose recovery value in 0-24 h bile of BDC rats was 65.1% and in the corresponding feces of these rats was 0.55%. The radioactive dose recovery values in 0-48 h bile of three BDC monkeys were 15.4, 16.9, and 75.2%, and in corresponding feces of these three monkeys were 30.6, 6.7, and 7.7%. The concentrations of muraglitazar equivalents (parent drug plus metabolites) in rat, mouse, monkey, and human bile within specified collection periods are shown in Table 2B. The concentrations of drug-related radioactivity in rat, mouse, and monkey bile were approximately 4, 5.5-190, and 0.1 times of that in human bile. Human subjects seemed to show a fast biliary elimination since approximately 40% of dose was found in bile during the 3-8 h collection after dose.

Pharmacokinetic Results

The pharmacokinetic parameters for the unchanged drug and radioactivity are presented in Table 3. The animal and human plasma concentration time profiles for radioactivity and muraglitazar are presented in Figure 2. Following oral administration, the radioactivity and muraglitazar concentrations reached a maximum at 0.5 to 1 h in rats, dogs, monkeys, and humans. While the trends declined in dogs and humans after reaching C_{max} , the radioactivity and muraglitazar concentrations reached a second peak at approximately 6-8 h in rats and monkeys, suggesting the

DMD012450

potential of enterohepatic recycling of muraglitazar and radioactivity in rats and monkeys. The mean terminal elimination $T_{1/2}$ values of plasma radioactivity and muraglitazar were longer in monkeys and humans (18-35 h) than those from rats and dogs (4-10 h). The longer $T_{1/2}$ values for the parent compound relative to total radioactivity in rats, dogs, and humans were probably due to truncation of the radioactivity AUC curves due to lower assay sensitivity of radioactivity compared to LC/MS-based parent determination. The overall kinetic profiles and AUC values for radioactivity are similar to those of muraglitazar in all species.

Identification of [^{14}C]Muraglitazar Metabolites

Table 4 shows the proposed structures for the muraglitazar metabolites identified in rats, dogs, monkeys, and humans following oral administration of [^{14}C]muraglitazar. Most of the metabolite identification was done from LC/MS/MS analysis. Seven of muraglitazar metabolite standards were also synthesized and matched the metabolites in human feces by retention times and mass fragmentation patterns. Detailed LC/MS/MS identification of the metabolites in mice and humans have been presented previously (Zhang et al., 2006; Wang et al., 2006) and identification of muraglitazar metabolites in rats, dogs, and monkeys matched those identified in mice and humans. Typical fragmentation patterns of muraglitazar in full scan MS/MS analysis showed a cleavage adjacent to the carbamate to give a fragment at m/z 292 and cleavage at the ether bond to give a fragment at m/z 186. Similar fragmentation patterns were observed with muraglitazar metabolites M2-M16. All monohydroxylation metabolites (M2, M5, M6, M7, M8a, M10, M11, and M14) showed a fragmentation ion at m/z 202 ($202 = 186 + 16$) or its dehydrated ion at m/z 184 ($184 = 202 - \text{H}_2\text{O}$), indicating that oxygenation was on the oxazole-ring side of the molecules.

Metabolite M1: This radioactive peak is a minor metabolite in *in vivo* samples but a major metabolite in hepatocyte incubations. M1 had poor ionization properties in a mass spectrometer (the parent and all other metabolites do not show ionization of this part of the molecule). M1 was

DMD012450

assigned based on comparing retention time of the radioactive peak with the metabolite standard as *O*-dealkyl muraglitazar.

Metabolite M22: M22 had an accurate mass of 549.1864 Da and a formulae of $C_{29}H_{29}N_2O_9$, consistent with a dioxygenation of muraglitazar. Since M22 was only observed in *in vitro* incubations and not observed in *in vivo* samples, M22 was not further characterized.

Metabolite M23: From hepatocyte incubations, 5-methyl-2-phenyl oxazolyl-4-acetic acid was also identified as a non-radiolabeled metabolite resulting from the dealkylation of muraglitazar. This metabolite had a measured accurate mass of 218.0820 and a formulae of $C_{12}H_{12}NO_3$ (expected mass of 218.0817). M23 was identified as 5-methyl-2-phenyl oxazolyl-4-acetic acid. The same dealkylation reaction also produced M1.

Metabolite Profiles in Plasma

The extraction recovery of radioactivity from all plasma samples was >98%. The HPLC radiochromatographic profiles of 1-h plasma from rat, dog, monkey, and human are shown in Figure 3. The relative distribution of radioactive metabolites in pooled plasma is summarized in Table 5. The metabolic profiles of plasma were qualitatively similar at different time points within species and across species. Muraglitazar was the major component at 1, 4 and 12 h, accounting for more than 85% of plasma radioactivity at 1 h in all four species.

The prominent metabolite in rat plasma was M15 which accounted for 2-4% of the sample radioactivity. Several metabolites were in rat plasma and each accounted for less than 0.4% of the radioactivity. The parent compound in dog plasma represented 90.5% at 1 h, 81.3% at 4 h, and 33.0% at 12 h of the sample radioactivity. Prominent metabolites in dog plasma included M1, M5, M15, and M20. Major metabolites in monkey plasma were M5, M11, and M18. M11 accounted for 8.0% of the plasma radioactivity at 1 h, and increased to 16.3% at 4 h and 21.4% at 12 h. M5 accounted for 1.5-2.9% of the plasma radioactivity. Metabolite M18 represented 3-4% of plasma radioactivity at 4 and 12 h in monkeys. Other metabolites observed in monkey plasma

DMD012450

were minor. M13 was a major metabolite in human plasma, but no metabolite accounted for more than 4.5% of the plasma radioactivity at all time points. Similarly, the parent drug was the major circulating drug-related component in mice (Li et al., 2006). All metabolites (M1, M2, M5, M8, M8a, M10-13, M15, and M18) observed in human plasma were also present in rats, dogs, or monkeys.

