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Studies on the Metabolic Activation of Mefenamic Acid

Characterization of the Acyl-Linked Metabolite, Mefenamyl Adenylate

by

Howard T. Horng

DISSERTATION

Submitted in partial satisfaction of the requirements for the degree of

DOCTOR OF PHILOSOPHY

in

Pharmaceutical Sciences and Pharmacogenomics

in the

GRADUATE DIVISION

of the

UNIVERSITY OF CALIFORNIA, SAN FRANCISCO

Acknowledgements

I would like to thank my advisor, Dr. Leslie Z. Benet, for his great mentorship and support. During my time as a graduate student, Les has always been there to inspire, support, and make me laugh during the good and the bad times. Les has always made me feel like I can do anything and as a result, is one of the few people in my life who has made an important impact on me. Les has taught me the art of pharmacokinetics, drug metabolism, and transporters and I aspire to live my life with the same passion, confidence, and dedication towards the world as he does.

I would also like to thank Dr. Deanna Kroetz and Dr. Tony McDonagh for their fruitful discussions and advice during the course of this graduate work. They are also members of my thesis committee and I am very grateful for their time.

Special thanks to numerous members (past and present) of the Benet group and the PSPG program for their friendship, support, and help: Dr. Hideaki Okochi, Frances Peterson, Dr. Sarah Shugarts, Dr. Jennifer Yokoyama, Dr. Stacy Musone, Dr. Jason Fernandes, Dr. Somayeh Ahmadiantehrani, Anita Grover, Mark Yago, Connie Remsberg, and Chelsea Hosey. Graduate school would not be fun without these wonderful friends.

Heartfelt thanks also to my family: Cheng (dad), Nancy (mom), and Grace (sister), for their love, encouragement and unyielding support throughout the years.

Howard Horng

January 2012

Abstract

Studies on the Metabolic Activation of Mefenamic Acid

Characterization of the Acyl-Linked Metabolite, Mefenamyl-Adenylate

Howard Horng

Mefenamic acid, (MFA), a nonsteroidal anti-inflammatory drug, is metabolized to MFA-1-*O*-acyl-glucuronide (MFA-1-*O*-Gluc), a chemically-reactive conjugate implicated in the formation of protein-adducts and the potential toxicity of the drug. We investigated the ability of mefenamic acid to become bioactivated to reactive metabolites that transacylate glutathione (GSH) forming MFA-*S*-acyl-glutathione (MFA-GSH) *in vitro* in incubations with rat hepatocytes. Mefenamic acid (100 μM) incubations, followed by LC-MS/MS analysis, led to the detection of MFA-GSH. The initial formation of MFA-GSH was rapid reaching a concentration of 1.7 µM after 60-min of incubation. The product MFA-GSH was shown to be unstable in incubations with rat hepatocytes ($t_{1/2}$ ~10 min). MFA-*S*-acyl-CoA (MFA-CoA) was undetectable until the 4-min time point, reaching a concentration of 45.6 nM at the 60-min time point. MFA-1-*O*-Gluc reached a Cmax of 42.2 µM after 1-h of incubation. Co-incubation of MFA (10 μM, 10-min) with (-)-borneol (100 μM), an inhibitor of glucuronidation, led to a 91.1% decrease in MFA-1-*O*-Gluc formation, however no inhibition of MFA-SG formation was observed. By contrast, co-incubation with lauric acid (1 mM), an inhibitor of acyl-CoA formation, led to a 66.1% inhibition of MFA-GSH formation. Since these data does not completely explain the formation of MFA-GSH, we predicted that the intermediate MFA-acyl-adenylate (MFA-AMP) may be responsible in mediating the formation of MFA-GSH. MFA-AMP was

detected in rat hepatocyte incubation extracts forming a concentration of 90.1 nM at the 20-sec time point. MFA-AMP was shown to be reactive with GSH, but ~10-fold less reactive than MFA-CoA, however, in the presence of GST, MFA-AMP mediated formation of MFA-GSH increased 6-fold. MFA-1-*O*-Gluc did not react with GSH to form MFA-GSH. MFA-GSH has also been shown to be reactive in itself toward N-acetyl-cysteine. ABT and temperature dependent inhibition of hepatocyte timecourse of formation incubations reveal that MFA-GSH formation is mediated by GST via MFA-AMP. These results demonstrate that mefenamic acid becomes bioactivated *in vitro* in rat hepatocytes to reactive transacylating derivatives into MFA-AMP and MFA-CoA that contribute to the transacylation of GSH forming MFA-GSH.

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Chapter 1

Introduction

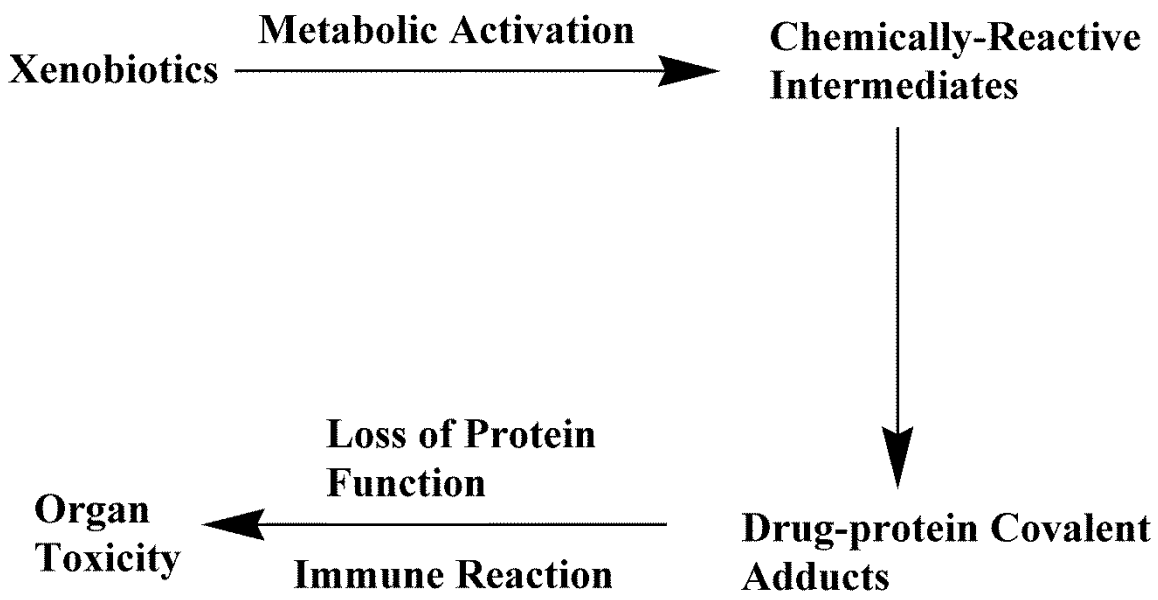

Figure 1.1 Metabolic activation of xenobiotics to chemically-reactive and potentially toxic products.

1.1 Bioactivation

The bioactivation of drugs to chemically reactive metabolites is considered by most to be an unwanted feature. Therefore, it is imperative that we as pharmaceutical scientists eliminate, if not minimize, this bioactivation liability in order to produce safer drugs. To accomplish this task, we must gain a better understanding of how these reactive metabolites are formed and how they interact with macromolecules of the body. From there, we can then make wise

judgments about the hidden risks associated with compounds to prevent potentially toxic drugs from ever reaching the patient.

Therapeutic compounds can be metabolized into reactive intermediates to elicit an idiosyncratic toxicity (Uetrecht, 2003; Kalgutkar and Soglia, 2005; Baillie, 2006; Williams, 2006). This phenomenon occurs at a very low rate, approximately one in fifty thousand (Zimmerman, 1999; Calvey, 2005; Primohamed, 2006). However, when it does occur, toxicity can be severe and sometimes fatal and as a result this has become a major concern clinically. Toxins can be classified into two simple categories, intrinsic and idiosyncratic toxins (Pumford and Halmes, 1997). Intrinsic toxins (Type A) have a high capacity for organ damage at high doses but tend to be safe when taken appropriately. Intrinsic toxins, therefore, produce a predictable and dose dependent toxicity. An example of an intrinsic toxin is acetaminophen. Idiosyncratic toxins (Type B) do not produce a predictable or dose dependent toxicity. Idiosyncratic toxicities do not occur in most patients taking the drug, the toxic effect does not relate to the pharmacological activity of the drug, and the onset of the reaction tends to occur at least one week after initial exposure, but latency can be up to one year (Ju and Uetrecht, 2002). An example of an idiosyncratic toxin is the nonsterodial anti-inflammatory drug (NSAID) diclofenac. Unfortunately, there are currently no animal models that can help us predict an idiosyncratic toxicity.

Two major reasons why drugs fail in development are preclinical toxicity and human adverse reactions (Kola and Landis, 2004; Guengerich and MacDonald, 2007). One hypothesis regarding intrinsic drug-induced hepatotoxicity is that many therapeutic compounds are

metabolically activated in the liver into reactive metabolites that can covalently bind onto proteins of the body (Figure 1.1), (Nelson, 1992; Baille, 2008). One pathway of drug-induced toxicity is that these drug-protein adducts can induce a toxic reaction by directly impairing the normal function of a critical protein. For example, there are many mitochondrial proteins that play a role in energy production and calcium homeostasis (Welch et al., 2005). The formation of a drug-protein adduct in the mitochondria is believed to lead to the loss of mitochondrial function and thus cell death and tissue necrosis. Studies have shown that acetaminophen (APAP) undergoes bioactivation by cytochrome P450 2E1, 1A2, 3A4, and 2D6 into the chemically reactive electrophilic intermediate, N-acetyl-*p*-benzoquinone imine (NAPQI). NAPQI can spontaneously or catalytically, via glutathione *S*-transferase (GST), form a glutathione-thioether-linked conjugate detoxification product, 3-(glutathione-*S*-yl) APAP (Dahlin et al., 1984; Nelson, 1990; Dong et al., 2000) This glutathione conjugate can be degraded into mercapturic acid conjugates in the kidney and then excreted in the urine. However, in an overdose situation, the detoxification of APAP, either by glucuronidation and/or sulfation becomes overwhelmed such that an increasing amount of APAP is converted into the reactive NAPQI intermediate. As a result, endogenous stores of GSH become depleted and the high concentration of NAPQI exhausts the hepatocyte defense system by covalently binding onto, and thus destroying, critical protein in the mitochondria (Park et al., 2005). The resulting loss of function of these proteins triggers events that lead to apoptosis and cell death (Wong and Liebler, 2008). Two-dimensional gel electrophoresis and mass spectrometric analysis have identified more than 20 APAP-labeled hepatic protein-adducts in [^{14}C]APAP treated mice (Qui et

al., 1998). Thus, the challenge has been and continues to be the ability to identify those protein targets that are critical for the onset of an idiosyncratic hepatotoxicity.

Another hypothesized drug-induced toxicity occurs through bioactivation where carboxylic acid-containing drugs are metabolized into Phase-II-reactive metabolites, acyl glucuronides and acyl-CoA. These electrophilic acyl-linked metabolites are capable of reacting with tissue protein nucleophiles (Benet et al., 1993). It is believed that covalent modification of proteins cause immune reactions by acting as antigens that mediate the formation of an allergic reaction in hypersensitive individuals (Boelsterli, 2002; Skonberg et al., 2008). The immune system is a highly complex and developed system that acts as our natural defense against pathogenic organisms. However, the immune system is susceptible to diseases especially when it cannot differentiate between host cells/macromolecules and foreign substances, resulting in auto-immune type disorders like multiple sclerosis, rheumatoid arthritis, systemic lupus, type I diabetes, and inflammatory bowel disease. Allergic reactions to drugs can also result in life disabling diseases such as asthma, anaphylaxis, dermatitis, hepatitis, hemolytic anemia, nephritis, Stevens-Johnson syndrome, toxic epidermal necrolysis, urticaria, and vasculitis (Ewan, 1998; Dansette et al., 1998; Zimmerman, 1999; Fung et al., 2001). The top four toxicological reasons for drug withdrawal from the market today are hepatic (26.2%), hematologic (10.5%), dermatologic (6.3%), and anaphylactic (3.3%). These toxicities can lead to organ failure and ultimately fatalities.

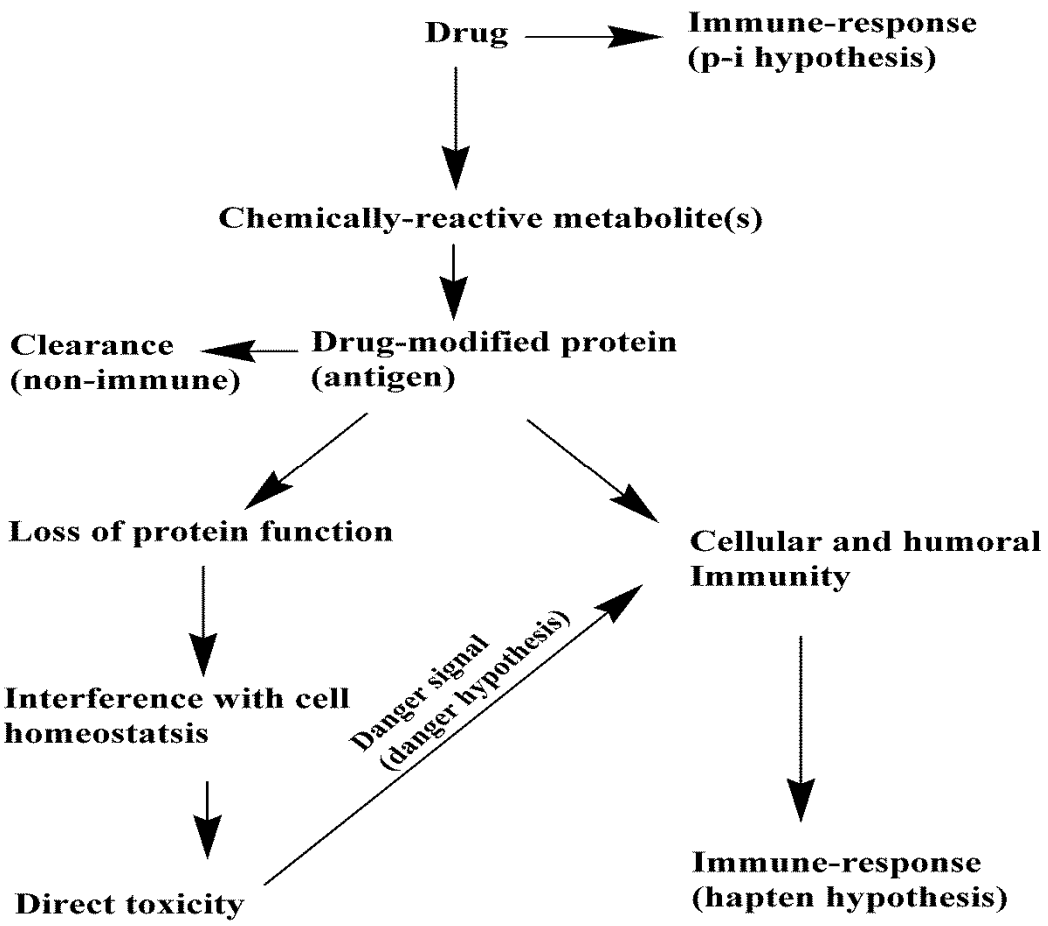

Figure 1.2 Scheme for the metabolism of drugs to chemically-reactive intermediates leading to direct toxicity or immune-mediated toxicity.

A principal working hypothesis still used today since its inception in 1935 is the hapten hypothesis. The hapten hypothesis states that a drug or its metabolite are not immunogenic in themselves. However, once they covalently bind onto macromolecules, these drug-protein adducts are recognized by the immune system as foreign and thus lead to an immune response toward the adducted protein (Figure 1.2) (Landsteiner and Jacobs, 1935; Park et al., 1987; Uetrecht, 2007). Tolmetin glucuronide protein conjugates have been shown to be immunogenic in mice by inducing antibodies specific for both the drug and the drug

glucuronide and it is believed that some degree of structural similarity is required for drug and drug glucuronide specific antibodies to cross-react with other NSAIDs and their respective glucuronides (Zia Amirhosseini et al., 1995). There are four stages of allergic hepatitis mediated by a reactive metabolite. The first stage involves metabolic activation of a drug into a reactive intermediate that covalently binds onto a protein to form a drug-protein adduct. The second stage involves subcellular trafficking and then cell surface expression of the protein-adduct fragments. The third stage involves antigen presentation and immune recognition, which then promotes an antibody response and cellular sensitization. The fourth stage involves an immune attack on the adducted hepatocyte resulting in immune-mediated liver injury. More recent drug-induced allergic reaction hypotheses include the danger hypothesis and the p-i hypothesis. The danger hypothesis states that when a reactive metabolite covalently binds onto a protein, it causes cellular damage inducing the cell to release an endogenous signal, referred to as the “danger” signal that leads to the activation of antigen-presenting cells and thus an immune response (Figure 1.2) (Matzinger, 1994; Uetrecht, 1999). Diclofenac is an example of a drug-induced allergic reaction via the danger hypothesis. The p-i (pharmacological interaction) hypothesis states that the parent drug, not the drug-protein adduct, is recognized by the immune system leading to an immune response (Figure 1.2) (Pichler, 2002). The anticancer drug oxaliplatin (Maindrault-Goebel et al., 2005) and the anticonvulsant drug lamotrigine (Naisbitt et al., 2003) are believed to have a direct effect on the immune system.

