Metabolic Activation of Mefenamic Acid Leading to Mefenamyl-S-acyl-glutathione Adduct Formation in Vitro and in Vivo in Rat

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Abbreviations: MFA, mefenamic acid; MFA-SCoA, MFA-\(S\)-acyl-CoA thioester; MFA-SG, mefenamyl-\(S\)-acyl-glutathione thioester; MFA-1-\(\beta\)-O-G, mefenamyl-1-\(\beta\)-O-acyl glucuronide; I-SCoA, ibuprofen-\(S\)-acyl-CoA; I-SG, ibuprofen-\(S\)-acyl-GSH; D-SG, diclofenac-\(S\)-acyl-glutathione; CBZ, carbamazepine; CID, collision-induced dissociation; CoASH, coenzyme A; GST, glutathione \(S\)-transferase; NSAID, nonsteroidal antiinflammatory drug; LC-MS/MS, liquid chromatography-tandem mass spectrometry; MRM, multiple reaction monitoring.
Abstract: Carboxylic acid-containing nonsteroidal antiinflammatory drugs (NSAIDs) can be metabolized to chemically-reactive acyl glucuronide and/or S-acyl-CoA thioester metabolites capable of transacylating glutathione (GSH). We investigated the metabolism of the NSAID mefenamic acid (MFA) to metabolites that transacylate GSH leading to MFA-S-acyl-GSH thioester (MFA-SG) formation in incubations with rat and human hepatocytes and in vivo in rat bile. Thus, incubation of MFA (1-500 \( \mu M \)) with rat hepatocytes led to the detection of MFA-\( 1-\beta-O \)-acyl glucuronide (MFA-\( 1-\beta-O \)-G), MFA-S-acyl-CoA (MFA-SCoA), and MFA-SG by liquid chromatography-tandem mass spectrometric analysis. The \( C_{\text{max}} \) of MFA-SG (330 nM; 10-min incubation with 100 \( \mu M \) MFA) was 120- to 1400-fold higher than the \( C_{\text{max}} \) of drug-S-acyl-GSH adducts detected from studies with other carboxylic acid drugs to date. MFA-SG was also detected in incubations with human hepatocytes, however at much lower concentrations. Inhibition of MFA acyl glucuronidation in rat hepatocytes had no effect on MFA-SG formation, whereas a 58±1.7% inhibition of MFA-SCoA formation led to a corresponding 66±3.5% inhibition of MFA-SG production. Reactivity comparisons with GSH in buffer showed MFA-SCoA to be 80-fold more reactive than MFA-\( 1-\beta-O \)-G forming MFA-SG. MFA-SG was detected in MFA-dosed (100 mg/kg) rat bile, where 17.4 \( \mu g \) was excreted post-administration. In summary, MFA exhibited bioactivation in rat and human hepatocytes and in vivo in rat and leading to reactive acylating derivatives that transacylate GSH. The formation of MFA-SG in hepatocytes was shown not to be mediated by reaction with MFA-\( 1-\beta-O \)-G, and not solely by MFA-SCoA, but perhaps also by intermediary MFA-acyl-adenylate formation, which is currently under investigation.
Introduction

The fenamate-type NSAID mefenamic acid (2-(2,3-dimethylphenyl)aminobenzoic acid; Ponstel; MFA; Fig. 1), an inhibitor of cyclooxygenase used for the treatment of pain and inflammation (Winder et al., 1962), has been shown to lead to instances of rare nephrotoxicity and hepatotoxicity in dosed patients (Robertson et al., 1980; Somchit et al., 2004; Chan et al., 1991). A hypothesis used to explain drug-induced hepatotoxicity is that some toxic drugs become metabolically activated in the liver to reactive metabolites that bind covalently to tissue proteins leading to cellular dysfunction or immunotoxicity (Nelson, 2001; Baillie, 2008). Carboxylic acid-containing drug-protein-adducts are proposed to function as antigens that mediate the formation of idiosyncratic allergic reactions (Zia-Amirhosseini et al., 1995; Boelsterli et al., 1995). Therefore, a proposed mechanism for the onset of MFA-mediated toxicities suggests that MFA is bioactivated to chemically-reactive metabolites that become covalently bound to protein in liver and kidney tissues leading to adverse immunological responses (McGurk et al., 1996; Boelsterli, 2002). MFA is metabolized to mefenamyl-1-O-acyl glucuronide (MFA-1-β-O-G; Fig. 1), which has been proposed to play a key role in MFA-mediated idiosyncratic toxicity (Sato et al., 1993; McGurk et al., 1996). Results from in vitro studies with MFA-1-β-O-G showed it to be very stable in phosphate buffer (16.5-h half-life at pH 7.4 and 37°C); however it was also shown to be chemically-reactive leading to covalent binding to human serum albumin in vitro (McGurk et al., 1996). Acyl glucuronide metabolites of acidic drugs are known to bind covalently to protein by two different mechanisms. These mechanisms include transacylation reactions of protein nucleophiles by the 1-O-acyl glucuronide isomer, and by a glycation mechanism involving the reaction of the open-chain aldehyde form of the acyl migration glucuronide isomers with protein amino-groups. Then it is proposed that drug-protein adducts are recognized by the immune system resulting in an immune response leading to potential allergic reactions (Zia-Amirhosseini et al., 1995). In addition to reactive acyl glucuronides, thioester-linked acyl-CoA derivatives (Fig. 1) are also electrophilic, and can
transacylate biological nucleophiles including protein and glutathione (GSH) forming S-acyl-GSH thioester adducts (Li et al., 2002; Boelsterli, 2002; Skonberg et al., 2008, Grillo, 2011). Prior to the present studies, experiments on the detection of S-acyl-CoA and S-acyl-GSH metabolites formed in vivo or in vitro of fenamic acid-type NSAIDs, including MFA, have not been performed.

Thioester-linked GSH-adducts are known metabolites of a range of carboxylic acid-containing drugs, for example clofibric acid (Grillo and Benet, 2002), zomepirac (Grillo and Hua, 2003), diclofenac (Grillo et al., 2003a), tolmetin (Olsen et al., 2007), flunoxaprofen (Grillo et al., 2010), and ibuprofen (Grillo and Hua, 2008). Mechanistic in vitro studies performed to investigate the relative contribution of acyl glucuronidation and S-acyl-CoA formation pathways leading to S-acyl-GSH thioester formation have indicated the S-acyl-CoA formation pathway to predominate in this regard and therefore may also be the bioactivation pathway predominating in vivo leading to the acylation of protein-nucleophiles by varied carboxylic acid-containing drugs.

