DMD Fast Forward. Published on August 23, 2013 as DOI: 10.1124/dmd.113.053223
This article has not been copyedited and formatted. The final version may differ from this version.

DMD Fast Forward. Published on August 23, 2013 as doi:10.1124/dmd.113.053223

The non-enzymatic reactivity of the acyl-linked metabolites of mefenamic acid towards amino and thiol functional group bionucleophiles

Howard Horng and Leslie Z. Benet

Department of Bioengineering and Therapeutic Sciences, University of California, San Francisco

DMD Fast Forward. Published on August 23, 2013 as DOI: 10.1124/dmd.113.053223
This article has not been copyedited and formatted. The final version may differ from this version.

Running title: Reactivity of the acyl-linked metabolites of mefenamic acid

Address correspondence to:

Leslie Z. Benet, Ph.D.

University of California, San Francisco

Department of Bioengineering and Therapeutic Sciences

533 Parnassus Ave., Room U-68

San Francisco, CA 94143-0912

Telephone: 415-476-3853 Fax: 415-476-8887 Email: Leslie.Benet@ucsf.edu

Number of text pages: 22

Number of tables: 1

Number of figures: 8

Number of references: 44

Number of words in Abstract: 250

Number of words in Introduction: 657

Number of words in Discussion: 1498

Non-standard abbreviations used are:

ACN, acetonitrile; ACS, acyl-CoA synthetase; AMP, adenosine monophosphate; ATP, adenosine triphosphate; CA, cholic acid; CBZ, carbamazepine; CoA, coenzyme A; DMSO, dimethylsulfoxide; ECF, ethyl chloroformate; ESI, electrospray ionization; Gly, glycine; GSH, L-glutathione; GST, glutathione-*S*-transferase; HPLC, high performance liquid chromatography; IS, internal standard; Kpi, potassium phosphate buffer; LC-MS/MS, liquid chromatography mass spectrometry; MFA, mefenamic acid; MFA-AMP, mefenamic acid-acyl-adenylate; MFA-CoA, mefenamic acid-*S*-acyl-Coenzyme A; MFA-Gly, mefenamic acid-glycine; MFA-GSH, mefenamic acid-*S*-acyl-glutathione; MFA-1-*O*-G, mefenamic acid-1-*O*-acyl-glucuronide; MFA-Tau, mefenamic acid-taurine; MFA-NAC, mefenamic acid- *N*-acetylcysteine; SRM, single reaction monitoring; NAC, *N*-acetylcysteine; NSAID, nonsteroidal anti-inflammatory drug; Tau, taurine; TEA, triethylamine; THF, tetrahydrofuran; Tol, tolmetin; UGT, uridine 5'-diphospho-glucuronosyltransferase; ZP, zomepirac

Abstract:

Mefenamic acid, (MFA), a carboxylic acid-containing nonsteroidal anti-inflammatory drug (NSAID) is metabolized into the chemically-reactive, MFA-1-*O*-acyl-glucuronide (MFA-1-*O*-G), MFA-acyl-adenylate (MFA-AMP), and the MFA-*S*-acyl-CoA (MFA-CoA), all of which are electrophilic and capable of acylating nucleophilic sites on biomolecules. In this study, we investigate the non-enzymatic ability of each MFA acyl-linked metabolite to transacylate amino and thiol functional groups on the acceptor biomolecules glycine (Gly), taurine (Tau), glutathione (GSH), and *N*-acetylcysteine (NAC). In vitro incubations with each of the MFA acyl-linked metabolites (1 µM) in buffer under physiological conditions with Gly, Tau, GSH, or NAC (10 mM) revealed that MFA-CoA was 11.5- and 19.5-fold more reactive than MFA-AMP towards the acylation of cysteine-sulfhydryl groups of GSH and NAC, respectively. However, MFA-AMP was more reactive towards both Gly and Tau, 17.5-fold more reactive towards the *N*-acyl-amidation of taurine than its corresponding CoA thioester, while MFA-CoA displayed little reactivity towards glycine. Additionally, MFA-GSH was 5.6- and 108-fold more reactive towards NAC than MFA-CoA and MFA-AMP, respectively. In comparison to MFA-AMP and MFA-CoA, MFA-1-*O*-G was not significantly reactive towards all four bionucleophiles. MFA-AMP, MFA-CoA, MFA-1-*O*-G, MFA-GSH, and MFA-Tau were also detected in rat in vitro hepatocyte MFA (100 µM) incubations while MFA-Gly was not. These results demonstrate that MFA-AMP selectively reacts nonenzymatically with the amino functional groups of glycine and lysine, MFA-CoA selectively reacts nonenzymatically with the thiol functional groups of GSH and NAC, and MFA-GSH reacts nonenzymatically with the thiol functional group of GSH, all of which may potentially elicit an idiosyncratic toxicity in vivo.

Introduction

Mefenamic acid (MFA), (2',3')-dimethyl-*N*-phenyl-anthranilic acid, is a carboxylic acid-containing nonsterodial anti-inflammatory drug (NSAID) associated with a rare, but sometimes serious idiosyncratic nephrotoxicity (Robertson et al., 1980; Drury et al., 1981; Woods, 1981; Taha et al., 1985) and possibly hepatotoxicity (Somchit et al., 2004). A proposed mechanism for the occurrence of these MFA induced toxicities suggests that MFA undergoes bioactivation into chemically-reactive acyl-linked metabolites that ultimately become covalently bound to tissue proteins resulting in adverse immunological responses (Figure 1). MFA is metabolized to 3-hydroxy-MFA (Glazko, 1966) and 3-carboxy-MFA (Sato et al., 1993) via cytochrome P450 2C9. MFA also undergoes glucuronidation via uridine 5'-diphospho-glucuronosyltransferase (UGT) into the unstable, reactive acyl glucuronide metabolite, MFA-1-*O*-acyl-glucuronide (MFA-1-*O*-G) (Somchit et al., 2004; McGurk et al, 1996). Acyl glucuronides of acidic drugs are proposed to bind covalently to protein via a direct transacylation reaction in which protein nucleophiles react with the facile carbonyl-carbon of the acyl glucuronide, resulting in the liberation of the glucuronic acid, and via the formation of a drug-protein conjugate or a glycation mechanism involving prior acyl migration of the drug on the glucuronic acid moiety permitting ring opening of the sugar resulting in an exposed reactive aldehyde group that reversibly forms an imine (Schiff's base) with an amine group on proteins. Subsequent Amadori rearrangement results in a stable ketoamine derivative in which both the drug and the glucuronic acid moiety become covalently bound onto the protein (Benet et al., 1993). MFA is also metabolized into the reactive MFA-acyl-adenylate (MFA-AMP) and MFA-*S*-acyl-CoA (MFA-CoA) via acyl-CoA synthetase (ACS), both of which have been demonstrated to form glutathione (GSH)-adducts and are proposed to play a role in MFA mediated idiosyncratic toxicity (Grillo et al., 2012; Horng and

Benet, 2013). This pathway occurs when the adenosine monophosphate (AMP) moiety of ATP is covalently transferred to the carboxyl group of MFA to form MFA-AMP, followed by the displacement of the AMP with coenzyme A (CoA) to form MFA-CoA thioesters (Kelley and Vessey, 1994; Mano et al., 2001). Upon their formation, the carbonyl carbon of both MFA-AMP and MFA-CoA increase in electrophilicity enabling them to transacylate the biological nucleophile GSH (Horng and Benet, 2013). It is proposed that these drug-protein adducts could act as haptens and are recognized by the immune system as foreign, illiciting an autoimmune type response resulting in the associated idiosyncratic toxicity (Uetrecht, 2007). Previous in vitro incubations with the model nucleophile glutathione (GSH) under physiological conditions showed MFA-AMP to be reactive towards GSH, but 11-fold less reactive than MFA-CoA, while MFA-1-*O*-G exhibited little GSH reactivity (Horng and Benet, 2013). In vitro rat hepatocyte incubations have also resulted in the detection of MFA-AMP, MFA-CoA, MFA-1-*O*-G, and MFA-GSH (Horng and Benet, 2013), all of which could be more reactive than MFA per se and potentially involved in the formation of drug-protein adducts. Additionally, studies involving the acyl-linked metabolites of the bile acid, cholic acid (CA), revealed that CA-AMP selectively reacts non-enzymatically with amino functional groups while CA-CoA preferentially reacts nonenzymatically with thiol functional groups (Mitamura et al., 2011).

The following experiments were designed to characterize the non-enzymatic acylation of the nucleophilic biomolecules containing the amino functional groups of glycine (Gly) and taurine (Tau) and the thiol functional groups of GSH and *N*-acetylcysteine (NAC) by MFA-AMP, MFA-CoA, MFA-1-*O*-G, and MFA-GSH (NAC only) as well as the detection of these adducts in rat hepatocyte in vitro incubations. We propose that MFA-AMP, MFA-CoA, and MFA-1-*O*-G are all selective towards their acylation of nucleophilic functional groups on biological molecules, all of which can contribute to the formation of MFA adducts with amino acids, peptides, and proteins. We also hypothesize that MFA-GSH is reactive in its own right due to its structural similarity to MFA-CoA via the thioester bond. If this proposal is correct, the reactive acyl-linked metabolites of MFA would be anticipated to be selective in their formation of drug-protein adducts in vivo, which may potentially mediate the idiosyncratic toxicities associated with MFA and other carboxylic acid-containing drugs.

Materials and Methods

Materials

MFA, AMP, CoA, anhydrous tetrahydrofuran (THF), triethylamine (TEA), ethyl chloroformate (ECF), *N,N'*-dicyclohexylcarbodiimide, pyridine, potassium phosphate, potassium carbonate, dimethyl sulfoxide (DMSO), carbamazepine (CBZ), L-glutathione (GSH), Gly, NAC, and Tau were all purchased from Sigma-Aldrich Chemical Co (St. Louis, MO). Acetonitrile (ACN), methanol, acetone, ammonium acetate, and ethyl acetate were all purchased from Fisher Scientific (Pittsburgh, PA). MFA-1-*O*-G was purchased from Toronto Research Chemicals (TRC) Inc. (North York, Ontario). All solvents used for HPLC and LC-MS/MS analysis were of chromatographic grade. Williams Medium E and L-glutamine were purchased from Gibco (Grand Island, NY). Male Sprague-Dawley rats were purchased from Charles River (Wilmington, MA). Stock solutions of MFA-AMP, MFA-CoA, MFA-GSH, MFA-1-*O*-G, MFA-Gly, MFA-Tau, and MFA-NAC were all prepared as 1 mM solutions in DMSO.

