IN VITRO METABOLISM OF MK-0767 [(±)-5-[(2,4-DIOXOTHIAZOLIDIN-5-YL)METHYL]-2-METHOXY-N-[[(4-TRIFLUOROMETHYL) PHENYL]METHYL]BENZAMIDE], A PEROXISOME PROLIFERATOR-ACTIVATED RECEPTOR α/γ AGONIST. I. ROLE OF CYTOCHROME P450, METHYLTRANSFERASES, FLAVIN MONOOXYGENASES, AND ESTERASES

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ABSTRACT:

The metabolism of MK-0767, (±)-5-[(2,4-dioxothiazolidin-5-yl)-methyl]-2-methoxy-N-[[(4-trifluoromethyl) phenyl]methyl]benzamide, a thiazolidinedione (TZD)-containing peroxisome proliferator-activated receptor α/γ agonist, was studied in liver microsomes and hepatocytes from humans and rat, dog, and rhesus monkey, to characterize the enzyme(s) involved in its metabolism. The major site of metabolism is the TZD ring, which underwent opening catalyzed by CYP3A4 to give the mercapto derivative, M22. Other metabolites formed in NADPH-fortified liver microsomes included the TZD-5-OH derivative (M24), also catalyzed by CYP3A4, and the O-desmethyl derivative (M28), whose formation was catalyzed by CYP2C9 and CYP2C19. Metabolite profiles from hepatocyte incubations were different from those generated with NADPH-fortified microsomal incubations. In addition to M22, M24, and M28, hepatocytes generated several S-methylated metabolites, including the methyl mercapto (M25), the methyl sulfoxide amide (M16), and the methyl sulfone amide (M20) metabolites. Addition of the methyl donor, S-adenosyl methionine, in addition to NADPH, to microsomal incubations enhanced the turnover and resulted in metabolite profiles similar to those in hepatocyte incubations. Collectively, these results indicated that methyltransferases played a major role in the metabolism of MK-0767. Using enzyme-specific inhibitors, it was concluded that microsomal thiol methyltransferases play a more important role than the cytosolic thiopurine methyltransferase. Baculovirus-expressed human flavin-containing monooxygenase 3, as well as CYP3A4, oxidized M25 to M16, whereas further oxidation of M16 to M20 was catalyzed mainly by CYP3A4. Esterases were involved in the formation of the methyl sulfone carboxylic acids, minor metabolites detected in hepatocytes.

MK-0767, (±)-5-[(2,4-dioxothiazolidin-5-yl)methyl]-2-methoxy-N-[[4-trifluoromethyl) phenyl]methyl]benzamide, is a novel thiazolidinedione (TZD) derivative (Fig. 1) that was being developed as an oral antidiabetic agent and was recently discontinued due to preclinical toxicity. MK-0767 has been shown to exert potent hypoglycemic and hypolipidemic actions in obese Zucker fatty rats by virtue of its potent binding to the peroxisome proliferator-activated receptors (PPARs) α and γ (Murakami et al., 1998). PPARs are members of the nuclear receptor family and have recently been proposed to play a key role in lipid and carbohydrate homeostasis. Three PPAR isoforms have been identified: PPARα, PPARγ, and PPARδ. PPARα is expressed in several tissues that have a high lipid catabolism activity, such as the liver, and has been shown to be activated by compounds such as fibrates, resulting in oxidation and stimulation of the uptake of fatty acids and synthesis of lipoproteins. PPARγ is expressed abundantly in adipose tissues and has been shown to be activated by TZD

derivatives, resulting in the stimulation of lipolysis of circulating triglycerides and subsequent uptake of fatty acids into the adipose cell. PPARδ is expressed ubiquitously in many tissues, but the highest expression is in the gut, kidney, and heart. However, it currently lacks connection to any important clinical manifestation (Kersten et al., 2000).

The objective of this study was to delineate the in vitro metabolic pathways and identify the enzymes involved in the metabolism of MK-0767. A combination of different approaches was used, namely, enzyme-specific chemical inhibitors and monoclonal antibodies, as well as immunoinhibition and metabolism by recombinant enzymes.

Materials and Methods

Chemicals and Reagents. MK-0767 and synthetic standards of its major metabolites (Fig. 1), the mercapto derivative, M22, the methyl mercapto, M25, the methyl sulfoxide amide M16, the methyl sulfone amide, M20, the O-desmethyl metabolite, M28, and the methyl sulfoxide carboxylic acids, M5 and M9, were synthesized at Kyorin Pharmaceutical Co., Ltd. (Tokyo, Japan). [TZD-5-^{14}C]MK-0767 (Fig. 1) was synthesized by the Labeled Compound Synthesis Group at Merck Research Laboratories (Rahway, NJ). [^{3}H]SAM

ABBREVIATIONS: MK-0767, (±)-5-[(2,4-dioxothiazolidin-5-yl)methyl]-2-methoxy-N-[[(4-trifluoromethyl) phenyl]methyl]benzamide; PPAR, peroxisome proliferator-activated receptor; TZD, thiazolidinedione; BNPP, bis-p-nitrophenylphosphate; P450, cytochrome P-450; TMT, thiol methyltransferase; TPMT, thiopurine methyltransferase; FMO, flavin monooxygenase; SAM, S-adenosyl methionine; SAH, S-adenosyl homocysteine; DCMB, 2,3-dichloro-α-methylbenzylamine.
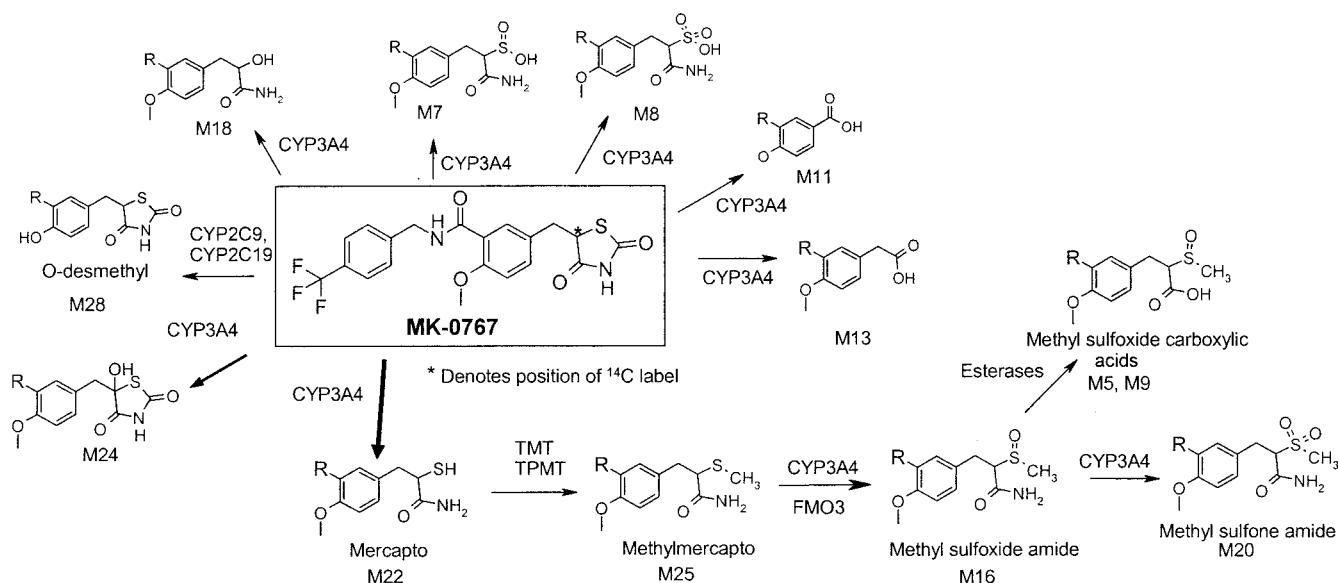
FIG. 1. Biotransformation pathways for MK-0767.