Metabolite Profiles in Urine

Urinary excretion was a minor pathway, representing less than 5% of the dose in all studied intact species following oral administration of [¹⁴C]muraglitazar. The HPLC radiochromatographic profiles of pooled urine from rats, dogs, monkeys, and humans are shown in Figure 4 and metabolite distribution in rat, dog, monkey, and human urine was listed in Table 6. Several very minor components (0.3 to 0.8% of the dose) were not sufficiently abundant for characterization. Rat urine contained many radioactive peaks. The prominent rat urinary metabolites included M1, M2, M10, M15 and M21. One major radioactive peak M1 was observed in dog urine. Metabolites M1, M21, and M11 were prominent radioactive peaks in monkey urine, accounting for about 1, 0.3, 0.6% of the dose, respectively. The low level of radioactivity in human urine was distributed in several radioactive peaks, of which the glucuronide M13 accounted for 1% of the dose and the parent accounted for 0.3% of the dose. All human urinary metabolites were found in animal urine samples.

Metabolite Profiles in Feces

Feces was the major elimination route following oral administration of [¹⁴C]muraglitazar to intact species. The HPLC radiochromatographic profiles of pooled feces are shown in Figure 5 and metabolite distribution in rat, dog, monkey, and human feces was listed in Table 6. Muraglitazar was one of the abundant components present in feces from all species. The most abundant metabolite was different in each species; however, each of these metabolites were hydroxylated and O-demethylated products found in the plasma of that species.

DMD012450

Rat feces contained prominent radioactive peaks M2, M5, M8a, M9, M15 and parent. The parent drug (P) accounted for 13% of the dose in the rat feces. M15, the most abundant peak, accounted for about 34% of the dose. Dog feces contained two major radioactive peaks, M15 and P, representing 25 and 53% of the dose, respectively. No other radioactive peaks accounted for more than 2% of the dose in dog feces. Prominent metabolites in monkey feces included M6, M8, M10, M11, M15 and P, each representing 5-26% of the dose. Major metabolites in the 0-240 h pooled human feces included M4, M5, M10, M11, M15, and M16, each of which accounted for 3-11% of the dose. The parent compound accounted for 9.6% of the dose. Radioactivity distribution of 0-48 h pooled human feces was also determined and the parent compound represented approximately 40% of the sample radioactivity that was higher than that in 0-240 h pooled human feces (data not shown).

Metabolite Profiles in Bile Samples

The HPLC-radiochromatograms of pooled bile samples from rats, monkeys, and humans are shown in Figure 6. Within 24 h following oral administration of [^{14}C]muraglitazar to BDC rats, 65% of the dose was excreted in bile and <1% of the dose was excreted in feces. The parent drug represented <5% of the dose in the rat bile, suggesting extensive metabolism of muraglitazar in rats. The glucuronide of *O*-demethyl muraglitazar (M20), the glucuronide of muraglitazar (M13), and *O*-demethyl muraglitazar (M15) were the major metabolites in rat bile, each accounting for 11-40% of the dose (Figure 6). Under the conditions used for sample collection and storage (2-5% acetic acid), the acylglucuronide metabolites appeared to be chromatographically separated as single peaks, which suggesting that minimal acyl migration occurred.

Within 48 h following oral administration of [^{14}C]muraglitazar to BDC monkeys, the average excretion of radioactive dose was 36% (range of 15-75%) in bile, and 15% in feces. Metabolism of [^{14}C]muraglitazar was extensive with small amounts of unchanged [^{14}C]muraglitazar in the monkey bile. M13 was the prominent metabolite in bile accounting for about 14% of the dose; all

DMD012450

other metabolites were less than 5% of the dose in bile. M15 and [^{14}C]muraglitazar were the major radioactive components corresponding to about 2.6 and 3.3% of the dose in the BDC monkey feces. Glucuronides were not present in feces; the major metabolites in bile were glucuronide conjugates. Metabolites M9, M10, and M11 were also prominent components in feces (data not shown), suggesting bile leakage to intestines in which the glucuronides were hydrolyzed to muraglitazar and oxidative metabolites.

Following oral administration of [^{14}C]muraglitazar to human subjects, approximately 40% of the dose was found in the bile collected over 3-8 h post dose using an oral-gastro-duodenal tube (Wang et al., 2006). More than 60% of the radioactivity in bile was present as the glucuronide of [^{14}C]muraglitazar (M13). Fecal samples contained mostly oxidative metabolites and little glucuronide of the parent drug.

Similarly, muraglitazar glucuronide (M13) was also a major metabolite in the mouse bile but was not found in the BDC mouse feces (Li et al., 2006). In addition to those metabolites (M1-M21) identified in other species (rats, dogs, monkeys, and humans), unique metabolites identified in mice included the taurine conjugates of muraglitazar and its metabolites formed from *O*-demethylation, hydroxylation and dihydroxylation, and glutathione conjugates (Li et al., 2006).

Metabolite Profiles in Hepatocyte Incubations

The distribution of radioactive peaks presented in each hepatocyte incubation is summarized in Table 7. The parent drug was the major component in all incubations. The control incubation of [^{14}C]muraglitazar also contained M1, M22, and a minor peak that had the same retention time as M10. Identification of *in vitro* metabolites were confirmed by LC/MS/MS analyses. Twenty one radioactive metabolites (M1-M8, M10-M15, M17-M22, and M8a) were detected in the hepatocyte incubations and the most prominent metabolites were M1, M6, M10, M11, M12, M13, M14, and M15. Eleven radioactive peaks, M1, M6-7, M10-15, M18, and M22, were observed from the incubation with human hepatocytes. Qualitatively similar metabolic profiles

DMD012450

were obtained from the incubations with hepatocytes from mice, rats, and monkeys. Figure 7 shows the LC/UV profiles of [^{14}C]muraglitazar metabolites in hepatocytes of mice, rats, monkeys, and humans. The LC/UV profiles were qualitatively similar to the radioactivity profiles of these hepatocyte incubations. All human metabolites of [^{14}C]muraglitazar were detected in incubations in hepatocytes from at least one toxicological animal species. Subsequent studies examining the stability of M13 in neutral pH aqueous buffer systems demonstrated that the metabolite hydrolyzed (Data not shown). Although the rate of hydrolysis was not measured under the exact conditions of the hepatocyte incubations, it is likely that there would be significant hydrolysis of M13 leading to under-representation of this pathway.