Instrumentation and Analytical Methods

HPLC/UV analysis was performed on a Hewlett Packard 1100 series binary pump HPLC system (Santa Clara, CA) coupled to a Hewlett Packard 1100 UV-Vis detector, utilizing HP Chemstation software for the acquisition of all HPLC/UV data. LC-MS/MS analyses of synthetic standards and in vitro samples were performed on a Shimadzu LC-20AD (Kyoto, Japan) HPLC system coupled to an Applied Biosystem/MDS Sciex API (Framingham, MA) 4000 triple quadrupole mass spectrometer outfitted with a Turbo V ion source using positive ionization mode. All LC-MS/MS analyses were performed on a reverse phase column (XTerra

C-18, 5.0 µm, 4.6 x 150 mm, Milford, MA). The detection of MFA, MFA-AMP, MFA-CoA, MFA-1-*O*-G, MFA-GSH, MFA-Gly, MFA-Tau, and MFA-NAC were obtained using electrospray (ESI) positive ionization and a gradient system of either aqueous ammonium acetate (10 mM, pH 5.6) and acetonitrile (MFA-CoA) or aqueous solution (0.1% formic acid) and acetonitrile (0.1% formic acid) (MFA, MFA-AMP, MFA-1-*O*-G, MFA-GSH, MFA-Gly, MFA-Tau, and MFA-NAC), 5% ACN to 100%, over 15 min at a flow rate of 0.5 ml/min. The high pH and ion strength afforded by the aqueous ammonium acetate is necessary to elute MFA-CoA from the column. Electrospray positive ionization was employed with a needle potential held at 5.5 kV. MS/MS tandem conditions utilized 2 mTorr argon collision gas and a collision potential of 89 eV. Mass spectral data were acquired with Analyst software (version 1.5.2, AB Sciex, Framingham, MA).

Synthesis of MFA-AMP, MFA-Gly, MFA-NAC, and MFA-Tau Derivatives

The synthesis of MFA-AMP, MFA-Gly, MFA-NAC, and MFA-Tau was carried out with a solution consisting of 110 mg *N,N'*-dicyclohexylcarbodiimide in 0.4 ml pyridine (Ikegawa et al., 1999; Horng and Benet, 2013). Briefly, an *N,N'*-dicyclohexylcarbodiimide solution was added to a solution containing MFA (0.49 mmol), and either AMP, Gly, Tau, or NAC (0.49 mmol) separately in 75% pyridine/25% water. The reaction mixture was stirred at 4^{o}C for 7 hr and then centrifuged at 3000 rpm for 5 min to remove any *N*-acylurea derivatives. The supernatant was transferred to another culture tube for precipitation by the addition of acetone (10 ml). The resulting precipitate was isolated by centrifugation at 3000 rpm for 5 min followed by further washes with acetone (10 x 10 ml) and acidified water (pH 4-5) (10 x 10 ml). For MFA-AMP, the precipitate was dissolved in 0.1 M potassium phosphate buffer (pH 6) and underwent continued liquid-liquid washes with ethyl acetate (10 x 10 ml). Following precipitation via 1M HCl, the MFA-AMP was further washed with acetone (10 x 10 ml). The MFA-AMP precipitate was blown down to dryness using N_2 gas and weighed out for preparation of a 1 mM MFA-AMP solution in DMSO. For MFA-Gly, MFA-NAC, and MFA-Tau, the initial acetone derived precipitate was dissolved in DMSO and subjected to purification via HPLC/UV-mass spectrometry. The correct HPLC eluent fractions, as determined by UV-MS, of each acyl-linked metabolite were collected, blown down to dryness, weighed, and then prepared as 1 mM solutions in DMSO. MFA-AMP eluted at a retention time of 7.6 min and showed no impurities when analyzed by HPLC/UV (wavelengths: 220, 254, 262, and 280 nm) and LC-MS via reverse-phase gradient elution (as described above), and ^{1}H NMR (Horng and Benet, 2013). Tandem LC-MS/MS analysis of MFA-AMP revealed (CID of MH^+ ion at m/z 571), *m/z* (%) yielded: *m/z* 224($[M + H – AMP]^+$, 100%), *m/z* 207 ($[M + H –364]^+$, 25%), and *m/z* 136($[M + H - adenine]^+$, 28%). MFA-Gly eluted at a retention time of 8.7 min (Figure 2C) and showed no impurities when analyzed by HPLC/UV (wavelengths: 220, 254, 262, and 280 nm) and LC-MS via reverse-phase gradient elution (as described above). Tandem LC-MS/MS analysis of MFA-Gly (CID of MH^+ ion at *m/z* 299), *m/z* (%) : *m/z* 224($[M + H - Gly]^+$, 99%), *m/z* 209 ($[M + H –90]^+$, 20%), *m/z* 180($[M + H – 119]^+$, 18%), *m/z* 152($[M + H –147]^+$, 4%), *m/z* 127($[M + H –172]^+$, 2%), *m/z* 77($[Gly + H]^+$, 1%) (Figure 2A and 2B). MFA-Tau eluted at a retention time of 9.1 min (Figure 3C) and showed no impurities when analyzed by HPLC/UV (wavelengths: 220, 254, 262, and 280 nm) and LC-MS via reverse-phase gradient elution (as described above). Tandem LC-MS/MS analysis of MFA-Tau (CID of MH^+ ion at *m/z* 349), *m/z* (%) : *m/z* 332 ($[M + H – H_2O]^+$, 10%), *m/z* 224($[M + H - Tau]^+$, 99%), *m/z* 209 ($[M + H –140]^+$, 25%), *m/z* 180($[M + H – 169]^+$, 16%), *m/z* 152([M + H –197]$^+$, 4%), and *m/z* 126([Tau + H$^+$]$^+$, 2%) (Figure 3A and 3B). MFA-NAC eluted at a retention time of 9.3 min (Figure 4C) and showed no impurities when analyzed by HPLC/UV (wavelengths: 220, 254, 262, and 280 nm) and LC-MS via reverse-phase gradient elution (as described above). Tandem LC-MS/MS analysis of MFA-NAC (CID of MH$^+$ ion at *m/z* 387), *m/z* (%) : *m/z* 309 ([M + H – 78]$^+$, 30%), *m/z* 224([M + H - NAC]$^+$, 99%), *m/z* 209 ([M + H –178]$^+$, 18%), *m/z* 180([M + H - 207]$^+$, 13%), and *m/z* 165([NAC + H]$^+$, 3%) (Figure 4A and 4B).

Synthesis of MFA-CoA and MFA GSH Thioester Derivatives

The synthesis of MFA-CoA and MFA-GSH thioesters was accomplished by a method employing ECF (Stadtman, 1957; Grillo et al., 2002; Horng and Benet, 2013) as described previously. Briefly, MFA (1.6 mmol) was dissolved in anhydrous THF (25 ml). While stirring at room temperature, TEA (1.6 mmol) was added to the solution followed by the addition of ECF (1.6 mmol). After 30 min, the resulting triethylamine hydrochloride was removed by passing the reaction mixture through a glass funnel fitted with a glass wool plug. The filtered solution was then added to a solution containing CoA (0.13 mmol, 100 mg) or GSH (1 g) and $KHCO_3$ (1.6 mmol) in nanopure water (10 ml) and THF (15 ml). The solution was stirred continuously at room temperature for 2 hr, after which the reaction was terminated by acidification (pH 4-5) through the addition of 1 M HCl. THF was then removed by evaporation under N_2 gas, followed by further solvent washes: acidified water (pH 5) (3 x 10 ml) and ethyl acetate (3 x 10 ml) for MFA-CoA or acetone (3 x 10 ml) for MFA-GSH. MFA-CoA and MFA-GSH precipitate was blown down to dryness using N_2 gas and then weighed out for preparation of a 1 mM MFA-CoA or 1 mM MFA-GSH solution in DMSO. HPLC analysis of MFA-CoA thioester resulted in an elution time of 7.3 min and showed no impurities when analyzed by HPLC/UV (wavelengths: 220, 254, 262, and 280 nm) and LC-MS via reverse-phase gradient elution (as described above). Tandem LC-MS/MS analysis of MFA-CoA standard yielded (CID of MH^+ ion at *m/z* 991), *m/z* (%): *m/z* 582 ([M + H-adenosine diphosphate – $H_2O]^+$, 20%), *m/z* 484([M + H – adenosine triphosphate]$^+$, 94%), *m/z* 428([adenosine diphosphate + $H^+]^+$, 40%), *m/z* 382 ([M + H –609]$^+$, 25%), *m/z* 330([adenosine monophosphate + H – $H_2O]^+$, 3%), *m/z* 224([M + H –CoA]$^+$, 99%). Synthetic MFA-GSH eluted at a retention time of 7.7 min and showed no detectable impurities when analyzed by HPLC/UV (wavelengths: 220, 254, 262, and 280 nm) and LC-MS via reverse-phase gradient elution (as described above). Tandem LC-MS/MS analysis of MFA-GSH standard yielded product in mass spectrum under CID of the protonated molecular ion at MH^+ *m/z* 531, *m/z* (%): *m/z* 456 ([M+H– GSH]$^+$, 10%), *m/z* 384 ([M + H – pyroglutamic acid – water]$^+$, 82%), *m/z* 224 ([MFA + H – $H_2O]^+$, 73%).

Stability and Reactivity Incubation Conditions and Quantitative Analysis of Reaction Products

Chemical stability was assessed by incubating MFA-AMP, MFA-CoA, MFA-GSH, MFA-Gly, MFA-Tau, and MFA-NAC (1 µM) with CBZ (internal standard) in 0.1 M potassium phosphate buffer (Kpi) (pH 7.4) in 2 ml HPLC vials (n=3). Each solution was then placed into an HPLC autosampler warmed to 37°C and injections were taken every 15 min for 3 or 24 hrs for LC-MS/MS analysis to determine each metabolite’s chemical stability. The stability of each sample was determined by comparing the analyte peak area to peak area ratios of CBZ, which we previously found to be stable for at least 72 hours (data not shown). Chemical reactivity experiments for MFA-AMP, MFA-CoA, and MFA-1-*O*-G were performed by incubating each acyl-linked metabolite (1 µM) separately in 0.1 M Kpi (pH 7.4) containing Gly, Tau, GSH, or NAC and MFA-GSH with NAC (10 mM) (n=3) at 37^{o}C in screw-capped glass vials in a shaking incubator (Figure 5). Aliquots (100 µl) of the incubation mixture were taken at 0, 2, 5, 10, 30, and 60 min and quenched with 1 µM CBZ/ACN solution and then injected onto the column for LC-MS/MS analyses. Quantitative measurements were performed by plotting peak area ratios of MFA-GSH, MFA-Gly, MFA-Tau, or MFA-NAC to CBZ versus the concentration of each acyl-linked MFA metabolite.

In Vitro Studies with Rat Hepatocytes

Freshly isolated rat (250-300 g, male Sprague-Dawley) hepatocytes were prepared according to the method of Moldeus et al. (1978) and greater than 85% viability was achieved routinely as determined by trypan blue exclusion testing. Incubations of hepatocytes (2 million viable cells/mL) with MFA (100 µM) were performed in Williams Medium E fortified with L-glutamine (4 mM) in a 50 ml round bottom flask. Incubations (n=3) were performed with continuous rotation and gassed with 95% O_2/5% CO_2 at 37^{o}C. Aliquots were taken at 0, 0.2, 0.5, 1, 2, 4, 8, 10, 20, 30, and 60 min and analyzed for MFA-AMP, MFA-CoA, MFA-GSH, MFA-1-*O*-G, MFA-Gly, MFA-Tau, and MFA-NAC by LC-MS/MS.