1.2 Carboxylic Acid Drugs

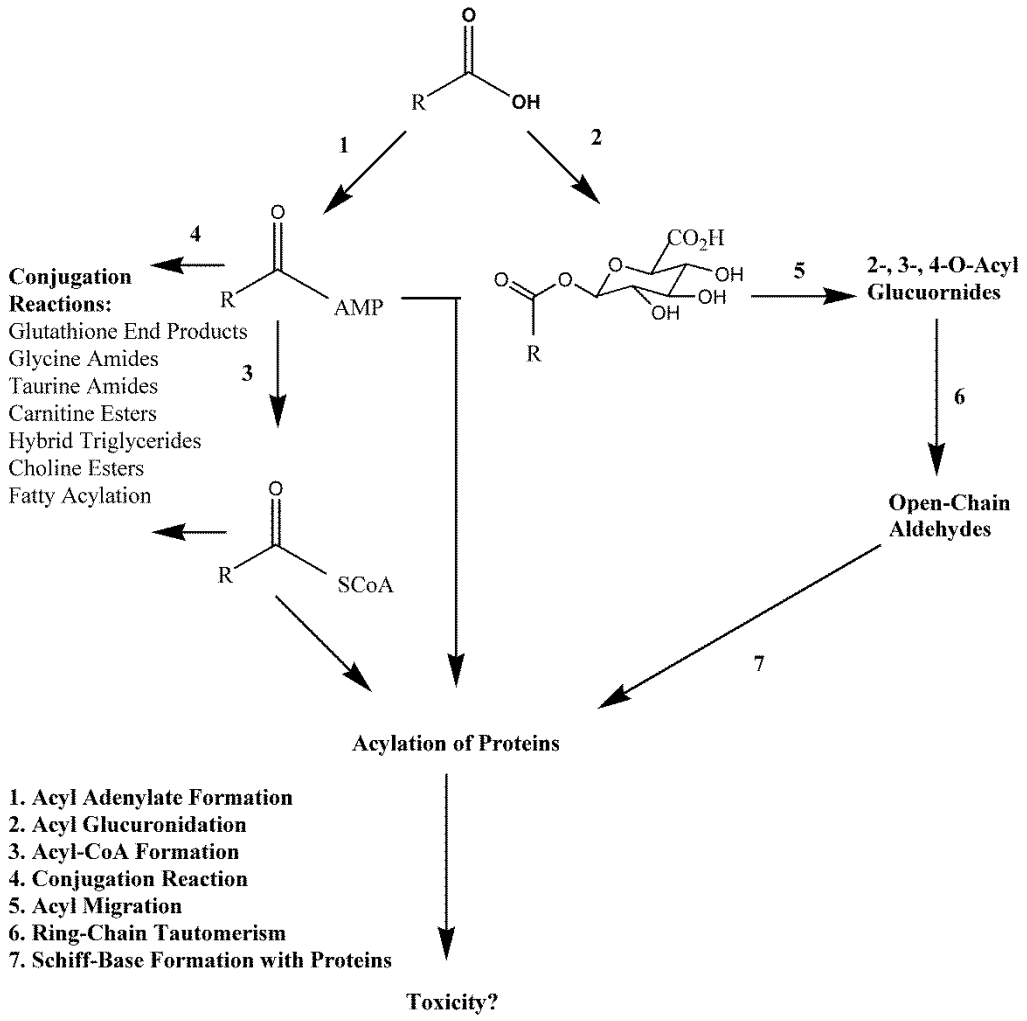

Figure 1.3 Proposed scheme for the metabolic activation of carboxylic acid-containing drugs to reactive intermediates and to non-toxic metabolites.

Xenobiotic carboxylic acid drugs are used as non-sterodial anti-inflammatory drugs (NSAIDs, ibuprofen, diclofenac, and mefenamic acid), diuretics (furosemide and ethacrynic acid), antiepileptic agents (valproic acid), hypolipidemic agents (fibric acid derivatives, clofibric acid and gemfibrozil) and HMG-CoA reductase inhibitors (simvastatin and cerivastatin). Although carboxylic acid drugs are generally well tolerated, they are associated with severe, sometimes

fatal idiosyncratic toxicities. These toxicities are believed to occur when carboxylic acid-containing drugs are bioactivated into chemically-reactive metabolites that are capable of transacylating protein nucleophiles (Figure 1.3). Between 1964-1993, 47 drugs were withdrawn from the U.S., British, and Spanish markets due to severe toxicity and 10 of those withdrawn were carboxylic acid containing drugs (alclofenac, bendazac, benoxaprofen, fenclofenac, ibufenac, indoprofen, pirprofen, suprofen, ticrynafen, and zomepirac) (Bakke et al., 1984; Bakke et al., 1995). The primary reason for their withdrawal was the formation of an idiosyncratic toxicity associated with liver damage, skin reaction, renal toxicity, fever, rash, and eosinophilia (Bakke et al., 1984; Zimmerman, 1990; Zimmerman, 1994; Bakke et al., 1995). More recently, cerivastatin has also been taken off the market due to a high risk for muscle toxicity (SoRelle, 2001; Thompson, 2001). Other carboxylic acid drugs still on the market that are associated with an idiosyncratic toxicity include diclofenac (Boelsterli, 2003), diflunisal (McKinnon, 1989), valproic acid (Williams, 1992), tolmetin (Zia-Amirhosseini, 1994), clofibric acid (Sallustio, 1991), ibuprofen (Castillo, 1995), and salicylic acid (Dickinson, 1994).

The overall incidence of acidic drug induced idiosyncratic toxicity is low, one in one thousand to one in one hundred thousand patients (Zimmerman, 1999; Calvey, 2005; Pirmohamed, 2006). Therefore, it is easy to miss these reactions even in a large clinical study consisting of ten thousand patients. However, when they do occur the result can be severe and sometimes fatal. This phenomenon has become a major concern clinically because presently, there does not exist an animal model that can predict the onset of an idiosyncratic toxicity. It is believed that the covalent modification of tissue proteins by reactive metabolites from the bioactivation of acidic drugs is a possible mechanism. What is known is that these types of reactions are host

(Boelsterli et al., 1995) and dose dependent. There are very few drugs taken off the market for toxicity reasons for which the approved dose was less than 10 milligrams/day (Uetrecht, 2001; Kalgutkar et al., 2002). Therefore, the current approach in drug discovery is to develop high potency drugs that can be administered to patients at low doses.

1.3 Acyl Glucuronides

Acyl glucuronidation is a major route of elimination of carboxylic acid drugs (Spahn-Langguth and Benet, 1992; Li and Benet, 2002a). Although glucuronidation primarily leads to detoxification for many drugs, acyl glucuronides are also capable of promoting cellular injury, hepatotoxicity or carcinogenesis. This is believed to occur because acyl glucuronides are reactive electrophilic intermediates that are capable of forming adducts with nucleophilic centers of proteins and glutathione (GSH) (Sphan-Langguth et al., 1992; Berkes, 2003; Bock, 1991; Boelsterli, 1993). Unlike glucuronides produced from phenols and alcohols, acyl glucuronides chemically are susceptible to intramolecular acyl migration to yield β-glucuronidase resistant isomers (Compernolle et al., 1978; Spahn-Langguth and Benet, 1992; Li and Benet, 2002a), and are involved in nucleophilic substitution reactions. Acyl glucuronides have been shown to form covalent bonds with serum albumin *in vitro* and with plasma and tissue proteins *in vivo* to produce drug-protein adducts (Etter-Kjelsaas and Kuenzle, 1975; Spahn-Lanngguth et al., 1997; Li and Benet, 2002a).

Esterfication of the hemiacetal hydroxyl group of glucuronic acid to a carboxylic acid produces ester or acyl glucuronides. The transfer of a glucuronic acid moiety to the carboxyl group of the aglycone is mediated by uridine-5'-diphospho (UDP)-glucuronosyltransferases (UGTs). This

occurs via a S_N2-type reaction where the anomeric center undergoes inversion during the enzyme transfer of α-D-glucuronic acid of UDPGA to the acceptor substrate resulting in the formation of the β-configuration (Burchell, 1999).

UGTs are membrane bound enzymes that are located primarily in the liver but also exist in nonhepatic tissue. Their location in the endoplasmic reticulum is of pharmacological importance in the detoxification of reactive intermediates produced by P450s. There are more than 50 known isoenzymes of UGTs found in humans and they are primarily located in the liver, lung, kidneys, intestines, brain, skin, and olfactory epithelium. However, the liver is the primary source of UGTs (Radominska-Pandya et al., 1999). Human UGTs 1A3, 1A9, and 2B7 and rat 2B1 have been shown to catalyze the acyl glucuronidation of many carboxylic acid drugs, including the NSAIDs (e.g. diclofenac[King et al., 2001], ibuprofen, ketoprofen, naproxen, pirprofen, flurbiprofen, diflunisal, mefenamic acid), antiepileptic agent (valproic acid), diuretic agents (e.g. furosemide) and fibric acid derivatives (clofibric acid, benzafibrate, ciprofibrates) (Ritter, 2000). Human UGTs 1A1 and 1A3 have been shown to catalyze the acyl glucuronidation of HMG-CoA reductase inhibitors (simvastatin, lovastatin, and cerivastatin) (Prueksaritanont et al., 2002).

1.3.1 Hydrolysis and Acyl Migration

Under physiological or weakly alkaline conditions, acyl glucuronides can be hydrolyzed back into the pharmacologically active parent drug. Potential catalysts include hydroxide ion, β-glucuronidases, serum albumin, and esterases. Rates of degradation are also affected by pH and temperature; the rate of hydrolysis increases at physiological and alkaline pH as opposed to acidic conditions. There is a significant decrease in hydrolysis under cold and acidic conditions

(pH 3-4). However, hydrolysis will still occur during freezing and thawing (Upton et al., 1982). Physiologically, β-glucuronidases and nonspecific esterases can also contribute in the hydrolysis of acyl glucuronides. Acyl glucuronide excreted into the intestinal tract via the bile may undergo enterohepatic cycling in which β-glucuronidases in the intestinal flora hydrolyze the acyl glucuronide back to the parent drug or its phase I metabolite, thereby allowing reabsorption into the portal circulation or excretion into the feces.

Drug-acyl glucuronides are reactive conjugates under physiological conditions that are able to undergo intramolecular acyl migration, a well-established phenomenon in carbohydrate chemistry (Haine, 1976), which is similar to alkaline hydrolysis. Migration occurs when the acyl group of the C-1 hydroxyl of the acyl glucuronide moves to the C-2, C-3, or C-4 positions. The resulting positional isomers are not hydrolyzable by β-glucuronidases and the isomers exhibits different chromatographic properties from one another. Studies have shown that all xenobiotic carboxylic acid glucuronides undergo intramolecular acyl migration (Spahn-Langguth and Benet, 1992; Li and Benet, 2002a).

Acyl migration occurs via a nucleophilic attack on the neighboring hydroxyl group resulting in the formation of an ortho-ester intermediate (Haine, 1976; Bradow et al., 1989;). *In situ* mechanistic studies of HPLC-purified isomers using ^{1}H-NMR spectroscopy show the order of migration to be from the 1-*O*-isomer to the 2-*O*-isomer followed by the formation of the 3-*O*-isomer and the 4-*O*-isomers. Migration is reversible between the 2, 3, and 4-*O*-isomers. However the 1-*O*-β-acyl glucuronide cannot be regenerated due to mutarotation of the C-1

after the initial migration. There is also no evidence for rearrangements beyond the nearest neighbor hydroxyl groups (Bradow et al., 1989).

The hydrolysis and acyl migration of 1-*O*-acyl glucuronides follows apparent first-order kinetics. Stability studies show that intramolecular migration under physiological conditions is the predominanting reaction in the early stages of *in vitro* incubations, while hydrolysis becomes more important at later time points or under alkaline conditions (Hasegawa et al., 1982). Acyl glucuronides are most stable under low temperatures, acidic conditions (pH 2-4), and in the presence of esterase inhibitors.

The presence of protein also affects the stability of acyl glucuronides. Human serum albumin (HSA) has been shown to accelerate the hydrolysis, intramolecular acyl migration, and covalent binding of oxaprozin glucuronide (Ruelius et al., 1986). These effects have been shown to vary for each drug. HSA has been shown to increase the degradation rates of acyl glucuronides for zomepirac (Smith et al., 1985), oxaprozin (Ruelius et al., 1986; Wells et al., 1987), fenoprofen (Volland et al., 1991), etodolac (Smith et al., 1992), ketoprofen (Hayball et al., 1992), naproxen (Bischer et al., 1995), clofibric acid (Grubb et al., 1993), and gemfibrozil (Sallustio et al., 1997). However, a stabilizing effect of HSA has been observed for tolmetin acyl glucuronide while bovine serum albumin (BSA) caused an increased hydrolysis rate (Munafo et al., 1990). HSA presence has also been shown to retard the degradation of diflunisal (Watt and Dickinson, 1990), salicylic acid (Dickinson et al., 1994), mefenamic acid (McGurk et al., 1996), and furosemide (Mizuma et al., 1999) glucuronides, while ibufenac glucuronide showed no significant degradation in the presence or absence of HSA (Castillo and Smith, 1995).

1.3.2 Covalent Binding of Acyl Glucuronides

Carboxylic acid acyl glucuronides are widely known to be able to form covalent linkages with proteins *in vitro* and *in vivo*. Acyl glucuronides of drugs that have been shown to covalently bind onto albumin *in vitro* include the NSAID benoxaprofen (van Breemen and Fenselau, 1985), indomethacin (van Breemen and Fenselau, 1985), flufenamic acid (van Breeman and Fenselau, 1985), oxaprozin (Ruelius et al., 1986), zomepirac (Smith et al., 1986), tolmetin (Munafo et al., 1990), carprofen (Iwakawa et al., 1988), fenoprofen (Volland et al., 1991), naproxen (Bischer et al., 1995), diflunisal (Watt and Dickinson, 1990), salicylic acid (Dickinson et al., 1994), etodolac (Smith et al., 1992), suprofen (Smith and Liu, 1993), ibuprofen (Castillo and Smith, 1995), ibufenac (Castillo and Smith, 1995), ketoprofen (Dubois et al., 1993), and mefenamic acid (McGurk et al., 1996), the uricosuric drug probenecid (Hansen-Moller and Schmit, 1991), the hypolipoproteinemic reagents clofibric acid (van Breemen and Fenselau, 1985; Grubb et al., 1993), fenofibric acid (Grubb et al., 1993), gemfibrozil (Sallustio et al., 1997), beclobric acid (Mayer et al., 1993), the diuretic agent furosemide (Mizuma et al., 1990) and the antiepileptic drug valproic acid (Williams et al., 1992). *In vivo* formation of covalently bound plasma proteins by acyl glucuronides include beclobric acid (Mayer et al., 1993), clofibric acid (Saullustio et al., 1991), carprofen (Iwakawa et al., 1988), diclofenac (Evans et al., 1993), diflunisal (McKinnon and Dickinson, 1989), fenoprofen (Volland et al., 1991), gemfibrozil (Sallustio and Foster, 1995), ketoprofen (Dubois et al., 1993), probenecid (McKinnon and Dickinson, 1989), salicylic acid (Dickinson et al., 1994), tolmetin (Munafo et al., 1993; Zia-Amirhosseini et al., 1994), valproic acid (Williams et al., 1992), and zomepirac (Smith et al., 1986).

In vitro covalent binding studies of various acyl glucuronides have shown that the extent of adduct formation is dependent on time (van Breemen and Fenselau, 1985; Wells et al., 1987), pH (Smith et al., 1986; Munafo et al., 1990; Smith and Liu, 1993), glucuronide concentration (Dubois et al., 1993), and origin of albumin (Munafo et al., 1990; Watt and Dickenson, 1990). The highest amount of protein adduct occurred at a pH of 7 for 1 hour at 37°C when oxaproxin glucuronide was incubated with HSA (Ruelius et al., 1986; Wells et al., 1987). However, covalent binding to HSA by zomepirac glucuronide occurred after 1 hour of incubation at a pH of 9 while higher adduct concentrations were observable after 6 hours at pH 7 and 8 at 37°C (Smith et al., 1986). Covalent binding of ketoprofen glucuronide to albumin was shown to be directly proportional to acyl glucuronide concentration over the concentration range of 11.62 to 69.72 µM (Dubois et al., 1993). The origins and purity of albumin also affects the amount of covalent binding of acyl glucuronides. Diflunisal glucuronide has a greater reactivity with fatty-acid-free HSA than with rat serum albumin (RSA), human and rat proteins (Dickinson, 1990). Tolmetin glucuronide has also been shown to be more reactive with BSA than HSA (Munafo et al., 1990).

Under physiological conditions, 1-*O*-acyl glucuronides are capable of undergoing intramolecular rearrangement to produce β-glucuronidase resistant positional isomers. These isomeric conjugates are also capable of covalently binding with proteins. Zomepirac glucuronide isomeric conjugates have been shown to covalently bind onto HSA (Smith et al., 1986; Smith et al., 1990). The rank order of zomepirac isomeric conjugate formation is as follows: C1>C2>C4>C3. The positional isomers of suprofen glucuronide exhibited 38% more covalent binding than that of the β-1-*O*-acyl glucuronide (Smith and Liu, 1993). Valproic acid (Williams et al., 1992), salicyclic acid (Dickinson et al., 1994), etodolac (Smith et al., 1992) and

diflunisal (Dickinson and King, 1991) isomeric conjugates have all been shown to be more reactive than their corresponding 1-β-*O*-acyl glucuronides. However, oxaprozin-1-*O*-glucuronide has been shown to be the only isomer contributing to covalent binding to proteins (Ruelius et al., 1986).