Summary on Metabolite Profiling Results

Parent compound was the major circulating drug-related component in rats, dogs, monkeys, and humans following oral administration of [^{14}C]muraglitazar. Fecal excretion of [^{14}C]muraglitazar and its metabolites was the major elimination pathway (>95% of the recovered dose) in intact species, principally via biliary elimination when with bile collections. The results suggested that biliary elimination (as glucuronides) was the major clearance pathway of [^{14}C]muraglitazar and that the glucuronide metabolites were hydrolyzed in the intestine before excretion in feces. Urinary excretion represented a minor elimination pathway (<5% of the recovered dose) in intact species. Biotransformation profiles of [^{14}C]muraglitazar were qualitatively similar in rats, dogs, monkeys, and humans. Major metabolic pathways of muraglitazar were glucuronidation, aliphatic/aryl hydroxylation, O-dealkylation, and a combination of O-demethylation and hydroxylation, and oxazole-ring opening. Biotransformation profiles of [^{14}C]muraglitazar were qualitatively similar in hepatocytes of mice, rats, monkeys, and humans. *In vitro* metabolism of [^{14}C]muraglitazar was qualitatively similar to its *in vivo* metabolism.

DMD012450

LC/MS Profiling of Muraglitazar Metabolites

Figure 8 shows the ion chromatograms of total mass spectral signals versus HPLC run time after mass defect filtering treatment of LC/accurate mass analysis of human, monkey, and rat feces. The relative intensity of the total ion peak of each metabolite and the parent resemble qualitatively those peaks in the radiochromatograms of these samples. Full scan mass spectra and MS/MS spectra were derived from these ion chromatograms for identification of metabolites. MDF-treated total ion chromatograms were specially useful to identify molecular ions of each metabolite due to the effective removal of endogenous interfering ions (Zhang et al., 2003).

PPAR α/γ Activities of Muraglitazar Metabolites

Table 8 shows the PPAR α/γ activities of muraglitazar metabolites. Based on these data from the PPAR α and γ binding and transactivation assays, the muraglitazar metabolites M1, M5, M9, M15 and M16 show little or no functional PPAR α or PPAR γ agonist activity in the PPAR α and PPAR γ receptor transactivation assays in the HEK cell line in comparison to the parent compound muraglitazar. Metabolite M10 shows no functional PPAR γ agonist activity in the transactivation assay, but did have partial PPAR α agonist activity in the PPAR α transactivation assay. Metabolite M11, which also had minimal functional PPAR γ agonist activity in the transactivation assay, showed low levels of reasonable PPAR α agonist activity in the PPAR α transactivation assay, with an EC₅₀ value of 0.260 μ M, seven-fold less potent than muraglitazar, (PPAR γ EC₅₀ = 0.035 μ M). In conclusion, none of the seven muraglitazar metabolites showed significant PPAR γ agonist functional activity, and only one metabolite showed any significant PPAR α agonist functional activity (M11, which is 7-fold less potent than muraglitazar based on a comparison of EC₅₀ values from the transactivation assay). The lack of significant PPAR α/γ activities for these seven synthetic metabolites suggests that these metabolites will have little or no contribution to the clinical efficacy of muraglitazar.

DMD012450

DISCUSSION

Following oral administration of [^{14}C]muraglitazar, the parent compound was the major circulating component in plasma of rats, dogs, monkeys, and humans. AUC values for muraglitazar and total radioactivity are similar in rats, dogs, and humans although the parent AUC represented approximately 40% of the radioactivity AUC in monkeys, suggesting that the radioactivity was mainly attributed to parent compound. HPLC/radioactivity profiling of selected plasma samples confirmed that muraglitazar was the major circulating component and there were limited amounts of circulating metabolites. Biliary elimination followed by fecal excretion was the major elimination pathway in all species while urinary elimination was minor. All human metabolites were found in samples from at least one of the toxicological animals (rats or monkeys). The metabolic pathways of muraglitazar included glucuronidation, aliphatic/aryl hydroxylation, *O*-dealkylation, a combination of *O*-demethylation and hydroxylation, and oxazole-ring opening. Although the mechanism is not known, oxazole-ring opening appeared to lead to the formation of two stable metabolites, M9 and M16, in all species tested. Both muraglitazar glucuronide (M13) and *O*-demethyl muraglitazar glucuronide (M20) were major metabolites in the bile of BDC rats. Muraglitazar glucuronide (M13) was the major metabolite in the bile of BDC monkeys. However, oxidative metabolites were predominant in feces of intact animals, suggesting that the glucuronides were hydrolyzed in the intestine. Similarly, most of the radioactive components in human bile were glucuronide metabolites, while oxidative metabolites were found in human feces (Wang et al., 2005). The biliary excretion and intestinal hydrolysis of the glucuronide metabolites provide a potential for enterohepatic recycling of muraglitazar and its oxidative metabolites. However, no obvious evidence for this process was observed in the plasma radioactivity time profiles for humans and dogs, although a second peak for both muraglitazar and total radioactivity appeared at 6-8 h post dose in rats and monkeys. The second peak for the parent and radioactivity in rats is likely not due to enterohepatic recirculation as the rat is a

DMD012450

continuous bile secretor. The effect may be due to regional differences in absorption or in changes of drug solubility in the rat intestines after food was reintroduced to the fasted animals at approximately 4 h post dose. Enterohepatic recycling may lead to the second peak in monkeys and may occur in all species, but it appeared to be minor in humans (Wang et al., 2006).

The biotransformation of [^{14}C]muraglitazar was also investigated in intact and BDC CD-1 mice following oral administration of 1 and 40 mg/kg (Li et al., 2006). Similar to rats, monkeys, and humans, biliary excretion was the major route of elimination, accounting for >66% of the dose during a 0-48 h collection period. Urinary excretion accounted for 7-16% of the dose in mice. Besides those metabolites (M1-M21) identified in other species (rats, dogs, monkeys, and humans), metabolites identified only in mice included the taurine conjugates of muraglitazar and its metabolites formed from *O*-demethylation, hydroxylation and dihydroxylation, and glutathione conjugates. None of these taurine and glutathione conjugates were found in the bile samples of rats, monkeys, or humans. The major drug-related component circulating in plasma was the parent compound for up to 4 h post dose.