For the analyses of MFA-AMP, MFA-GSH, MFA-1-*O*-G, MFA-Gly, MFA-Tau, and MFA-NAC, aliquots (200 µl) of the incubation mixture were added directly into microcentrifuge tubes

(2 ml) followed by quenching with a solution of ACN containing 3% formic acid/2 μM CBZ (200 μl). Samples were then centrifuged (14,000 rpm, 5 min) and the supernatant fractions (200 μl) were transferred to HPLC autosampler vials for LC-MS/MS analysis.

For the analyses of MFA-CoA formation, aliquots (200 μl) from the same incubations were transferred directly into microcentrifuge tubes and quenched with a solution of ACN/ 2 μM CBZ (400 μl) followed by the addition of hexane (600 μl). The samples were vortexed (1 min), centrifuged (14,000 rpm, 5 min), and aliquots (300 μl) of the aqueous layer were transferred to an HPLC autosampler vial followed by a 1 hr evaporation of residual hexane under the fume hood. Samples were then analyzed by LC-MS/MS.

Identification and Quantification of MFA-AMP, MFA-CoA, MFA-GSH, and MFA-1-*O*-G

MFA treated rat hepatocyte extracts were analyzed by LC-MS/MS for MFA-AMP, MFA-CoA, MFA-GSH, and MFA-1-*O*-G as previously described (Horng and Benet, 2013). Single Reaction Monitoring (SRM) in positive ionization mode with the chromatographic conditions described above were used for the quantitation with the following mass transitions: MH^+ *m/z* 571 to *m/z* 224 (MFA-AMP), MH^+ *m/z* 991 to *m/z* 224 (MFA-CoA), MH^+ *m/z* 531 to *m/z* 224 (MFA-GSH), MH^+ *m/z* 418 to *m/z* 224 (MFA-1-*O*-G) and MH^+ *m/z* 237 to *m/z* 194 for CBZ. The elution times of each acyl-linked metabolite were as follows: 7.6 min (MFA-AMP), 7.3 min (MFA-CoA), 7.7 min (MFA-GSH), 7.9 min (MFA-1-*O*-G), and 9.3 min (CBZ). No chromatographic peaks corresponding to each acyl-linked metabolite were detected in blank incubation extracts lacking

MFA. The concentration of each MFA-acyl-linked metabolite was determined by plotting peak area ratios of each metabolite to CBZ versus the concentration.

Identification and Quantification of MFA-Gly

The identification and quantitation of MFA-Gly by LC-MS/MS was carried out by single reaction monitoring (SRM) using the mass transitions MH^+ *m/z* 299 to *m/z* 224 and MH^+ *m/z* 237 to *m/z* 194 for CBZ detection using ESI positive ionization mode and the chromatographic methods described above. The elution times of 8.7 min (Figure 2C) and 9.3 min were obtained for authentic MFA-Gly and CBZ, respectively. No chromatographic peaks corresponding to MFA-Gly were detected in blank incubation extracts lacking MFA. The concentration of MFA-Gly was determined by plotting peak area ratios of MFA-Gly to CBZ versus the concentration of MFA-Gly.

Identification and Quantification of MFA-Tau

The identification and quantitation of MFA-Tau by LC-MS/MS was carried out by SRM using the mass transitions MH^+ *m/z* 349 to *m/z* 224 and MH^+ *m/z* 237 to *m/z* 194 for CBZ detection using ESI positive ionization mode and the chromatographic methods described above. The elution times of 9.1 min (Figure 3C) and 9.3 min were obtained for authentic MFA-Tau and CBZ, respectively. No chromatographic peaks corresponding to MFA-Tau were detected in blank incubation extracts lacking MFA. The concentration of MFA-Tau was determined by plotting peak area ratios of MFA-Tau to CBZ versus the concentration of MFA-Tau.

Identification and Quantification of MFA-NAC

The identification and quantitation of MFA-NAC by LC-MS/MS was carried out by SRM using the mass transitions MH^+ *m/z* 387 to *m/z* 224 and MH^+ *m/z* 237 to *m/z* 194 for CBZ detection using ESI positive ionization mode and the chromatographic methods described above. The elution times of 9.25 min (Figure 4C) and 9.3 min were obtained for authentic MFA-NAC and CBZ, respectively. No chromatographic peaks corresponding to MFA-NAC were detected in blank incubation extracts lacking MFA. The concentration of MFA-NAC was determined by plotting peak area ratios of MFA-NAC to CBZ versus the concentration of MFA-NAC.

Results

Identification of MFA-AMP, MFA-CoA, MFA-1-*O*-G, and MFA-GSH

Analysis of rat hepatocyte extracts incubated with MFA by LC-MS/MS detection allowed for the identification of MFA-AMP, MFA-CoA, MFA-1-*O*-G, and MFA-GSH formed in incubations with MFA, as previously described (Horng and Benet, 2013). MFA-AMP, MFA-CoA, MFA-GSH, and MFA-1-*O*-G formed in rat hepatocyte extracts and authentic standards coeluted at retention times of 7.6, 7.3, 7.7, and 7.9 min, respectively, and the product ion spectrum of each conjugate was consistent with its chemical structure and identical to its corresponding authentic standard.

Identification of MFA-Tau

Analysis of rat hepatocyte extracts incubated with MFA by LC-MS/MS detection allowed for the identification of MFA-Tau formed in incubations with MFA. MFA-Tau formed in rat hepatocyte extracts and authentic standard coeluted at a retention time of 9.1 min (Figure 3C) and the product ion spectrum of the conjugate was consistent with its chemical structure and identical to its corresponding authentic standard. MFA-Gly and MFA-NAC were not detected in MFA rat hepatocyte incubations.

Chemical Stability of MFA-CoA, MFA-AMP, MFA-GSH, MFA-NAC, MFA-Gly, and MFA-Tau in Buffer

In vitro incubation of each acyl-linked metabolite of MFA in Kpi under physiological conditions (pH 7.4, 37°C) revealed that MFA-AMP, MFA-CoA, and MFA-GSH were chemically stable for at least 24 hr while MFA-NAC, MFA-Gly, and MFA-Tau were chemically stable with no detectable hydrolysis for at least 3 hr of incubation (data not shown). Previous studies carried out under the same conditions have shown MFA-1-*O*-G possesses a half life of degradation of ~16 h in buffer under physiological conditions (McGurk et al., 1996; Grillo et al., 2012).

Chemical Reactivity of MFA-CoA, MFA-AMP, MFA-1-*O*-G, and MFA-GSH with Gly, Tau, GSH, and NAC

Incubation of MFA-AMP (1 µM) with Gly and Tau (10 mM) in Kpi (0.1 M) under physiological conditions (i.e., 37°C, pH 7.4) resulted in the *N*-amidation of both glycine and taurine, producing 23.2±4.2 nM and 20.1±1.8 nM of MFA-Gly and MFA-Tau conjugates, respectively, after 60 min of incubation (Figure 6A and 6B). Incubations of MFA-CoA (1 µM) with glycine and taurine (10 mM) resulted in minimal *N*-amidation, producing no MFA-Gly (limit of detection~ 0.5 nM for all MFA-conjugates) and 0.93±0.65 nM of MFA-Tau at the 60 min time point. MFA-1-*O*-G (1 µM) exhibited no reactivity towards both Gly and Tau. In vitro GSH (10 mM) reactivity studies with MFA-AMP, MFA-CoA, and MFA-1-*O*-G (1 µM) resulted in 12.8±1.0 nM, 145±40 nM, and 1.3±0.97 nM of MFA-GSH formation, respectively at the 60 min time point (Figure 7A). The incubation of NAC (10 mM) with MFA-AMP, MFA-CoA, and MFA-GSH (1 µM) under physiological conditions resulted in the formation of 7.45±4.8 nM, 141±4.9 nM, and 780±26 nM of MFA-NAC conjugates, respectively at 60 min (Figure 7B). While MFA-1-*O*-G continued to show no reactivity towards the nucleophile NAC.

Time course of Formation of MFA-AMP, MFA-Tau, and MFA-Gly in Rat Hepatocyte Incubations

The incubation of MFA (100 μM) in rat hepatocytes under physiological conditions (37°C, pH 7.4) resulted in the detection of MFA-AMP, MFA-CoA, MFA-GSH, MFA-1-*O*-G, and MFA-Tau. The initial rise of MFA-AMP formation was very rapid attaining a concentration of 107.7 nM at ~30 sec (Figure 8A). MFA-CoA was not detectable until the 4 min time point, reaching a concentration of 45.6 nM at 60 min. MFA-Tau levels were undetectable until 4 min, reaching a concentration of 15.7 nM at 60 min. The formation of MFA-GSH was linear, reaching a concentration of 2.1 μM at 60 min, while the formation of MFA-1-*O*-G increased to a concentration of 30.0 μM at 60 min (Figure 8B). MFA-Gly and MFA-NAC conjugates were undetectable during the 60 min incubation period.

Discussion

Carboxylic acid-containing drugs are metabolized into reactive electrophilic acyl-linked metabolites that can form irreversible adducts with proteins, potentially causing an allergic reaction in hypersensitive individuals (Stogniew and Fenselau, 1982). MFA is an NSAID prescribed for its analgesic and anti-inflammatory activity via inhibition of cyclooxygenase-dependent prostanoid formation (Hawkey, 1999). Commonly used to treat pain, MFA has been implicated in several cases of hepatic and renal disturbances and hypersensitivity reactions (Handisurya et al., 2011). These toxicities are proposed to occur via bioactivation of MFA into reactive acyl-linked metabolites covalently binding onto macromolecules. MFA undergoes conjugation via the free carboxyl group into the 1-*O*-acyl glucuronide, MFA-1-*O*-G (Glazko, 1966; Sato et al., 1993), which has been shown to irreversibly bind to albumin (McGurk et al., 1996). MFA also undergoes further conjugation into MFA-AMP, MFA-CoA and MFA-GSH (Grillo et al., 2012; Horng and Benet, 2013) in rat hepatocytes, all of which possess an increased chemical electrophilicity and are reactive towards protein nucleophiles.

Electrophilicity assessment of metabolites in drug development involve screens utilizing nucleophilic trapping agents. Glutathione is a commonly used biomarker of reactivity for bioactivation studies. Presumably, the greater the in vitro nucleophilic adduct formation, the greater the probability it will covalently bind onto proteins and elicit a toxic reaction. However, not all protein covalent binding result in the onset of toxicity, and thus the challenge is to identify those protein targets that are critical for the onset of a drug induced toxicity. In addition to thioesters (Van Breemen and Fenselau, 1985), acyl-linked metabolites have also been shown to react with proteins via oxygen ester (Wells and Janssen, 1987) and amide-linkage (van Breemen and Fenselau, 1986; Mitamura et al., 2011). In the present study, we investigate the selective non-enzymatic acylation of amino and thiol functional groups of four biological nucleophiles: Gly, Tau, GSH, and NAC.