(S-adenosyl-L-methionine-methyl-^{3}H), SAM, S-adenosyl homocysteine (SAH), bis-p-nitrophenylphosphate (BNPP), paraoxon, anisic acid, and 2,3-dichloro-α-methylbenzylamine (DCMB) were obtained from Sigma-Aldrich (St. Louis, MO). All other chemicals and reagents were obtained from commercial sources and were of analytical grade.

Liver Preparations, Recombinant and Purified Enzymes, and Antibodies. Human liver microsomal preparations were prepared from six individual livers by differential centrifugation (Raucy and Lasker, 1991). Aliquots from each preparation were pooled on the basis of equivalent protein concentrations. Rat, dog, and rhesus monkey liver microsomes were prepared from fresh or frozen control tissue. Microsomes containing recombinant P450 isozymes, with coexpressed P450 reductase, were obtained from Dr. Tom Rushmore (Merck Research Laboratories, West Point, PA). Microsomes containing baculovirus-expressed human recombinant FMO3 were obtained from BD Gentest (Woburn, MA). Human and rat hepatocytes were isolated, based on a two-step perfusion procedure (Pang et al., 1997), and exhibited a viability greater than 80%, as determined by the trypan blue exclusion test. The cells were suspended in Krebs-bicarbonate buffer. Frozen human, rat, and dog liver cytosol and S9 preparations were purchased from In Vitro Technologies (Baltimore, MD). Ascites fluid containing monoclonal antibodies to P450 isoforms were obtained from Dr. T. Rushmore (Merck Research Laboratories, West Point, PA). Porcine and rabbit carboxy esterases were purchased from Sigma-Aldrich.

Liver Microsomal Incubations. [^{14}C]MK-0767 (1–5 μM) was incubated with human, rat, dog, and rhesus liver microsomes at a protein concentration of 1 to 2 mg/ml in 0.1 M potassium phosphate buffer pH 7.4, containing 1 mM EDTA and 5 mM MgCl$_2$. Incubations were initiated by the addition of an NADPH-regenerating system, consisting of 10 mM glucose 6-phosphate, 10 mM NADP, and 1.4 units/ml of glucose-6-phosphate dehydrogenase. The reactions were carried out for 30 to 60 min at 37°C in a shaking water bath. [^{14}C]MK-0767 was added as an acetonitrile solution, the final solvent concentration not exceeding 2%. To generate methylated metabolites, microsomal incubations were fortified with a 100 μM concentration of the methyl donor, SAM (solutions of 10 mM prepared in water), before addition of the NADPH-regenerating system. The microsomal metabolism of some of the intermediate metabolites of MK-0767 was also studied. The mercapto metabolite, M22, was incubated at a concentration of 10 μM in the presence of 100 μM [^{3}H]SAM and in the presence or absence of an NADPH-regenerating system at 37°C for 30 min. The methylmercapto metabolite, M25, and the methyl sulfoxide amide, M16, were incubated at 10 μM in the presence of an NADPH-regenerating system at 37°C for 30 min. Typically, the total volume of the incubations was 200 μl and reactions were quenched with 50 μl of acetonitrile.

Hepatocyte Incubations with [^{14}C]MK-0767. Rat, dog, and human hepatocytes (1–2 million cells/ml) were incubated with 1–5 μM [^{14}C]MK-0767.

Reactions were carried out for 30 to 60 min at 37°C. Typically, the incubation volumes were 500 μl and the reactions were terminated with 100 μl of acetonitrile.

Incubations of [^{14}C]MK-0767 and M22 with Human Liver S9 Fraction. The compounds were incubated with human liver S9 fraction (2–5 mg of protein/ml) in a total volume of 200 μl, for 60 min. Reactions were carried out in the presence and absence of 1 mM NADPH plus or minus 100 μM SAM. Typically, the total volume of the incubations was 200 μl and reactions were quenched with 50 μl of acetonitrile.

Incubations of MK-0767 and M22 with Rat, Dog, and Human Liver Cytosol. The compounds (1–10 μM) were incubated with 2 to 5 mg/ml cytosolic protein in a total volume of 0.5 ml in the presence of [^{3}H]SAM for 60 min. Typically, the total volume of the incubations was 200 μl and reactions were quenched with 50 μl of acetonitrile.

Metabolism of MK-0767, M25, and M16 by Heterologously Expressed P450 Isoforms and FMO3. [^{14}C]MK-0767 (5 μM) was incubated with microsomes from cells containing recombinant P450 isoforms (80 pmol/ml CYP3A4, CYP2C8, CYP2C9, CYP2C19, CYP2D6, CYP2E1, and CYP1A2) in the presence of an NADPH-regenerating system at 37°C for 60 min. Both M25 and M16 (10 μM) were incubated with recombinant CYP3A4 microsomes for 60 min. MK-0767, M25, and M16 (10 μM) were incubated with microsomes (1 mg/ml) containing baculovirus-expressed human FMO3 for 60 min in the presence of an NADPH-regenerating system. Typically, the total volume of the incubations was 200 μl and reactions were quenched with 50 μl of acetonitrile.