When muraglitazar was incubated with hepatocytes of mice, rats, monkeys, and humans at a concentration of 2-10X C_{max} values determined in these species, the majority of metabolites resulted from oxidation pathways including hydroxylation and *O*-demethylation which were also observed *in vivo*. A relatively small fraction of metabolites resulted from glucuronidation to form M13 and M18 in these hepatocyte incubations, which underestimated the *in vivo* importance of the glucuronidation pathway. This is likely due to the instability of the glucuronide metabolite in the hepatocyte incubations. In contrast, the glucuronide metabolites in plasma and bile samples were relatively stable under the acidified and low temperature conditions.

In general, both quantitative and qualitative metabolite profiles are important to understand the relative abundance and identities of metabolites in various matrices from different species. The metabolites of muraglitazar in plasma, urine, feces, and bile as well as hepatocyte incubations

DMD012450

were profiled quantitatively and qualitatively through LC/radioactivity detection, LC/UV, LC/MS, and LC/MS/MS analysis. Metabolites identified in each species were assigned as same structure based on HPLC retention time and MS/MS spectral properties. Qualitatively and quantitatively similar metabolite profiles of muraglitazar in rats, dogs, monkeys, and humans support that these animal species were valid models to evaluate the safety profile of muraglitazar. Mass defect filtering analysis not only generates metabolite identification data (mass spectra with minimal endogenous interferences) (Zhang et al., 2003), but also provides total ion chromatograms that qualitatively resemble the metabolite radiochromatograms of fecal extracts of human, monkeys, and rats (Figure 8). Although total ion chromatograms obtained after the mass defect filtering analysis of high resolution MS data, representing mass spectral signals or total ion signals acquired at each time point plotted against HPLC run time, resemble the radiochromatograms of human, monkey, and rat feces, these ion chromatograms should not be used to fully quantify the amounts of metabolites. However, the close resemble to the radiochromatograms of the fecal samples provides the first potentially useful LC/MS-based estimation method for relative abundance of metabolites. This metabolite quantity estimation method would be specially useful when a validated quantitation method is not available yet quantity information is important to know in certain phases of drug development such as the first-in-man study for metabolite profile in plasma. Therefore, LC/high resolution MS and LC/MS/MS methods were complementary to quantitative profiling using radioactivity detection when the existence of a metabolite need to be qualified.

In summary, the current study describes the disposition and comparative metabolism of [^{14}C]muraglitazar in rats, mice, dogs, monkeys, and humans by qualitative and quantitative metabolite profiling. [^{14}C]Muraglitazar was rapidly absorbed and circulated as the major drug-related component in all species. The compound was extensively metabolized by both oxidative

DMD012450

and conjugative pathways, and eliminated mainly through biliary excretion as glucuronide conjugates of the parent and its oxidative metabolites.

ACKNOWLEDGEMENT: We would like to thank Kamelia Behnia for preparation of rat and mouse hepatocytes and Geoffrey Kinnel for technical assistance.

DMD012450

REFERENCES

- Albericio F, and Barany G (1985) Improved approach for anchoring N^{α} -9-fluorenylmethoxycarbonylamino acids as *p*-alkoxybenzyl esters in solid-phase peptide synthesis. *Int J Peptide protein Res* **26**: 92-97.
- Al-Rubaie AZ, Yousif LZ, Al-Hamad AJH (2002) Palladium-catalyzed formation of 3,5-diaryl-1,2,4-selenadiazoles from arylselenocarboxamide. *J Organomet Chem* **656**: 274-280.
- Balfour JA, McTavish D, and Heel RC (1990) Fenofibrate. A review of its pharmacodynamic and pharmacokinetic properties and therapeutic use in dyslipidaemia. *Drugs* **40(2)**:260-290.
- Balfour JA, and Plosker GL (1999) Rosiglitazone. *Drugs* **57(6)**:921-930.
- Berry MN and Friend DS (1969) High-yield preparation of isolated rat liver parenchymal cells, *J Cell Biol* **43**: 506-520.
- Cheng-Lai A, and Levine A (2000) Rosiglitazone: an agent from the thiazolidinedione class for the treatment of type 2 diabetes. *Heart Dis* **2(4)**:326-333.
- Davies B and Morris T (1993) Physiological parameters in laboratory animals and humans. *Pharm Res (NY)* **10**: 1993-1996.
- Devasthale PV, Chen S, Jeon Y, Qu F, Shao C, Wang W, Zhang H, Cap M, Farrelly D, Golla R, Grover G, Harrity T, Ma Z, Moore L, Ren J, Seethala R, Cheng L, Sleph P, Sun W, Tieman A, Wetterau JR, Dowsyko A, Chandrasena G, Chang SY, Humphreys WG, Sasseville VG, Biller SA, Ryono DE, Selan F, Hariharan N, and Cheng PTW (2005) Design and synthesis of *N*-[(4-methoxyphenoxy)carbonyl]-*N*-[[4-[2-(5-methyl-2-phenyl-4-oxazolyl)ethoxy]phenyl]methyl]glycine, muraglitazar /BMS-298585], a novel peroxisome proliferator-activated receptor α/γ dual agonist with efficacious glucose and lipid-lowering activities. *J Med Chem* **48**: 2248-2250.
- Despres JP (2001) Increasing high-density lipoprotein cholesterol: an update on fenofibrate. *Am J Cardiol* **88(12A)**:30-36N.
- Gibaldi M and Perrier D. (1982) Pharmacokinetics, 2nd Ed. New York: Marcel Dekker, Inc..
- Goldstein BJ (2000) Rosiglitazone. *Int J Clin Pract* **54(5)**:333-337.

DMD012450

Gillies PS, and Dunn CJ (2000) Pioglitazone. *Drugs* **60**(2):333-343 and 344-345.

Harrity T, Farrelly D, Tienman A, Chu C, Kunselman L, Gu L, Cap M, Wu F, Shao C, Wang W, Zhang H, Fenderson W, Chen S, Devasthale P, Jeon Y, Seethala R, Yang WP, Ren J, Zhou M, Ryono D, Biller S, mookhtiar KA, Wetterau J, Gregg R, Cheng PT, and Harihara N (2006) Muraglitazar, a novel dual (alpha/gamma) peroxisome proliferator-activated receptor activator, improves diabetes and other metabolic abnormalities and preserves beta-cell function in db/db mice. *Diabetes* **55**(1): 240-248.