In vitro GSH reactivity assessment of MFA-AMP, MFA-CoA, and MFA-1-*O*-G revealed MFA-CoA to be 11.5-fold more reactive than MFA-AMP toward the thiol functional group of GSH (Figure 7A), consistent with our previous studies (Horng and Benet, 2013), while MFA-CoA was 19.5-fold more reactive toward the thiol groups of NAC than its corresponding MFA-AMP (Figure 7B). Alternatively, incubations with Gly and Tau revealed that MFA-AMP is more reactive towards *N*-acyl-amidation than its corresponding MFA-CoA, producing a significant amount of MFA-Gly while MFA-CoA did not react with Gly (Figure 6A). The amidation of Tau by MFA-AMP was also 17.5-fold greater than MFA-CoA (Figure 6B). MFA-1-*O*-G exhibited little to no reactivity towards all four bionucleophiles. Reactivity data were linear during the incubation and therefore the 60 min time point was used to calculate the slope of conjugate formation (Table 1). Reactivity studies utilizing cholic acid have also demonstrated a greater reactivity of CA-AMP compared to CA-CoA towards the *N*-acyl-amidation of glycine and taurine, while CA-CoA exhibited a greater reactivity towards the acylation of the cysteine-sulfhydryl group of GSH and NAC (Mitamura et al., 2011). Studies in buffer also show that acyl-adenylates can spontaneously react with the amino groups of substance P (Goto et al., 2001), lysozomes (Goto et al., 2005), and histones (Mano et al., 2004), further suggesting that acyl-adenylates have a high reactive affinity towards amino functional groups, and that acyl-AMPs and acyl-CoAs are selective in their non-enzymatic acylation of amino versus thiol functional groups. This selectivity may be attributed to differences in the degree of electrophilicity of MFA-AMP versus MFA-CoA and the nucleophilicity of the amino versus the thiol functional groups, suggesting different reactivity mechanisms towards protein nucleophilic sites.

Acyl-GSH conjugates share a common structural moiety, thioester, to that of the acyl-CoA. Therefore, it is conceivable that MFA-GSH is just as, if not more reactive than its corresponding acyl-CoA derivative. NAC reactivity assessments show that MFA-GSH is indeed highly reactive, exhibiting a 5.5-fold and 108-fold greater reactivity towards NAC than MFA-CoA and MFA-AMP, respectively (Figure 7B). Grillo and Benet (2002) have demonstrated that the reactive potential of *S*-acyl-glutathione conjugates with NAC correlates to the degree of α-carbon substitution of the acyl-linkage, increasing α-carbon substitution results in decreasing reactivity, which is in agreement with the relative degradation rates associated with acyl glucuronides and assumed to be identical for that of their respective *S*-acyl-CoA thioesters. Therefore, if MFA toxicity is the result of covalent binding by reactive intermediates, then it is conceivable that the unusually high formation of MFA-GSH in rat hepatocytes (Grillo et al., 2012; Horng and Benet, 2013), compared to diclofenac (Grillo et al., 2003), zomepirac (Olsen et al., 2005), (R)-ibuprofen (Grillo and Hua, 2008), and (R)-flunoxaprofen (Grillo et al., 2010), may be responsible for the tissue injury associated with MFA. This assumption is based on the high covalent binding values in animals dosed with drugs known to cause hepatotoxicity in humans, such as isoniazid (Nelson et al., 1978) and acetaminophen (Matthews, 1997).

Acyl-CoA thioester synthesis is a two step reaction. This reaction occurs when the AMP moiety of ATP is transferred to the acyl group of the carboxylic acid via ACS forming an acyl-adenylate intermediate. The enzyme bound activated intermediate is then displaced by coenzyme A to yield the associated acyl-CoA product and free AMP (Vlahcevic et al., 1999). Acyl-CoA and acyl-AMP metabolites both spontaneously and enzymatically, via glutathione-*S*-transferase, form GSH-conjugates (Li et al.,2002; Grillo et al., 2012; Horng and Benet, 2013). In addition to GSH, glycine (Keller and Keller, 1842) and taurine (James et al., 1971; Hutson and Casida, 1978) are two of the most commonly cited metabolic amino acid conjugation reactions. Amino acid conjugation occurs through the transfer of the acyl group from the acyl-CoA to an amino acid via *N*-acetyltransferase. However, previous and our current studies have shown that acyl-adenylates are capable of spontaneously reacting to both Gly and Tau (Mitamura et al., 2011) suggesting that acyl-adenylate intermediates have a greater inherent chemical affinity towards amino groups than both the acyl-CoAs and acyl-1-*O*-G. Rat hepatocyte MFA incubation resulted in rapid MFA-AMP formation, (Cmax 107 nM at ~30 sec) while MFA-CoA was undetectable until 4 mins, achieving a concentration of 45.6 nM at 60 min (Figure 8A). This sequence is in agreement with the acyl-CoA biosynthetic pathway. MFA-1-*O*-G and MFA-GSH were also shown to increase linearly, achieving concentrations of 30.0 μM and 2.1 μM at 60 min, respectively (Figure 8B). Our experiments also revealed the presence of MFA-Tau, undetectable until 4 min, reaching a concentration of 15.7 nM at 60 min. It was not determined if the formed MFA-Tau primarily occurs nonenzymatically from MFA-AMP or enzymatically via the MFA-CoA thioester. MFA-Gly was not detected in these incubations, possibly due to an insufficiency in analytical sensitivity (limit of detection for all MFA-conjugates was ~0.5 nM). Previous zomepirac (ZP) rat hepatocyte incubations revealed the presence of ZP-CoA , ZP-1-*O*-G, ZP-

Tau, and ZP-Gly (Olsen et al., 2005). The high concentration of ZP-1-*O*-G compared to ZP-Gly and ZP-Tau may be reflective of dose, a determinant of whether or not a drug undergoes glucuronidation or amino acid conjugation (Hutt and Caldwell, 1990). At lower doses, carboxylic acids tend to undergo amino acid conjugation while at higher doses, glucuronidation dominates. Amino acid conjugation is a high-affinity, low capacity system while glucuronidation is a high-capacity pathway with a broader substrate selectivity. Dose also influences the type of amino acid conjugate formed (Hirom et al., 1977), with dosage increases of phenylacetic acid resulting in decreases in glycine:taurine conjugate ratios. Rat liver experiments with tolmetin (Tol) also identified the acyl-CoA derived conjugates, Tol-Gly and Tol-Tau conjugates in rat urine (Olsen et al., 2003), whose concentrations were unaffected by clofibric acid, confirming a high-affinity/low-capacity metabolic pathway (Hutt and Caldwell, 1990). Oral administration of RS-ibuprofen to humans also led to the detection of ibuprofen-Tau but not the glycine conjugate (Shirley et al., 1994), signifying a minor biotransformation pathway. Gly and Tau conjugates are believed to be formed enzymatically (*N*-acyltransferases) via the acyl-CoA, however we have demonstrated that Gly and Tau conjugates also form spontaneously via acyl-AMP conjugates. MFA-NAC conjugates were not detected in rat hepatocyte incubations, due to the lack of detectable *N*-acylcysteinylglycine and glutamyl transpeptidase (γ-GT) activity in rat livers (Hinchman and Ballatori, 1990). However, in humans, NAC formation would be expected to occur.

In conclusion, MFA-AMP selectively reacts nonenzymatically with the amino functional groups of glycine and lysine, while MFA-CoA selectively reacts nonenzymatically with the thiol functional groups of GSH and NAC. MFA-GSH is also reactive towards NAC, which may be of toxicological significance considering that MFA produces a high amount of GSH adducts in hepatocytes. MFA-1-*O*-G was not chemically reactive toward both amino and thiol functional groups. This preferential reactivity between MFA-AMP and MFA-CoA provides MFA the ability to covalently bind onto a broad range of nucleophilic sites on proteins, which increases its probability of covalently modifying critical protein targets and inducing a toxic reaction. Therefore, acyl-linked metabolites may be important in the formation of drug-protein adducts and the onset of an idiosyncratic toxicity, suggesting that the bioactivation of carboxylic acid-containing drugs into acyl-linked metabolites should be further evaluated to allow for structural modification of drug candidates thereby reducing bioactivation and improving drug safety.

Acknowledgements

We would like to thank Dr. Mark Grillo (Amgen Inc., Department of Pharmacokinetics and Drug Metabolism, South San Francisco, CA) for many helpful discussions during the course of this work and Chris Her (UCSF Liver Center Cell Biology Core) for his assistance in the rat hepatocyte preparations.

Authorship Contributions

Participated in research design: Horng, Benet

Conducted experiments: Horng

Performed data analysis: Horng, Benet

Wrote or contributed to the writing of the manuscript: Horng, Benet

References

Benet LZ, Spahn-Langguth H, Iwakawa S, Volland C, Mizuma T, Mayer S, Mutchler E, and Lin ET (1993) Predictability of the covalent binding of acidic drugs in man. *Life Sci* **53**: PL141-146.

Drury, PL, Asirdas LG, and Bulger GV (1981). Mefenamic acid nephropathy: further evidence. *Br Med J (Clin Res Ed)* **282**: 865-866.

Glazko AJ (1966) Experimental observations on flufenamic, mefenamic and meclofenamic acids metabolic disposition. *Ann Phys Med* Suppl: 23-36.

Goto J, Nagata M, Mano N, Kobayashi N, Ikegawa S, and Kiyonami R (2001) Bile acid acyl adenylate: a possible intermediate to produce a protein-bound bile acid. *Rapid Commun Mass Spectrom* **15**: 104-109.

Goto T, Shibata A, Sasaki D, Suzuki N, Hishinuma T, Kakiyama G, Iida T, Mano N, and Goto J (2005) Identification of a novel conjugate in human urine: bile acid acyl galactosides. *Steroids* **70**: 185-192.

Grillo MP and Benet LZ (2002) Studies on the reactivity of clofibryl-S-acyl-CoA thioester with glutathione in vitro. *Drug Metab Dispos* **30**: 55-62.

Grillo MP and Hua F (2008) Enantioselective formation of ibuprofen-S-acyl-glutathione in vitro in incubations of ibuprofen with rat hepatocytes. *Chem Res Toxicol* **21**: 1749-1759.

Grillo MP, Hua F, Knutson CG, Ware JA, and Li C (2003) Mechanistic studies on the bioactivation of diclofenac: identification of diclofenac-S-acyl-glutathione in vitro in incubations with rat and human hepatocytes. *Chem Res Toxicol* **16**: 1410-1417.

Grillo MP, Tadano Lohr M, and Wait JC (2012) Metabolic activation of mefenamic acid leading to mefenamyl-*S*-acyl-glutathione adduct formation in vitro and in vivo in rat. *Drug Metab Dispos* **40**: 1515-1526.