MFA is known to be metabolized in humans by cytochrome P450 on the 3-methyl group forming 3-hydroxy-MFA (Glazko, 1966). Subsequent oxidation of the 3-hydroxy group leads to the formation of the 3-carboxy-MFA metabolite. The major metabolites isolated from human urine are the acyl glucuronides of MFA, 3-hydroxy-MFA, and 3-carboxy-MFA (Sato et al., 1993). Thioether-linked GSH-adducts of MFA have recently been detected from incubations with human liver microsomes fortified with NADPH by employing hybrid triple quadruple linear ion trap mass spectrometric techniques for screening and identification of GSH-trapped reactive metabolites (Zheng et al., 2007). The formation of the reactive metabolites leading to GSH-adducts with [M+H]⁺ ions m/z 547 and m/z 563 was proposed to be mediated by cytochrome P450-mediated bioactivation of MFA to epoxide and quinone imine intermediates, respectively, that react with GSH. The present studies were designed to examine the contribution
of acyl glucuronidation and potential acyl-CoA formation pathways on the bioactivation of MFA
to reactive intermediates that transacylate GSH in vitro in rat and human hepatocytes and in vivo
in rat leading to the formation of the thioester-linked GSH-adduct, mefenamyl-S-acyl-glutathione
(MFA-SG, Fig. 1).

Materials and Methods

Materials. MFA, carbamazepine (CBZ), GSH, lauric acid sodium salt, (-)-borneol,
(R)-ibuprofen, coenzyme A, L-glutamate, β-glucuronidase (type IX-A from E. coli), William’s
Media E, and glutathione S-transferase (GST, from rat liver, 31 units/mg protein) were purchased
from Sigma Chemical Co. (St. Louis, MO). MFA-SG (Khera et al., 2010), ibuprofen-S-acyl-
glutathione (I-SG) and ibuprofen-S-acyl-CoA (I-SCoA) (Grillo and Hua, 2008), and diclofenac-S-
acyl-glutathione (D-SG) (Grillo et al., 2003a) were available from previous studies. Authentic
standards of MFA-SCoA and MFA-1-β-O-G were synthesized as described below. Rat
hepatocytes were isolated as described below. Human cryopreserved hepatocytes (pooled from
20-donors; product no. X008000, lot DET) were purchased from Celsis-In vitro Technologies
(Baltimore, MD). Human suspension hepatocytes (male, single donor) were purchased from
Invitrogen (Carlsbad, CA). All solvents used for liquid chromatography-tandem mass
spectrometry (LC-MS/MS) analyses were of chromatographic grade. Stock solutions of MFA,
MFA-SCoA, MFA-SG, MFA-1-β-O-G, D-SG, I-SCoA, and I-SG were freshly-prepared as 1 mM
solutions in dimethyl sulfoxide for each experiment.

Instrumentation and Analytical Methods. MFA-SCoA, MFA-SG, and MFA-1-β-O-G
derivatives were characterized by LC-MS/MS on a Thermo Electron TSQ Quantum Discovery
Max mass spectrometer (Thermo Electron Corporation, Waltham, MA) linked to an Agilent 1100
HPLC (Agilent Technologies, Santa Clara, CA) and a CTC HTS PAL Autosampler (Leap
Technologies, Carrboro, NC). LC-MS/MS analysis of MFA-SCoA, MFA-SG, and
MFA-1-\(\beta\)-O-G derivatives was performed with a Phenomenex Luna, 5\(\mu\)m, C18(2), 100Å, 150x2.00 mm, reverse-phase column (Torrance, CA), and eluted with a mobile phase flow rate of 0.3 ml/min. The mobile phase used for the analysis of MFA-SCoA consisted of ammonium acetate (10 mM, pH 6.0) in water (solvent-A) and acetonitrile (solvent-B). The mobile phase used for the analysis of MFA-SG and MFA-1-\(\beta\)-O-G derivatives consisted of 0.1% formic acid in water (solvent-C) and 0.1% formic acid in acetonitrile (solvent-D). For these LC-MS/MS analyses, gradient elution was achieved by increasing solvent-B, or solvent-D, from 0 to 95% over 13-min. Electrospray ionization was employed with the needle potential held at 4.5 kV.

The tandem MS/MS conditions used were 2 mTorr argon collision gas and a collision potential of 35 eV. Positive ion mode full scan (\(m/z\) 50 to \(m/z\) 1000) LC-MS/MS analysis was conducted with a scan time of 0.73-sec and source collision energy of 10 V. Xcalibur software (version 2.0, Thermo Electron Corporation, Waltham, MA) was used to acquire all data. NMR spectra were acquired in methanol-d\(_4\) on a 600-MHz spectrometer equipped with a 5-mm cryoprobe (Bruker, Newark, DE).

**Synthesis of MFA-SCoA Thioester.** MFA-SCoA thioester was obtained in 30% yield by a synthetic procedure using ethyl chloroformate and analogous to that previously reported for the synthesis and purification of clofibryl-S-acyl-CoA thioester (Grillo and Benet, 2002). The MFA-SCoA thioester eluted at a retention time of 7.1-min and showed no detectable impurities when analyzed by both positive and negative ion LC-MS scan modes via reverse-phase gradient elution as described above. Tandem LC-MS/MS analysis of synthetic MFA-SCoA by collision-induced dissociation (CID) of the protonated molecular ion at \(m/z\) 991 yielded a product ion mass spectrum, \(m/z\) (%): \(m/z\) 768 ([CoASH+H]\(^{+}\), 3%), \(m/z\) 582 ([M+H-409]\(^{+}\), 58%), \(m/z\) 508 ([adenosine triphosphate-2H]\(^{+}\), 21%), \(m/z\) 484 ([M+H-507]\(^{+}\), 100%), \(m/z\) 428 ([adenosine diphosphate + 2H]\(^{+}\), 54%), \(m/z\) 382 ([M+H-609]\(^{+}\), 33%), \(m/z\) 224 ([MFA+H-H\(_2\)O]\(^{+}\), 20%), and \(m/z\) 136 ([adenine+H]\(^{+}\), 8%).
Synthesis of MFA-1-β-O-G. MFA-1-β-O-G was synthesized by the method of Baba and Yoshioka (2006) by employing commercially available methyl 2,3,4-tri-O-acetyl-1-bromo-1-deoxy-α-D-glucopyranuronate to obtain the methyl 2,3,4-tri-O-acetyl derivative of MFA-1-β-O-acyl glucuronide followed by chemo-selective enzymatic removal of the methyl and acetyl protecting groups. Tandem LC-MS/MS analysis of HPLC purified synthetic MFA-1-β-O-G by CID of the protonated molecular ion at m/z 418 yielded a product ion mass spectrum, m/z (%): m/z 242 ([MFA+H]^+, 14%) and m/z 224 ([MFA+H-H2O]^+, 100%). 1H NMR analysis of MFA-1-β-O-G (CD3OD): δ 2.16 (3H, s, 2-methyl), δ 2.34 (3H, s, 3-methyl), δ 3.50–3.69 (3H, m, glucuronosyl C2-, C3- and C4-), δ 4.05 (1H, d, J 9.6, glucuronosyl C5-H), δ 5.79 (1H, d, J 7.9, glucuronosyl C1-H), δ 6.66 (1H, d, J 8.5, Ar-H), δ 6.74 (1H, dt, J 0.9 and 7.5, Ar-H), δ 7.07–7.18 (3H, m, Ar-H), δ 7.34 (1H, dt, J 7.9 and 1.6, Ar-H), δ 8.08 (1H, dd, J 1.6 and 8.1, Ar-H) and δ 9.14 (1H, s, NH). β-Glucuronidase enzyme (1000 units/ml, 30-min, 2 ml total volume, pH 5.0 and 37°C, employed as per the manufacturer's instructions), which is known to specifically cleave 1-β-O-acyl-linked acyl glucuronides, and not the 2-, 3-, or 4-O-acyl glucuronide migration isomers, was used to further confirm the 1-β-O-acyl glucuronide linkage to MFA.