Effect of Inhibitory Monoclonal Antibodies on Metabolism of MK-0767, M25, and M16. To determine the contribution of specific P450 isozymes to the metabolism of MK-0767, human liver microsomes (2 mg/ml) were preincubated with anti-CYP3A4, CYP2C8, CYP2C9, or CYP2C19 (0–25 μl/mg microsomal protein) monoclonal antibody-containing ascites fluid at room temperature for 10 min. Reactions were initiated with the addition of 5 μM [^{14}C]MK-0767 and an NADPH-regenerating system, and were carried out in the absence or presence of SAM. The effect of CYP3A4 monoclonal antibody on the metabolism of M25 and M16 in human liver microsomes (1 mg/ml) was determined in a similar manner. Reactions were initiated by the addition of 10 μM substrate and incubations were carried out for 30 min at 37°C. Typically, the total volume of the incubations was 200 μl and reactions were quenched with 50 μl of acetonitrile.

Chemical Inhibition Studies. To establish the role of enzymes other than cytochrome P450 and FMOs in the metabolism of MK-0767, specific enzyme inhibitors were used. The effect of the following inhibitors was studied in microsomal incubations of MK-0767 and M22: ketoconazole (CYP3A4), DCMB [microsomal thiol methyltransferase (TMT)], and SAH (microsomal and cytosolic methyltransferase). Solutions of these compounds were prepared

in acetonitrile, water, and 0.1 M potassium phosphate, pH 7.4, respectively. Final concentrations in the incubations ranged from 0.1 to 1000 μM. The incubations contained 2 mg/ml of microsomal protein in 0.1 M potassium phosphate, pH 7.4, with 1 mM EDTA, 5 mM MgCl$_2$, 100 μM SAM, and 5 μM [^{14}C]MK-0767 or 10 μM M22. Reactions were initiated with an NADPH-regenerating system. Also, the effect of M22 and ketoconazole on the metabolism of MK-0767 was studied in the absence of SAM under similar conditions.

The effect of the following inhibitors was studied in incubations of MK-0767 and M22 with rat and human hepatocyte suspensions: BNPP and paraoxon (esterase), anisic acid (cytosolic methyltransferase), and SAH (microsomal and cytosolic methyltransferase). Solutions of BNPP, paraoxon, and anisic acid were prepared in methanol, and SAH was dissolved in 0.1 M potassium phosphate, pH 7.4. Final concentrations of the inhibitors ranged from 1 to 1000 μM. The incubations consisted of 1 to 2 million hepatocyte cells/ml and substrate concentrations ranging from 1 to 5 μM.

The effect of the esterase inhibitors, BNPP and paraoxon, on testosterone 6β-hydroxylation (a CYP3A4-mediated reaction) was studied in human liver microsomal incubations. Testosterone (100 μM) was incubated with 0.5 mg/ml microsomal protein in the presence of BNPP and paraoxon at concentrations ranging from 0.1 to 1 mM. Incubations were carried out in the presence of an NADPH-regenerating system for 20 min at 37°C in a shaking water bath. To study the inhibitory effect of M22, the compound was incubated at concentrations ranging from 0.01 to 100 μM with 2 μM MK-0767 or 50 μM testosterone in the presence of an NADPH-regenerating system. Reactions were terminated with 5 volumes of acetonitrile.

Incubations of M16 and M25 with Heat-Treated Human Liver Microsomes. Microsomes were incubated first with 50 mM sodium pyrophosphate, pH 9, at 55°C for 55 s in the absence of NADPH, and then cooled over ice. Reactions were initiated by the addition of the substrates, M25 or M16, and an NADPH-regenerating system.

Data Analysis. The IC$_{50}$ values were calculated by nonlinear regression analysis using KaleidaGraph software (Synergy Software, Reading, PA).

Analytical Methods. *HPLC.* In vitro incubations of MK-0767 quenched with acetonitrile were centrifuged at 14,000 rpm at 4°C to precipitate proteins. The acetonitrile supernatants were analyzed on a Zorbax XDB-C8 column (3 × 150 mm, 3.5 μm; MAC-MOD Analytical, Chadds Ford, PA) at ambient temperature. The column was eluted at a flow rate of 0.5 ml/min with a 30-min linear gradient from 73% A (10 mM ammonium acetate) to 50% B (93% acetonitrile and 7% methanol in 1 mM ammonium acetate). The HPLC system included a Shimadzu 10A system controller, two LC-10 AD pumps, an SIL 10A automatic sample, an SPD 10A UV-visible spectrophotometric detector, and an on-line radiometric detector (PerkinElmer Life and Analytical Sciences, Boston, MA). The wavelength for UV detection was 220 nm.

Mass Spectrometry. LC-MS analyses were conducted on a PE Sciex API 3000 mass spectrometer (PerkinElmerSciex Instruments, Boston, MA) using the Turbo-Ionspray interface operated in the positive ion mode, which was interfaced with a PerkinElmer HPLC system. The temperature of the auxiliary gas was 350°C and the ionization voltage was 4500 V. The orifice and ring voltages were set to 48 V and 240 V, respectively. Tandem mass spectrometry experiments were performed using a collision energy of 42 eV (laboratory frame) and a collision cell gas pressure of 4 (N$_2$). A Zorbax XDB-C8 column (3.0 × 150 mm, 3.5 μm) was used for chromatographic separation with a gradient of 10 mM ammonium acetate in water (A) and 7 mM ammonium acetate in acetonitrile with 7% methanol (B) for elution at a flow rate of 0.5 ml/min. The gradient was started at 26% B, and increased linearly to 30% B in 5 min, followed by a hold at 30% B for 7 min. It was then increased linearly to 55% B in 20 min, followed by a linear ramp to 90% B in 1 min. Accurate mass measurements were conducted on a Micromass Q-ToF II mass spectrometer (Waters, Milford, MA) operated in the positive electrospray ionization mode. The desolvation gas temperature was 80°C and the capillary voltage was 3000 V. The cone voltage and collision energy were 48 V and 10 V, respectively.