Kendall DM, Rubin CJ, Mohideen P, Ledezine LM, Beller R, Gross J, Norwood P, O'Mahony M, Sall K, Sloan GG, Roberts A, Fiedorrek FT, Defronzo RA (2006) Improvement of glycemic control, triglycerides, and HDL cholesterol levels with muraglitazar, a dual ([alpha]/[gamma]) peroxisome proliferator-activated receptor activator, in patients with type 2 diabetes inadequately controlled with metformin monotherapy: A double-blind, randomized, pioglitazone-comparative study, *Diabetes Care* **29**(5): 1016-1023.

Mudaliar S, and Henry RR (2001) New oral therapies for type 2 diabetes mellitus: The glitazones or insulin sensitizers. *Annu Rev Med* **52**:239-257.

Packard KA, Backes JM, Lenz TL, Wurdeman RL, Destache C, and Hilleman DE (2002) Comparison of gemfibrozil and fenofibrate in patients with dyslipidemic coronary heart disease. *Pharmacotherapy* **22**(12):1527-1532.

Wang L, Zhang D, Swaminathan A, Cheng PTW, Xue Y, Mosqueda-Garcia R, Everett DW, and Humphreys WG (2006) Glucuronidation as a major metabolic clearance pathway of [¹⁴C]muraglitazar in humans: Different metabolic profiles in subjects with or without bile collection. *Drug Metab Dispos* **34**: 427-439.

Zhang, HY, Zhang DL, and Ray K (2003) A software filter to remove interference ions from drugs metabolites in accurate mass liquid chromatography/mass spectrometric analyses. *J. Mass Spectrom* **38**: 1110-1112.

Zhang D, Zhang H, Aranibar N, Hanson R, Cheng PTW, Huang Y, Bonacorsi S, Swaminathan A, Everett DW, and Humphreys WG (2006) Structure elucidation of human oxidative metabolites of muraglitazar: use of microbial bioreactors in the biosynthesis of metabolite standards. *Drug Metab Dispos* **34**: 267-280.

DMD012450

Legends for Figures

Figure 1. [^{14}C]Muraglitazar dose excreted in urine (A) and feces (B) during each collection interval from rats, dogs, monkeys, and humans following oral administration.

Figure 2. Total plasma radioactivity (A) and muraglitazar concentration (B) versus time profiles following a single administration of [^{14}C]muraglitazar in rats (11 mg/kg), dogs (1.9 mg/kg), monkeys (2 mg/kg), and humans (10 mg).

Figure 3. Radiochromatograms of pooled plasma samples (4 h) after an oral administration of [^{14}C]muraglitazar to rats, dogs, monkeys, and humans (1, 2, 3....p = M1, M2, M3....parent).

Figure 4. Radiochromatograms of pooled urine samples after oral administration of [^{14}C]muraglitazar to rats (0-168 h), dogs (0-168 h), monkeys (0-168 h), and humans (0-240 h) (1, 2, 3....p = M1, M2, M3....parent).

Figure 5. Radiochromatograms of pooled fecal samples after oral administration of [^{14}C]muraglitazar to rats (0-168 h), dogs (0-168 h), monkeys (0-168 h), and humans (0-240 h) (1, 2, 3....p = M1, M2, M3....parent).

Figure 6. Radiochromatograms of pooled bile samples after oral administration of [^{14}C]muraglitazar to rats (0-24 h), monkeys (0-48 h), and humans (3-8 h) (1, 2, 3....p = M1, M2, M3....parent).

Figure 7. Comparative LC/UV profiles of [^{14}C]muraglitazar incubations with hepatocytes from mice, rats, monkeys, and humans.

Figure 8. Comparative ion chromatograms of fecal extracts of humans (0-48 h), monkeys (0-168 h), and rats (0-168 h) following oral doses of [^{14}C]muraglitazar (1, 2, 3....p = M1, M2, M3....parent) after mass defect filtering analysis of a high resolution spectral set.

DMD012450

Table 1: Mass balance results in rats, mice, dogs, monkeys, and humans following a single oral administration of [¹⁴C]muraglitazar

Species	No. Subjects	Dose ^a	Matrix	Sampling Time (h)	Recovery (% of dose)	Total Recovery (% of dose)
Rat	3	11 mg/kg	Urine	0-168	1.0	
			Feces	0-168	87.7	88.7
	3 BDC	10 mg/kg	Urine	0-24	0.98	
			Bile	0-24	65.1	
			Feces	0-24	0.55	66.6
Mouse ^b	5 BDC	1 mg/kg	Urine	0-24	8.3	
			Bile	0-24	75	
			Feces	0-24	8.2	91.5
	5 BDC	40 mg/kg	Urine	0-24	16.1	
			Bile	0-24	66	
			Feces	0-24	12	94.1
Dog	3	1.9 mg/kg	Urine	0-168	0.5	
			Feces	0-168	89.0	89.5
Monkey	3	2 mg/kg	Urine	0-168	3.7	
			Feces	0-168	80.4	84.1
	3 BDC	5 mg/kg	Urine	0-48	5.0	
			Bile	0-48	35.7	
			Feces	0-48	15.0	55.7
Human ^b	6	10 mg	Urine	0-240	2.6	
			Feces	0-240	61.6	64.2
	4 ^c	20 mg	Urine	0-240	3.7	
			Bile	3-8	39.9	
			Feces	0-240	50.7	94.3

DMD012450

^a Radiospecific activity of [¹⁴C]muraglitazar used was: 8.4 and 5 $\mu\text{Ci}/\text{mg}$ for rats, 11.2 $\mu\text{Ci}/\text{mg}$ for mice, 2.8 $\mu\text{Ci}/\text{mg}$ for dogs, 6.6 and 6 $\mu\text{Ci}/\text{mg}$ for monkeys, and 10 and 5 $\mu\text{Ci}/\text{mg}$ for humans.

^b The human and mouse data with bile collection were presented previously (Wang et al., 2006; Li et al., 2006) and were included here to allow a complete species comparison.

^c Bile was collected for 3-8 h post dose (Wang et al., 2006).