1.3.3 Acyl Glucuronide Mechanisms of Covalent Binding to Proteins

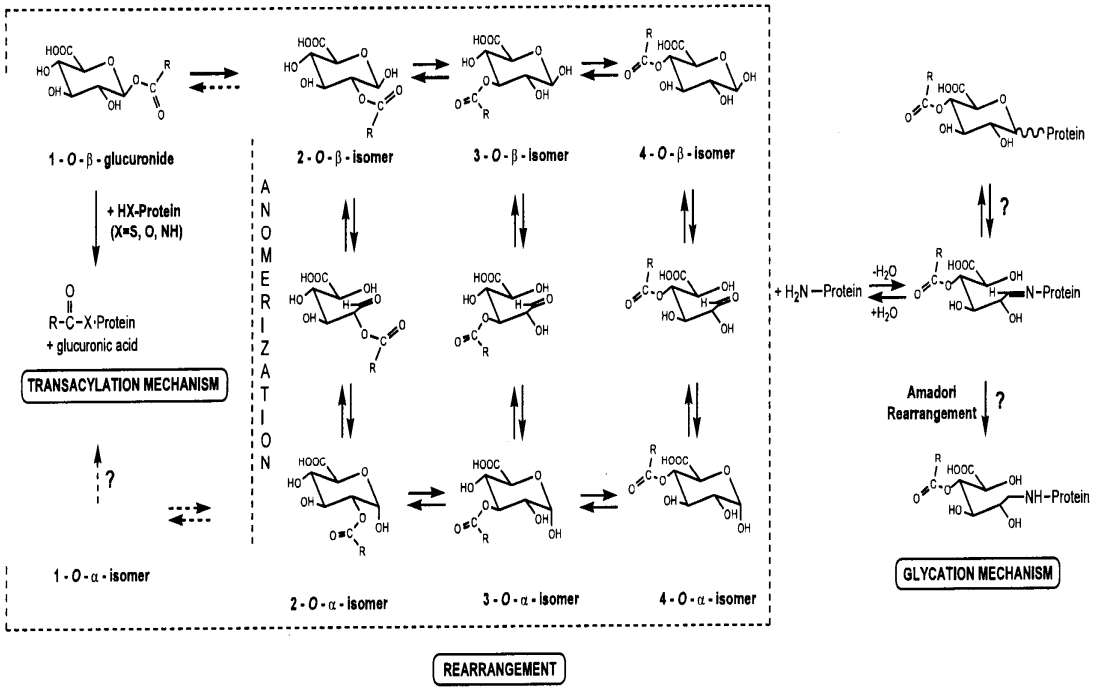

Figure 1.4 Mechanism for the covalent binding of acyl-glucuronides to proteins. (adapted from Bailey and Dickinson, 2003)

Acyl glucuronides are proposed to covalently bind onto proteins by one of two mechanisms (Figure 1.4). The first mechanism is a direct transacylation reaction with protein nucleophiles of the 1-*O*-acyl glucuronides. This nucleophilic displacement occurs when nucleophilic acceptors (-NH, O, S) attack the carbonyl carbon resulting in the formation of a new bond (amide, ester,

or thioester) between the nucleophilic proteins and the drug, and the liberation of the glucuronic acid moiety. Covalent binding to proteins can also occur through a second glycation mechanism involving the reaction of the protein amino-group with the open-chain aldehyde form of the acyl migrated isomers of the 1-*O*-acyl-glucuronide (Spahn-Langguth and Benet, 1992). This process first requires the acyl migration of the drug moiety away from the C1 position allowing for the opening of the sugar ring. The resulting exposed aldehyde then reversibly forms an imine (Schiff's base) with the amine group attached to the protein, followed by a proposed Amadori rearrangement to yield a stable ketoamine derivative. In this scenario, the protein, drug, and glucuronide moiety continue to be linked together.

1.3.4 The Basis of Covalent Binding of Carboxylic Acid Drugs

Studies have shown that the extent of covalent binding by carboxylic acid drugs *in vitro* depends on the rate of degradation, hydrolysis and acyl migration of the glucuronide conjugate. Specifically, the extent of alpha carbon substitution effects the stability and reactivity of the acyl-linked metabolites of carboxylic acid drugs (Grillo et al., 2002). There appears to be a linear correlation between the first-order disappearance rate constant, which is proportional to its chemical reactivity, of the acyl glucuronide in buffer and the extent of covalent binding with HSA (Figure 1.5) (Benet et al., 1993). Arylacetic acid (α-unsubstituted), tolmetin and zomepirac exhibited the highest amounts of covalent binding and the lowest stability. The 2-arylpropionic acids (mono α-substituted), carprofen and fenoprofen, displayed less covalent binding than the α-unsubstituted carboxylic acid drugs, while the fully substituted alpha carbon carboxylic acid drugs, beclobric acid and furosemide, displayed the lowest amount of covalent binding. The

data in Figure 1.5 summarizes the data from our laboratory over a 6-year period with respect to *in vitro* degradation rates of the β-1-*O*-acyl glucuronides and the extent of their *in vitro* covalent binding for nine drugs. These data suggest that the extent of *in vitro* covalent binding to albumin can be predicted by the degree of alpha carbon substitution of the corresponding carboxylic acid.

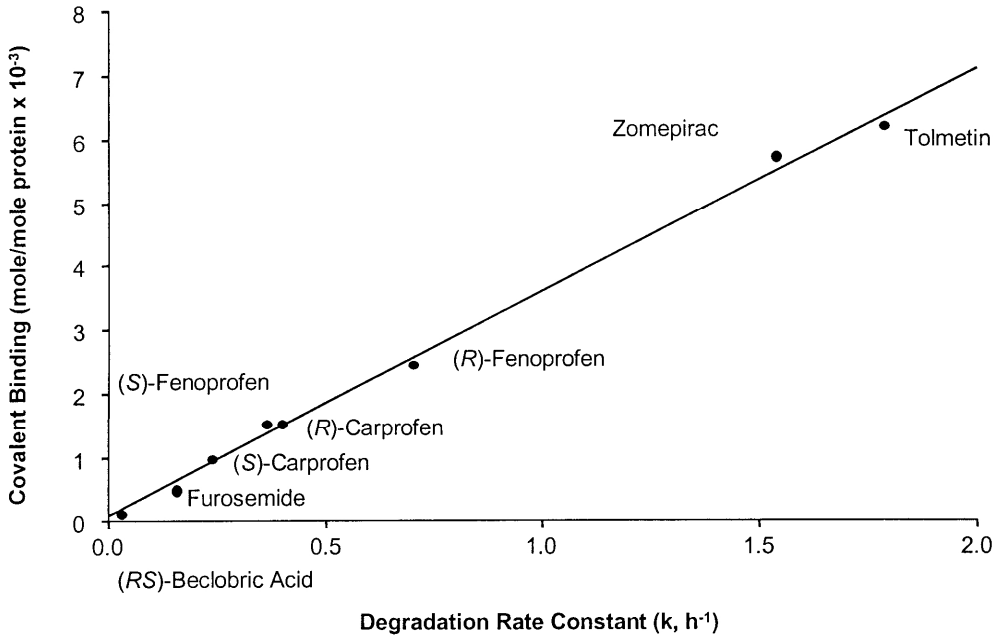

Figure 1.5 Plot of maximum epitope density (moles drug covalently bound per mole of protein x 10^{-3}) versus degradation rate constant (h^{-1}) for the in vitro incubation of various acyl glucuronides (1 µM) in the presence of human serum albumin (0.5 mM). Degradation rates reflect both acyl migration and hydrolysis. (adapted from Li, 2002)

The degree of *in vivo* covalent binding also depends on the concentration of the acyl glucuronide and the degradation of each conjugate. Plasma concentrations of each acyl glucuronide vary with each drug and are determined by the rate of formation, degradation,

elimination, as well as the administered dose. Many carboxylic acids such as zomepirac (Smith et al., 1985) and diflunisal (McKinnon and Dickinson, 1989) have yielded a significant amount of acyl glucuronides in human plasma. Table 1.1 shows the *in vivo* studies of five different carboxylic acid drugs administered at their therapeutic doses in five different groups of healthy volunteers. The data show a 30-fold variation in acyl glucuronide AUC and a 25-fold variation in the maximum amount of plasma protein binding. Normalizing the bound drug to the AUC of the corresponding acyl glucuronide yields a significant linear correlation suggesting that the *in vivo* covalent binding of carboxylic acid drugs to albumin in humans is also predictable by the degradation rate constant of the glucuronide conjugate when the extent of covalent binding is corrected for the levels of the glucuronide present in plasma (AUC).

Parent Compound	Bound drug (mole/mole protein x 10^4	AUC Glucuronide (mole x h/L) x 10^6	Bound AUC (mole drug x L/(mole protein x mole glucuronide x h)) x 10^{-2}	k/h
Tolmetin	2.77 ±1.54	3.72 ±0.95	0.75	1.78
Zomepirac	2.33 ±0.45	6.41 ±2.14	0.36	1.54
(*R*)-fenoprofen	1.02 ±0.32	6.31 ±5.65	0.16	0.71
(S)-fenoprofen	3.23 ±0.85	60.4 ±24.7	0.054	0.36
Racemic carprofen	1.92 ±1.28	40.9 ±7.3	0.047	0.32
(+)-beclobric acid	0.12 ±0.03	8.16 ±1.34	0.015	0.031
(-)-beclobric acid	0.20 ±0.11	8.31 ±1.63	0.024	0.027

*Measurement of maximum amount of drug covalently bound to human serum albumin and area under the plasma concentration time curve (AUC) for the glucuronide conjugates measured in five different groups of healthy volunteers following oral dosing of either 400 mg of tolmetin (Hyneck et al., 1988), 100 mg of zomepirac (Smith et al., 1986), 600 mg of racemic fenoprofen (Volland et al., 1991), 50 mg of racemic carprofen (Iwakawa et al., 1988), or 100 mg of racemic beclobric acid (Mayer et al., 1993). When covalently bound drug is normalized to area under the curve for the representative glucuronide conjugates, an excellent correlation with the in vivo degradation rate constant (k) is obtained with an r^2 of 0.873.

Table 1.1 *In vivo* bound drug, area under the plasma drug glucuronide concentration time curve

(AUC) and in vitro acyl glucuronide degradation rates. (adapted from Li, 2002)

1.4 Acyl-Coenzyme A Thioesters (Acyl-CoA)

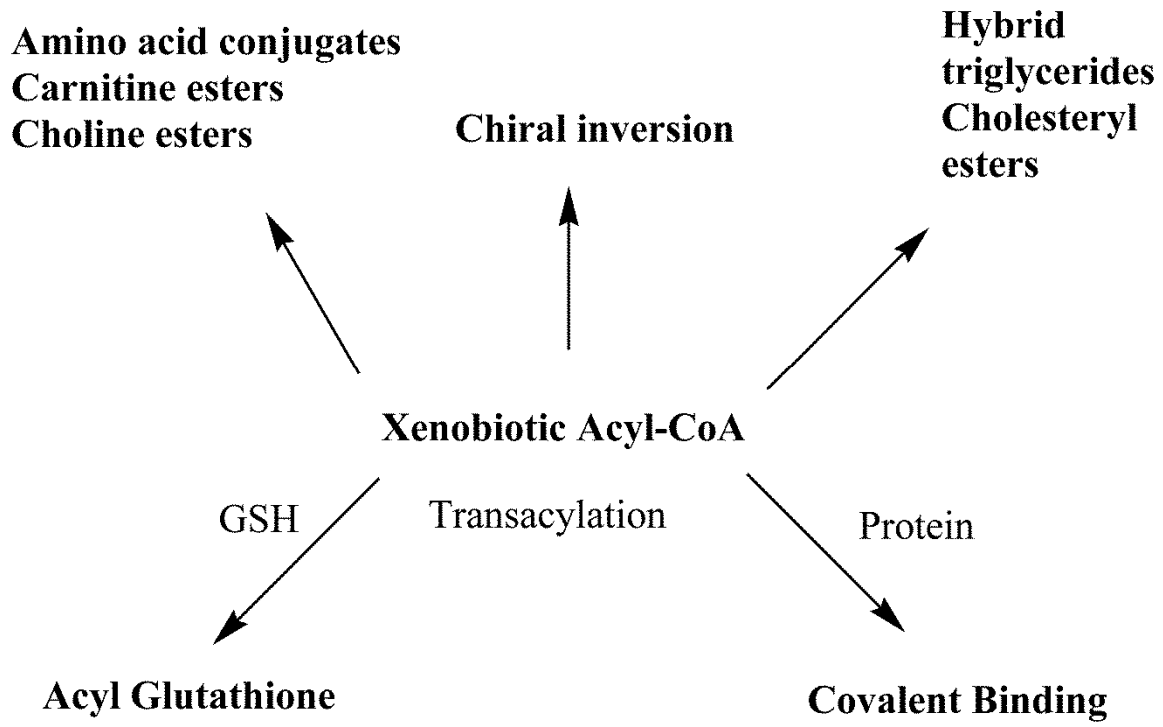

Figure 1.6 Potential routes of disposition of xenobiotic acyl-CoA thioesters.

Endogenous fatty acids can undergo enzymatic activation to high energy acyl coenzyme A thioester intermediates. When carbohydrate supplies run low, the body uses fats as an energy source. Fats are stored in adipose tissue where they are converted into fatty acids and glycerol by lipases that are then transported by the blood to the tissues where they are oxidized. Fatty acids are first activated in the cytoplasm prior to transport into the endoplasmic reticulum or the mitochondria where they are then converted into *S*-acyl-CoA thioester (CoA) intermediates. Acyl CoA thioesters play a role in fatty acid synthesis, β-oxidation, triacylglycerol formation, protein acylation, as well as other intermediate processes (Figure 1.6). Carboxylic acid-containing xenobiotics can also be converted into *S*-acyl-CoA thioester intermediates. The

formation of these acyl-CoA intermediates may serve an important role in phase II metabolism by amino acid conjugation (Hutt et al., 1990), formation of acyl carnitine (Knights, 1998), and choline derivatives (Sastry et al., 1997). Xenobiotic acyl-CoA intermediates may also play a part in lipid metabolism resulting in the formation of hybrid triacylglycerides and cholesteryl esters (Fears, 1985). 2-Arylpropionic acids show *in vivo* inversion from the pharmacologically inactive (*R*)-isomer to the active (*S*)-isomer via the acyl-CoA thioester intermediate (Hutt and Caldwell, 1983; Caldwell et al., 1988). Endogenous fatty acid *S*-acyl CoA thioesters are efficiently degraded by fatty acid β-oxidation. However, xenobiotic *S*-acyl-CoA intermediates may not undergo fatty acid β-oxidation. It is possible that these xenobiotic *S*-acyl-CoA derivatives accumulate in the cell and transacylate nucleophilic centers of macromolecules resulting in the formation of cytotoxic and/or immunogenic drug-protein adducts.

1.4.1 Biochemical Aspects of Acyl-CoA Formation

S-Acyl-CoA thioester intermediate formation is catalyzed by acyl-CoA synthetases (ACS) located in the endoplasmic reticulum, the outer mitochondrial membrane (long-chain fatty acid acyl-CoA synthetases), and in the mitochondrial matrix (short-, medium-, and branched-chain acyl-CoA synthetases). The formation of an *S*-acyl-CoA thioester via acyl-CoA synthetase occurs through a two-step reaction mechanism that requires the cofactors ATP and coenzyme A (CoASH) to proceed. The reaction occurs via a ping-pong mechanism in which the adenylate (AMP) moiety of ATP is transferred to the acyl group forming an acyl-adenylate intermediate and a pyrophosphate (PPi) (Figure 1.7). This enzyme bound activated intermediate is then

attacked by CoASH to yield an acyl-CoA product and free AMP. The reaction is believed to be "pulled" by pyrophosphatases that hydrolyzes PPi into inorganic phosphates (Watkins, 1997).

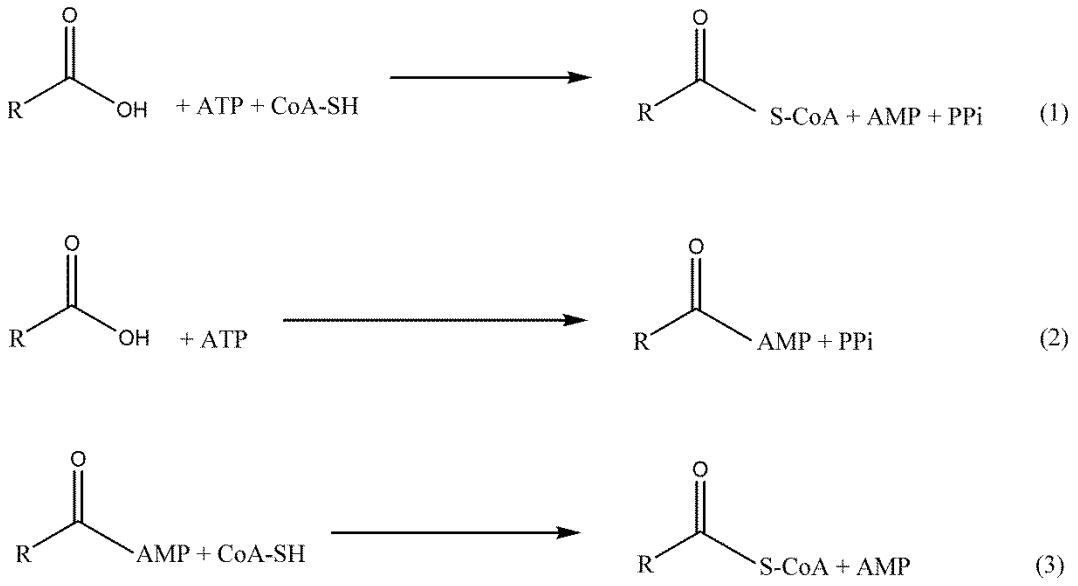

Figure 1.7 Mechanism of acyl-CoA thioester formation. (adapted from Li, 2002)

ACS enzymes are classified according to the length of the fatty acid carbon chains they react with; short-chain ACS (C_2-C_4), medium-chain ACS (C_4-C_{12}), long-chain ACS (C_{10}-C_{22}), and very long-chain ACS (>C_{22}) (Knights, 1998; Knights and Drogemuller, 2000). ACS enzymes are located throughout cells including the cytosol, endoplasmic reticulum, mitochondria, and peroxisomes with the highest activity associated with the liver and adipose tissue. Medium and long chain-ACS have been implicated in the metabolism of a number of carboxylic acid drugs. Carboxylic acid drugs that have been shown to be substrates for medium-chain ACS located on the mitochondrial matrix, are the arlyacetic and aromatic carboxylic acids, which include phenylacetic acid, 1-naphthylacetic acid, benzoic acid, and salicylic acid. 2,4-diclophenoxyacetic acid (2,4-D), ibuprofen (IB), trimethylacetic acid (TMA) and valproic acid

(VPA) have also been shown to be substrates for medium-chain ACS. Hypolipidemic and perioxisome-proliferating agents such as clofibric acid, ciprofibric acid, nafenopin, phthalate plasticizers, and 2-arylpropionic acid nonsteroidal anti-inflammatory drugs (ibuprofen, fenoprofen, ketoprofen, and benoxaprofen) have also been shown to be substrates for long-chain ACS (Li, 2002).

1.4.2 Chemical Reactivity of Acyl-CoA Thioesters

Xenobiotic *S*-acyl-CoA thioesters are considered reactive intermediates due to the presence of the relatively electrophilic thioester carbonyl carbon (soft electrophile). Early characterization of the chemical reactivity of *S*-acyl-CoA thioesters came from *in vitro* studies of the non-enzymatic acylation of glycine by salicylic acid-*S*-acyl-CoA yielding the glycine amide, salicylurate (Tishler and Goldman, 1970). Other studies have shown that endogenous long-chain fatty acid *S*-acyl-CoA thioesters are capable of non-enzymatically transacylating the sulfhydryl groups of cysteine residues found in proteins (Duncan and Gilman, 1996). Many xenobiotic *S*-acyl-CoA thioester intermediates have also been reported to transacylate GSH *in vitro* in buffer and incubations with hepatocytes.