Grillo MP, Wait JC, Tadano Lohr M, Khera S, and Benet LZ (2010) Stereoselective flunoxaprofen-S-acyl-glutathione thioester formation mediated by acyl-CoA formation in rat hepatocytes. *Drug Metab Dispos* **38**: 133-142.

Handisurya A, Moritz KB, Riedl E, Reinisch C, Stingl G, and Wohrl S (2011) Fixed drug eruption caused by mefenamic acid: a case series and diagnostic algorithms. *J Dtsch Dermatol Ges* **9**: 374-378.

Hawkey C J (1999) COX-2 inhibitors. *Lancet* **353**: 307-314.

Hinchman CA and Ballatori N (1990). Glutathione-degrading capacities of liver and kidney in different species. *Biochem Pharmacol* **40**: 1131-1135.

Hirom PC., Idle JR, Millburn P, and Williams RT (1977) Glutamine conjugation of phenylacetic acid in the ferret. *Biochem Soc Trans* **5**: 1033-5.

Horng H and Benet LZ (2013) Characterization of the acyl-adenylate linked metabolite of mefenamic acid. *Chem Res Toxicol* **26**: 465-476.

Hutson DH and Casida JE (1978) Taurine conjugation in metabolism of 3-phenoxybenzoic acid and the pyrethroid insecticide cypermethrin in mouse. *Xenobiotica* **8**: 565-571.

Hutt, A J and Caldwell J (1990). Amino acid conjugation, in: *Conjugation Reactions in Drug Metabolism* (Mulder G ed.) Taylor & Francis, New York

Ikegawa SH, Ishikawa H, Oiwa H, Nagata M, Goto J, Kozaki T, Gotowda M, and Asakawa N (1999) Characterization of cholyl-adenylate in rat liver microsomes by liquid chromatography/electrospray ionization-mass spectrometry. *Anal Biochem* **266**: 125-132.

James M, Smith RL, and Williams RT (1971) Conjugates of phenylacetic acid with taurine and other amino acids in various species. *Biochem J* **124**: 15P-16P.

Keller W and Keller M (1842) Conversion of benzoic into hippuric acid. *Prov Med J Retrosp Med Sci*, **4**: 256-257.

Kelley M and Vessey DA (1994) Determination of the mechanism of reaction for bile acid: CoA ligase. *Biochem J* **304**: 945-949.

Li C, Benet LZ, and Grillo MP (2002) Studies on the chemical reactivity of 2-phenylpropionic acid 1-O-acyl glucuronide and S-acyl-CoA thioester metabolites. *Chem Res Toxicol* **15**: 1309-1317.

Mano N, Goto T, Uchida M, Nishimura K, Ando M, Kobayashi, N, and Goto J (2004) Presence of protein-bound unconjugated bile acids in the cytoplasmic fraction of rat brain. *J Lipid Res* **45**: 295-300.

Mano N, Uchida M, Okuyama H, Sasaki I, Ikegawa S, and Goto J (2001). Simultaneous detection of cholyl adenylate and coenzyme A thioester utilizing liquid chromatography/electrospray ionization mass spectrometry. *Anal Sci* **17**: 1037-1042.

Matthews A M, Hinson JA, Roberts DW, and Pumford NR (1997). Comparison of covalent binding of acetaminophen and the regioisomer 3'-hydroxyacetanilide to mouse liver protein. *Toxicol Lett* **90**: 77-82.

McGurk KA, Remmel RP, Hosagrahara VP, Tosh D, and Burchell B (1996). Reactivity of mefenamic acid 1-*O*-acyl glucuronide with proteins in vitro and ex vivo. *Drug Metab Dispos* **24**: 842-849.

Mitamura K, Aoyama E, Sakai T, Iida T, Hogmann AF, and Ikegawa S (2011) Characterization of non-enzymatic acylation of amino or thiol groups of bionucleophiles by the acyl-adenylate or acyl-CoA thioester of cholic acid. *Anal Bioanal Chem* **400**: 2253-2259.

Moldeus P, Jones DP, Ormstad K, and Orrenius S (1978). Formation and metabolism of a glutathione-*S*-conjugate in isolated rat liver and kidney cells. *Biochem Biophys Res Commun* **83**: 195-200.

Nelson SD , Mitchell JR, Snodgrass JR, and Timbrell JA (1978). Hepatotoxicity and metabolism of iproniazid and isopropylhydrazine. *J Pharmacol Exp Ther* **206**: 574-585.

Olsen J, Bjornsdottir I, and Honore Hansen S (2003). Identification of coenzyme A-related tolmetin metabolites in rats: relationship with reactive drug metabolites. *Xenobiotica* **33**: 561-570.

Olsen J, Li C, Bjornsdottir I, Sidenius U, Hansen SH, and Benet LZ (2005). In vitro and in vivo studies on acyl-coenzyme A-dependent bioactivation of zomepirac in rats. *Chem Res Toxicol* **18**: 172917-36.

Robertson CE, Ford MJ, Van Someren V, Dlugolecka M, and Prescott LF (1980). Mefenamic acid nephropathy. *Lancet* **2**: 232-233.

Sato J, Yamane Y, Ito K, and Bando H (1993). Structures of mefenamic acid metabolites from human urine. *Biol Pharm Bull* **16**: 811-812.

Shirley MA, Guan X, Kaiser DG, Halstead GW, and Baille TA (1994). Taurine conjugation of ibuprofen in humans and in rat liver in vitro. Relationship to metabolic chiral inversion. *J Pharmacol Exp Ther* **269**: 1166-1175.

Somchit N, Sanat F, Gan EH, Shahrin IA, and Zuraini A (2004). Liver injury induced by the non-steroidal anti-inflammatory drug mefenamic acid. *Singapore Med J* **45**: 530-532.

Stadtman TC and Elliott P (1957). Studies on the enzymic reduction of amino acids. II. Purification and properties of D-proline reductase and a proline racemase from *Clostridium sticklandii. J Biol Chem* **228**: 983-97.

Stogniew M and Fenselau C (1982). Electrophilic reactions of acyl-linked glucuronides. Formation of clofibrate mercapturate in humans. *Drug Metab Dispos* **10**: 609-613.

Taha A, Lenton RJ, Murdoch PS, and Peden NR (1985). Non-oliguric renal failure during treatment with mefenamic acid in elderly patients: a continuing problem. *Br Med J (Clin Res Ed)* **291**: 661-662.

Uetrecht J (2007). Idiosyncratic drug reactions: current understanding. *Annu Rev Pharmacol Toxicol* **47**: 513-539.

Van Breemen RB and Fenselau C (1985). Acylation of albumin by 1-*O*-acyl glucuronides. *Drug Metab Dispos* **13**: 318-320.

Van Breemen, RB and Fenselau C (1986). Reaction of 1-*O*-acyl glucuronides with 4-(*p*-nitrobenzyl)pyridine. *Drug Metab Dispos* **14**: 197-201.

Vlahcevic ZR, Pandak WM, and Stravitz RT (1999). Regulation of bile acid biosynthesis. *Gastroenterol Clin North Am* **28**: 1-25.

Wells DS, Janssen FW, and Ruelius HW (1987). Interactions between oxaprozin glucuronide and human serum albumin. *Xenobiotica* **17**: 1437-1449.

Woods KL and Michael J (1981) Mefenamic acid nephropathy. *Br Med J (Clin Res Ed)* **282**: 1471.

Footnotes

This work was supported in part by the National Institute of Health Grant [GM36633] and by the University of California, San Francisc Liver Center Cell Biology Core through National Institute of Health grant [P30 DK026743]. The authors declare no competing financial interests.

Figure Legends:

Figure 1 Proposed conjugative bioactivation pathways of mefenamic acid.

Figure 2 Proposed identities of the fragment ions of MFA-Gly (A), tandem mass spectrum (B), and representative reverse-phase gradient LC-MS/MS Single Reaction Monitoring (SRM) (*m/z* 299 to *m/z* 224) (C) of MFA-Gly authentic standard.

Figure 3 Proposed identities of the fragment ion of MFA-Tau (A), tandem mass spectrum (B), and representative reverse-phase gradient LC-MS/MS SRM (*m/z* 349 to *m/z* 224) (C) of MFA-Tau authentic standard.

Figure 4 Proposed identities of the fragment ions of MFA-NAC (A), tandem mass spectrum (B), and representative reverse-phase gradient LC-MS/MS SRM (*m/z* 387 to *m/z* 224) (C) of MFA-NAC authentic standard.

Figure 5 Scheme of the transacylation of MFA-AMP, MFA-CoA, and MFA-1-*O*-G with the bionucleophiles Gly, Tau, GSH, and NAC in buffer (0.1 M Kpi (pH 7.4, 37^{o}C)).

Figure 6 Mean reactivity assessment ± standard deviation at 60 min of MFA-AMP, MFA-CoA, and MFA-1-*O*-G toward Gly (A) and Tau (B) in buffer (0.1 M Kpi (pH 7.4, 37^{o}C)).

Figure 7 Mean reactivity assessment ± standard deviation at 60 min of MFA-AMP, MFA-CoA, MFA-1-*O*-G, and MFA-NAC toward GSH (A) and NAC (B) in buffer (0.1 M Kpi (pH 7.4, 37^{o}C)).

Figure 8 Mean time-dependent formation ± standard deviation of MFA-AMP, MFA-CoA, and MFA-Tau (A), MFA-1-*O*-G (right axis units) and MFA-GSH (left axis units) (B) in rat hepatocyte incubations.

DMD Fast Forward. Published on August 23, 2013 as DOI: 10.1124/dmd.113.053223
This article has not been copyedited and formatted. The final version may differ from this version.

Table 1. Mean Rates±Standard Deviations of Formation of MFA Conjugates incubated with Gly, Tau, GSH, and, NAC (10 mM) in Physiological Buffer (pH 7.4, 37°C) (n=3)

Conjugate (1 μM)	**MFA-Gly Formation (nM/min)**	**MFA-Tau Formation (nM/min)**	**MFA-GSH Formation (nM/min)**	**MFA-NAC Formation (nM/min)**
MFA-AMP	0.39±0.07	0.35±0.01	0.21±0.02	0.12±0.08
MFA-CoA	N.D.	0.02±0.01	2.42±0.67	2.34±0.08
MFA-GSH	-	-	-	13.0±0.44
MFA-1-*O*-G	N.D.	N.D.	N.D.	N.D.