DMD012450

Table 2: Approximate concentrations of [¹⁴C]muraglitazar and metabolites in urine and bile from rats, mice, dogs, monkeys, and humans following oral administration

A: Urine

	Rat	Dog	Monkey	Human
Dose (mg/kg or mg/subject)	11	2	1.9	10
Body Mass (kg) ^a	0.25	10	5	70
Urine collection interval (h) ^b	0 - 12	0 - 24	0 - 12	0 - 8
Concentration in urine (μg eq/mL) ^c	1.60	0.20	0.57	0.17
% Dose in urine of interval collection	0.51	0.49	1.7	1.3

B: Bile

	Rat	Mouse	Monkey	Human
Dose (mg/kg or mg/subject)	10	1, 40	5	20
Body Mass (kg) ^a	0.25	0.02	5	70
Dose/subject (mg)	2.5	0.02, 0.8	25	20
Bile Flow (mL/h) ^a	22.5	2	125	350
Bile collection interval (h)	0 - 24	0 - 24	0 - 48	3 - 8
Concentration in bile (mg-eq/mL) ^d	0.289	0.375, 13.2	0.007	0.068
% Dose in bile of interval collection	65.1	75, 66	36	40

^a Literature values from Davies and Morris (1993).

^b Closest matched intervals; highest concentrations were excreted in these intervals.

^c Total concentration of muraglitazar plus all metabolites (muraglitazar equivalents).

^d Total concentration of muraglitazar plus all metabolites (muraglitazar equivalents). Calculated based on theoretical bile excretion volume in 0-24 or 0-48 h for animals and 0-8 h for humans. The bile concentration in rats was an assumption only since there is no gallbladder in the rat.

DMD012450

Table 3: Mean pharmacokinetic parameters of muraglitazar in rats, dogs, monkeys, and humans following oral administration of [¹⁴C]muraglitazar

Species (n)	Dose	Analyte	C _{max} ^a (μg/mL)	T _{max} (h)	AUC _{0-t} ^a (μg.h/mL)	T _{1/2} ^b (h)
Rat (3)	10 mg/kg	Muraglitazar	12.8 ± 3.0	0.5 ^d	101 ± 20	10.3 ± 0.4
		Radioactivity	13.5 ± 2.6	0.5 ^d	105 ± 19	3.8 ± 1.8
Dog (3)	2 mg/kg	Muraglitazar	0.8 ± 0.4	0.5	1.3 ± 0.4	5.7 ± 4.6
		Radioactivity	1.0 ± 0.4	1	1.1 ± 0.4	- ^c
Monkey (3)	1.9 mg/kg	Muraglitazar	6.9 ± 2.5	1 ^e	41 ± 9	18.0 ± 2.1
		Radioactivity	7.1 ± 2.1	1 ^e	71 ± 8.3	23.5 ± 11.7
Human (6)	10 mg	Muraglitazar	1.8 ± 0.4	1	12 ± 2.8	35.1 ± 9.7
		Radioactivity	1.4 ± 0.4	1	9.0 ± 3.1	19.8 ± 8.5

^a Units for C_{max}: muraglitazar (μg/mL) and radioactivity (μg-equivalents/mL); Units for AUC: muraglitazar (μg•h/mL) and radioactivity (μg-equivalents•h/mL). Last detectable time points for respective parent compound and the radioactivity were 96 and 24 h for rat, 12 and 4 h in dog, 144 and 24 h for monkey, and 192 and 72 h for human.

^b The T_{1/2} values for radioactivity were lower than the parent for rats, dogs, and monkeys because the detection limit for radioactivity was approximately 15-30 times higher than LC/MS-based parent determination.

^c Not determined due to limited time points.

^d Second peaks were observed at 8 h in rats.

^e Second peaks were observed for at 6 h in monkeys.

DMD012450

Table 4: Structures of [¹⁴C]muraglitazar metabolites

Drug or Metabolite	Proposed Structure ^a	Source
Muraglitazar	* indicates C-14 labels	Rat: Plasma, urine, feces, bile Dog: Plasma, urine, feces Monkey: Plasma, urine, feces, bile Human: Plasma, urine, feces, bile
M1 (O-dealkyl muraglitazar)		Rat: Plasma, urine, feces Dog: Plasma, urine, feces Monkey: Plasma, urine, feces, bile Human: Plasma, urine, feces
M2 (8-hydroxy-O-demethyl muraglitazar)		Rat: Plasma, urine, feces, bile Dog: ND ^b Monkey: Plasma, urine, feces, bile Human: Plasma, urine, feces, bile
M3 (8,12-dihydroxy muraglitazar)		Rat: Urine Dog: ND Monkey: Feces, bile Human: Plasma, urine, feces
M4 (9,12-dihydroxy muraglitazar)		Rat: ND Dog: Feces Monkey: Feces, bile Human: Plasma, urine, feces
M5 (12-hydroxy-O-demethyl muraglitazar)		Rat: Plasma, urine, feces, bile Dog: Plasma, urine, feces Monkey: Plasma, urine, feces, bile Human: Plasma, urine, feces, bile
M6 (17-hydroxy-O-demethyl muraglitazar)		Rat: Plasma, urine Dog: Plasma, urine, feces Monkey: Plasma, urine, feces, bile Human: Plasma, urine, feces
M7 (9-hydroxy-O-demethyl muraglitazar)		Rat: Feces, bile Dog: Feces Monkey: Feces, bile Human: Feces, bile