Clofibric acid undergoes bioactivation into the chemically reactive clofibryl-*S*-acyl-CoA (CA-*S*-acyl-CoA) intermediate, which is capable of transacylating GSH to form clofibryl-*S*-acyl-GSH (CA-SG) (Grillo and Benet, 2002). The rate of formation of CA-SG from CA-*S*-acyl-CoA was determined to be 40-fold higher compared to the incubations involving the corresponding clofibryl-1-*O*-glucuronide (CA-1-*O*-Gluc). However, unlike the CA-1-*O*-Gluc, which has a half life of ~7.3 hrs due to acyl migration and whose isomers are less able to transacylate GSH, CA-*S*-

acyl-CoA was shown to be highly stable (half life ~ 20 days) *in vitro* in potassium phosphate buffer under physiological conditions. These early studies show that xenobiotic-*S*-acyl-CoA thioester intermediates are not only reactive with GSH but also significantly more reactive than their corresponding 1-β-*O*-glucuronides toward the transacylation of GSH and potentially protein nucleophiles.

The model profen 2-phenylpropionic acid (2-PPA) yielded results where the 2-PPA-*S*-acyl-CoA was 70-fold more reactive with GSH than its corresponding 2-PPA-1-*O*-glucuronide in buffer under physiological conditions. Stability studies have also shown that 2-PPA-1-*O*-glucuronide possessed a $t_{1/2}$ of 2.4 hours while 2-PPA-*S*-CoA was completely stable after 24 hours (Li et al., 2002a). No enantioselective differences were observed in the rate of reaction of 2-PPA-*S*-acyl-CoA with GSH. Covalent binding studies in rat hepatocytes showed that the radioactive time-course profile of binding correlates to the 2-PPA-*S*-acyl-CoA formation and not with the 2-PPA-1-β-*O*-G (Li et al., 2002b). Rat hepatocyte metabolic activation studies have also shown that enantioselective covalent binding to proteins (*R/S* = 4.5) was similar to the 2-PPA-*S*-acyl-CoA (*R/S* = 7.0) and not to the corresponding 2-PPA-1-*O*-glucuronide (R/S = 0.7) clearly showing that 2-PPA-*S*-acyl-CoA contributes more to the covalent binding of proteins than the corresponding 2-PPA-1-*O*-G.

Studies with ibuprofen have shown that the formation of ibuprofen-*S*-acyl-GSH (I-*S*-acyl-GSH) in rat hepatocyte incubations is selective for the (*R*)-ibuprofen isomer (Grillo and Hua, 2008). Ibuprofen-*S*-acyl-CoA (I-*S*-acyl-CoA) is known to be highly enantioselective for the (*R*)-antipode. Therefore, incubations utilizing enantiomerically pure isomers of ibuprofen show that 1-*S*-acyl-

GSH formation is primarily due to the 1-*S*-acyl-CoA and not ibuprofen-1-*O*-acyl glucuronide. The time course of formation of 1-*S*-acyl-GSH was also shown to be similar to the formation of 1-*S*-acyl-CoA and not the corresponding glucuronide. Further evidence of the involvement of the *S*-acyl-CoA towards glutathione formation is also demonstrated from the stereoselective transacylation of GSH by (*R*)-flunoxaprofen forming flunoxaprofen-*S*-acyl-GSH (Grillo et al., 2010). In another study, naproxen-*S*-acyl-CoA was 100-fold more reactive with GSH forming (*S*)-naproxen-*S*-acyl-GSH than with the corresponding (*S*)-naproxen-1-β-*O*-glucuronide, which is consistent with the relative ability of the naproxen acyl-linked derivatives to covalently bind onto human serum albumin *in vitro* (Olsen et al., 2002).

Studies on the reactivity of tolmetin-*S*-acyl-CoA thioester, a derivative of the α-unsubstituted arylacetic acid NSAID tolmetin, have shown that tolmetin-*S*-acyl-CoA thioester is ~15 fold more reactive toward the transacylation of GSH in potassium phosphate buffer than the *S*-acyl-CoA thioesters of (*S*)-naproxen and clofibric acid (Olsen et al., 2007). These results are consistent with the structure/chemical reactivity relationships in which arylacetic acid derivatives are more reactive than the profens and 2-methyl-2-phenoxypropionic acid derivatives for both acyl-glucuronides and *S*-acyl-GSH-thioesters.

1.4.3 The Basis of Covalent Binding of S-Acyl-CoA

The chemical structure of a carboxylic acid moiety can be correlated to the reactivity of the corresponding *S*-acyl-CoA reactivity. A study involving eight structurally different synthetic *S*-acyl-CoA derivatives, i.e. ibuprofen, clofibric acid, indomethacin, fenbufen, tolmetin, salicylic acid, 2-phenoxyproprionic acid, and 4-chloro-2-methyl-phenoxyacetic acid (MCPA), were

incubated with GSH *in vitro* (Sidenius et al., 2004). Each carboxylic acid derivative differed in one of three ways: substitution at the α-carbon, the presence of an oxygen atom at the β-position, or the placement of an *o*-hydroxy group on the benzoic acid moiety. The results of this study showed that the reactivity of the *S*-acyl-CoA derivative with GSH is dependent on the α-carbon substitution (increased substitution resulted in decreased reaction rates), the placement of an oxygen atom at the β-position carbon resulted in increased reactivity, and the substitution of an *o*-hydroxy-group on the benzoic acid led to increased reaction rate of the corresponding *S*-acyl-CoA. The rank order of reactivity of the *S*-acyl-CoA with GSH was phenoxyacetic acid > *o*-hydroxybenzoic acid ~ phenoxypropionic acid > arylacetic acid > 2-methyl-2-phenoxypropionic acid ~ 2-phenylpropionic acid derivatives. More specifically, phenoxyacetic acid-containing MCPA *S*-acyl-CoA derivatives were ~4-fold more reactive than salicylic acid-S-acyl-CoA and 120-fold more reactive than ibuprofen-*S*-acyl-CoA towards glutathione adduct formation.

The anticonvulsant agent valproic acid (VPA) has been shown to induce a rare but sometimes fatal hepatotoxicity. Valproic acid is metabolized by P450 to the unsaturated 4-ene-VPA (Rettie et al., 1987) that is further metabolized by fatty acid β-oxidation enzymes to form the (*E*)-2,4-diene-VPA-CoA and the 3-keto-4-ene-VPA-CoA, which are reactive and capable of forming glutathione intermediates. However, studies have shown that α-fluorination of 4-ene-VPA resulted in no hepatotoxicity (Tang et al., 1995), which is consistent with the *S*-acyl-CoA structure/chemical relationship. Interestingly, further mechanistic studies have shown that the α-fluorinated derivative of VPA did not form any acyl-CoA thioester derivatives (Grillo et al.,

2001) suggesting that the VPA-acyl-CoA derivatives may in part mediate some of the idiosyncratic hepatotoxicity associated with valproic acid.

Not all xenobiotic *S*-acyl-CoA derivatives have been shown to form *S*-acyl-GSH adducts. When phenylacetic acid (PAA) was incubated in hepatocytes, no corresponding PAA-*S*-GSH was detected despite the fact that PAA-*S*-acyl-CoA was present (Grillo and Lohr, 2009). However, covalent binding studies utilizing [^{14}C]PAA did result in radiolabeled covalent binding to proteins. One possible hypothesis to explain this phenomenon is that the PPA-*S*-acyl-CoA is unable to escape its site of formation and react with glutathione pools in the cytosol or mitochondrial matrix.

1.5 Acyl-Adenylates

The activation of carboxylic acid functional groups into the *S*-acyl-CoA thioester occurs through the formation of a high energy intermediate, the mixed anhydride adenosine 5'-monophosphate adenylate (AMP) (Mao et al., 1992; Menzel et al., 1994; Hall and Xiaotao, 1994 Ikegawa et al., 1999). Acyl-AMP derivatives, which have a tendency to accumulate in cells, are considered to be reactive in nature because they possess an electrophilic carbonyl-carbon capable of reacting non-enzymatically with taurine amino groups (Ikegawa et al., 1999), nucleophilic centers of proteins, and sulphydryl groups found in cysteine residues of GSH (Goto et al., 2001). The acyl-AMP intermediates of the endogenous fatty acids of acetic and butyric acid have been shown to build up in cells but the formation of protein adducts from these acyl-AMPs via a transacylation reaction was not determined (Webster and Campagnari, 1962). Xenobiotic carboxylic acids can also undergo bioactivation into their corresponding acyl-AMP

intermediates by the same mechanism as fatty acids. The anticonvulsant drug valproic acid has been shown to be metabolized into valproyl-AMP when incubated in rat hepatocytes. The valproyl-AMP exists in the non-bound form to the acyl-CoA synthetase enzymes (Mao et al., 1992). The release of valproyl-AMP from the acyl-CoA synthetase active site was proposed to occur by the ability of the di-*n*-propyl branched-chain structure of valproic acid to sterically hinder the transacylation of CoASH. However, in those studies, the chemical reactive potential of valproyl-AMP was not investigated. The NSAID ibuprofen has also been shown to form an acyl-AMP intermediate, but its reactivity was also not determined (Menzel et al., 1994). More recent studies on cholic acid have shown that endogenous bile acids are effectively transformed into chemically reactive acyl-AMP intermediates and are capable of undergoing transacylation reactions with peptides, proteins, and thiol groups of GSH resulting in the formation of bile acid protein and bile acid *S*-acyl-GSH conjugates (Goto et al., 2001; Mitamura et al., 2007; Mitamura et al., 2009a; Mitamura et al., 2009b).

Studies have shown that cholic acid is transformed into reactive *S*-acyl-CoA thioesters, which then go on to form amino acid conjugates. The mechanism of formation of acyl-CoA proceeds via a ping-pong mechanism catalyzed by the acyl-CoA synthetase enzyme. The first step involves an adenylyl transfer from ATP to the carbonyl carbon of the carboxylic acid functional group forming an acyl-adenylate as an obligatory enzyme bound intermediate and PPi. The activated intermediate is then attacked by CoA-S^- yielding the corresponding acyl-CoA thioester and a free AMP moiety. When cholic acid was incubated in rat liver microsomal fractions, acyl-AMP derivatives were detected and are thus considered to be the activated form of carboxylic acids. These cholyl-adenylates have been shown to react in buffer with compounds having

amino groups via a nonenzymatic condensation of cholyl-adenylate with taurine to produce taurocholate (Ikegawa et al., 1999). Additional studies involving the incubation of cholyl-adenylate with lysozyme produced protein bound bile acids. More specifically, cholic acid covalently bound onto lysine residues 1, 33, 97, and 116 suggesting that bile acid adenylates may act as reactive intermediates to produce bile acid-protein adducts with peptides by nucleophilic displacement of the 5'-adenylic acid through the free amino group (Goto et al., 2001). Cholyl-AMP and cholyl-CoA have also been shown to covalently modify DNA suggesting the possibility of acyl adenylate genotoxicity (Takamura-Enya et al., 2005). In order to determine the rate of formation of acyl-AMPs in subcellular fractions, five common natural bile acids [cholic acid (CA), chenodeoxycholic acid (CDCA), deoxycholic acid (DCA), ursodeoxycholic acid (UDCA), and lithocholic acid (LCA)] were incubated in rat liver microsomal fractions, mitochondrial fractions, and cytosol (Ikegawa et al., 2009). These incubations showed that the enzymes catalyzing the transformation of all five bile acids into their acyl-AMP derivatives were primarily present in the microsomal fractions. Acyl-AMP formation by the mitochondria was only observed for CDCA, DCA, and LCA, while no ACS activity was detected in the cytosol. Post-administration of a mixture of cholic acid derivatives to biliary fistula rats revealed the presence of *S*-acyl-GSH adducts, furthering the notion that the metabolism of bile acids to *S*-acyl-GSH thioesters occurs in the liver prior to excretion in the bile (Mitamura et al., 2007). The relative transacylating properties of cholyl-AMP compared to cholyl-CoA with GSH, NAC (*N*-acetylcysteine), taurine, and glycine in buffer under physiological conditions revealed that cholyl-AMP was preferentially more reactive than cholyl-CoA towards the amino groups found in glycine and taurine. In contrast, cholyl-CoA was more reactive than cholyl-AMP with the thiol

groups of GSH and NAC (Mitamura et al., 2011). These differences in the reactivity of cholyl-AMP and cholyl-CoA towards amino and thiol groups may be due to different acylation reaction mechanisms (SN1 versus SN2) attributed to electrophilicity differences of the carbonyl carbon of these acyl-linked cholic acid metabolites and the nucleophilicity of the amino and thiol groups located in the bionucleophiles studied. All of this evidence strongly suggests that in addition to the acyl-CoA, and acyl-glucuronide metabolites, xenobiotic carboxylic acids can undergo bioactivation into a reactive acyl-AMP derivative that has the potential to react with macromolecules in the body to cause an idiosyncratic toxicity.

1.6 Mefenamic Acid

Mefenamic acid (Ponstel ®), (*N*-(2,3-xylyl) anthranilic acid, is a carboxylic acid containing NSAID that possesses analgesic, and antipyretic effects. Mefenamic acid is an anthranilic acid derivative belonging to the fenamate class of NSAIDs. Mefenamic acid, first marketed in the late 1960s, is most often used to treat the pain associated with dysmenorrhea in the short term (seven days or less) as well as mild to moderate pain including headache, dental pain, postoperative and postpartum pain. Mefenamic acid is available worldwide, however, in the US mefenamic acid is only licensed for the treatment of moderate pain in adults and its use is not recommended for a period longer than seven days at a time, while in Germany, mefenamic acid is no longer prescribed due to possible severe side effects such as hematologic disturbance. A typical oral dose for an adult is up to 500 mg three times daily. Upon oral administration, mefenamic acid is absorbed rapidly and has a short half life of approximately two hours.

NSAIDs are a routinely prescribed class of drugs used to treat mild to moderate pain and they are the most commonly prescribed analgesic medication worldwide. NSAIDs work by reversibly inhibiting cyclooxygenases which mediate prostaglandin and thromboxane A2 synthesis (Fitzgerald and Loll, 2001). Prostaglandins maintain the gastric mucosal barrier, regulate renal blood flow, and regulate endothelial tone. NSAIDs' ability to inhibit cyclooxygenase-dependent prostanoid formation (Hawkey, 1999) allows them to regulate inflammation and nociception.

The use of mefenamic acid has been implicated in several cases of hematologic disturbances including agranulocytosis, hepatic and renal disturbances, nephritis, renal failure, renal papillary necrosis and severe intolerance reactions. There have also been a few reported cases of mefenamic acid induced hypersensitivity reactions of the skin and the mucous membranes, commonly known as fixed drug eruptions. Fixed drug eruptions are drug-induced reactions of the skin and mucous membranes characterized by the sudden appearance of one or several round lesions after repeated exposure to the responsible drug (Sehgal and Srivastava, 2006; Ozkaya, 2008). The reason for these lesions is currently unknown, however an association with HLA-B22 has been reported (Pellicano et al., 1994). Intraepidermal αβTRC+ CD8+T cells possessing a phenotype resembling effector memory T cells may play a central role in the pathogenesis of epidermal damage (Mizukawa and Shiohara, 2009). These T cells persist for years and can be reactivated after renewed exposure to the eliciting drug, possibly through TNF-α-dependent upregulation of the expression of ICAM-1 on keratinocytes. Subsequently, the IFN-γ release results in an inflammatory response and the reappearance of the lesions. Fixed drug eruptions tend to occur more frequently after oral administration and are less likely to occur after intravenous or intramuscular administration or topical application.

Several cases of nephrotoxicity, including acute renal failure and tubulointerstitial nephritis, have also been reported following the use of mefenamic acid (Robertson et al., 1980; Drury et al., 1981; Woods, 1981; Taha et al., 1985). Patients experiencing induced mefenamic acid renal failure and impaired renal function recovered after withdrawal of the drug in most documented cases (Robertson et al., 1980; Drury et al., 1981). In addition to renal failure, tubulointerstitial nephritis has also been associated with the use of mefenamic acid. The direct cause of the drug-induced tubulointerstitial nephritis is still unknown but evidence suggests that the basis of this disorder is immunological in nature rather than toxicological. Mefenamic acid has also been shown to induce a dose dependent hepatocyte degeneration in mouse liver parenchyma, including hepatocellular necrosis, massive degeneration, inflammation, a significant increase in plasma alanine aminotransferase activity, and an increase in liver weight, suggesting that mefenamic acid is capable of inducing hepatotoxicity (Somchit et al., 2004). The current hypothesis of this idiosyncratic toxicity is that mefenamic acid or its reactive metabolite covalently binds onto proteins stimulating an immunological response in hypersensitive individuals (McCluskey and Colvin, 1978).