N.D., not detected

Figure 1

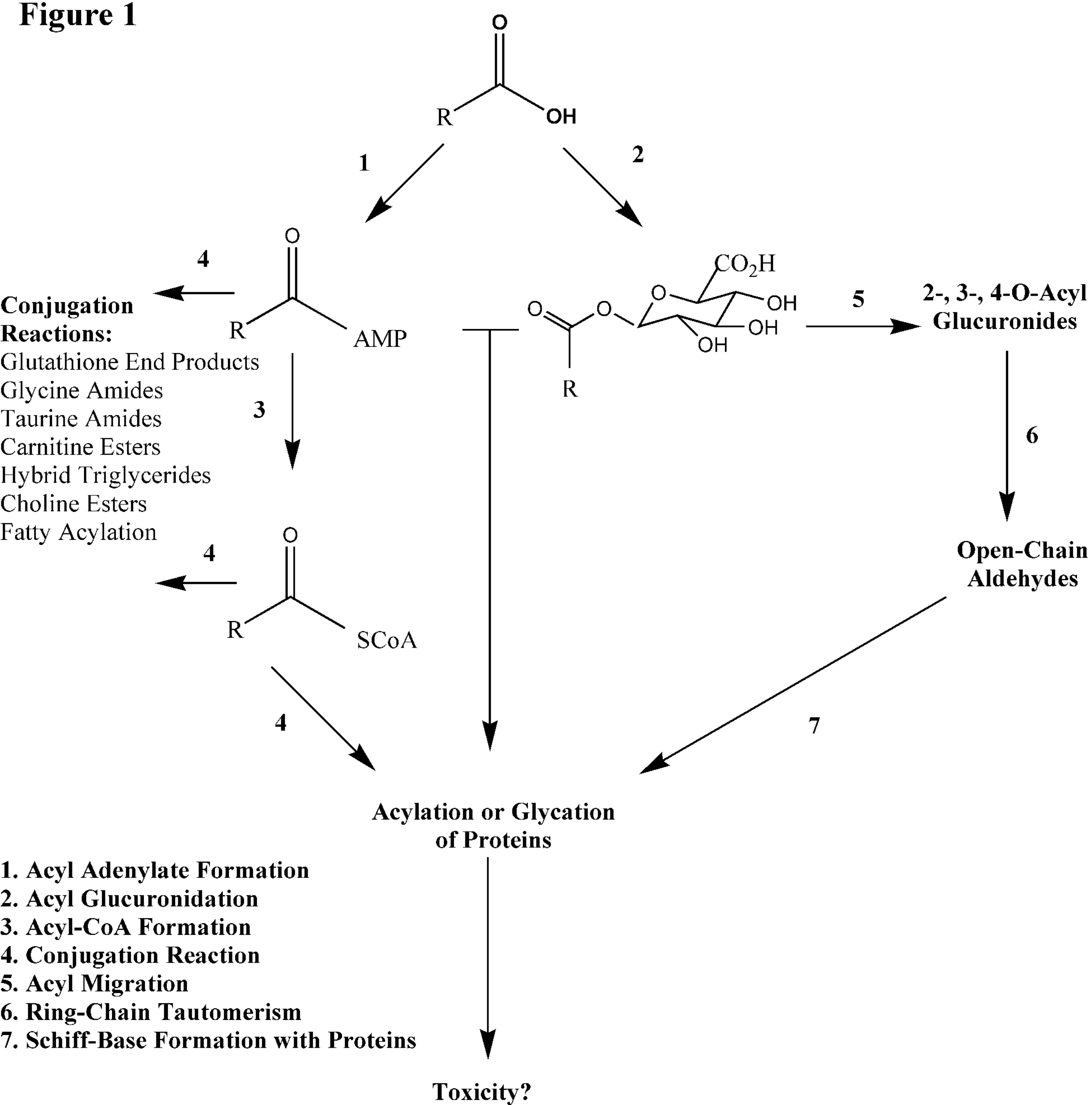
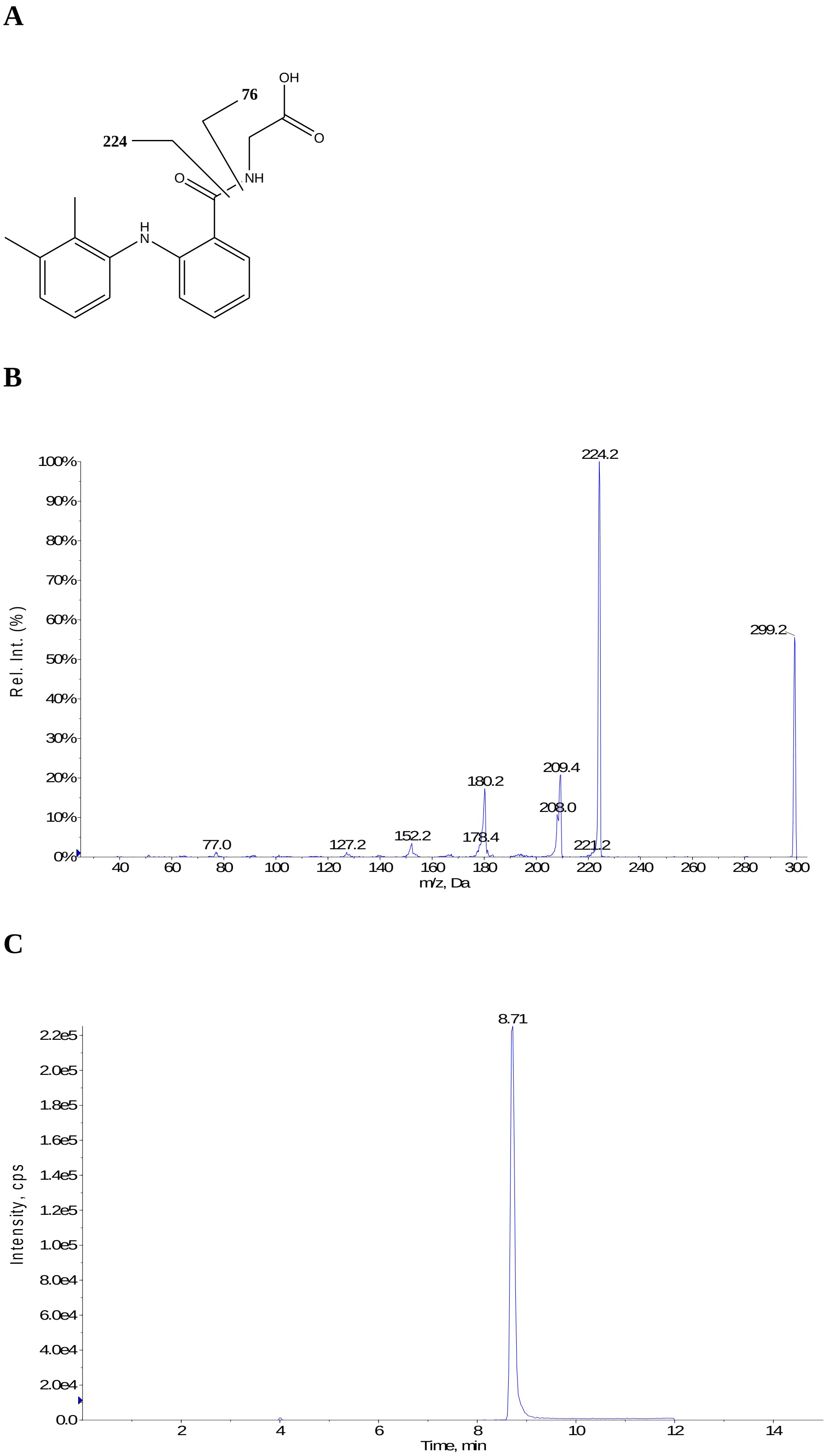
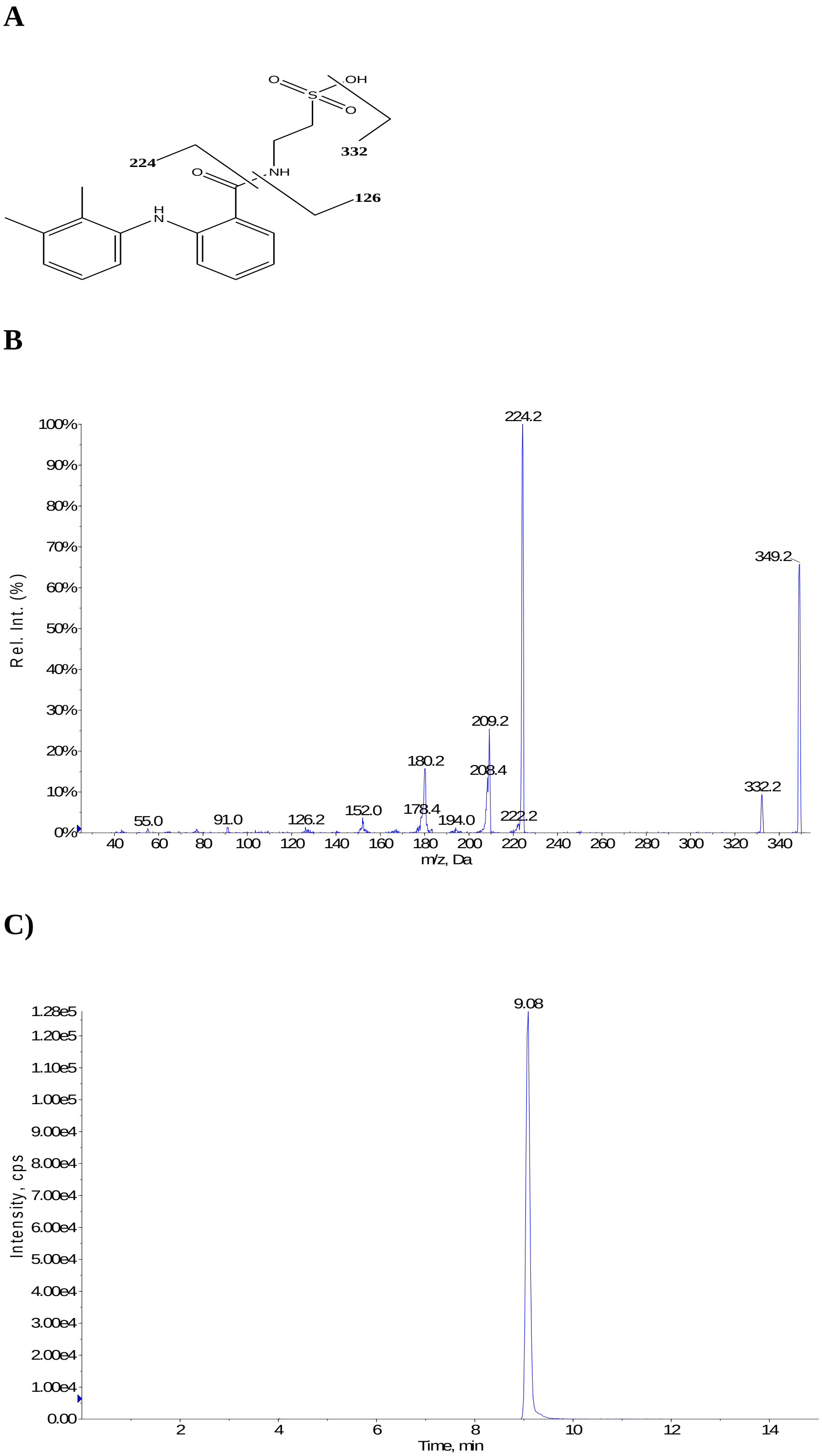

O
R
OH
1
2
O
R
AMP
4
Conjugation Reactions:
Glutathione End Products
Glycine Amides
Taurine Amides
Carnitine Esters
Hybrid Triglycerides
Choline Esters
Fatty Acylation
3
O
R
SCoA
4
4
CO2H
O
O
O
OH
OH
OH
R
5
2-, 3-, 4-O-Acyl Glucuronides
6
Open-Chain Aldehydes
7
Acylation or Glycation of Proteins
Toxicity?
1. Acyl Adenylate Formation
2. Acyl Glucuronidation
3. Acyl-CoA Formation
4. Conjugation Reaction
5. Acyl Migration
6. Ring-Chain Tautomerism
7. Schiff-Base Formation with Proteins

Figure 2


A
76
OH
224
O
O
NH
H
N
B
224.2
299.2
209.4
180.2
208.0
152.2
178.4
77.0
127.2
221.2
100%
90%
80%
70%
60%
50%
40%
30%
20%
10%
0%
Rel. Int. (%)
40
60
80
100
120
140
160
180
200
220
240
260
280
300
m/z, Da
C
8.71
2.2e5
2.0e5
1.8e5
1.6e5
1.4e5
1.2e5
1.0e5
8.0e4
6.0e4
4.0e4
2.0e4
0.0
Intensity, cps
2
4
6
8
10
12
14
Time, min

DMD Fast Forward. Published on August 23, 2013 as DOI: 10.1124/dmd.113.053223
This article has not been copyedited and formatted. The final version may differ from this version.