DMD012450

Table 4: (Cont) Structures of muraglitazar metabolites

Drug or Metabolite	Proposed Structure ^a	Source
M8 (12,17-dihydroxy muraglitazar)		Rat: Plasma, bile Dog: Feces Monkey: Plasma, urine, feces, bile Human: Plasma, feces, bile
M8a (8-hydroxy muraglitazar)		Rat: Plasma, urine, feces, bile Dog: Feces Monkey: Plasma, urine, bile Human: Plasma, urine, feces, bile
M9 (urea-derivative of muraglitazar)		Rat: Plasma, feces Dog: Feces Monkey: Feces, bile Human: Feces
M10 (12-hydroxy muraglitazar)		Rat: Plasma, urine, feces, bile Dog: Feces Monkey: Plasma, urine, feces, bile Human: Plasma, urine, feces, bile
M11 (17-hydroxy muraglitazar)		Rat: Plasma, urine, feces, bile Dog: Feces Monkey: Plasma, urine, feces, bile Human: Plasma, urine, feces
M12 (muraglitazar 12-carboxylic acid)		Rat: Plasma, urine, bile Dog: Feces Monkey: Plasma, urine, feces, bile Human: Plasma, feces, bile
M13 (muraglitazar glucuronide)		Rat: Plasma, urine, bile Dog: Plasma Monkey: Plasma, urine, feces, bile Human: Plasma, urine, bile
M14 (9-hydroxy muraglitazar)		Rat: Urine, feces, bile Dog: Feces Monkey: Urine, feces, bile Human: Feces, bile
M15 (O-demethyl muraglitazar)		Rat: Plasma, urine, feces, bile Dog: Plasma, urine, feces Monkey: Plasma, urine, feces, bile Human: Plasma, feces, bile
M16 (acetylurea-derivative of muraglitazar)		Rat: Plasma, feces Dog: Feces Monkey: Feces Human: Plasma, feces

DMD012450

Table 4: (Cont) Structures of muraglitazar metabolites

Drug or Metabolite	Proposed Structure ^a	Source
M17a (hydroxy-O-demethyl muraglitazar glucuronide)		Rat: Bile Dog: ND Monkey: bile Human: Bile
M17b (hydroxy-O-demethyl muraglitazar glucuronide)		Rat: Urine, bile Dog: ND Monkey: Urine, bile Human: Urine, bile
M17c (hydroxy-O-demethyl muraglitazar glucuronide)		Rat: Bile Dog: ND Monkey: Bile Human: Bile
M18a (hydroxy muraglitazar glucuronide)		Rat: Plasma, bile Dog: ND Monkey: Bile Human: Bile
M18b (hydroxy muraglitazar glucuronide)		Rat: Plasma, urine, bile Dog: ND Monkey: Plasma, urine, bile Human: Plasma, bile
M18c (hydroxy muraglitazar glucuronide)		Rat: Bile Dog: ND Monkey: Bile Human: Bile
M20 (O-demethyl muraglitazar glucuronide)		Rat: Plasma, urine, bile Dog: Plasma, urine Monkey: Plasma, urine, bile Human: Bile
M21 (O-dealkyl-O-demethyl muraglitazar)		Rat: Urine, feces Dog: Urine Monkey: Plasma, urine, feces, bile Human: Urine, feces
M22 (di-oxygenated muraglitazar)		In incubations of hepatocytes from mice, rats, monkeys, and humans and in the control incubation without hepatocytes
M23 (5-methyl-2-phenyloxazolyl 4-acetic acid)		In incubations of hepatocytes from mice and monkeys

^a Structures were proposed based on MS data and comparison with synthetic metabolites.

^b ND = not detected.

DMD012450

Table 5: Distribution of radioactive metabolites in pooled plasma after an oral dose of [¹⁴C]muraglitazar to intact rats, dogs, monkeys, and humans

Meta	Distribution of Muraglitazar Metabolites in Plasma (% of sample) ^b											
	Rat			Dog			Monkey			Human		
	1 h	4 h	12 h ^a	1 h	4 h	12 h	1 h	4 h ^a	12 h	1 h	4 h ^a	12 h
M1	ND ^c	ND	0.05	2.0	3.0	9.0	0.3	0.3	ND	ND	trace	0.2
M2	ND	ND	0.05	ND	ND	ND	ND	0.1	ND	ND	trace	NA
M5	ND	ND	trace ^d	1.0	1.0	15.0	1.5	2.9	2.7	ND	0.3	1.0
M6	ND	ND	trace	ND	ND	ND	ND	trace	ND	ND	ND	ND
M8	ND	ND	trace	ND	ND	ND	1.0	1.5	1.0	ND	trace	ND
M8a	ND	ND	trace	ND	ND	ND	0.9	1.3	1.0	ND	trace	ND
M10	ND	ND	0.2	1.0	ND	9.0	0.9	1.6	0.4	ND	1.0	1.0
M11	ND	ND	trace	trace	ND	ND	8.0	16.2	21.4	ND	0.3	ND
M12	ND	ND	trace	ND	ND	ND	ND	trace	0.5	ND	0.2	ND
M13	ND	ND	0.05	trace	ND	ND	0.1	0.2	0.1	2.5	4.2	2.5
M15	2.3	2.1	3.5	1.0	3.0	9.0	0.1	0.3	ND	0.2	0.5	0.4
M18	ND	ND	trace	ND	ND	ND	ND	3.1	4.0	ND	0.3	ND
M20	ND	ND	0.4	ND	3.0	ND	0.1	0.2	0.4	ND	ND	ND
P	95.9	95.3	92.2	90.5	81.3	33.0	85.1	67.6	65.9	97.1	92.2	94.0
Other ^e	6.8	10.5	1.6	2.0	6.7	21.0	5.5	1.1	0.6	1.3	0.6	0.2
Total	98.2	97.4	96.6	97.5	98.0	96.0	98.1	92.9	97.1	99.8	99.0	98.1

Note: Metabolites are assigned based on retention times of radioactive peaks and LC/MS analysis.

^a LC/MS analysis was performed on this plasma sample.

^b Radioactive peaks are reported as a percentage of the total radioactivity eluted from the column. Column recoveries were >98%.

^c ND = not detected. Criteria are no discernible radioactive peak, <0.05% radioactivity and not detected by LC/MS.

DMD012450

^d Trace = <0.05% radioactivity and detected by LC/MS.

^e Other = not identified peaks.