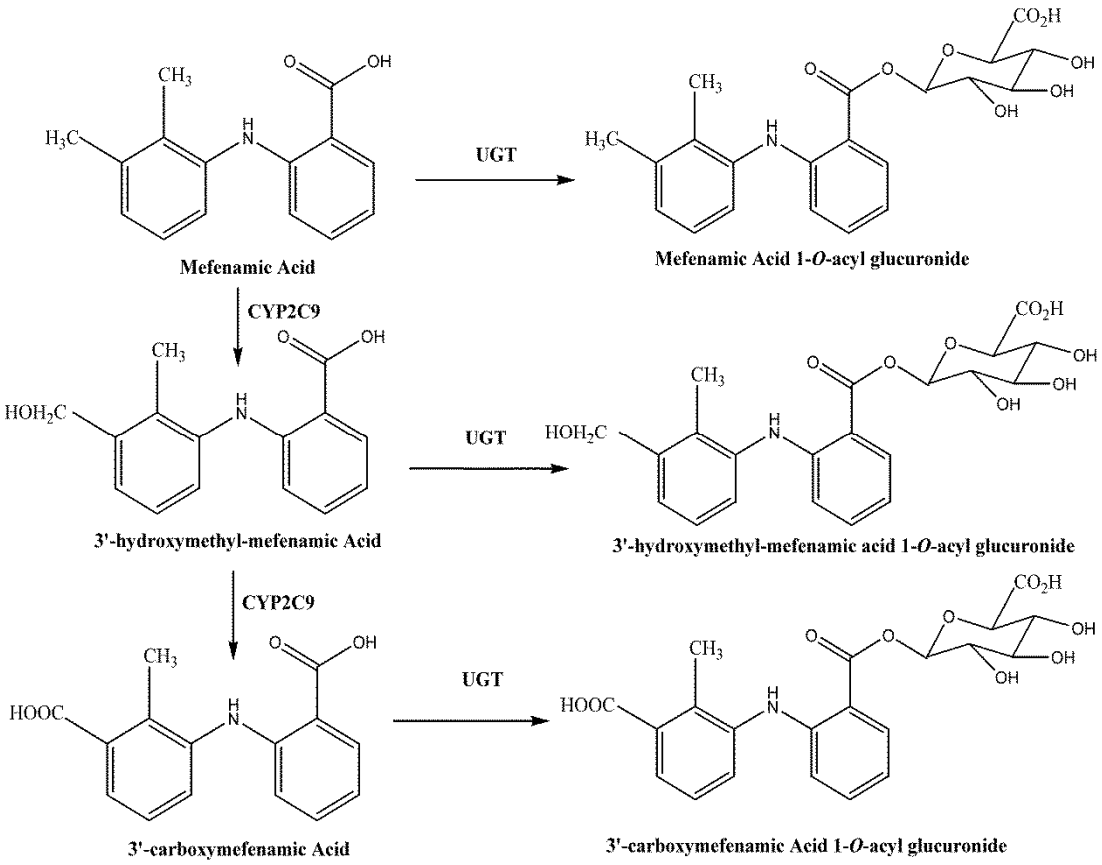

Figure 1.8 Metabolic scheme of mefenamic acid.

Mefenamic acid (MFA) undergoes phase II metabolism via the free carboxyl group into an acyl glucuronide, mefenamyl-1-*O*-glucuronide (MFA-1-*O*-Gluc) *in vitro* and *in vivo* (Figure 1.8) (Glazko, 1968; Sato et al., 1993). In addition to being glucuronidated, mefenamic acid is also metabolized by cytochrome P450 2C9 at the 3-methyl position prior to glucuronidation to form the 3-*OH*-MFA, which can undergo further oxidation of the 3-hydroxy functional group into a 3-carboxyl, resulting in a dicarboxylic acid metabolite (Glazko, 1968), both of which can be glucuronidated. The dicarboxylic acid metabolite and the monoglucuronide are excreted unchanged in the urine.

Studies characterizing the reactivity of MFA-1-*O*-Gluc have shown that under physiological conditions, MFA-1-*O*-Gluc irreversibly binds to human serum albumin *in vitro* (McGurk et al., 1996). Covalent binding to cellular proteins in culture was also shown to occur when mefenamic acid was incubated in the heterologous Chinese hamster lung fibroblast cell line V79 expressing UGT1A2. This irreversible binding was directly correlated to MFA-1-*O*-Gluc because nontransfected cells exhibited no covalent binding. Stability studies also revealed that MFA-1-*O*-Gluc has a half life of ~16.5 hours in buffer under physiological conditions, with degradation increasing under alkaline conditions ($t_{1/2}$~ 5 hours at pH 8.0) (McGurk et al., 1996).

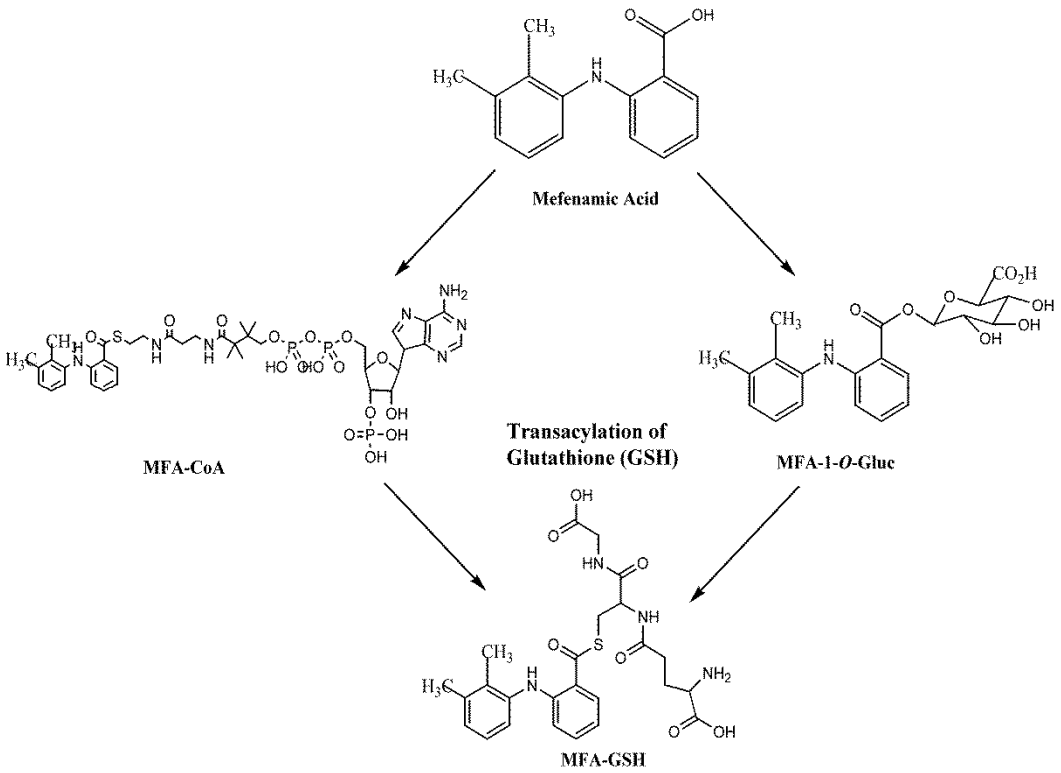

Figure 1.9 Scheme for the phase II bioactivation of mefenamic acid.

Preliminary studies comparing the reactivities of the phase II acyl-linked metabolites of mefenamic acid, mefenamyl-CoA (MFA-CoA) and MFA-1-*O*-Gluc, with glutathione in buffer

under physiological conditions have shown that MFA-CoA is 79 fold more reactive than its corresponding glucuronide in the transacylation of glutathione (Figure 1.9). When 100 µM of mefenamic acid was incubated in rat hepatocytes (2 million cells/ml), mefenamic acid produced a maximum concentration of mefenamyl-GSH (MFA-GSH) 0f 1.2 µM at 20 minutes. This concentration of glutathione adducts has been shown to be significantly higher than with the corresponding NSAIDs ibuprofen (~1.3 nM) (Grillo and Hua, 2008), diclofenac (~1.0 nM) (Grillo et al., 2003), 2-PPA (~0.3 nM) (Li et al., 2002a), zomepirac (~0.25 nM) (Olsen et al., 2005), and phenylacetic acid (~0.2 nM). Mefenamic acid has also been shown to form a maximum concentration of 50 nM MFA-CoA at 30 minutes and a maximum concentration of 3 µM of MFA-1-*O*-Gluc at 60 minutes under the same conditions in rat hepatocytes. The time profiles and maximum concentrations of both MFA-CoA and MFA-1-*O*-Gluc do not account for the time course of formation and the maximum concentration of MFA-GSH. Inhibition studies in rat hepatocytes have also shown that borneol, an inhibitor of glucuronidation, exhibited 91.1% inhibition of MFA-1-*O*-Gluc formation and 3.3% inhibition of MFA-CoA formation. However, MFA-GSH increased 16%, suggesting that MFA-GSH formation is not due to the transacylation of GSH by MFA-1-*O*-Gluc. Additional studies using lauric acid, an inhibitor of medium-chain acyl-CoA synthetase, resulted in a 68% inhibition of MFA-1-*O*-Gluc formation and a 58.4% inhibition of MFA-CoA formation further suggesting that the transacylation of MFA-GSH is not entirely due to MFA-CoA reactivity. Further studies assessing the effects of borneol on the cytotoxicity of NSAIDs in rat hepatocytes have shown that mefenamic acid has a borneol factor (LD_{50} with borneol/LD_{50} without borneol) of 2.7, suggesting that most of the cytotoxicity associated with mefenamic acid is not due to MFA-1-*O*-Gluc (Siraki et al., 2005).

Mefenamic acid is a NSAID associated with several cases of nephrotoxicity and skin lesions in humans and hepatotoxicity in mice. Studies thus far characterizing the transacylation properties of the phase II acyl-linked metabolites, MFA-CoA and MFA-1-*O*-Gluc, of mefenamic acid have failed to account for all of the MFA-GSH formed in rat hepatocytes. However, a third acyl linked metabolite, mefenamyl-adenylate (MFA-AMP), the intermediate to acyl-CoA formation, is hypothesized to exist and be reactive in rat hepatocytes.

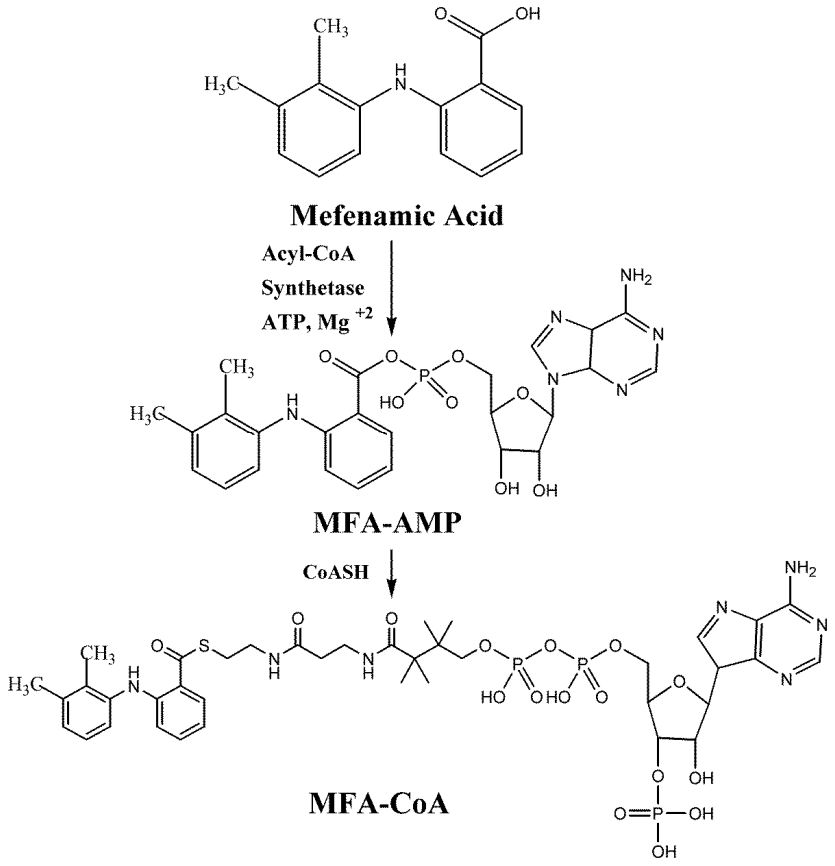

Figure 1.10 Biosynthesis of mefenamyl-*S*-Acyl-CoA via the chemically-reactive intermediate acyl-adenylate MFA-AMP.

Studies attempting to characterize the structural requirements for the hepatotoxicity of NSAIDs in isolated rat hepatocytes show that mefenamic acid exhibits a high percentage of LDH leakage (a marker for cytotoxicity) and high ATP consumption (a cofactor for AMP and *S*-CoA synthesis) (Masubuchi et al., 1998), which may be consistent with the presence of an reactive acyl-AMP pathway (Figure 1.10). Therefore, we hypothesize that the acyl-linked metabolite of mefenamic acid, MFA-AMP is responsible for the high concentration of MFA-GSH formation in rat hepatocytes and potentially the toxicity induced by mefenamic acid.

1.7 Objective and Overview of Thesis

The overall aim of this thesis is to investigate the chemical reactivity of the acyl adenylate-linked metabolite of mefenamic acid (MFA-AMP) and to compare its reactivity and time course of formation with that of its MFA-CoA and MFA-1-*O*-glucuronide metabolite. We predict that xenobiotic acyl-AMP derivatives are capable of acylating nucleophilic sites of proteins. We believe this reactivity may contribute to the covalent binding of mefenamic acid to macromolecules and stimulate some of the idiosyncratic toxicity associated with acidic drugs. This study focuses on the phase II acyl-adenylate metabolite of mefenamic acid and compares its contribution to the acylation of glutathione in rat hepatocytes to those of the acyl-CoA thioesters and the acyl-glucuronides. The specific aims of this study are as follows:

1. **Synthesize and characterize MFA-AMP, MFA-CoA, and MFA-GSH by UV, LC/MS/MS, and NMR.** In order to investigate the reactivity and time course of formation of the acyl-linked metabolites of mefenamic acid, which are not commercially available, all of these derivatives must be synthesized and purified for use as authentic standards.

2. Determine the chemical stability and reactivity with GSH of MFA-AMP, MFA-CoA, and MFA-1-*O*-Gluc in buffer. Glutathione is an atypical endogenous tripeptide that is believed to play a cytoprotective role in the body by neutralizing reactive electrophilic compounds. For our purposes, glutathione serves as a nucleophilic biomarker for comparing the electrophilicity of reactive metabolites of xenobiotics. Presumably, the more GSH adducts that are formed the greater the reactivity of that acylating metabolite, and hence the higher probability that the metabolite will covalently bind onto a protein and stimulate an idiosyncratic reaction.

3. Measure the timecourse of formation of MFA-AMP, MFA-CoA, MFA-1-*O*-Glucuronide, and MFA-GSH in rat and human hepatocytes *in vitro*. Glutathione adduct formation and covalent binding to proteins is a function of the amount of reactive metabolites formed, the time profile of formation of each metabolite, and each metabolite's intrinsic reactivity. The time profile of formation (t_{max} and C_{max}) of each metabolite will be compared to the time profile of GSH in order to determine which acyl-linked metabolite of mefenamic acid contributes most to mefenamic acid glutathione adduct formation.

4. Study the mechanism of formation of MFA-AMP, MFA-CoA, and MFA-1-*O*-Glucuronide in rat hepatocytes. The inhibition of acyl-CoA synthetases (ACS) and UGT with specific inhibitors will allow us to determine which enzymes are responsible for the formation of each acyl-linked metabolite. Inhibition studies will also allow us to compare how the relative changes in the time course of formation for each acyl-linked metabolite, providing further insight into which metabolite contributes most to the formation of glutathione adducts.

5. Determine the temperature dependence of GST mediated reactivity and the rate of formation of MFA-AMP, MFA-CoA, and MFA-1-*O*-Glucuronides in rat hepatocytes. The rates of enzyme catalyzed reactions have a tendency to double for every ten degree increase in temperature until the optimal temperature has been reached. By varying the temperature at which we run our GST mediated reactivity and the time course of formation of each metabolite in rat hepatocyte incubations, we will gain further understanding about the formation of glutathione adduct formation.

6. Determine the relative abilities of MFA-1-*O*-Glucuronide, MFA-AMP, and MFA-CoA to nonenzymatically form MFA-Glycine and MFA-Taurine conjugates. The synthesis and characterization of mefenamic acid glycine and taurine conjugates will provide authentic standards to determine the acylating abilities of each acyl-linked metabolite with amino groups.

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Chapter 2

Synthesis and Characterization of MFA-AMP, MFA-CoA, MFA-1-*O*-Gluc and MFA-GSH using UV, LC/MS/MS, and NMR

2.1 Introduction

Carboxylic acid-containing drugs can undergo bioactivation into chemically-reactive metabolites that are capable of transacylating nucleophilic centers found in macromolecules of the body as well as the sulfur anion of glutathione (GSH) (Smith et al., 1986; Benet et al., 1993; Zia Amirhosseini et al., 1994). Acyl glucuronides and *S*-acyl-CoA derivatives are believed to form adducts with proteins and glutathione via transacylation. Many carboxylic acid drugs can form one or both of these metabolites. The reactive transacylating properties possessed by these acyl-linked metabolites are proposed to cause hypersensitivity reactions through the formation of immunogenic drug-protein adducts (Spahn-Langguth and Benet, 1992; Boelsterli, 2002; Uetrecht, 2007).

1-β-*O*-acyl glucuronides are the major metabolites of carboxylic acid drugs and are known to form covalent linkages with proteins *in vitro* and *in vivo*. These drug-protein adducts are formed by one of two mechanisms. The first mechanism involves a direct transacylation of the 1-β-*O*-acyl glucuronide with nucleophilic sites (serine-, tyrosine-hydroxyls, cysteine-sulfhydryls, and lysine-amines) found in proteins. The second mechanism occurs via a Schiff-base formation

with lysinyl-amines from the open aldehyde chain of the acyl migrated isomer. *S*-acyl-CoA thioester intermediates of many structurally varied carboxylic acid drugs have also been shown to transacylate nucleophilic sites such as –SH, -OH, and -NH_2 groups located on proteins due to the electrophilic nature of their carbonyl-carbon at the thioester linkage (Ding et al., 1995; Qiu et al., 1998). Examples of reactive xenobiotic *S*-acyl-CoA thioesters include those formed from the lipid lowering drug clofibric acid (Grillo and Benet, 2002) and nafenopin (Sallustio et al., 2000), and the non-sterodial anti-inflammatory drugs tolmetin (Olsen et al., 2007) and zomepirac (Olsen et al., 2005). The endogenous bile acid, cholic acid, has been shown to form the reactive *S*-acyl-CoA thoiesters as well as the acyl-adenylate intermediate (Mano et al., 2001). Acyl-adenylate derivatives are also believed to be reactive in nature because, like the *S*-acyl-CoA, the electrophilic nature of the carbonyl-carbon at the phosphodiester bond of the acyl-adenylates can readily react with nucleophilic centers found in proteins and GSH (Goto et al., 2001; Mitamura et al., 2007). In order to investigate and compare the reactive potential of the phase II acyl-linked metabolites of mefenamic acid (MFA), MFA-1-*O*-Gluc, MFA-CoA, MFA-AMP, and MFA-GSH, authentic standards must be obtained for further experiments. The present studies include the synthesis, purification, and analytical characterization of MFA-CoA, MFA-AMP, MFA-GSH, and MFA-1-*O*-Gluc.