In Vitro Studies with Rat and Human Hepatocytes. Freshly-isolated hepatocytes were prepared and incubated according to the method of Moldéus et al. (1978). Hepatocytes were isolated from male Sprague-Dawley rats (250-300 g, Charles River Laboratories, Worcester, MA) and >95% viability was achieved as assessed by trypan blue exclusion testing. Incubations with human hepatocytes were conducted separately with cryopreserved human hepatocytes (20-donor pooled, product no. X008000, lot DET, In Vitro Technologies, Baltimore, MD) and with human hepatocytes in suspension (single donor, male, Invitrogen, Carlsbad, CA). Cryopreserved human hepatocytes (one tube, 5 million cells/tube) were thawed in a water bath at 37°C for 5-min without shaking. Human hepatocytes in suspension (60 million cells/tube) were warmed to 37°C for 5-min without shaking. Cells were centrifuged (26g, 1-min) and the isolated pellets were
gently resuspended in William’s Media E buffer (pH 7.4; containing 4 mM L-glutamate). Hepatocytes were washed twice more in this fashion, with resuspension of the final cell pellets in William’s Media E buffer to a cell concentration of 2 million viable cells/ml. Greater than 95% viability was achieved as assessed by trypan blue exclusion testing. Hepatocytes were warmed to 37°C in a water bath under an atmosphere of 95% O₂ and 5% CO₂ for 15-min prior to the initiation of metabolism experiments. Incubations of hepatocytes (2 million viable cells/ml; 0.5 ml total volume; n=3) with MFA were performed in William’s Media E (pH 7.4, containing 4 mM L-glutamate) in 96-well Costar® standard non-sterile deep-well polypropylene plates (2 ml well volume, Corning Inc., Corning, NY) and with continuous orbital shaking (20 rpm/min) under an atmosphere of 95% O₂ and 5% CO₂ at 37°C in a Model ORS-200 Boekel/Grant orbital and reciprocating water bath water (Expotech USA, Houston, TX).

For time-dependent experiments (n=3 separate studies), hepatocytes were incubated with MFA (100 µM, n=3 incubations/time-point) and analyzed for MFA-SCoA, MFA-SG, and MFA-1-β-O-G formation over a 60-min time-period. For the analysis of MFA-SG and MFA-1-β-O-G derivatives, incubations (500 µl) were quenched at 0-, 1-, 2-, 4-, 6-, 8-, 10-, 20-, 30-, and 60-min with a quench solution (500 µl) consisting of acetonitrile, 3% formic acid, and 1 µM CBZ internal standard, then sealed with a silicone sealing mat for 96-well deep well plates (Fisher Scientific, Pittsburgh, PA), followed by vortex-mixing (10 min) in a VWR DVX-2500 Multi-tube Vortexer (VWR, Brisbane, CA). Samples then were centrifuged (4,500 rpm, 30-min, 8-10°C) and aliquots (300 µl) of the supernatants transferred to SPEware 96-well plates (V bottom, 0.8 ml, Fisher Scientific, Pittsburgh, PA) prior to LC-MS/MS analysis. Incubations (n=3) of (R)-ibuprofen (100 µM, 10-min) with rat hepatocytes were performed and processed as described above for the quantification of I-SG.

For the analysis of MFA-SCoA formation, separate incubations were performed in triplicate as described above for MFA-SG except that incubations were quenched with a solution
consisting of acetonitrile (without formic acid, 500 µl) and CBZ (1 µM), followed by the addition of hexane (500 µl). The sample plates then were capped, vortex-mixed (1-min), centrifuged (4,500 rpm, 10-min, 8-10ºC), and aliquots (300 µl) of the aqueous layer transferred to SPEware 96-well plates for LC-MS/MS analysis of MFA-SCoA.

Concentration-dependent experiments (n=3 separate studies) were performed as described above with increasing concentrations of MFA (0.8, 1.5, 3.9, 7.8, 15.6, 31.3, 62.5, 100, 125, 250, and 500 µM) incubated with rat hepatocytes (2 million cells/ml) for 10-min and processed for LC-MS/MS analysis of MFA-SG, MFA-1-β-O-G, and MFA-SCoA derivatives as described above.

Inhibition experiments were performed with MFA (100 µM) incubated with rat hepatocytes (2 million cells/ml) in the presence or absence of (-)-borneol (1000 µM), for the inhibition of MFA-1-β-O-G formation, or lauric acid (1000 µM), for the inhibition of MFA-SCoA formation. Hepatocyte incubations (0.5 ml total volume; n=3 incubations/treatment) were performed for 10-min and then quenched as described above for the analysis of MFA-SG, MFA-1-β-O-G, and MFA-SCoA derivatives. A stock solution of (-)-borneol (1000 mM) was prepared in dimethyl sulfoxide, and control incubations included the same final concentration of dimethyl sulfoxide (0.1%, v/v). A stock solution of lauric acid sodium salt was prepared as a 100 mM solution in distilled water (pH 7).

An in vitro experiment was performed to assess the stability of MFA-1-β-O-G and MFA-SG, I-SG, and D-SG thioesters (1 µM) in separate incubations with rat hepatocytes (2 million cells/ml, n=3 incubations/time-point). Incubations (500 µl) were quenched at 0-, 0.5, 1-, 2-, 3-, 4-, 5-, 10-, 20- and 30-min and processed as described above for the LC-MS/MS analysis of MFA-SG, MFA-1-β-O-G, I-SG, and D-SG as described below.

**Stability of MFA-SG, I-SG, and D-SG in Buffer in the Presence and Absence of GST.** An in vitro study was performed to determine the stability of MFA-SG, I-SG, and D-SG in incubations with phosphate buffer (0.1 M, pH 7.4, 37ºC, 0.2 ml incubation volume) in the
presence and absence of rat liver GST (3 units/ml). Incubations were performed in duplicate for each time-point in 96-well plates as described above and quenched with acetonitrile (containing 3% formic acid, v/v, 1 μM CBZ, 200 μl) at 0-, 5-, 10-, 20-, 30-, and 60-min time-points. The quenched incubation mixtures were analyzed directly by LC-MS/MS for MFA-SG, I-SG, and D-SG as described below.

**Identification and Quantification of MFA-SG.** Extracts of MFA-treated rat hepatocyte incubations were analyzed by LC-MS/MS for MFA-SG and CBZ by using the multiple reaction monitoring (MRM) transitions MH+ m/z 531 to m/z 224, for MFA-SG detection, and MH+ m/z 237 to m/z 194, for CBZ detection, in the positive ion mode and using the chromatographic method described above. Authentic MFA-SG standard eluted at a retention time of 9.1-min, while CBZ eluted at 8.4-min. The concentration of MFA-SG thioester was determined from a linear standard curve generated from MFA-SG/CBZ peak area ratios. The LC-MS/MS quantification of I-SG and D-SG was performed using published procedures (Grillo and Hua, 2008; Grillo et al., 2003a) except using the LC-MS/MS system and chromatography parameters described above. Under these conditions, the retention times of I-SG and D-SG were 7.0- and 7.2-min, respectively. The concentrations of I-SG and D-SG thioesters were determined from linear standard curves generated from I-SG/CBZ and D-SG/CBZ peak area ratios.