DMD012450

Table 6: Relative distribution (percent of dose) of radioactive metabolites in pooled urine and feces after an oral dose of [¹⁴C]muraglitazar to intact rats, dogs, monkeys, and humans

Metab ^a	Metabolite Distribution (% of dose) in Urine and Feces ^b							
	Rat		Dog		Monkey		Human	
	Urine	Feces	Urine	Feces	Urine	Feces	Urine	Feces
M1	0.22	1.75	0.44	0.89	1.04	2.81	0.06	0.80
M2	0.05	9.65	ND	ND	0.08	1.29	ND	0.56
M3	trace ^d	ND	ND	ND	ND	2.41	ND	1.36
M4	ND ^e	ND	ND	0.34	ND	0.16	ND	2.83
M5	0.01	2.63	ND	0.38	0.26	1.61	0.16	11.45
M6	0.01	ND	ND	ND	0.01	3.22	ND	1.48
M7	ND	0.43	ND	0.18	ND	0.32	ND	2.21
M8	ND	ND	ND	0.89	0.13	6.43	ND	0.56
M8a	trace	4.39	ND	0.18	0.12	ND	0.03	0.56
M9	ND	2.63	ND	0.89	ND	0.56	ND	2.46
M10	0.03	0.88	ND	1.10	0.01	7.24	0.03	8.20
M11	trace	0.88	ND	1.78	0.59	25.73	0.07	5.92
M12	trace	ND	ND	0.10	0.04	1.61	ND	0.13
M13	trace	0.88	ND	ND	0.02	0.80	1.00	0.06
M14	trace	0.88	ND	0.85	0.03	0.56	ND	1.60
M15	0.12	34.20	ND	24.92	0.08	7.24	ND	3.33
M16	ND	0.88	ND	0.18	ND	0.16	ND	3.21
M18	trace	ND	ND	ND	trace	ND	0.20	ND
M20	trace	ND	ND	ND	trace	ND	ND	ND
M21	0.21	0.88	0.01	ND	0.33	0.40	0.04	0.40
Parent	0.02	13.16	0.01	52.51	0.11	7.24	0.29	9.62

DMD012450

Others ^c	0.3	13.6	0.02	NA	0.79	4.02	0.58	2.40
Total	0.97	74.1	0.48	85.2	3.63	73.49	2.46	59.20
% Excretion	1.0	87.7	0.5	89.0	3.7	80.4	2.56	61.59

^a Metabolites are assigned based on retention times of radioactive peaks and LC/MS analysis.

^b Radioactive peaks are reported as a percentage of dose (% of the sample multiplied by % excretion).

^c Uncharacterized peaks.

^d Trace = <0.01% radioactivity and detected by LC/MS.

^e ND = not detected. Criteria are no discernable radioactive peak, <0.01% radioactivity and not detected by LC/MS.

DMD012450

Table 7: Relative distribution (% of sample) of radioactive metabolites in incubation of [¹⁴C]muraglitazar with hepatocytes from mice, rats, monkeys, and humans

Metab ^a	Distribution (%) of Metabolites in Hepatocyte Incubations ^b				
	Control	Mouse	Rat	Monkey	Human
M1	9.5	23.6	12	7.6	5.7
M2	ND ^f	4.8	trace	trace	trace
M3	ND	0.3	trace	ND	trace
M5	ND	3.0	trace	trace	trace
M6	ND	0.3	trace	11	1.6
M7	ND	1.2	trace	0.5	trace
M8	ND	0.8	0.3	trace	0.2
M8a	ND	0.3	trace	trace	trace
M10	1.0	11	1.3	0.8	1.9
M11	ND	5.4	2.0	7.9	4.4
M12	ND	0.8	trace	13	0.9
M13	ND	5.2	0.5	1.7	1.1
M14	ND	1.3	0.3	0.6	0.5
M15	ND	1.2	2.2	2.1	2.3
M17b	ND	trace ^e	trace	trace	trace
M18a/b ^g	ND	2.7	0.5	2.8	0.2
M20	ND	trace	trace	trace	trace
M21	ND	5.7	0.3	trace	trace
M22	3.5	1.4	7.5	1.3	4.5
Parent	83	20	69	44	73
Other ^c	0.5	6.1	1.5	1.7	0.5
Total ^d	97	95	97	95	96

^a Metabolites are assigned based on retention times of radioactive peaks and LC/MS analysis.

^b Metabolites are reported as a percentage of the total radioactivity eluted from the column.

^c Unidentified peaks.

^d Total is less than 100% because remainder of the radioactivity is distributed in small amounts (< 0.2%) in various fractions throughout the radiochromatogram.

^e <0.2% radioactivity and detectable by LC/MS.

^f Not detectable by LC/MS.

^g M18a/b contained a and b isomers in approximately the same amounts.

DMD012450

Table 8: The *in vitro* PPAR α/γ binding and transactivation activities of seven synthetic metabolites of muraglitazar (n=3)

Metabolite	PPAR α IC ₅₀ (μ M)	PPAR α EC ₅₀ (μ M) Intrinsic Activity (%)	PPAR γ IC ₅₀ (μ M)	PPAR γ EC ₅₀ (μ M) Intrinsic Activity (%)
Muraglitazar	0.188 $\pm$ 0.073	0.035 $\pm$ 0.017 IA = 120 $\pm$ 16% @ 1 μ M 139 $\pm$ 26% @ max	0.247 $\pm$ 0.008	1.41 $\pm$ 0.27 IA = 57 $\pm$ 38% @ 1 μ M 107 $\pm$ 40% @ max
M1	>15	>25	>15	>20
M5	>15 (n = 3)	18.1 $\pm$ 1.4 IA = 2 $\pm$ 1% @ 1 μ M 69 $\pm$ 3 % @ max	>15	>25
M9	>15	>25	>15	>25
M10	3.09 $\pm$ 0.85	1.94 $\pm$ 0.06 IA = 24 $\pm$ 2 % @ 1 μ M 88 $\pm$ 12 % @ max	>15	>25
M11	0.099 $\pm$ 0.008	0.260 $\pm$ 0.01 IA = 78 $\pm$ 5% @ 1 μ M 93 $\pm$ 4 % @ max	2.05 $\pm$ 0.43	19.8 $\pm$ 2.2 IA = 4 $\pm$ 1% @ 1 μ M 133 $\pm$ 5 % @ max
M15	3.74 $\pm$ 1.32	6.89 $\pm$ 0.93 IA = 6 $\pm$ 2% @ 1 μ M 78 $\pm$ 2% @ max	>15	>25
M16	>15	>25	>15	>25

IA represents intrinsic activity relative to that of GW2331 for PPAR α and rosiglitazone for PPAR γ (Devasthale et al., 2005).

Figure 1

Figure 2

Figure 3

Figure 4

Figure 5

Figure 6

Figure 7

Figure 8