Results

Metabolism of [^{14}C]MK-0767 in NADPH-Enriched Liver Microsomes. Incubations of MK-0767 carried out in the presence of an NADPH-regenerating system yielded qualitatively similar metabolite
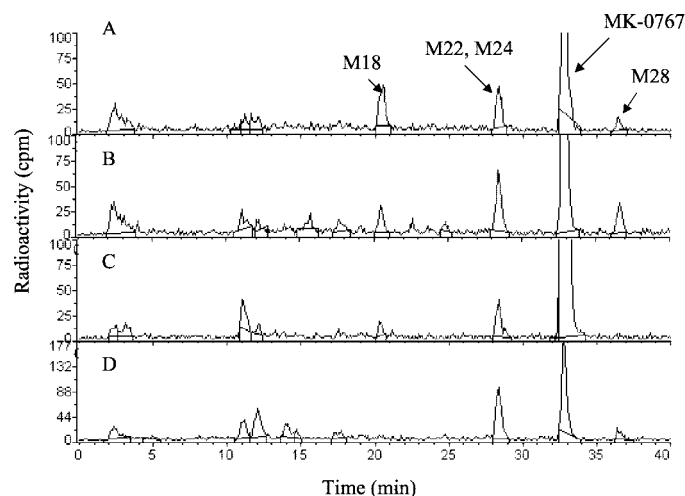

FIG. 2. HPLC radiochromatograms of MK-0767 incubations with human (A), rhesus monkey (B), dog (C), and rat (D) liver microsomes in the presence of an NADPH-regenerating system.

profiles in human, rhesus monkey, dog, and rat liver microsomes (Fig. 2). Two of the metabolites formed in these incubations, the mercapto (M22) and the *O*-desmethyl derivative (M28), were identified by comparison of their HPLC retention times and product ion spectra with those of synthetic standards, as described elsewhere (Liu et al., 2004). Two other major metabolites formed in NADPH-enriched microsomes were M24, the hydroxylated TZD derivative of MK-0767, and M18, a hydroxy amide. The identification of M24, which was achieved by quadrupole time-of-flight mass spectral and NMR analysis, M18, and several minor metabolites, including M7, M11, and M13 (Fig. 2) are described elsewhere (Liu et al., 2004). Formation of these metabolites was NADPH-dependent.

Metabolism of [^{14}C]MK-0767 in Liver Microsomes in the Presence of SAM. Results from microsomal incubations of MK-0767 in the presence of an NADPH-regenerating system and the methyl donor, SAM, are shown in Fig. 3. Metabolite profiles were similar between species, and a severalfold increase in the metabolism of MK-0767 was noted upon addition of SAM. Also, in addition to the metabolites observed in incubations with NADPH alone, several other metabolites were formed, all of which were *S*-methylated. These metabolites were the methylated forms of the mercapto derivative, M25, the methyl sulfoxide amide, M16, the methyl sulfone amide, M20, and the isomeric carboxylic acids, M5 and M9. Identification of these metabolites was based on comparison of their HPLC retention times and product ion spectra with those of authentic standards (Liu et al., 2004). Incubation of MK-0767 with microsomes in the absence of NADPH did not result in metabolism, irrespective of the presence or absence of SAM.

Metabolism of M22, M25, and M16 in Human Liver Microsomes. To identify the various steps involved in the biotransformation of MK-0767, the microsomal metabolism of the intermediate metabolites, M22 and M16, was studied. Incubation of the mercapto metabolite, M22, in microsomes in the presence of [^{3}H]SAM alone (no NADPH) resulted in the formation of the radiolabeled methylmercapto product, M25. In the presence of both NADPH (as the regenerating system) and SAM, M22 was metabolized to the oxidative metabolites, M16 and M20 as well as M25 (Fig. 4). Also, incubations starting with M25 as a substrate gave rise to the *S*-oxidation products, M16 and M20 (data not shown).

Hepatocyte Incubations of [^{14}C]MK-0767. Metabolite profiles of MK-0767 in rat, dog, rhesus monkey, and human hepatocytes were
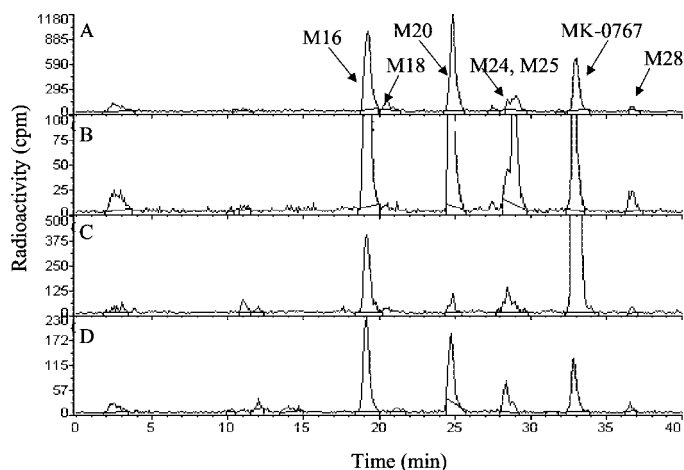
FIG. 3. HPLC radiochromatograms of MK-0767 incubations with human (A), rhesus monkey (B), dog (C), and rat (D) liver microsomes in the presence of an NADPH-regenerating system and SAM.

FIG. 4. HPLC radiochromatograms of M22 incubations with human liver microsomes in the presence of [³H]SAM (A), and [³H]SAM and NADPH-regenerating system (B).

similar across species and resembled profiles obtained from microsomal incubations carried out in the presence of NADPH and SAM. Metabolism in human and dog hepatocyte preparations was less extensive than in other species. Major metabolites included M25, M16, M20, M9, and M5 (data not shown). Also, no phase II metabolites, such as glucuronide or sulfate conjugates, were detected.

Metabolism of [¹⁴C]MK-0767 with Human Liver S9 Fractions. Incubation of MK-0767 with previously frozen human liver S9 yielded metabolite profiles similar to those of human liver microsomal incubations carried out under similar cofactor conditions (data not shown). The major metabolites formed in the presence of NADPH only were the mercapto metabolite, M22, the hydroxyl TZD metabolite, M24, and the *O*-desmethyl derivative, M28. No phase II metabolites, such as glucuronide or sulfate conjugates, were detected. Additional metabolites formed in the presence of both NADPH and SAM were the methylmercapto metabolite, M25, the methyl sulfone amide, M20, and the carboxylic acids, M9 and M5. As in microsomal incubations, MK-0767 was stable in incubations with human liver S9 carried out in the absence of NADPH and SAM.

Incubations of [¹⁴C]MK-0767 and M22 with Rat, Dog, and Human Liver Cytosol. To determine whether cytosolic methyltransferases could mediate the methylation of MK-0767 and its mercapto metabolite, M22, incubations of these two compounds were carried out in rat, dog, and human liver cytosol in the presence of [³H]SAM. HPLC analysis of incubation extracts showed that there was no detectable metabolism of MK-0767. The mercapto metabolite, on the other hand, gave rise to a radioactive peak that coeluted with the methyl mercapto derivative, M25 (Fig. 5).