Figure 3



DMD Fast Forward. Published on August 23, 2013 as DOI: 10.1124/dmd.113.053223
This article has not been copyedited and formatted. The final version may differ from this version.

Figure 4

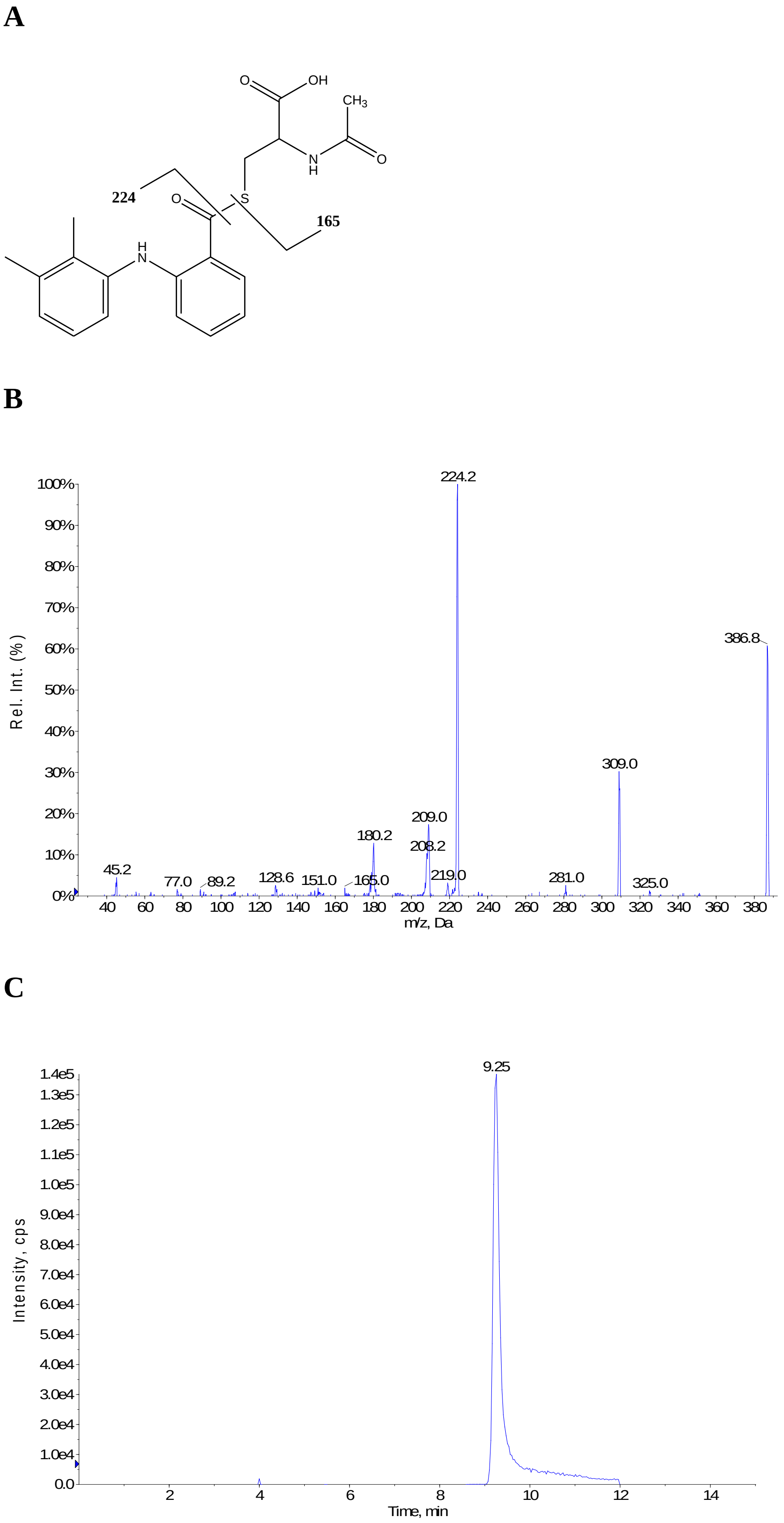


Figure 5

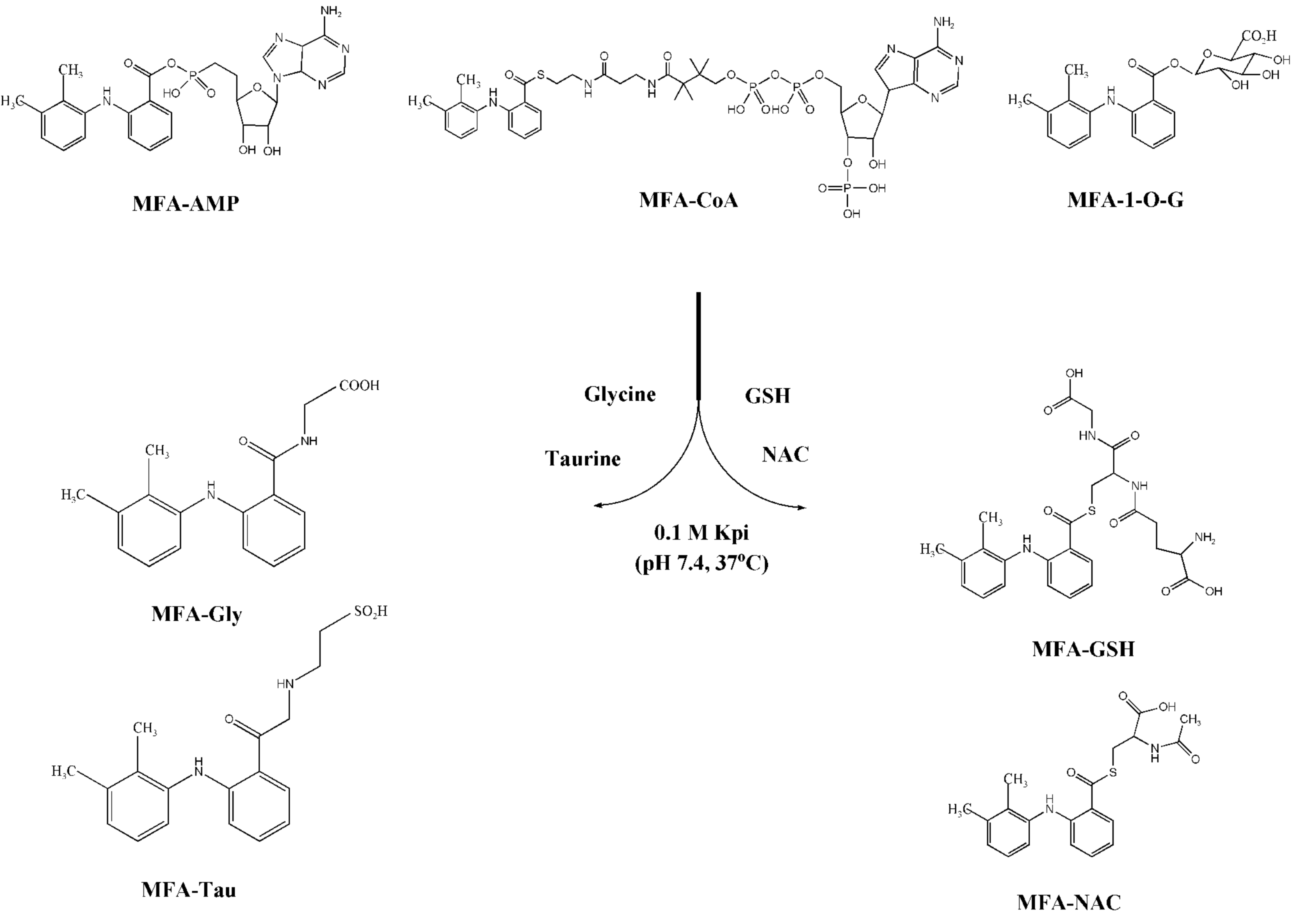

MFA-AMP
MFA-CoA
MFA-1-O-G
Glycine
Taurine
GSH
NAC
0.1 M Kpi
(pH 7.4, 37°C)
MFA-Gly
MFA-Tau
MFA-GSH
MFA-NAC