**Identification and Quantification of MFA-SCoA.** Extracts of MFA-treated rat hepatocyte quenched incubations were analyzed by LC-MS/MS for MFA-SCoA and CBZ by using the MRM transitions MH+ m/z 991 to m/z 224 and m/z 484, and MH+ m/z 237 to m/z 194, respectively, in the positive ion mode and by employing the chromatographic method for the analysis of MFA-SCoA as described above. Authentic MFA-SCoA standard eluted at a retention time of 6.2-min, while CBZ eluted at 7.1-min. The concentration of MFA-SCoA thioester was determined from a linear standard curve generated from MFA-SCoA/CBZ peak area ratios.

**Identification of MFA-1-O-G.** Analysis for the formation of MFA-1-β-O-G in incubations of MFA with rat hepatocytes was performed by LC-MS/MS in the positive ion mode.
with the MRM transitions MH⁺ m/z 418 to m/z 224 for MFA-1-β-O-G detection, and MH⁺ m/z 237 to m/z 194 for CBZ detection, using the same LC-MS/MS chromatography method as described above for the analysis of MFA-SG. Authentic MFA-1-β-O-G standard eluted at retention time of 11-min. The concentration of MFA-1-β-O-G was determined from a linear standard curve generated from MFA-1-β-O-G/CBZ peak area ratios.

**Reactions of MFA-SCoA and MFA-1-β-O-G with GSH in Buffer.** Incubations (0.5 ml, n=3) containing MFA-SCoA (1 μM) or MFA-1-β-O-G (1 μM) were performed in phosphate buffer (0.1 M, pH 7.4) at 37°C and fortified with 10 mM GSH in 96-well Costar® standard non-sterile deep-well polypropylene plates (2 ml well volume, Corning Inc., Corning, NY). Corresponding incubations were also performed with I-SCoA in order to compare relative reactivity with GSH forming I-SG. Incubations were conducted for 0-, 15-, 30-, 45-, and 60-min followed by quenching with acetonitrile (0.5 ml) containing 3% formic acid (v/v) and 1 μM CBZ. Corresponding incubations were also performed in the presence of rat liver GST (3 units/ml). Quenched mixtures then were directly analyzed by LC-MS/MS for MFA-SG (as described above). An in vitro study on the degradation of MFA-SCoA and MFA-1-β-O-G in phosphate buffer (0.1 M, pH 7.4, 37°C) was also performed where the time-dependent degradation of MFA-SCoA and MFA-1-β-O-G (1 μM, separate incubations) was examined as above except in the absence of GSH. Incubations (n=3 incubations/time-point) were quenched at the 0-, 5-, 8- and 15-h time-points and quantified as described above for analysis of MFA-SCoA and MFA-1-β-O-G derivatives.

**Identification and quantification of MFA-SG Thioester in Rat Bile.** Three male Sprague-Dawley rats (300-330 g) fitted with exteriorized bile duct cannulas (obtained from Charles River, Hollister, CA) were housed individually in metabolic cages for the collection of bile. Rats received MFA (100 mg/kg, in water, pH 7.0) by intraperitoneal administration. An artificial bile salt supplement (13.4 mg/ml taurocholic acid, 0.5 mg/ml KCl, 9.0 mg/ml NaCl) was infused (1 ml/h) via a duodenal cannula during a 6-h collection period. Bile was collected in
plastic 50 ml Corning® clear polypropylene conical bottom centrifuge tubes (Fisher Scientific, Pittsburgh, PA) containing 600 μl of phosphoric acid on wet ice. Collected bile samples then were stored frozen (-20ºC) until further processing for LC-M/MS analysis. Frozen bile samples (~10-15 ml collections) were thawed on wet ice followed by the addition of an equal volume of quench solution consisting of acetonitrile containing 3% formic acid (v/v) and 1 μM CBZ internal standard. The samples then were vortex-mixed, centrifuged (14,000 rpm, 10-min) and the supernatants analyzed and quantified by LC-MS/MS for MFA-SG and MFA-1-β-O-G derivatives as described above. MFA concentration in rat bile was also determined from these same extracts by LC/MS positive ion scanning and extracted ion analysis for the protonated molecular ion of MFA (MH+ m/z 242) and from a linear standard curve generated from MFA/CBZ peak area ratios.

Results

Identification of MFA-SCoA. Analysis of incubation extracts by LC-MS/MS MRM detection allowed for the identification of MFA-SCoA formed in rat hepatocyte incubations (Fig. 2). The transitions used for this analysis were MH+ m/z 991 to m/z 224 and MH+ m/z 991 to m/z 484, which were chosen because of being the major fragmentation pathways for authentic MFA-SCoA as assessed by positive ion LC-MS/MS CID of the MH+ ion (Fig. 2B). Reverse-phase LC-MS/MS analysis showed the presence of MFA-SCoA in incubations of MFA (100 μM) with rat hepatocytes, and which co-eluted with authentic MFA-SCoA standard at a retention time of 7.1-min (Fig. 2A). LC-MS/MS analysis of MFA-SCoA formed in hepatocytes provided a product ion spectrum from CID of the MH+ ion at m/z 991 that was identical to the authentic MFA-SCoA standard and consistent with its chemical structure (Figs. 2B and 2C; Sidenius et al., 2004). MFA-SCoA was not detected in incubations of MFA with human hepatocytes.

Identification of MFA-SG. Analysis of extracts by a sensitive LC-MS/MS MRM detection technique allowed for the identification of MFA-SG formed in hepatocyte incubations.
(Figs. 3 and 4). The transition used for this analysis was MH$^+$ m/z 531 to m/z 224, and was chosen due to it being major the fragmentation pathway for MFA-SG as assessed by positive ion LC-MS/MS CID of the MH$^+$ ion of authentic MFA-SG (Fig. 3B). LC-MS/MS analysis showed the presence of MFA-SG in incubations of MFA (100 μM) with rat hepatocytes, and which co-eluted with authentic MFA-SG standard at a retention time of 9.2-min (Fig. 3A). LC-MS/MS analysis of MFA-SG formed in hepatocytes provided a product ion spectrum that displayed fragment ions identical to the authentic MFA-SG standard and also consistent with its chemical structure (Figs. 3B and 3C; Baillie and Davis, 1993). The formation of MFA-SG in incubations with human hepatocytes was also shown and provided LC-MS/MS properties in agreement with standard MFA-SG (Fig. 4). Small differences in retention time and tandem mass spectra between the LC-MS/MS data for the MFA-SG detected in rat and human hepatocytes were observed even though the same LC-MS/MS parameters and chromatography system were used, except that the two analysis were performed ~4 months apart.