Metabolism of [¹⁴C]MK-0767, M25, and M16 by Heterologously Expressed P450 Isoforms. MK-0767 was incubated with microsomes containing recombinant CYP3A4, 2C8, 2C9, 2C19, 2D6, 2E1, and 1A2. CYP3A4-containing microsomes exhibited the highest metabolizing activity for MK-0767 (~60% metabolism at 5 μM). The major metabolites formed in CYP3A4 incubations were the mercapto metabolite, M22, the hydroxylated TZD derivative, M24, and the hydroxy amide, M18. The CYP3A4 metabolite profile was similar to the human microsomal profile with one exception: the *O*-desmethyl derivative, a minor metabolite of MK-0767, was not formed in CYP3A4 incubations but was formed in recombinant CYP2C9 and CYP2C19 microsomal incubations. These incubations did not produce any other metabolites except the *O*-desmethyl derivative (data not shown). Incubations with CYP2C8 microsomes generated a me-
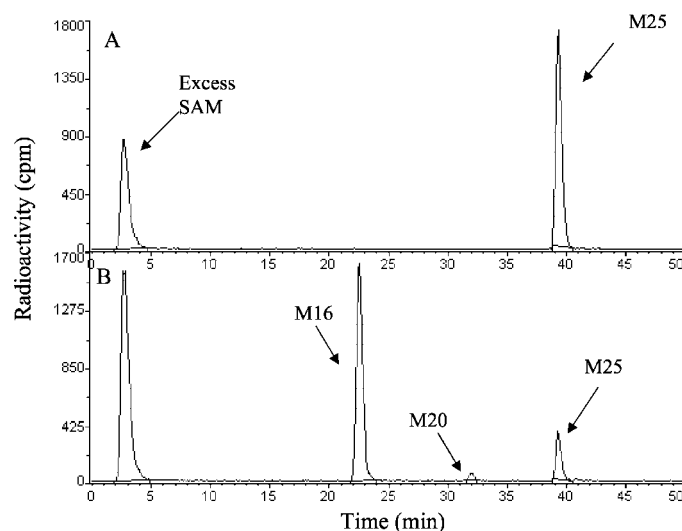
tabolite profile that was similar to that of CYP3A4; however, the turnover of the parent compound was less than 5% (data not shown). There was no detectable metabolism in incubations with CYP2D6, 2E1, and 1A2.

Incubation of the methyl mercapto metabolite, M25, with recombinant CYP3A4 microsomes yielded M16 and M20. Incubation of M16 with CYP3A4 microsomes gave rise to M20 (data not shown).

Effect of Inhibitory Monoclonal Antibodies on Microsomal Metabolism of MK-0767. To identify the P450 isozymes involved in metabolism of MK-0767, human liver microsomes were preincubated with inhibitory monoclonal antibodies raised against the major P450s in human liver microsomes, CYP3A4, 2C8, 2C9, 2C19, and 2D6. As shown in Fig. 6, CYP3A4 monoclonal antibody inhibited, in a concentration-dependent manner, the formation of most of the metabolites in NADPH-enriched incubations, irrespective of the presence of the methyl donor, SAM. At a concentration of 25 μl per mg of microsomal protein, there was a 98% inhibition of metabolism. The only metabolite whose formation was not inhibited completely by CYP3A4 monoclonal antibody was M28, the *O*-desmethyl derivative. Formation of M28 was inhibited by monoclonal antibodies raised against CYP2C9 and 2C19 (data not shown).

Effect of Monoclonal Antibodies on Metabolism of M25 and M16. To identify the enzymes involved in the metabolism of intermediate metabolites of MK-0767, the metabolism of the methylmercapto metabolite, M25, in human liver microsomes was studied in the presence of monoclonal antibody against CYP3A4. HPLC analysis of control incubations with ascites fluid showed that M25 was metabolized to the *S*-oxidation products, M16 and M20. In the presence of 1 μl of CYP3A4 antibody, formation of M16 and M20 was inhibited by 60% and 100%, respectively. There was no further inhibition in the formation of M16 by increasing amounts (5 and 10 μl) of antibody (Fig. 7), indicating possible involvement of an additional enzyme in this reaction step.

Chemical Inhibition Studies in Microsomes. Ketoconazole inhibited the NADPH- and SAM-dependent metabolism of MK-0767 in human liver microsomes, in a concentration-dependent manner (Fig. 8A). The IC_{50} value, 62 nM, was similar to the values reported in the literature for inhibition of CYP3A4-catalyzed reactions (Sheets et al., 1986; Maurice et al., 1992). DCMB, a specific inhibitor of microsomal methyltransferase (Glauser et al., 1993b; Lill et al., 1996;
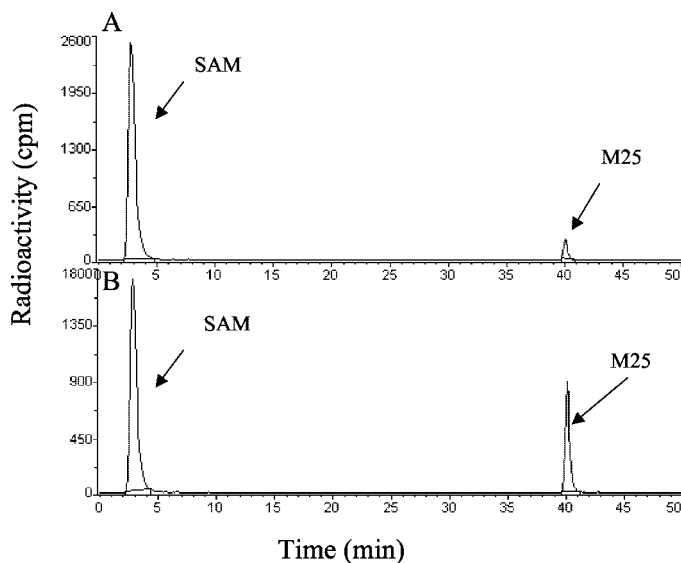
FIG. 5. HPLC radiochromatograms of M22 incubations with human (A) and rat (B) liver cytosol in the presence of [³H]SAM.
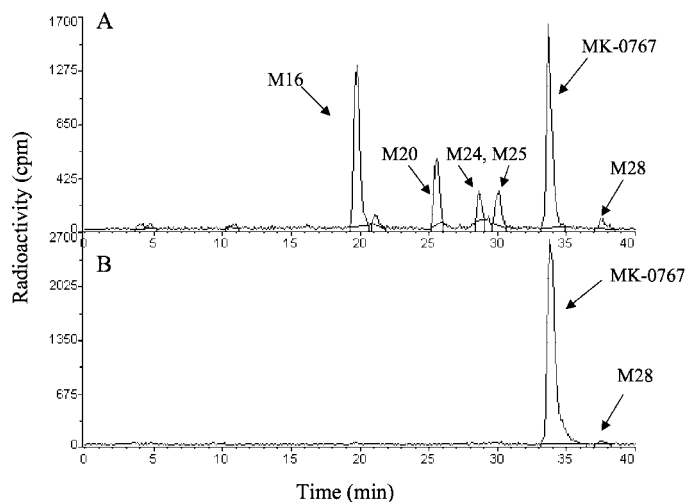

FIG. 6. HPLC radiochromatograms of MK-0767 incubations with NADPH- and SAM-fortified human liver microsomes (A), and in the presence of anti-CYP3A4 monoclonal antibody (B).