2.2 Experimental Section

2.2.1 Chemicals

Mefenamic acid, glutathione, adenosine monophosphate, coenzyme A, tetrahydrofuran (anhydrous), triethylamine, ethyl chloroformate, N,N'-dicyclohexylcarbodiimide, pyridine,

monobasic and dibasic potassium phosphate, DMSO, methyl-2, 3, 4,-tri-*O*-acetyl-1-bromo-1-deoxy-α-D -glucopyranuronate, lipase AS Amano (LAS), and porcine liver esterase (PLE) were all purchased from Sigma-Aldrich Chemical Co (St. Louis, MO). Acetonitrile, methanol, acetone, and ethyl acetate were all purchased from Fisher Scientific (Pittsburgh, PA). All solvents used for HPLC and LC/MS/MS analysis were of chromatographic grade. Stock solutions of MFA-AMP, MFA-CoA, MFA-GSH and MFA-1-*O*-Gluc were all prepared as 1 mM solutions in DMSO.

2.2.2 Instrumentation

HPLC/UV was carried out on a Hewlett Packard 1100 series binary pump HPLC system (Santa Clara, CA) coupled to a Hewlett Packard 1100 UV-Vis detector. LC/MS/MS analyses of synthetic standards and *in vitro* samples was 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. ^{1}H NMR spectra were recorded on an Avance II spectrometer (Bruker Deltronics, Billerica, MA) operating at 500 MHz and using a 5-mm, general purpose, cryogenically cooled probe (QNP CryoProbe; Bruker BioSpin Corporation, Fremont, CA).

2.2.3 Synthesis of MFA-GSH

The synthesis of mefenamyl glutathione (MFA-GSH) thioesters was performed by conventional procedures employing ethyl chloroformate (Figure 2.1) (Stadtman, 1957); 1.6 mmol MFA was dissolved in anhydrous THF (25 ml); 220 µl of triethylamine (1.6 mmol) was added to the solution followed by the activation of MFA by the addition of 160 µl of ethylchloroformate (1.6 mmol). This solution was stirred for 30 minutes at room temperature.

The resulting precipitate, triethylamine hydrochloride, was removed by passing the reaction mixture through a glass funnel fitted with a glass wool plug. The filtered mixture was then added to a solution containing 1 g of GSH and 100 mg $KHCO_3$ in 10 ml of filtered nanopure water and 15 ml of THF. The solution was stirred continuously at room temperature for 2 hr, after which the reaction was terminated by acidification (pH 4-5) with the addition of 1M HCl. THF was then removed by evaporation under N_2 gas and the water was removed by aspiration following centrifugation for 10 min at 3000 rpm. The resulting precipitate was then washed with acidified water (pH 4-5)(3 x 10 ml) and acetone (3 x 10 ml). The precipitate was then isolated by aspirating the remaining wash liquid after centrifugation for 10 min at 3000 rpm to yield pure MFA-GSH. The MFA-GSH solid was blown down to dryness using N_2 gas and then weighed out for preparation of a 1 mM MFA-GSH solution in DMSO. The reaction yield for MFA-GSH was ~12%.

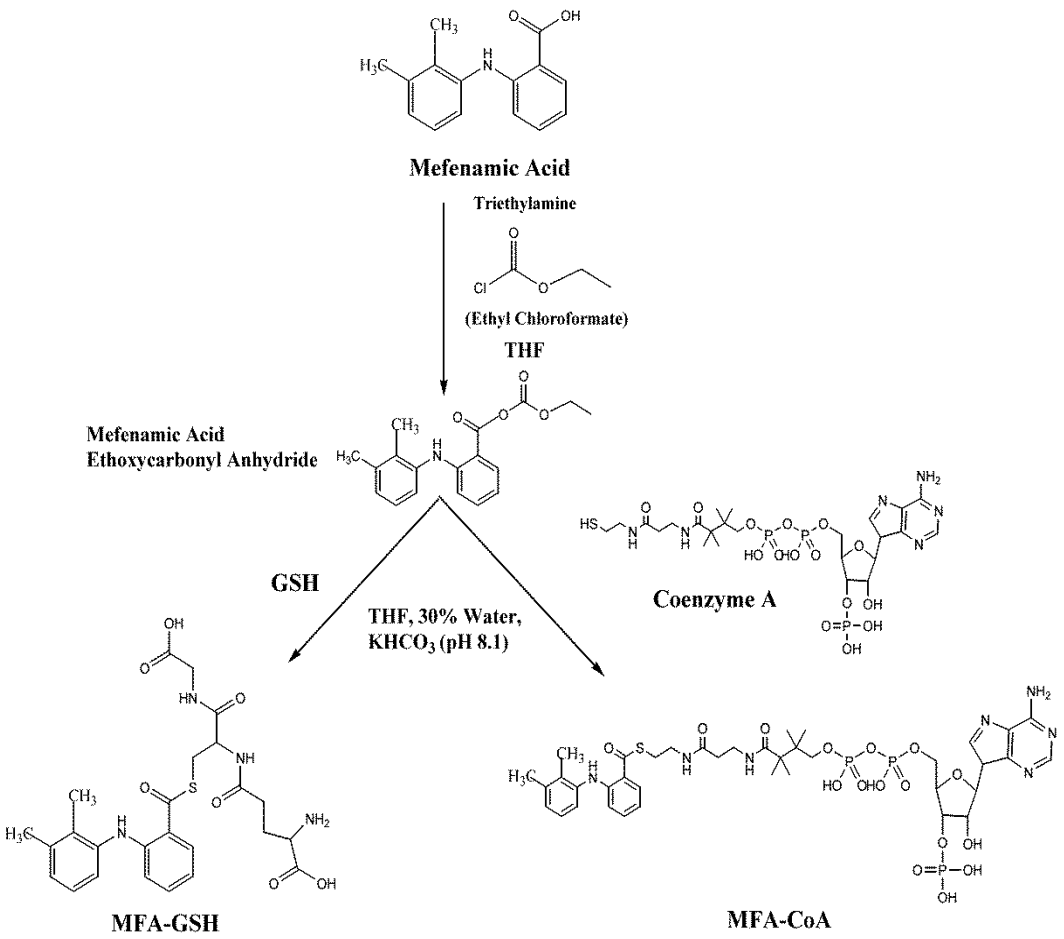


Figure 2.1 Synthesis of MFA-GSH and MFA-CoA.

The MFA-GSH solution was then checked for purity by HPLC/UV and LC/MS/MS analysis. HPLC/UV analysis was accomplished using a reverse phase gradient system of 10 mM aqueous ammonium acetate to acetonitrile over 10 minutes at a flow rate of 0.5 ml/min using an XTerra (Milford, MA) C-18 (5.0 µm, 4.6 x 150 mm) column. Samples were analyzed at three wavelengths: 220 nm, 254 nm, and 262 nm. MFA-GSH eluted at a retention time of 9.2 minutes (Figure 2.2) and was shown to contain no other peaks at all three wavelengths. LC/MS/MS analysis was accomplished using the same liquid chromatography parameters followed by positive ionization mass spectrometry. Full ion positive mode scan revealed only MFA-GSH and

no other contaminants. Tandem LC/MS/MS analysis of MFA-GSH (CID of MH^+ ion at m/z 531), m/z (%) yielded: m/z 456 ([M + H – Gly]$^+$, 10%), m/z 384 ([M + H – pyroglutamic acid – water]$^+$, 82%), m/z 224 ([M + H – GSH – water]$^+$, 73%) (Figure 2.3). The mass transition used for quantitative LC/MS/MS analysis was MH^+ m/z 531 to 224. LC/MS/MS chromatograms and product ion spectra of MFA-GSH formed in rat hepatocyte incubations showed retention times and fragment ions identical to the authentic MFA-GSH standard and were consistent with its chemical structure (Figure 2.2 and 2.3). Purity and chemical structure were also confirmed by ^{1}H NMR.

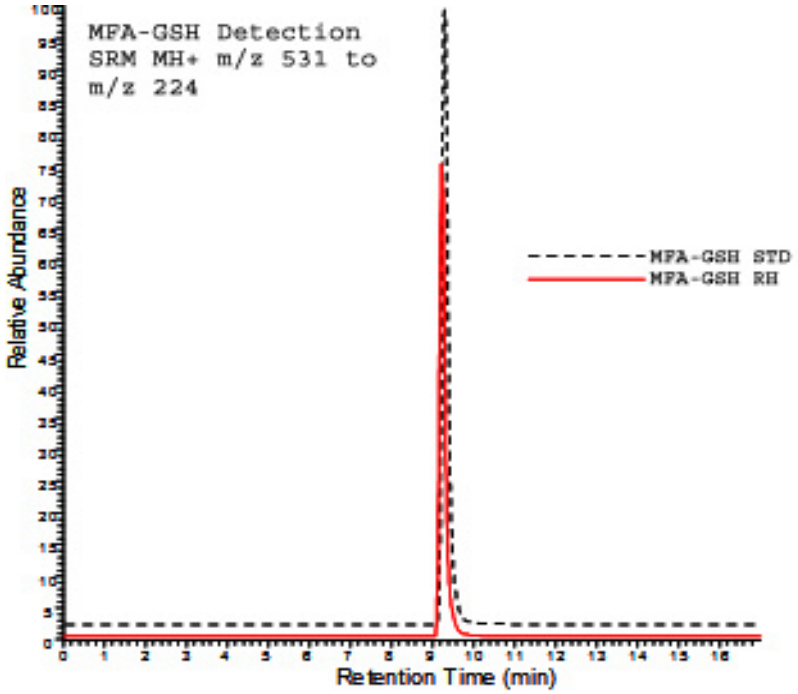
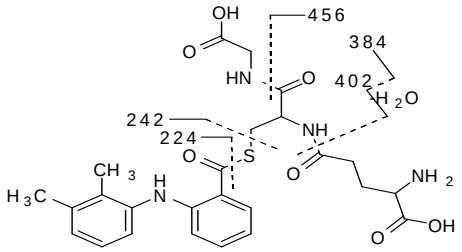
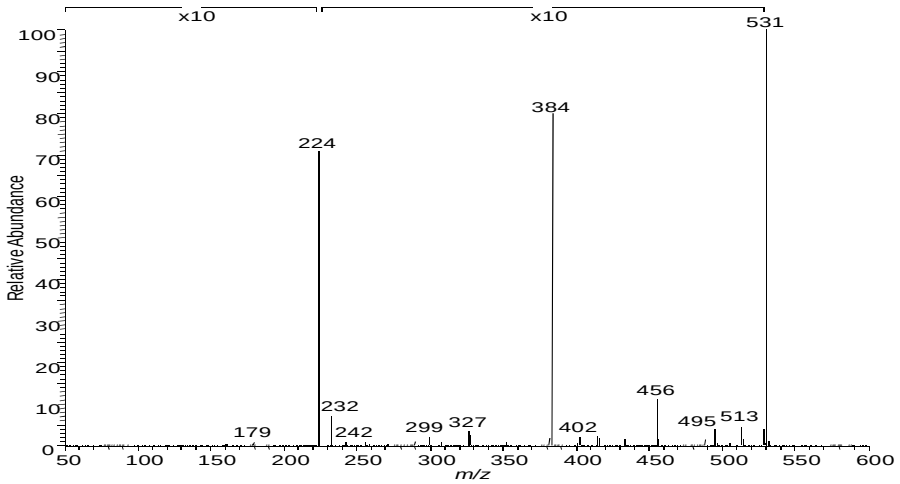


Figure 2.2 Representative reverse-phase gradient LC/MS/MS Single Reaction Monitoring (SRM) of MFA-GSH authentic standard (STD) and MFA-GSH biologically formed in rat hepatocytes (RH).



Mefenamyl-*S*-acyl-glutathione
MH^+ *m/z* 531

A) MFA-SG Standard



B) MFA-SG from Rat Hepatocyte Incubations

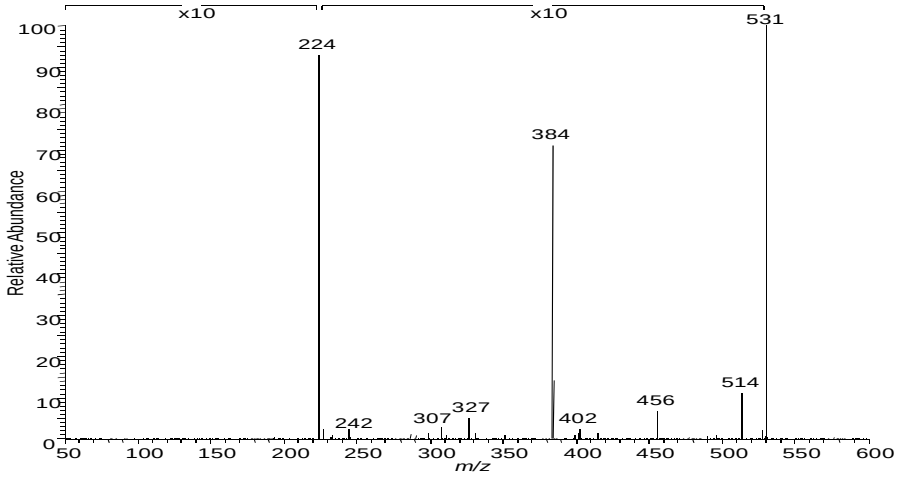


Figure 2.3 LC/MS/MS tandem mass spectra of (A) authentic MFA-GSH standard and (B) MFA-GSH biologically formed in rat hepatocyte incubations with MFA (31 µM, 9.2 min) obtained by CID of the protonated molecular ion MH^+ at m/z 531.

2.2.4 Synthesis of MFA-CoA

The synthesis of mefenamyl- *S*-CoA (MFA-CoA) thioesters was accomplished by a method employing ethyl chloroformate (Figure 2.1) (Stadtman, 1957). 1.6 mmol MFA was dissolved in anhydrous THF (25 ml). Triethylamine (1.6 mmol) was added to the solution followed by the activation of MFA by the addition of 160 µl of ethylchloroformate (1.6 mmol). This solution was stirred for 30 minutes at room temperature. The resulting precipitate, triethylamine hydrochloride, was removed by passing the reaction mixture through a glass funnel fitted with a glass wool plug. The filtered mixture was then added to a solution containing 100 mg of CoA and $KHCO_3$ in 10 ml of nanopure water and 15 ml of THF. 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 1M HCl. THF was then removed by evaporation under N_2 gas. Ethyl acetate (10 ml) was added to the remaining aqueous phase, vortexed for 60 seconds, and then centrifuged for 10 minutes at 3000 rpm. On centrifugation, flakes of MFA-CoA appear between the water and ethyl acetate layers. These flakes were removed using a Pasteur pipet followed by solvent washes: acidified water (pH 4-5)(3 x 10 ml) and ethyl acetate (3 x 10 ml). The remaining precipitate was then isolated by aspirating the remaining wash liquid following centrifugation yielding pure MFA-CoA. The MFA-CoA precipitate was blown down to dryness using N_2 gas and then weighed out for preparation of a 1 mM MFA-CoA solution in DMSO. The reaction yield was ~5%. The MFA-CoA solution was then checked for purity by HPLC/UV and LC/MS/MS analysis. HPLC/UV analysis was accomplished using a reverse phase gradient system of 10 mM aqueous ammonium acetate to acetonitrile over 10 minutes at a flow rate of 0.5 ml/min using an XTerra C-18 (5.0 µm, 4.6 x 150 mm) column. Samples were analyzed at three

wavelengths: 220 nm, 254 nm, and 262 nm. MFA-CoA eluted at a retention time of 7.1 minutes (Figure 2.4) and was shown to contain no additional peaks at all three wavelengths. LC/MS/MS analysis was accomplished using the same liquid chromatography parameters followed by positive ionization mass spectrometry. Full ion positive mode scan revealed only MFA-CoA and no other contaminants. Tandem LC/MS/MS analysis of MFA-CoA (CID of MH^+ ion at m/z 991), m/z (%) : m/z 582 ($[M + H – 409]^+$, 20%), m/z 484($[M + H - 507]^+$, 94%), m/z 382 ($[M + H –609]_+$, 25%), m/z 224($[M + H –CoA]^+$, 99%), m/z 428([adenosine diphosphate + $2H^+]^+$, 40%), m/z 330([adenosine phosphate + $H]^+$, 3%) (Figure 2.5). The mass transition used for quantitative LC/MS/MS analysis was MH^+ m/z 991 to 224. LC/MS/MS analysis of MFA-CoA formed in rat hepatocyte incubations provided a chromatogram and product ion spectrum that showed retention times and fragments ions identical to the authentic MFA-CoA standard and consistent with its chemical structure (Figures 2.4 and 2.5). Purity and chemical structure were also confirmed by ^1H NMR.