**LC-MS/MS Detection of MFA-1-β-O-G.** The 1-O-β-acyl glucuronide metabolite of MFA was detected by positive ion LC-MS/MS analysis of rat (Fig. 5) and human (data not shown) hepatocyte MFA incubation extracts. Results from these analyses showed MFA-1-β-O-G eluting at the same retention time as authentic MFA-1-β-O-G standard (11-min, Fig. 5A). LC-MS/MS analysis of the MFA-1-β-O-G metabolite formed in hepatocytes incubated with MFA provided product ion spectra containing fragment ions that were consistent with its chemical structure and identical to the authentic MFA-1-β-O-G standard (Figs. 5B and 5C). The product ion spectrum for MFA-1-β-O-G formed in hepatocytes showed that the major fragment ion (100% relative abundance) upon CID of the MH$^+$ ion at m/z 418 was m/z 224. Evidence for acyl migration isomers was not obtained during the LC-MS/MS analysis of MFA rat hepatocyte incubation extracts, which is consistent with the known chemical stability of the MFA-1-β-O-G metabolite (McGurk et al., 1996).
Time-Course of MFA-SCoA, MFA-SG, and MFA-1-β-O-G Formation in Incubations with Rat and Human Hepatocytes. When MFA (100 μM) was incubated with rat hepatocytes for up to 60-min, the formation of MFA-SCoA was determined to reach a maximum concentration (C_max) of 15±3 nM after 30-min of incubation (Fig. 6A). This concentration was much lower than the C_max observed for S-acyl-CoA metabolites of (R)-ibuprofen (2,600 nM; Grillo and Hua, 2008) and phenylacetic acid (1,300 nM; Grillo and Lohr, 2009) measured under similar incubation conditions with rat hepatocytes; however it was similar to the C_max of flunoxaprofen-S-acyl-CoA (42 nM) detected in recent studies with rat hepatocytes incubated with 100 μM (R)-flunoxaprofen (Grillo et al., 2010). In addition, the length of incubation time leading to C_max (T_max) of MFA-SCoA was 30-min in the present studies, whereas the reported T_max of formation of I-SCoA, phenylacetyl-S-acyl-CoA, and flunoxaprofen-S-acyl-CoA was 3- to 7.5-fold shorter at 10-, 4-, and 6-min, respectively (Grillo, 2011). The formation of MFA-SG thioester in rat hepatocyte incubations with MFA (100 μM) was more rapid than MFA-SCoA formation and reached a 22-fold higher C_max of 330±22 nM at the 10-min time-point (Fig. 6A). This C_max of MFA-SG was 120- to 1400-fold higher than the C_max of drug-S-acyl-GSH adducts detected from rat hepatocyte incubations with other carboxylic acid drugs (100 μM) studied to date. For example, the C_max of the S-acyl-GSH thioester detected in incubations with rat hepatocytes incubated with 100 μM carboxylic acid drugs from previous studies was 0.24 nM for zomepirac (Grillo and Hua, 2003), 0.8 nM for diclofenac (Grillo et al., 2003a), 1.3 nM for (R)-ibuprofen (Grillo and Hua, 2008), and 2.8 nM for (R)-flunoxaprofen (Grillo et al., 2010). After the 10-min time-point, the concentration of MFA-SG in rat hepatocyte incubations fell sharply in a fairly linear fashion reaching ~50 nM after 60-min of incubation. The time-course of MFA-1-β-O-G formation observed from the analysis of extracts from hepatocyte incubations with MFA (100 μM) showed that the concentration of MFA-1-β-O-G increased throughout the 60-min incubation period reaching 1560±120 nM (Fig. 6A). A significant consumption of the initial
MFA concentration was observed over the 60-min time-period leading to approximately 80 microM remaining (data not shown), and where MFA-1-β-O-G, MFA-SG, and MFA-S-CoA contributed to roughly 7.8, 0.25, and 0.05% of the amount of MFA consumed, respectively. Incubations of MFA (100 μM) with single donor male human hepatocytes in suspension showed a time-dependent formation of MFA-SG that reached a C_max of 0.83±0.03 nM at the 30-min time-point (Fig. 7). This C_max was ~400-fold lower that the C_max of MFA-SG observed in corresponding incubations with rat hepatocytes. In incubations with cryopreserved human hepatocytes (pooled from 20-donors), 10-min incubations were conducted with 100 μM MFA and led to the formation of 1.0±0.1 nM MFA-SG (data not shown). The MFA-1-β-O-G was also detected in the human hepatocyte incubations where a time-dependent formation led to 18.6±0.9 μM after 60-min of incubation with the single donor hepatocytes in suspension (Fig. 7). Incubations with the cryopreserved human hepatocytes for 10-min showed 3.5±0.25 μM MFA-1-β-O-G formed (data not shown).

**Concentration-Dependent Formation of MFA-SCoA, MFA-SG, and MFA-1-β-O-G in Incubations with Rat Hepatocytes.** When rat hepatocytes (2 million cells/ml) were incubated for 10-min with increasing concentrations of MFA, results showed a concentration-dependent formation of MFA-SCoA from 0.8 to 15.6 μM MFA reaching 6±2 nM MFA-SCoA at 15.6 μM MFA (Fig. 6B). No significant change in MFA-SCoA formation was observed at MFA concentrations studied above 15.6 μM. By comparison, from these same incubations, the concentration MFA-SG formation was also found to increase sharply with increasing concentration of MFA (Fig. 6B). The formation of MFA-SG reached a C_max of 308±6 nM at 100 μM MFA. Above 100 μM MFA, MFA-SG concentration did not increase, but decreased by 25% to 230±30 nM at the 500 μM MFA incubation concentration. From incubations with (R)-ibuprofen (100 μM, 10-min), the I-SG thioester was detected at 3±1 nM. The concentration of MFA-1-β-O-G in the rat hepatocyte incubations increased with increasing MFA concentration
up to 700±80 nM at 62.5 μM MFA, but then decreased to 460±85 nM at the 500 μM MFA incubation concentration.

**Time-Dependent Degradation of MFA-SG in Rat Hepatocytes.** The time-course of degradation of authentic MFA-SG (1 μM) in incubations with rat hepatocytes showed the thioester derivative to be degraded in a fairly rapid fashion (t1/2=8.5-min), whereas corresponding incubations with MFA-1-β-O-G (1 μM) showed complete stability after 60-min of incubation (Fig. 8). Although the MFA-SG degradation t1/2 in incubations with rat hepatocytes was rapid, it was approximately 2.5- to 10.5-fold longer than the degradation t1/2 reported for zomepirac-SG (t1/2=0.8-min; Grillo and Hua, 2003), D-SG (t1/2=1.0-min; Grillo et al., 2003a), flunoxaprofen-SG (t1/2=1.5-min; Grillo et al., 2010), and I-SG (t1/2=4.0-min; Grillo and Hua, 2008). In the present S-acyl-GSH thioester stability experiments, we also tested for the degradation t1/2 of (R,S)-I-SG and D-SG in incubations with rat hepatocytes where results showed their degradation half-lives to be 2.0- and 0.4-min, respectively. The degradation of (R)- and (S)-I-SG in incubations with rat hepatocytes is known not to be enantioselective (Grillo and Hua, 2008). The products of degradation of MFA-SG were not determined in this study, but are proposed to be due to hydrolysis of the thioester, similar to that determined for D-SG (Grillo et al., 2003a), and not due to γ-glutamyltranspeptidase activity which is known to be negligible in rat liver tissue (Hinchman and Ballatori, 1990). Noteworthy, S-acyl-GSH thioesters are known to be chemically-reactive species that can transacylate nucleophiles such as N-acetylcysteine and thus might also contribute to the transacylation of protein nucleophiles (Grillo and Benet, 2002); however, we did not characterize the chemical reactivity of MFA-SG in the present studies.