Lee and Kim, 1999) also inhibited MK-0767 metabolism in a concentration-dependent manner (Fig. 8B). However, the extent of inhibition was not as high as that obtained with ketoconazole. The maximum inhibition was 65% at a concentration of 100 to 200 μM DCMB. SAH, an endogenous inhibitor of SAM-dependent methylation by both microsomal and cytosolic methyltransferases (Lee et al., 1999), caused less inhibition, 50% at 2 mM (Fig. 8C).

Figure 9 shows the results from experiments carried out to determine the P450-inhibitory potential of the mercapto metabolite, M22. These data indicated that M22 is a potent inhibitor of MK-0767 metabolism in human liver microsomes, with an IC$_{50}$ value of 2 μM. M22 also inhibited the CYP3A4-catalyzed testosterone 6β-hydroxylation in a different preparation of microsomes with an IC$_{50}$ value of 12 μM (data not shown).

Chemical Inhibition Studies in Hepatocytes. Since the turnover of MK-0767 was low in human hepatocyte suspensions, the inhibitor studies were done in rat hepatocyte suspensions, which gave qualitatively similar metabolite profiles. Table 1 shows the effect of these inhibitors on the formation of individual metabolites. The effect of ketoconazole was similar to that observed in microsomal incubations. Inhibition of total metabolism ranged from ~63% at 1 μM to 91% at 10 μM. The microsomal methyltransferase inhibitor, DCMB, also had a substantial effect on the metabolism of MK-0767 (67% at 1 mM). Anisic acid, an inhibitor of cytosolic methyltransferase (Woodson and Weinshilboum, 1983), tested at concentrations ranging from 100 to 1000 μM, had no profound effect on the formation of the two methylated metabolites, M16 and M20, or on the metabolism of the parent compound. Also, as observed for microsomal metabolism, there was a minimal effect of SAH on the metabolism of MK-0767 in hepatocyte suspensions. These data suggested that the cytosolic methyltransferases may not play a significant role in the metabolism of MK-0767.

The effects of BNPP and paraoxon, two known inhibitors of esterases (McCracken et al., 1993), on the metabolism of MK-0767 in rat hepatocytes are shown in Table 1. At a concentration of 100 μM, both BNPP and paraoxon inhibited only the formation of the two carboxylic acids, M9 and M5. The formation of the other metabolites was inhibited at higher concentrations (500 and 1000 μM). However, at these higher concentrations, both BNPP and paraoxon also inhibited

the CYP3A4-mediated reaction, as monitored by testosterone 6β-hydroxylation, with IC$_{50}$ values of 171 and 356 μM, respectively (data not shown).

Incubations of M25 and M16 with Heat-Treated Human Liver Microsomes. To determine the contribution of human liver FMOs in the metabolism of M25 and M16, microsomes suspended in potassium phosphate buffer, pH 7.4 (optimum for P450 reactions), or sodium pyrophosphate, pH 9 (optimum for FMO reactions), were first heated at 55°C for 55 s in the absence of NADPH, to denature the FMOs. Reactions were then initiated with the substrates and an NADPH-regenerating system. Comparison of HPLC profiles of M25 (methyl-mercapto metabolite) incubations in phosphate buffer (pH 7.4) with heat-treated and untreated microsomes showed that the formation of M16 was reduced ~17% by the heat treatment (data not shown). At pH 9, formation of M16 from M25 was considerably lower than that at pH 7.4 (34 versus 73%). However, heat treatment had a greater effect at pH 9 than at pH 7.4, causing 80% inhibition of formation of M16. There was some formation of M20 in the pH 7.4 incubations, but no detectable levels were observed in incubations carried out at pH 9, consistent with conclusions from other experiments that M20 is primarily a CYP3A4 product.

Incubations of MK-0767, M25, and M16 with Recombinant FMO3 Microsomes. The ability of human FMO3 to catalyze the
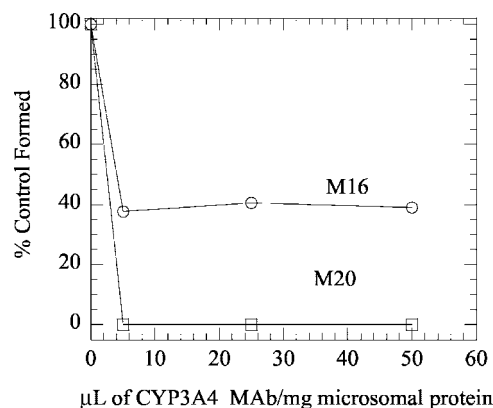

FIG. 7. Effect of anti-CYP3A4 monoclonal antibody on the formation of the methyl sulfoxide (M16) and methyl sulfone (M20) derivatives from methyl mercapto (M25) in human liver microsomes.
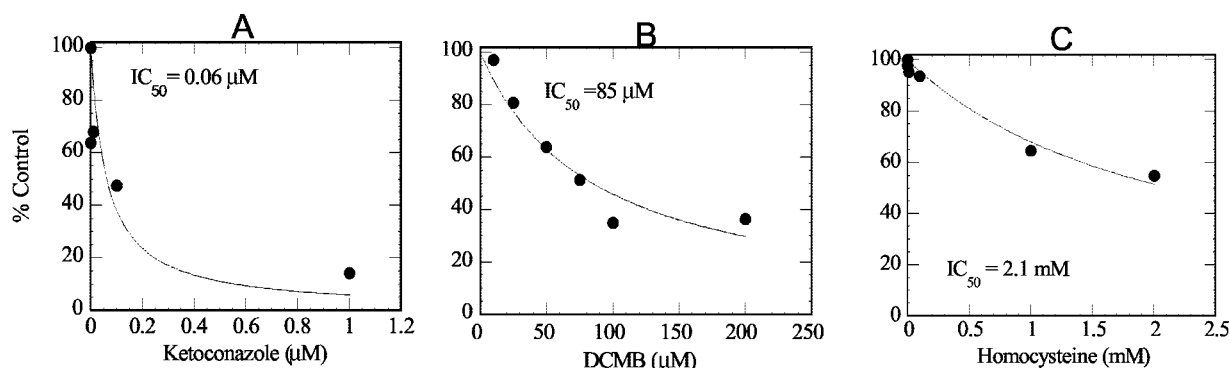
FIG. 8. Inhibition of [^{14}C]MK-0767 metabolism in NADPH- and SAM-fortified human liver microsomes by ketoconazole (A), DCMB (B), and SAH (C).