Figure 6

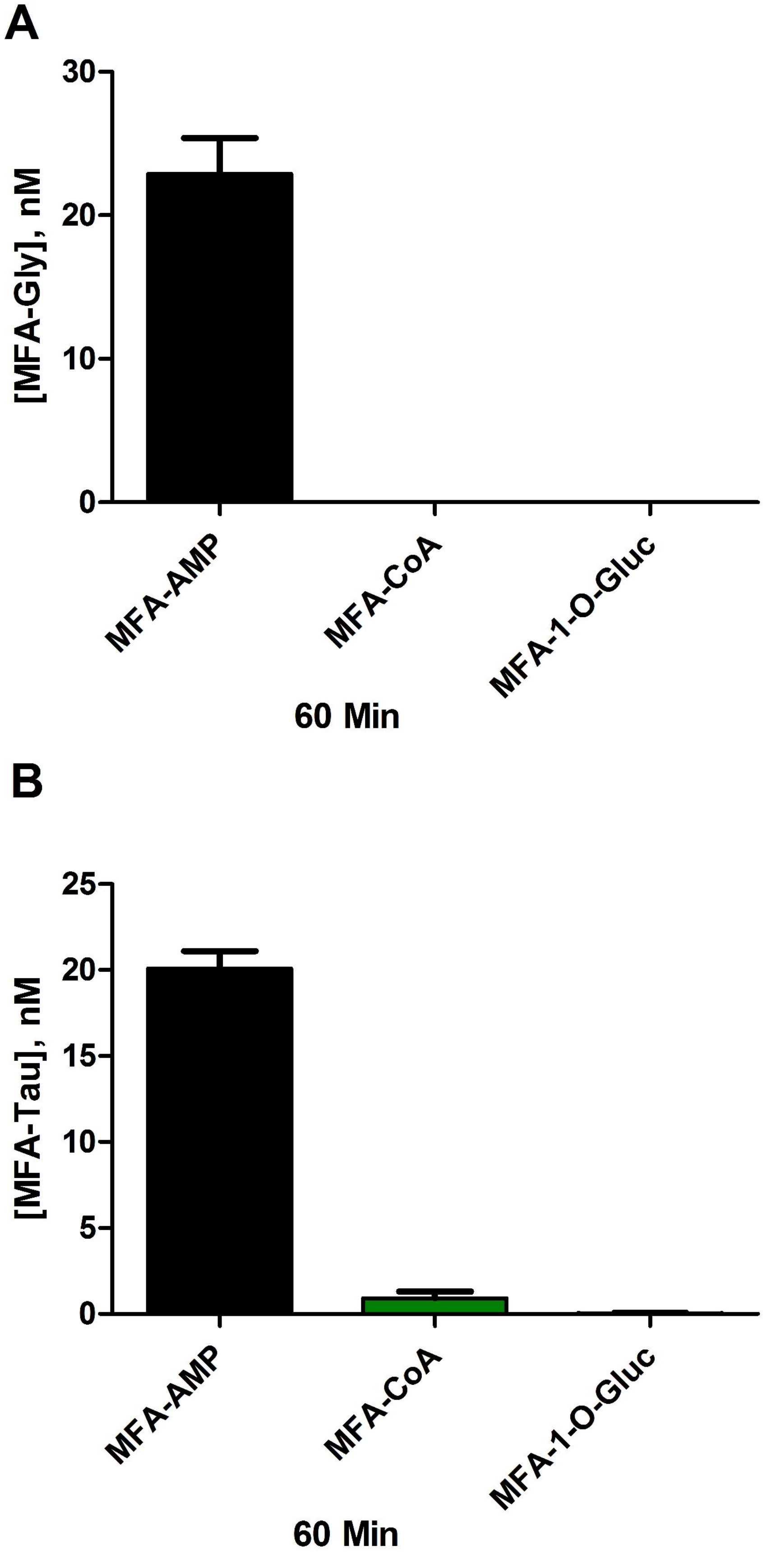

A
30
20
10
0
[MFA-Gly], nM
MFA-AMP
MFA-CoA
MFA-1-O-Gluc
60 Min
B
25
20
15
10
5
0
[MFA-Tau], nM
MFA-AMP
MFA-CoA
MFA-1-O-Gluc
60 Min

Figure 7

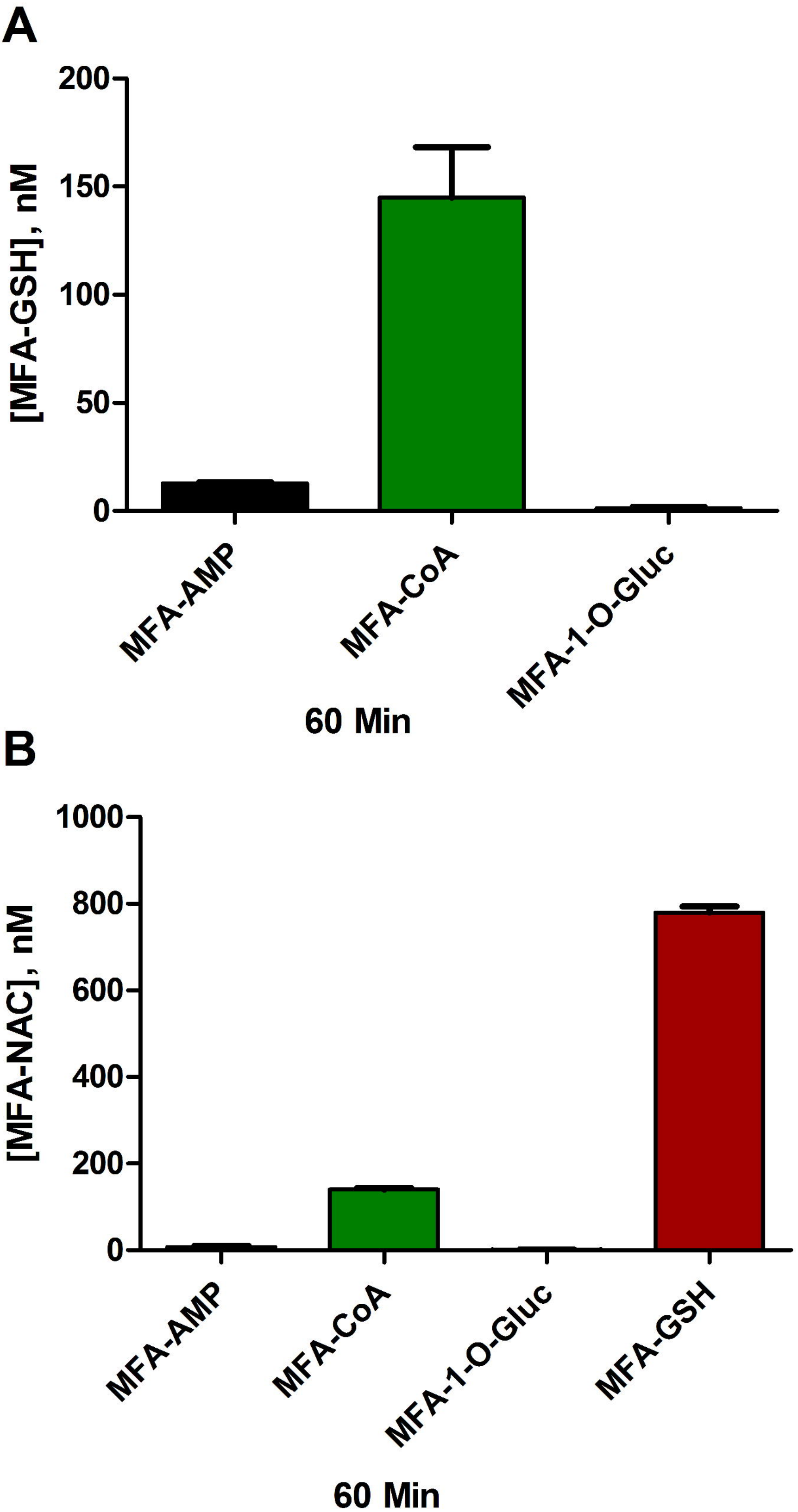
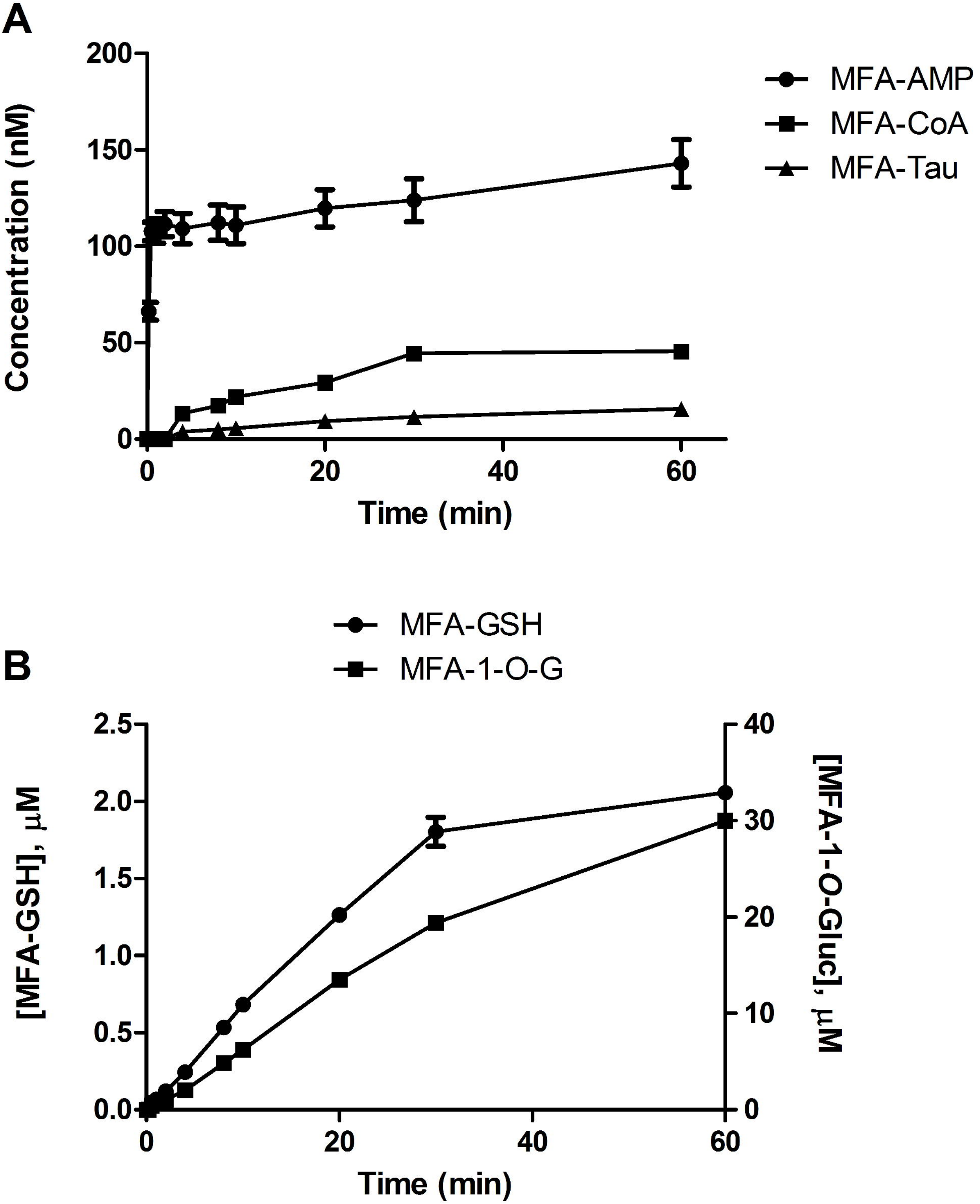

A
[MFA-GSH], nM
200
150
100
50
0
MFA-AMP
MFA-CoA
MFA-1-O-Gluc
60 Min
B
[MFA-NAC], nM
1000
800
600
400
200
0
MFA-AMP
MFA-CoA
MFA-1-O-Gluc
MFA-GSH
60 Min

Figure 8


A
Concentration (nM)
200
150
100
50
0
0
20
40
60
Time (min)
MFA-AMP
MFA-CoA
MFA-Tau
MFA-GSH
MFA-1-O-G
B
[MFA-GSH], μM
2.5
2.0
1.5
1.0
0.5
0.0
0
20
40
60
Time (min)
[MFA-1-O-Gluc], μM
40
30
20
10
0