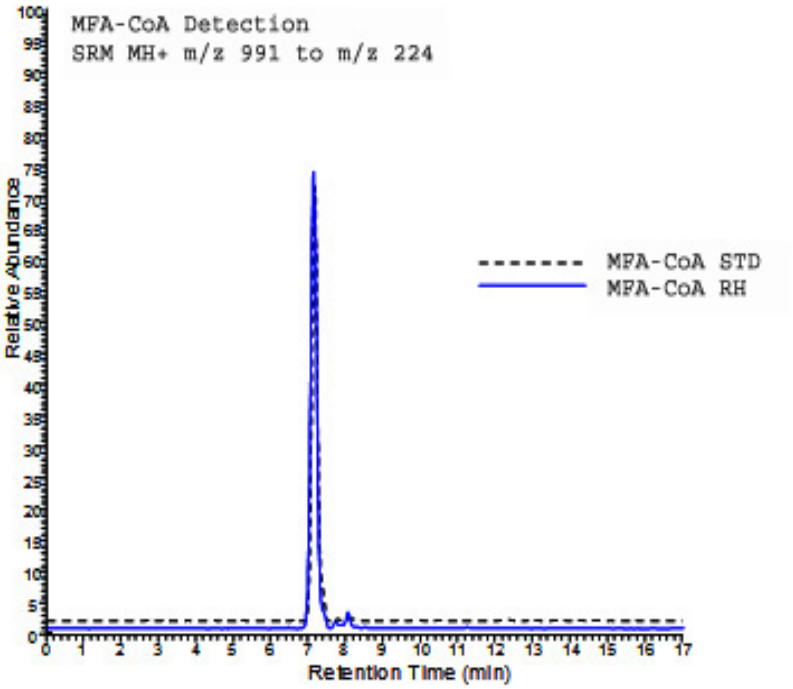
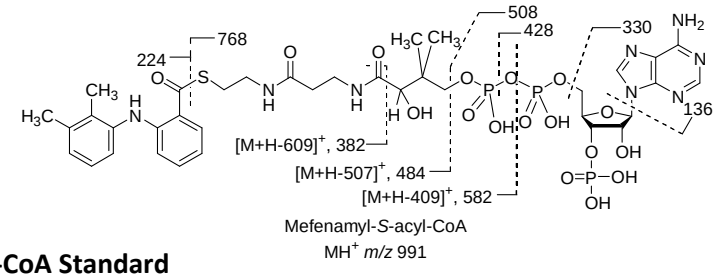
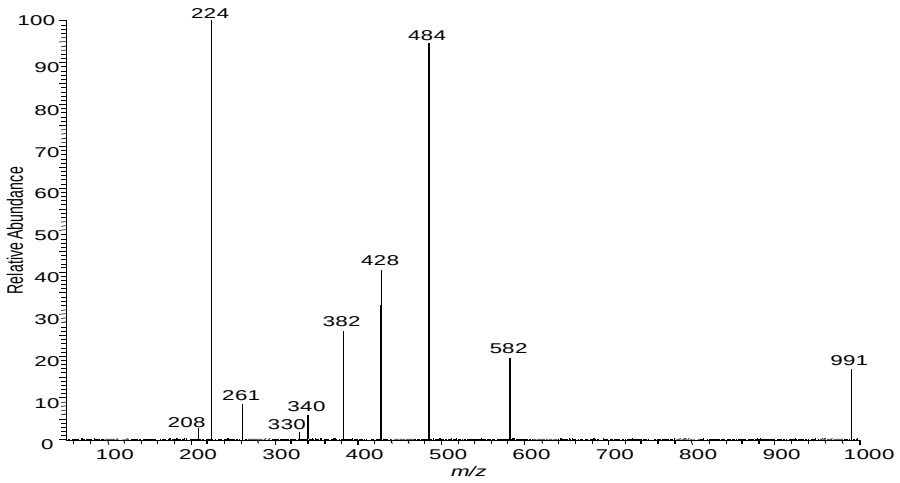


Figure 2.4 Representative reverse-phase gradient LC/MS/MS Single Reaction Monitoring (SRM) of MFA-CoA authentic standard (STD) and biologically formed MFA-CoA in rat hepatocytes (RH).



A) MFA-CoA Standard



B) MFA-CoA Formed in Rat Hepatocyte Incubations

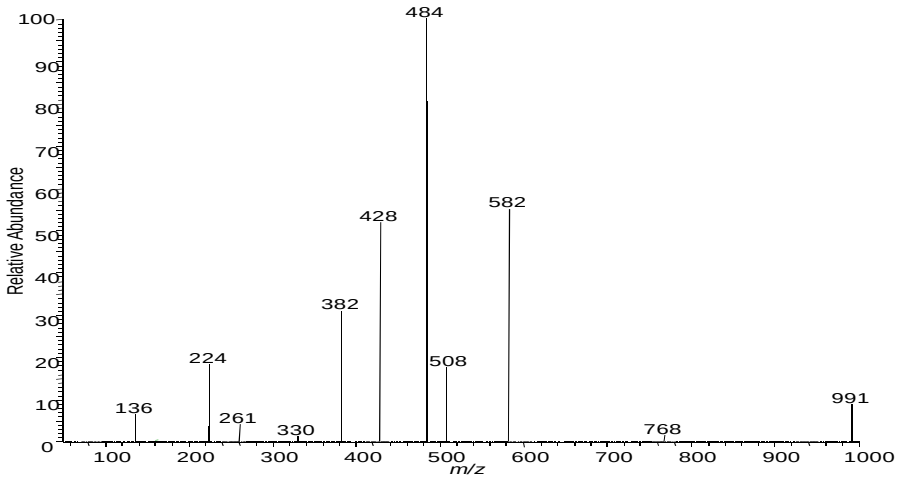


Figure 2.5 LC/MS/MS tandem mass spectra of (A) authentic MFA-CoA standard and (B) MFA-CoA biologically formed in rat hepatocyte incubations with MFA (31 µM, 7.1 min) obtained by CID of the protonated molecular ion MH^+ at m/z 991.

2.2.5 Synthesis of MFA-1-*O*-Glucuronide

The synthesis of mefenamyl-1-*O*-glucuronide (MFA-1-*O*-G) was carried out chemo-enzymatically (Baba and Yoshioka, 2006) by reacting 1.5 mmol of mefenamic acid with 1.0 mmol of methyl 2, 3, 4-tri-*O*-acetyl-1-bromo-1-deoxy-α-D-glucopyranuronate in 8 ml DMSO. The reaction was stirred at 30°C for 3 hrs. The reaction mixture was diluted with 100 ml of ethyl acetate and then washed with water (3 x 100 ml), saturated aqueous $NaHCO_3$ (3 x 40 ml) and saturated aqueous NaCl (3 x 40 ml). The organic solvent was dried over Na_2SO_4 and evaporated under N_2 to yield crude methyl-2, 3, 4-tri-*O*-acetyl derivatives of mefenamic acid 1-β-*O*-acyl glucuronide. The crude product was dissolved in hot methanol and filtered through filter paper and allowed to cool for recrystallization to yield pure methyl 2, 3, 4-tri-*O*-acetyl derivatives of mefenamyl-1-β-*O*-acyl glucuronide. Deacetylation was carried out through the addition of 0.072 mmol of methyl 2, 3, 4-tri-*O*-acetyl derivatives of mefenamyl-1-β-*O*-acyl glucuronide in 48 ml DMSO to 144 ml of 25 mM sodium phosphate buffer (pH 6) containing 3.6 g lipase AS Amano (15 mg/ml). The incubation mixture was stirred for 5 hr at 40°C. The reaction mixture was then filtered and the filtrate underwent HPLC/UV purification using a reverse phase gradient system of 0.1% formic acid/H_2O to 0.1% formic acid/ACN over 13 minutes at a flow rate of 0.5 ml/min using an XTerra C-18, (5.0 µm, 4.6 x 150 mm) column. The solvent from the collection fractions was then evaporated to yield the methyl ester of mefenamic acid 1-β-*O*-acyl glucuronide solid. Removal of the methyl protecting groups were carried out by dissolving 27 mg of the methyl ester in 32 ml DMSO, which was then poured into 96 ml of 25 mM sodium phosphate buffer (pH 6.0). To the resultant solution, 65 mg of porcine liver esterase dissolved in 32 ml of aqueous solution was added and incubated at 40°C for 1.5

hr. The reaction mixture was acidified with 6M HCl (pH 3.0) and then filtered. HPLC/UV purification of the residue was done using a reverse phase gradient system of 0.1% formic acid/H_2O to 0.1% formic acid/ACN over 13 minutes at a flow rate of 0.5 ml/min using an XTerra C-18 (5.0 µm, 4.6 x 150 mm) column. MFA-1-*O*-Gluc peaks were collected at 11 minutes and the resulting fractions were lyophilized to yield a pure solid. LC/MS/MS analysis was accomplished using the same liquid chromatography parameters followed by positive ionization mass spectrometry. A full ion positive mode scan revealed only MFA-1-*O*-Gluc and no other contaminants (Figure 2.6). Tandem LC/MS/MS analysis of MFA-1-*O*-Gluc (CID of MH^+ ion at m/z 418), m/z (%) yielded: m/z 224([M + H – glucuronide]$^+$, 100%), m/z 248([M + H - 170]$^+$, 35%), m/z 209([M + H –209]$_+$, 50%) (Figure 2.7). The mass transition used for quantitative LC/MS/MS analysis was MH^+ m/z 418 to 224. LC/MS/MS analysis of MFA-1-*O*-Gluc formed in rat hepatocyte incubations provided a chromatogram and product ion spectrum that showed retention times and fragment ions identical to the authentic MFA-1-*O*-Gluc standard and consistent with its chemical structure (Figure 2.6 and 2.7). Purity and chemical structure were also confirmed by ^{1}H NMR.

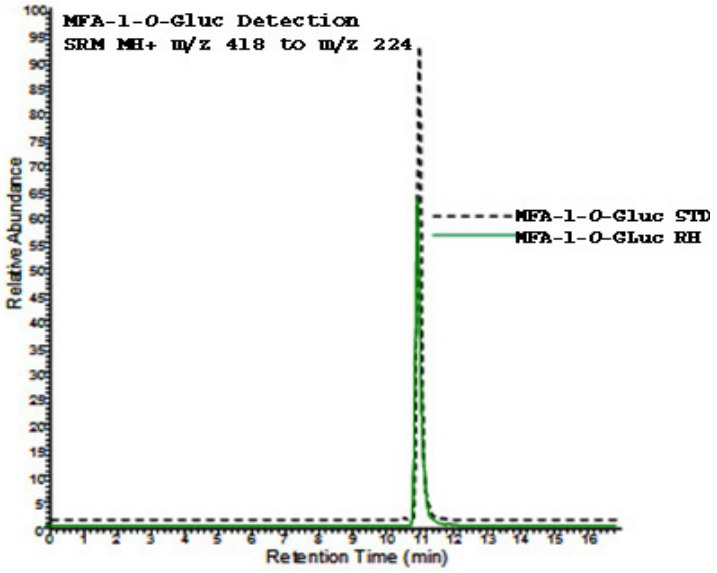
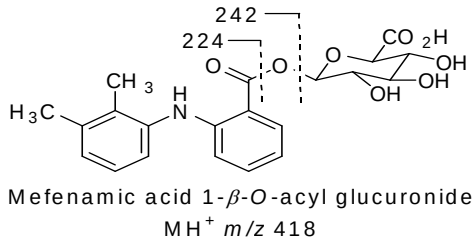
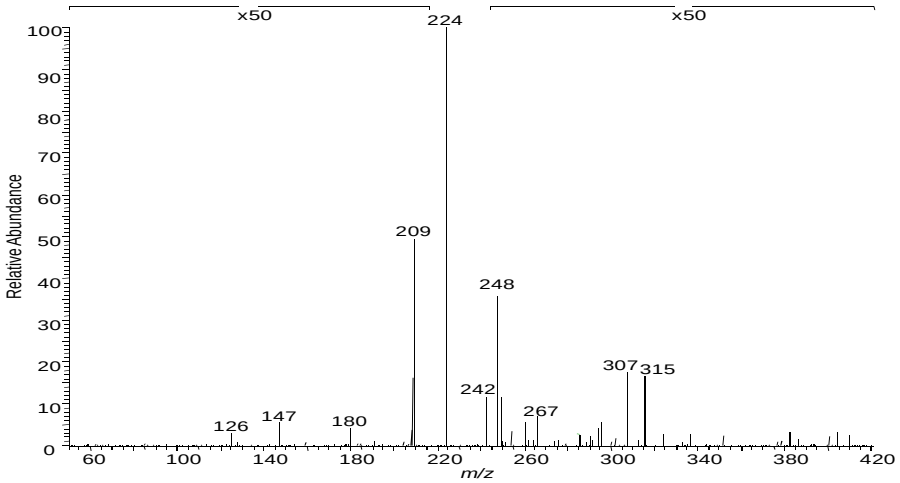


Figure 2.6 Representative reverse-phase gradient LC/MS/MS Single Reaction Monitoring (SRM) of MFA-1-*O*-Gluc authentic standards (STD) and biologically formed MFA-1-*O*-Gluc in rat hepatocytes (RH).



A) MFA-1-*O*-Gluc Standard



B) MFA-1-*O*-Gluc from Rat Hepatocyte Incubations

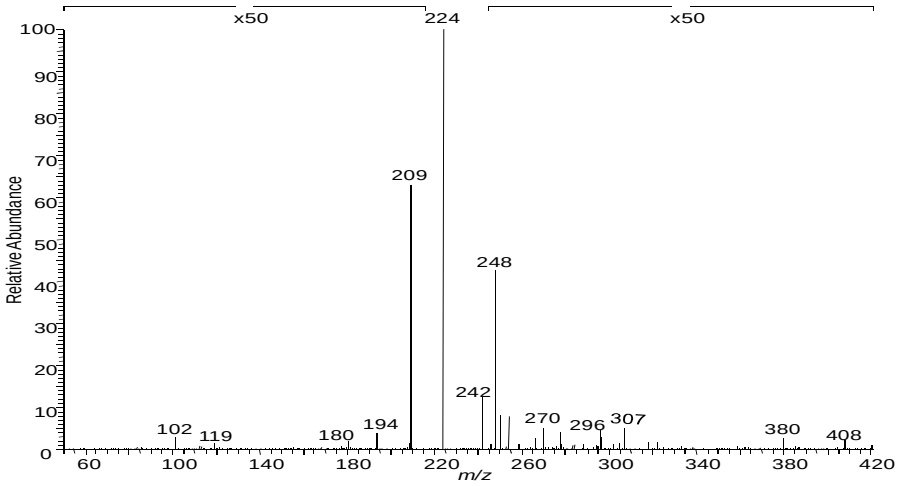


Figure 2.7 LC/MS/MS tandem mass spectra of (A) authentic MFA-1-*O*-Gluc standard and (B) MFA-1-*O*-Gluc biologically formed in rat hepatocyte incubations with MFA (31 µM, 11 min) obtained by CID of the protonated molecular ion MH^+ at m/z 418.

2.2.6 Synthesis of MFA-AMP

The synthesis of mefenamyl-adenylate (MFA-AMP) was carried out with a solution consisting of 110 mg N,N'-dicyclohexylcarbodiimide in 0.4 ml pyridine (Figure 2.8) (Ikegawa et al., 1999). The *N*,*N*'-dicylcohexylcarbodiimide solution was added to a solution consisting of 0.489 mmol of mefenamic acid, and 0.491 mmol of adenosine monophosphate(AMP) in 75% pyridine (1.2 ml pyridine/0.4 ml water). The reaction mixture was stirred at $4^{o}C$ for 7 hr. The reaction mixture was then centrifuged at 3000 rpm for 5 min to remove the *N*-acylurea derivatives. The supernatant was then transferred to another culture tube followed by the addition of 10 ml acetone. 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). The precipitate was then dissolved in 0.1 M potassium phosphate buffer (pH 6) and underwent liquid-liquid washes with ethyl acetate (10 x 10 ml). After removal of the ethyl acetate, one drop of 1M HCl was added to the potassium buffer to precipitate MFA-AMP. The MFA-AMP underwent further washes with acetone (10 x 10 ml). If HPLC/UV analysis revealed any contamination peaks, the wash processes was repeated.

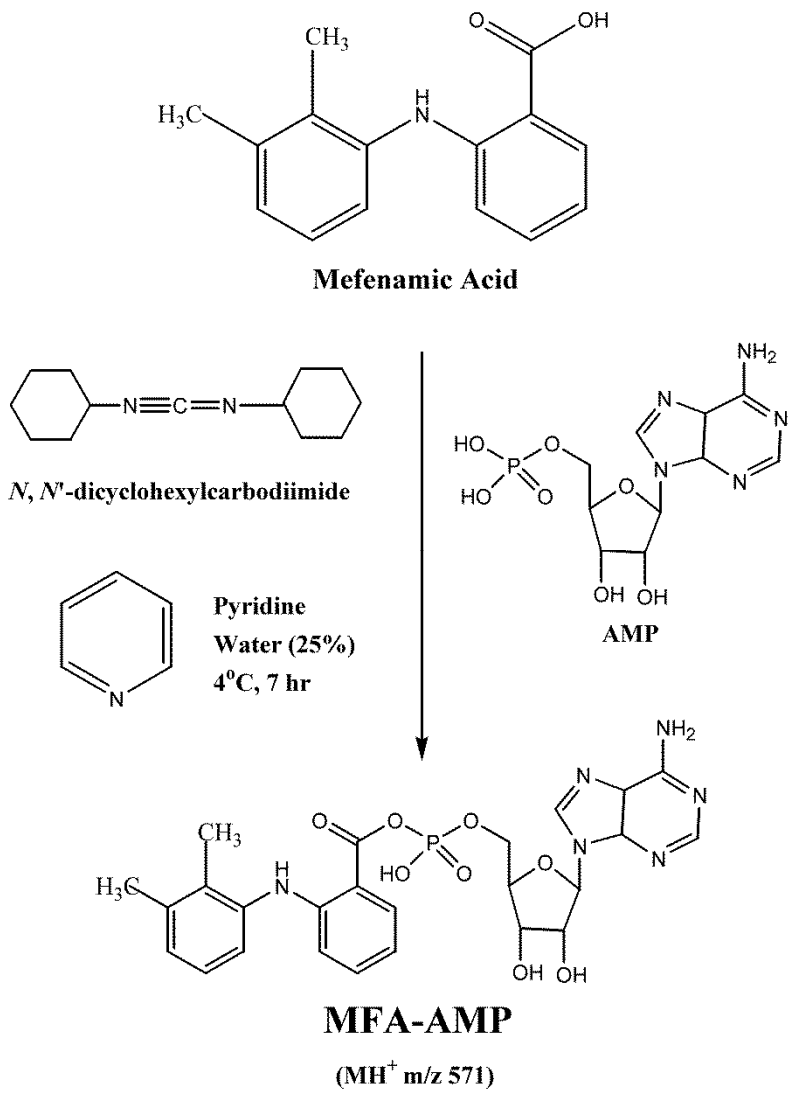


Figure 2.8 Synthesis of MFA-AMP.

HPLC/UV analysis was accomplished using a reverse phase gradient system of 10 mM aqueous ammonium acetate to acetonitrile over 10 minutes at a flow rate of 0.5 ml/min using an XTerra C-18 (5.0 µm, 4.6 x 150 mm) column. Samples were analyzed at three wavelengths: 220 nm, 254 nm, and 262 nm. MFA-AMP eluted at a retention time of 7.3 minutes (Figure 2.9) and was shown to contain no additional peaks at all three wavelengths. LC/MS/MS analysis was accomplished using the same liquid chromatography parameters followed by positive ionization

mass spectrometry. Full ion positive mode scan revealed only MFA-AMP and no other contaminants. Tandem LC/MS/MS analysis of MFA-AMP (CID of MH^+ ion at m/z 571), m/z (%) yielded: m/z 224($[M + H – AMP]^+$, 100%), m/z 136($[M + H - adenine]^+$, 28%), m/z 207 ($[M + H – 364]_+$, 25%) (Figure 2.10). The mass transition used for quantitative LC/MS/MS analysis was MH^+ m/z 571 to 224. LC/MS/MS analysis of MFA-AMP formed in hepatocyte incubations provided a chromatogram and product ion spectrum that showed retention times and fragment ions identical to the authentic MFA-AMP standard and consistent with its chemical structure (Figure 2.9 and 2.10). Purity and chemical structure were also confirmed by ^1H NMR.