oxidation of the sulfur group in MK-0767 and its metabolites, M25 and M16, was determined by carrying out incubations of these compounds with microsomes containing recombinant FMO3, in the presence of an NADPH-regenerating system. HPLC analysis of the products of these incubations indicated that the parent compound, MK-0767, did not undergo any metabolism by FMO3. On the other hand, the methylmercapto metabolite, M25, was converted to the methyl sulfoxide amide, M16, in these incubations. There was no formation of M20 when either M25 or M16 was used as the substrate (data not shown).

Discussion

MK-0767, a thiazolidinedione-containing PPAR dual α/γ agonist, was being developed for the control of diabetes and hyperlipidemia in humans. The present study was undertaken to identify the enzymes involved in the in vitro metabolism of MK-0767 and to elucidate its biotransformation pathways. Results from the present experiments demonstrated that the in vitro metabolism of MK-0767 was mediated primarily by CYP3A4, with the microsomal methyltransferases playing a major role in its secondary metabolism. Additionally, there was evidence for the involvement of FMOs and esterases in the metabolism of the intermediate metabolites of MK-0767.

In vitro metabolism studies showed that [^{14}C]MK-0767 was metabolized to several metabolites in liver microsomes from rat, dog, monkey, and human and yielded qualitatively similar metabolite profiles in all these species. With the exception of the O-desmethyl derivative (M28), all other metabolites formed in NADPH-fortified human liver microsomes involved CYP3A4-catalyzed opening of the TZD ring. Identification of CYP3A4 as the enzyme catalyzing TZD ring opening was based on the finding that significant inhibition of the formation of all metabolites, except M28, occurred in human liver microsomal incubations in the presence of CYP3A4 monoclonal antibodies. Also, incubations of MK-0767 with recombinant P450 microsomes indicated that of all the microsomes tested, only CYP3A4 microsomes generated the major metabolites, M18, M22, and M24. Moreover, ketoconazole, a known CYP3A4 inhibitor, significantly inhibited MK-0767 metabolism in human liver microsomes with an IC$_{50}$ value of 62 nM. The O-desmethyl metabolite, M28, appeared to be the only metabolite not formed by CYP3A4. Its formation was inhibited by antibodies to CYP2C9 and CYP2C19 and was the only metabolite generated in incubations of MK-0767 with recombinant CYP2C9 and CYP2C19 microsomes.

Metabolite profiles of [^{14}C]MK-0767 in hepatocyte suspensions, though similar across species (rat, dog, rhesus, and human), were distinctly different from the profiles in liver microsomal incubations conducted in the presence of NADPH. The main difference was that the major metabolites formed in hepatocyte incubations were meth-

ylated at the sulfur atom of the TZD ring, indicating an involvement of methyltransferases in the metabolism of MK-0767. The major metabolites formed in hepatocyte incubations were the methylated form of the mercapto derivative, M25, the methyl sulfoxide amide, M16, the methyl sulfone amide, M20, and the isomeric carboxylic acids, M9 and M5. Smaller amounts of the nonmethylated metabolites, M22 (mercapto), M24 (5-hydroxy TZD) and M28 (O-desmethyl) also were detected in these incubations. When microsomal incubations of MK-0767 were conducted in the presence of a methyl donor such as SAM plus an NADPH-regenerating system, an activation in the metabolism of MK-0767 was observed. This enhancement in MK-0767 metabolism is probably due to the depletion of M22, the mercapto metabolite, which was shown to be an inhibitor of CYP3A4, inhibiting the metabolism of MK-0767 (2 μM) with an IC$_{50}$ value of 2 μM. Metabolism of M22 by methylation and further oxidation in SAM- and NADPH-fortified microsomal incubations resulted in increased turnover of MK-0767 metabolism. Indeed, incubations of M22 in the presence of SAM and NADPH in liver microsomes resulted in the generation of M25, the methyl mercapto derivative, and its oxidation products, the methyl sulfoxide, M16 and the methyl sulfone, M20. It should be noted that there was no metabolism of MK-0767 in microsomal incubations or human liver S9 in the absence of NADPH, irrespective of whether SAM was present or not. Collectively, these findings suggest that in NADPH-fortified microsomal incubations, the TZD ring was the initial site of metabolism of MK-0767 giving rise to two major metabolites, the hydroxylated TZD
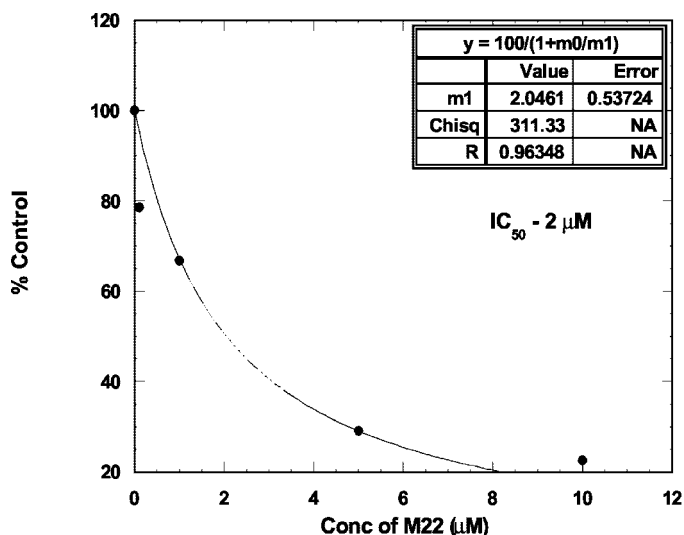

FIG. 9. Effect of M22 on the metabolism of [^{14}C]MK-0767 in NADPH-fortified human liver microsomes.

derivative, M24, and the ring-opened mercapto intermediate, M22. Both of these metabolites were formed solely by CYP3A4. In the presence of SAM, there was a shift in the metabolism, with the mercapto derivative being methylated by methyltransferases to M25 and further oxidized to the methyl sulfoxide amide, M16, and the methyl sulfone amide, M20, thereby resulting in an increase in the metabolism of MK-0767.