**Inhibition Study.** An inhibition experiment was performed with MFA (100 μM) in incubations with rat hepatocytes (2 million cells/ml, 10-min) in the presence or absence of (-)-borneol (1000 μM), for the inhibition of MFA-1-β-O-G formation (Watkins and Klaassen, 1982), or lauric acid (1000 μM), for the inhibition of MFA-SCoA formation (Xiaotao and Hall,
1993). Results showed that coincubation with (-)-borneol led to the inhibition of MFA-1-β-O-G formation by 91%, however no inhibition of MFA-SG production was observed (Table 1). By contrast, coincubation of MFA with lauric acid led to a 58±2% inhibition of MFA-SCoA formation and to a corresponding 66±4% inhibition MFA-SG formation (Table 1).

**Reaction of GSH with MFA-SCoA and MFA-1-β-O-G in Buffer.** Incubation of MFA-SCoA with GSH (10 mM) in buffer (0.5 mL incubation volume, 0.1 M potassium phosphate, pH 7.4, 37°C) resulted in a rapid transacylation of GSH (3.5 nM MFA-SG formed/min) generating 208±13 nM MFA-SG after 60-min of incubation (Fig. 9A). Compared to I-SCoA, MFA-SCoA was shown to be ~2-fold less reactive with GSH, where the reactivity of I-SCoA with GSH led to ~440 nM I-SG formed after 60-min of incubation. Corresponding incubations with MFA-1-β-O-G (1 μM) showed the acyl glucuronide to be 80-fold less reactive than MFA-SCoA with GSH (0.045 nM MFA-1-β-O-G formed/min) leading to 2.7±1.0 nM MFA-SG formed at the 60-min time-point (Fig. 9A). In a separate study, the reaction of MFA-SCoA or MFA-1-β-O-G (1 μM) with GSH (10 mM) in incubations fortified with rat liver GST led to a 1.3- and 6-fold increase in formation of MFA-SG, respectively (results not shown). MFA-SCoA (1 μM) was found to be completely stable in incubations with buffer (0.1 M potassium phosphate, pH 7.4, 37°C) in the absence of GSH, whereas MFA-1-β-O-G (1 μM) degraded in a linear fashion over time with a degradation t₁/₂ of 16.5-h (Fig. 9B).

**Stability of MFA-SG, I-SG, and D-SG in Buffer With and Without GST.** Results from an in vitro study to determine the ability of rat liver GST (0.1 M potassium phosphate buffer, pH 7.4, 37°C) to hydrolyze MFA-SG showed MFA-SG to be very stable, where ~10% was degraded after 30-min of incubation relative to incubations in the absence of GST (Fig. 10). The thioester D-SG, by contrast, was completely degraded by the 30-min time-point in incubations fortified with GST. Corresponding incubations with I-SG showed this thioester to be
similarly as stable as MFA-SG in GST-fortified incubations (11% degraded at 30-min relative to incubations in buffer in the absence of GST).

**Identification of MFA-SG in Rat Bile.** Post-administration of MFA to male Sprague-Dawley rats (100 mg/kg, i.p.), bile was collected, processed, and the extract analyzed for MFA-SG by LC-MS/MS. Results showed the presence of MFA-SG, where 17.4 µg excreted in bile over 6-h of collection. The total amounts of MFA-1-β-O-G and MFA excreted in bile 6-h post-administration of MFA were also determined and shown to be 2000 and 4 µg, respectively.

**Discussion**

Herein, we report MFA to undergo bioactivation to reactive transacylating species in rat and human hepatocytes and in vivo in rat that transacylate the cysteiny1-thiol of GSH forming MFA-S-acyl-GSH thioester. Results from the present studies provide the first evidence for the formation of S-acyl-CoA and S-acyl-GSH thioester metabolites of a fenamic acid-containing compound. LC-MS/MS analysis of extracts from MFA-treated rat hepatocyte incubations led to the detection of the MFA-SCoA and MFA-SG thioesters, where their tandem mass spectra showed product ions consistent with their chemical structures (Figs. 2 and 3) and with LC-MS/MS product ion spectra of S-acyl-CoA and S-acyl-GSH derivatives in general (Sidenius et al., 2004; Grillo, 2011). MFA-SG, but not the MFA-SCoA thioester, was detected incubations with human hepatocytes, but at much lower concentrations (Fig. 4). MFA-SG was also identified in MFA-dosed rat bile, where 17.4 µg was excreted in rat bile over a 6-h collection period; which was ~60- and 3-fold greater than the reported amount of biliarily excreted D-SG in 200 mg/kg diclofenac-dosed (Grillo et al., 2003b) and zomepirac-S-acyl-GSH in 100 mg/kg zomepirac-dosed (Grillo and Hua, 2003) rats.

Studies on the chemical reactivity of MFA-SCoA and MFA-1-β-O-G with GSH showed the S-acyl-CoA thioester to be ~80-fold more reactive than the acyl glucuronide leading to MFA-SG formation (Fig. 9), which is consistent with results from previous studies comparing the
relative chemical reactivity between \( S \)-acyl-CoA thioesters and their corresponding acyl glucuronides with GSH (Grillo, 2011; Li et al., 2002). The 1.3- and 6-fold GST-catalyzed increase in MFA-SG formation rates from reactions of MFA-SCoA and MFA-1-\( \beta \)-O-G with GSH is consistent with the weak catalysis observed in previous studies with clofibric acid derivatives, where rat liver GST-mediated catalysis was shown to occur in incubations of clofibryl-1-\( \beta \)-O-G (~8-fold; Shore et al., 1995) and clofibryl-\( S \)-acyl-CoA (~3-fold, Grillo and Benet, 2002) with GSH forming clofibryl-\( S \)-acyl-GSH thioester. In addition, we determined MFA-SCoA to be chemically stable in buffer at pH 7.4 and 37°C over 15-h of incubation, whereas MFA-1-\( \beta \)-O-G degraded with a \( t_{1/2} \) of 16.5-h, which was the same as previously reported (McGurk et al., 1996; Walker et al., 2007). The degradation of MFA-1-\( \beta \)-O-G in buffer is known to occur almost exclusively through acyl migration and not by hydrolysis (Walker et al., 2007).

In time-course rat hepatocyte studies, we determined the \( C_{\text{max}} \) of MFA-SCoA detected in incubations treated with 100 \( \mu \)M MFA to be 15 nM after 30-min of incubation, which was 170- and 90-fold lower than I-SCoA and phenylacetyl-\( S \)-acyl-CoA detected in similar studies with \( (R) \)-ibuprofen (Grillo and Hua, 2008) and phenylacetic acid (Grillo and Lohr, 2009), respectively. Differences in drug-\( S \)-acyl-CoA formation may be due to differences in the acyl-CoA synthetase(s) catalyzing their formation; however, the identity of the acyl-CoA synthetase(s) that mediate the formation of MFA-SCoA is not known. Thioester \( S \)-acyl-CoA formation is catalyzed by acyl-CoA synthetases located in the endoplasmic reticulum, the outer membrane of mitochondria (long-chain fatty acid acyl-CoA synthetases), and in the mitochondrial matrix (short-, medium-, and branched-chain acyl-CoA synthetases). Mitochondrial medium-chain acyl-CoA synthetase is primarily associated with the metabolism of salicylic acid, benzoic acid and phenylacetic acid, whereas hepatic microsomal and peroxisomal long-chain acyl-CoA synthetases are involved in the formation of \( S \)-acyl-CoA thioesters of hypolipidemic and peroxisomal proliferating drugs (e.g., clofibric acid) and of 2-arylpropionic acid drugs such as ibuprofen (Knights, 1998; Nelson and Cox, 2005).
From the same time-course experiments, incubations of 100 µM MFA with rat hepatocytes led to a MFA-SG C\textsubscript{max} of 330 nM after 10-min of incubation, which was 120- to 1400-fold higher than the C\textsubscript{max} of drug-S-acyl-GSH adducts detected from rat hepatocyte incubations with other carboxylic acid drugs (100 µM) studied to date. In the present work, incubations with (R)-ibuprofen led to the detection of 3 nM I-SG, which was ~100-fold less than the C\textsubscript{max} of MFA-SG formed in the same experiments. In contrast to results from previous studies with ibuprofen and flunoxaprofen, where the concentration-time profile of S-acyl-GSH formation correlated with S-acyl-CoA formation, the time-course of MFA-SG formation was not consistent with the time-course of MFA-SCoA formation, where the T\textsubscript{max} of MFA-SG formation occurred at 10-min of incubation (Fig. 5A), and the T\textsubscript{max} for MFA-SCoA formation occurred at the 30-min incubation time-point. From these results alone we proposed that MFA-SCoA was not solely responsible for the transacylation of GSH leading to MFA-SG formation in rat hepatocytes. As expected, the time-course of MFA-1-β-O-G formation was not consistent with the time-course for MFA-SG formation, where MFA-1-β-O-G increased in a time-dependent fashion throughout the entire incubation period reaching 1,560 nM after 60-min of incubation (Fig. 5A), and MFA-SG concentration at 60-min decreased by ~7-fold from its C\textsubscript{max} at 10-min. This lack of acyl glucuronide/S-acyl-SG time-course correlation has been observed before from similar studies with ibuprofen (Grillo and Hua, 2008), flunoxaprofen (Grillo et al., 2010), and diclofenac (Grillo et al., 2003a). We do not believe that the decrease in MFA-SG concentration after the 10-min time-point was due to a decrease in intracellular GSH concentration, since the method used for the incubation of rat hepatocytes allowed for maintained GSH concentration up to 3-h of incubation (Nakagawa and Moldéus, 1998). Rather, we propose that the decrease in MFA-SG concentration observed after the 10-min time-point occurred due to a decrease in the amount of reactive transacylating metabolite formed after the 10-min time point. Corresponding studies with human hepatocytes showed that MFA-SG formation (Fig. 7) occurred at a 400-fold lower C\textsubscript{max} compared to incubations with rat hepatocytes (Fig. 6A). We currently do not
understand the reason for the dramatic species difference in MFA-SG formation; however, similar studies with diclofenac showed no species difference, where the formation of D-SG was shown to reach maximum concentrations of 1 and 0.8 nM after 4-min in incubations with rat and human hepatocytes, respectively (Grillo et al., 2003).

Results from MFA concentration-dependent studies in rat hepatocytes (Fig. 6B) showed that the 100 \( \mu \)M MFA concentration where maximum MFA-SG also occurred did not coincide with the \( \text{C}_{\text{max}} \) of MFA-SCoA formation (15.6 \( \mu \)M MFA), again demonstrating a lack of correlation of MFA-SG formation with MFA-SCoA formation alone.

\( S \)-Acyl-GSH thioesters are degraded in incubations with rat hepatocytes to the corresponding free acids (Grillo, 2011). The degradation rates of MFA-SG, I-SG and D-SG in rat hepatocytes were examined in the present studies to determine if MFA-SG might be hydrolyzed at a significantly slower rate. We hypothesized that the substantially higher \( \text{C}_{\text{max}} \) of MFA-SG in MFA-treated rat hepatocytes, relative to other carboxylic acids examined to date, may have been due to a lower degradation rate. Results indicated that MFA-SG, I-SG, and D-SG were degraded with half-life values of 8.5-, 2.0-, and 0.4-min, respectively (Fig. 8). From these data, we propose that the 4.3-fold lower MFA-SG degradation rate did not contribute to the ~100-fold increase in MFA-SG concentration measured in rat hepatocyte incubations with MFA relative to \((R)\)-ibuprofen in the present experiments.

\( S \)-Acyl-GSH thioesters are also known to be degraded to the corresponding free acids by GST-mediated hydrolysis (Ibarra et al., 2003). Thus, we determined that MFA-SG and I-SG were relatively stable toward rat liver GST and degraded by only 10-11% after 30-min; whereas D-SG was completely hydrolyzed after 30-min of incubation (Fig. 10). Since MFA-SG and I-SG have similar stabilities in incubations with GST, then the differences in their respective \( S \)-acyl-GSH formation in incubations with rat hepatocytes may not be due to differences in rates of degradation catalyzed by intracellular GST.
Results from inhibition studies in rat hepatocytes where MFA-SCoA and MFA-1-β-O-G formation were inhibited by coincubation with lauric acid and (-)-borneol, respectively, showed that inhibition of S-acyl-CoA formation led to a corresponding inhibition of MFA-SG formation; whereas almost complete inhibition of acyl glucuronidation had no effect on MFA-SG formation (Table 1). These results are consistent with results from similar studies with ibuprofen and flunoxaprofen (Grillo and Hua, 2008; Grillo et al., 2010) and point to a general lack of importance for acyl glucuronides mediating the transacylation of GSH. Potentially, metabolism of MFA by acyl-CoA synthetase(s) leads to a buildup of an intermediate MFA-acyl-adenylate. Xenobiotic and endogenous carboxylic acids have been shown to be converted to chemically-reactive mixed anhydride adenosine 5-monophosphate-linked intermediates detected during the formation of their corresponding S-acyl-CoA thioesters (Mao et al., 1992; Menzel et al., 1994; Ikegawa et al., 1999; Hall and Xiaotao, 1994). Acyl-adenylate intermediates of endogenous bile acids such as cholic acid (Ikegawa et al., 1999) are known to be reactive acylating derivatives that undergo transacylation-type reactions the amino group of taurine, with peptides and proteins, and with the thiol group of GSH leading to the formation of bile acid S-acyl-GSH conjugates (Goto et al., 2001).