Thiol methylation is an important metabolic pathway for several xenobiotics with sulfhydryl groups (Bremer and Greenberg, 1961). In humans, there are at least two methyltransferases, TMT and thiopurine methyltransferase (TPMT), that are capable of catalyzing the *S*-methylation of xenobiotic compounds (Weinshilboum et al., 1978, 1979). TMT is primarily a membrane-associated enzyme and has been reported to catalyze the methylation of captopril (Keith et al., 1984; Ferroni et al., 1996), *N*-acetylcysteine and 7α-thio-spirolactone (Keith et al., 1984), and diethyldithiocarbamate (Glauser et al., 1993a). TPMT is a cytoplasmic enzyme with a substrate preference for thiopurines, thiopyrimidines, and aromatic sulfhydryl compounds (Remy, 1963; Woodson et al., 1983; Woodson and Weinshilboum, 1983). These two classes of enzymes are known to have distinct inhibitor sensitivity, and this property of the enzymes was used to determine the relative contribution of TMT (microsomal) and TPMT (cytosolic) on the metabolism of MK-0767. DCMB, a known inhibitor of TMT, caused significant inhibition of MK-0767 metabolism in human liver microsomes with an IC_{50} value of 85 μM. In rat hepatocyte suspensions, DCMB inhibited the metabolism of MK-0767 by ~67% at a concentration of 1 mM. *p*-Anisic acid, a known inhibitor of TPMT (cytosolic methyltransferase), did not have a significant effect on MK-0767 metabolism in rat hepatocyte suspensions, ~18% inhibition at 500 μM. These results indicate that, of the two methyltransferases, TMT, the microsomal enzyme, may play a more important role than TPMT, the cytosolic enzyme, in the overall metabolism of MK-0767.

Studies also were conducted to identify the enzymes involved in the metabolism of some of the intermediate metabolites of MK-0767. The metabolism of the methyl mercapto metabolite (M25) to the methyl sulfoxide amide (M16) and the methyl sulfone amide (M20) was mediated by CYP3A4. This conclusion was based on the observation that both human liver and recombinant CYP3A4 microsomal incubations of M25 generated M16 and M20. However, human liver microsomal incubations of M25, conducted in the presence of CYP3A4 monoclonal antibody, showed incomplete inhibition of the formation of M16 (~60% inhibition) but caused total inhibition of the formation of M20, indicating the possible involvement of another enzyme in the conversion of M25 to M16. Since FMOs are known to oxygenate nucleophilic sulfur heteroatom of xenobiotics (Cashman, 2000), the role of FMOs in the *S*-oxidation of M25 was investigated. Heat treatment of microsomes before incubations of M25 at pH 9 resulted in 80% inhibition of the formation of M16. At this pH, there was no formation of M20, in either control or heat-treated incubations. Also, incubations of recombinant human FMO3 with M25 generated M16, but not M20. Moreover, incubations of M16 in FMO3-containing microsomes did not generate M20. These results, taken together, indicated that the oxidation of the methylmercapto metabolite, M25, to M16 was mediated by both CYP3A4 and FMO3 enzymes, whereas further oxidation to M20 appeared to be catalyzed solely by CYP3A4.

The possible role of esterases in the TZD ring opening of MK-0767 was investigated by conducting incubations with purified porcine esterase and with human liver microsomes and S9 (previously frozen) in the absence of NADPH. Consistent with the observations and conclusions discussed above, no metabolism was observed. Moreover, BNPP and paraoxon, two known esterase inhibitors, did not inhibit MK-0767 metabolism in hepatocyte incubations. As expected, these compounds inhibited the formation of the carboxylic acids, M5 and M9. Although inhibition of some of the other metabolites was observed at higher BNPP and paraoxon concentrations, this observation was attributed to inhibition of CYP3A4. In a separate experiment, both BNPP and paraoxon were found to inhibit testosterone 6β-hydroxylation, with IC_{50} values of 171 and 356 μM, respectively. These results confirm that esterases are not involved in the opening of the TZD ring.

In conclusion, the present study shows that the major biotransformation pathway of MK-0767 involves CYP3A4-catalyzed opening of the TZD ring, followed sequentially by methylation, catalyzed by microsomal methyltransferase, and sulfur oxidation, catalyzed by CYP3A4 (major) and FMOs. These metabolite profiles are consistent with those observed in vivo following administration of MK-0767 in preclinical species and humans (S. Vincent, M. Creighton, C. Kochansky, B. Karanam, and R. Franklin, unpub-

TABLE 1

Effects of ketoconazole, BNPP, paraoxon, DCMB, anisic acid, and SAH on the metabolism of [^{14}C]MK-0767 in rat hepatocyte suspensions

Inhibitor	Concentration	Percentage Inhibition of Formation[a]				
		M5, M9	M16	M20	M24	M25
	μM					
Ketoconazole	1	75	85	100	100	100
	5	73	88	100	100	100
	10	62	85	100	100	100
BNPP	100	100	–[b]	–	–	–
	500	100	–	3	–	–
	1000	100	9	65	–	100
Paraoxon	100	100	–	–	–	–
	500	100	72	86	–	100
	1000	100	89	100	–	100
DCMB	100	23	25	26	–	–
	500	36	85	85	52	100
	1000	100	81	100	100	100
Anisic acid	100	–	3	–	–	–
	500	58	2	33	–	–
	1000	15	–	8	–	–
SAH	100	–	6	–	12	–
	1000	–	–	–	–	–
	2000	–	–	–	–	–

[a] Inhibition of metabolites was calculated relative to control incubations.
[b] –, no inhibition.

lished data). The major in vivo metabolites were similar to those observed in hepatocyte incubations and were M25, M16, and M20, all involving TZD-ring opening followed by methylation with and without oxidation. Thus, the major metabolism pathway for MK-0767 is very different from that of the other known TZD-containing PPAR agonists, troglitazone (Kawai et al., 1997), pioglitazone (Krieter et al., 1994; Maeshiba et al., 1997), and rosiglitazone (Bolton et al., 1996). TZD ring cleavage has been reported as a minor biotransformation pathway for troglitazone (Kassahun et al., 2001) and pioglitazone (Shen et al., 2003). Also, to our knowledge, *S*-methylated metabolites have not been reported for any of these TZD-containing compounds.

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