In summary, from these data we propose that the formation of MFA-SG is not mediated by MFA-1-β-O-G, and not solely by reaction with MFA-SCoA, but also mediated by other MFA-linked reactive acylating species that have not yet been identified. Investigations on the ability of MFA to form a reactive acyl-adenylate intermediate that might mediate the acylation of GSH in incubations with rat and human hepatocytes are ongoing.
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Authorship Contributions.

Participated in research design: Grillo

Conducted experiments: Grillo, Lohr, and Wait

Contributed new reagents or analytic tools: Grillo

Performed data analysis: Grillo, Lohr, and Wait

Wrote or contributed to the writing of the manuscript: Grillo
References


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Footnotes:

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Figure Legends:

**FIG. 1.** Proposed scheme for the metabolism of MFA by acyl glucuronidation and acyl-CoA formation leading to the transacylation of GSH forming MFA-SG.

**FIG. 2.** A) Representative reverse-phase gradient LC-MS/MS MRM chromatograms of MFA-SCoA from the analysis of extracts from rat hepatocytes incubated for 10-min with 100 μM MFA (solid line) and extract from rat hepatocytes spiked with MFA-SCoA authentic standard (10 nM, dashed line). The LC-MS/MS MRM transition used for the analysis of MFA-SCoA was MH⁺ m/z 991 to m/z 224 and m/z 484. B) LC-MS/MS tandem mass spectrum of MFA-SCoA formed in rat hepatocyte incubations with MFA (100 μM, 10-min) obtained by CID of the protonated molecular ion at m/z 991. C) Corresponding LC-MS/MS tandem mass spectrum of authentic MFA-SCoA standard. D) The chemical structure and origins of the characteristic fragment ions for MFA-SCoA are as shown.

**FIG. 3.** A) Representative reverse-phase gradient LC-MS/MS MRM chromatograms of MFA-SG from the analysis of extracts from rat hepatocytes incubated for 10-min with 100 μM MFA (solid line) and extract from rat hepatocytes spiked with MFA-SG authentic standard (500 nM, dashed line). The LC-MS/MS MRM transition used for the analysis of MFA-SG was MH⁺ m/z 531 to m/z 224. B) LC-MS/MS tandem mass spectrum of MFA-SG formed in rat hepatocyte incubations with MFA (100 μM, 10-min) obtained by CID of the protonated molecular ion at m/z 531. C) Corresponding LC-MS/MS tandem mass spectrum of authentic MFA-SG standard. D) The chemical structure and origins of the characteristic fragment ions for MFA-SG are as shown.
FIG. 4. A) Representative reverse-phase gradient LC-MS/MS MRM chromatograms of MFA-SG from the analysis of extracts from human hepatocytes incubated for 10-min with 100 μM MFA (solid line) and extract from rat hepatocytes spiked with MFA-SG authentic standard (1 nM, dashed line). The LC-MS/MS MRM transition used for the analysis of MFA-SG was MH⁺ m/z 531 to m/z 224. B) LC-MS/MS tandem mass spectrum of MFA-SG formed in rat hepatocyte incubations with MFA (100 μM, 10-min) obtained by CID of the protonated molecular ion at m/z 531. C) Corresponding LC-MS/MS tandem mass spectrum of authentic MFA-SG standard. D) The chemical structure and origins of the characteristic fragment ions for MFA-SG are as shown.

FIG. 5. A) Representative reverse-phase gradient LC-MS/MS MRM chromatograms of MFA-1-β-O-G from the analysis of extracts from rat hepatocytes incubated for 10-min with 100 μM MFA (solid line) and extract from rat hepatocytes spiked with MFA-1-β-O-G authentic standard (1000 nM, dashed line). The LC-MS/MS MRM transition used for the analysis of MFA-1-β-O-G was MH⁺ m/z 418 to m/z 224. B) LC-MS/MS tandem mass spectrum of MFA-1-β-O-G formed in rat hepatocyte incubations with MFA (100 μM, 10-min) obtained by CID of the protonated molecular ion at m/z 418. C) Corresponding LC-MS/MS tandem mass spectrum of authentic MFA-1-β-O-G standard. D) The chemical structure and origins of the characteristic fragment ions for MFA-1-β-O-G are as shown.

FIG. 6. A) Time-course for the formation of MFA-SCoA (open squares), MFA-SG (closed circles), and MFA-1-β-O-G (open triangles) in rat hepatocytes (2 million cells/ml) incubated with 100 μM MFA. B) MFA concentration-dependent formation of MFA-SCoA, MFA-SG and MFA-1-β-O-G in incubation with rat hepatocytes for 10-min. Values are expressed as the
mean±SD from three separate experiments and $n=3$ incubations/time-point and MFA concentration.

**FIG. 7.** Time-course for the formation of MFA-SG (closed circles), and MFA-1-$\beta$-O-G (open triangles) in human hepatocytes (single donor, 2 million cells/ml) incubated with 100 $\mu$M MFA. Values are expressed as the mean±SD from a single experiment and $n=3$ incubations/time-point. MFA-SCoA was not detected in extracts from human hepatocyte incubations with MFA.

**FIG. 8.** Time-dependent degradation of 1 $\mu$M MFA-SG, MFA-1-$\beta$-O-G, I-SG, and D-SG in incubations with rat hepatocytes. Values are expressed as the average of two incubations/time-point.

**FIG. 9.** A) Time-dependent formation of MFA-SG and I-SG obtained from separate incubations of MFA-SCoA (1 $\mu$M), MFA-1-$\beta$-O-G (1 $\mu$M), and I-SCoA (1 $\mu$M) with GSH (10 mM) in potassium phosphate buffer (0.1 M, pH 7.4, 37°C). B) Time-dependent degradation of MFA-1-$\beta$-O-G and MFA-SCoA in corresponding incubations in buffer in the absence of GSH. Values are expressed as the mean±SD from three incubations/time-point.

**FIG. 10.** Time-dependent degradation of MFA-SG, I-SG, and D-SG (1 $\mu$M) in potassium phosphate buffer (0.1 M, pH 7.4, 37°C) in the presence or absence of GST (3 units/ml). Values are expressed as the average of two incubations/time-point.
Table 1. Effect of inhibitors of MFA-1-β-O-G and MFA-SCoA formation on the production of MFA-SG in incubations of MFA (100 µM) with rat hepatocytes (2 million cells/ml, 10-min incubation time-period). Values are expressed as the mean±SD from triplicate incubations.

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Fig. 1
Fig. 2
Fig. 3

A) Chromatogram showing the retention time (min) and relative abundance of the compounds.

B) Mass spectrum at m/z range showing peak at 531.

C) Mass spectrum at m/z range showing peaks at 224, 384, 456, 514.

D) Structural formula of Mefenamyl-S-acyl-glutathione with its molecular formula (MH⁺ m/z 531).
Fig. 4
Fig. 5
Fig. 6
Fig. 7
Fig. 9