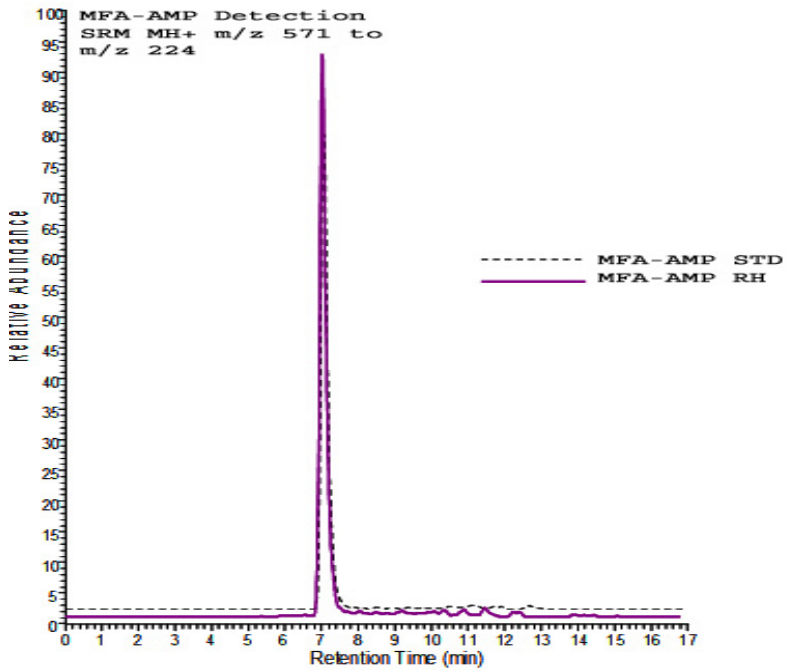
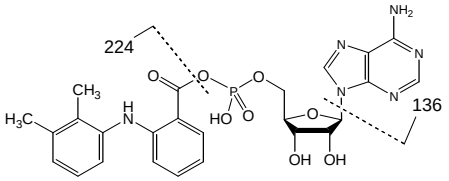
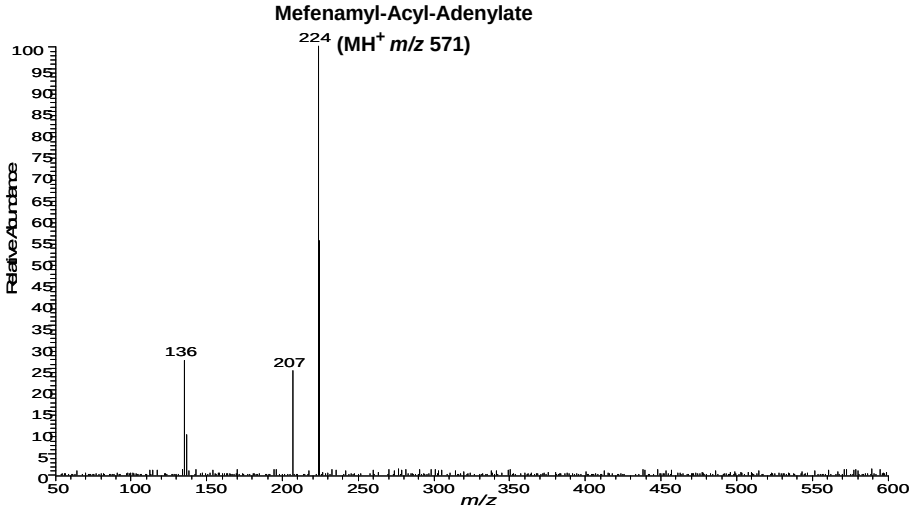


Figure 2.9 Representative reverse-phase gradient LC/MS/MS Single Reaction Monitoring (SRM) of MFA-AMP authentic standards (STD) and MFA-AMP biologically formed in rat hepatocytes (RH).

A) MFA-AMP Standard





B) MFA-AMP from Rat Hepatocyte Incubations

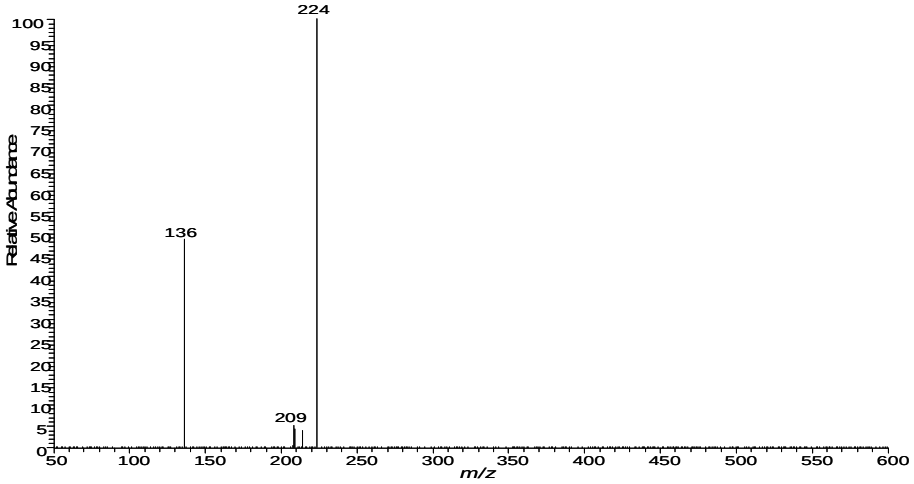


Figure 2.10 LC/MS/MS tandem mass spectra of (A) authentic MFA-AMP standard and (B) biologically formed MFA-AMP from rat hepatocyte incubations with MFA (31 µM, 7.3 min) obtained by CID of the protonated molecular ion MH^+ at m/z 571.

2.3 Discussion

In order to carry out mefenamic acid bioactivation experiments and determine the reactive potential of its acyl-linked metabolites, it is essential to use authentic standards. Therefore the first step was dedicated to the synthesis of MFA-AMP, MFA-CoA, MFA-1-*O*-Gluc, and MFA-GSH. The synthetic method for obtaining MFA-AMP pure standards required the selective condensation of the carboxy group of mefenamic acid with the phosphoric acid group of AMP. The use of *N*,*N*'-dicyclohexylcarbodiimide in aqueous pyridine for the selective condensation of a phosphoric acid group with carboxylic acids to produce an anhydride linkage had been previously demonstrated (Berg, 1958). Therefore, mefenamic acid was reacted with AMP in aqueous pyridine in the presence of *N*,*N*'-dicyclohexylcarbodiimide to synthesize MFA-AMP, whose structure was confirmed by MS and NMR. The ESI-MS/MS spectrum showed a mass transition of MH^+ m/z 571 to 224, indicating the covalently condensed product of mefenamic acid and AMP.

MFA-CoA and MFA-GSH were also chemically synthesized by conventional procedures using ethyl chloroformate. The ESI-MS/MS spectrum showed a mass transition of MH^+ m/z 991 to 224 for MFA-CoA and a mass transition of MH^+ m/z 531 to 224 for MFA-GSH, indicating the covalent linkage of mefenamic acid to coenzyme A and glutathione respectively. The structures of both compounds have been confirmed by MS and NMR.

MFA-1-*O*-Gluc was synthesized chemo-enzymatically by reacting mefenamic acid with commercially available methyl-2,3,4-tri-*O*-acetyl-1-bromo-1-deoxy-α-D -glucopyranuronate followed by the removal of the acetyl and methyl ester protecting groups with lipase and

esterases respectively. Pure MFA-1-*O*-Gluc was obtained following HPLC purification and yielded an ESI-MS/MS spectra mass transition of MH^+ m/z 418 to 224, whose structure was confirmed by MS and NMR.

The next effort involved the development of chromatographic conditions on a reverse-phase column for simultaneous determination of MFA-AMP, MFA-CoA, MFA-GSH, and MFA-1-*O*-Gluc. The chromatographic peaks for all four compounds were completely resolved using a reverse phase gradient system of 10 mM aqueous ammonium acetate to acetonitrile over 10 minutes at a flow rate of 0.5 ml/min using an XTerra C-18 (5.0 µm, 4.6 x 150 mm) column followed by MS/MS analysis. In summary, MFA-AMP, MFA-CoA, MFA-GSH, and MFA-1-*O*-Gluc authentic pure standards were obtained synthetically and a selective and sensitive LC/MS/MS method was developed to characterize and quantitate each acyl-linked metabolite of mefenamic acid for further experiments.

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Chapter 3

Chemical Stability and Reactivity with GSH of MFA-AMP, MFA-CoA, and MFA-1-*O*-Gluc Metabolites

3.1 Introduction

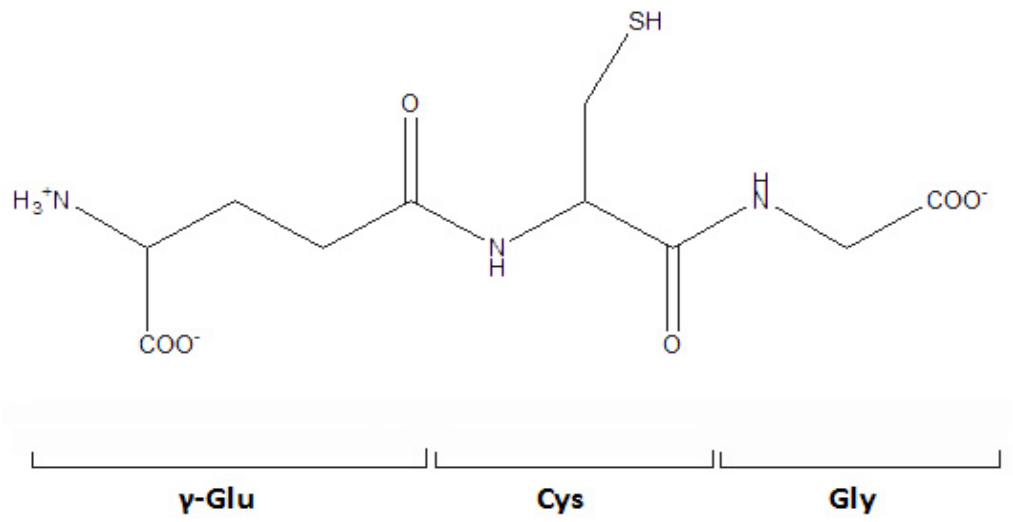


Figure 3.1 Structure of glutathione.

Carboxylic acid drugs can be bioactivated into chemically-reactive acyl-linked metabolites that are capable of transacylating cellular protein nucleophiles and cysteinyl-thiols of glutathione (GSH). In order to gain a better understanding of how bioactivation can illicit an idiosyncratic toxicity, the model nucleophile, GSH, is used as a biomarker of reactivity for reactive intermediates. Glutathione [N-(N-*L*-γ-glutamyl-*L*-cysteinyl) glycine] (Figure 3.1) is an endogenous atypical tripeptide that plays a protective role in the body by removing potentially

toxic electrophiles. GSH exists in its highest concentration in the liver (~10 mM) and is present in the cytosol, mitochondria, and nucleus (Zubay et al., 1994; Zubay et al., 1995). The concentration of GSH present in the blood is ~20 µM. GSH conjugation primarily results in detoxification since reaction of electrophiles with glutathione prevents their reaction with nucleophilic centers of macromolecules. Glutathione conjugates are mainly excreted in the bile but can also be excreted directly in the urine; *S*-substituted glutathione conjugates tend to undergo further modification prior to their excretion in the urine as mercapturic acid derivatives. Enzymatic metabolism is first mediated by the enzyme γ-glutamyl transferase (γ-GT) that hydrolyzes the GSH conjugate at the γ-glutamyl bond resulting in a glycylcysteine conjugate. Peptidases then remove the glycine from the dipeptide conjugate to form cysteine conjugates that may undergo further modification by *N*-acetylases to form *N*-acetylcysteine conjugates of mercapturic acid (Zubay et al., 1994). Mercapturic acid conjugates are excreted into the urine and can be used as biomarkers for reactive metabolites.

1-β-*O*-acyl glucuronides, *S*-acyl-CoA, and acyl-AMP derivatives have been shown to react with GSH to form *S*-acyl-GSH thioester products. *S*-acyl-GSH conjugates are formed when the acyl-linked metabolites of carboxylic acid containing drugs form thioester bonds between the cysteinyl-thiol of GSH and the carbonyl-carbon of the carboxylic acid functional group. *S*-acyl-GSH adducts have been shown to form from cholic acid (Mitamura et al., 2007; Mitamura et al., 2009), clofibric acid (Shore et al., 1995), zomepirac (Grillo and Hua, 2003), diclofenac (Grillo et al., 2003), tolmetin (Olsen et al., 2007), and ibuprofen (Grillo and Hua, 2008). Conjugation of reactive metabolites with GSH is often, but not solely, catalyzed by glutathione *S*-transferases (GST) (Armstrong, 1991).

Glutathione conjugation with reactive electrophilic drug metabolites most often results in the formation of conjugates with increased chemical stability and elimination. The elimination of P450-medited bioactivation of xenobiotics to GSH-adducts include acetaminophen (Nelson, 1990), diclofenac (Tang et al., 1999), and naphthalene (Buonarati et al., 1990). In contrast to most thioether-linked GSH-adducts, which are chemically stable and do not further react with nucleophiles, *S*-acyl-GSH conjugates are chemically reactive electrophiles on their own and have been shown to form covalent bonds with biological nucleophiles and transacylate *N*-acetylcysteine (NAC) *in vitro* (Grillo and Benet, 2002). *In vitro* studies comparing the reactivity of diclofenac-*S*-acyl-GSH thioester and diclofenac 1-β-*O*-acyl glucuronide with NAC revealed that the *S*-acyl-GSH thioester was ~200-fold more reactive than its corresponding 1-β-*O*-acyl glucuronide (Grillo et al., 2003). This reactivity difference is due to the tendency of the acyl glucuronide to migrate to less reactive isomers, which is consistent with the studies showing that degradation rates due to acyl migration and hydrolysis of acyl glucuronides correlates with drug *in vitro* covalent binding to proteins (Benet et al., 1993). When a number of structurally varied carboxylic acid glutathione conjugates were incubated with NAC in potassium phosphate buffer (pH 7.5, room temperature), the rate of formation of *S*-acyl-NAC conjugates was dependent on the degree of α-carbon substitution. The rank order of NAC transacylation is as follows: phenoxyacetyl- > arylacetyl- > 2-phenylproprionyl-, α,α-dimethyl-phenoxyacetyl- > α,α-dimethyl-substituted-S-acyl-GSH. Phenoxyacetyl-*S*-acyl-GSH conjugates were ~3- and 26-fold more reactive with NAC than the arylacetic acid- and profen-*S*-acyl-GSH, respectively, and no significant reaction occurred with the α,α-dimethyl-substituted *S*-acyl-GSH (gemfibrozil-*S*-acyl-

GSH). This α-carbon substitution reactivity trend is analogous to the 1-*O*-β-acyl glucuronide and *S*-acyl-CoA thioester reactivity with proteins and GSH (Benet et al., 1993; Sidenius et al., 2004).

A number of carboxylic acid containing drugs have been studied for their *in vitro* and *in vivo* transacylation of GSH potential from their corresponding phase II metabolites, 1-β-*O*-acyl glucuronide, *S*-acyl-CoA, and acyl-AMP. Clofibric acid-*S*-acyl-CoA has been shown to be 40-fold higher in reactivity toward GSH compared to its corresponding acyl glucuronide. However, unlike clofibric acid-1-*O*-glucuronide, which has a half-life of 7.3 hours, clofibric acid-*S*-CoA exhibited a half-life of 21 days *in vitro* in buffer (Grillo and Benet, 2002). Studies comparing the GSH reactivity potential of the 1-β-*O*-acyl glucuronide with the *S*-acyl-CoA of 2-phenylpropionyl-*S*-acyl-CoA thioester have shown that 2-PPA-*S*-acyl-CoA was 70-fold more reactive than the 2-PPA-1-β-*O*-glucuronide. Incubations in buffer under physiological conditions show that 2-PPA-1-β-*O*-glucuronide possessed a half-life of ~1.2 to 2.4 hours while 2-PPA-*S*-CoA was stable for at least one day (Li et al., 2002). Naproxen-*S*-acyl-CoA reactivity with GSH has been shown to be 100-fold more rapid than its corresponding (*S*)-naproxen-1-β-*O*-glucuronide (Olsen et al., 2002). All of these reveal that the transacylation of GSH by *S*-acyl-CoA is higher than the corresponding 1-β-*O*-acyl glucuronide and suggest that the *S*-acyl-CoA may be more important than the acyl glucuronides to the covalent binding to proteins *in vitro*.

The bile acid, cholic acid, has been shown to undergo bioactivation into the highly electrophilic acyl-linked metabolites acyl-adenylates and acyl-*S*-CoA thioesters. Both metabolites have also been shown to transacylate GSH and amino acids. The *in vitro* non-enzymatic acylation of GSH under physiological conditions (pH 7.4, 37°C) in buffer revealed that

cholyl-*S*-CoA was 3.3-fold and 4.9-fold more reactive toward the transacylation of GSH and NAC, respectively, than the corresponding cholyl-AMP. However, cholyl-AMP was found to be 5.5-fold and 8.2-fold more reactive towards glycine and taurine than MFA-*S*-CoA (Mitamura et al., 2011).

The present studies were designed to characterize the *in vitro* stability of MFA-1-*O*-glucuronide, MFA-*S*-CoA, MFA-AMP, and MFA-GSH in buffer under physiological conditions and to compare the relative *in vitro* reactivities of all three acyl-linked metabolites towards the transacylation of GSH with and without GST.

3.2 Experimental Section

3.2.1 Chemicals

MFA-AMP, MFA-CoA, MFA-GSH, and MFA-1-*0*-Gluc were obtained synthetically by the methods previously described (Chapter 2). Carbamazepine were purchased from Sigma-Aldrich Chemical Co (St. Louis, MO).

3.2.2 Instrumentation and Analytical Methods

All analytical LC/MS/MS analyses were performed using the previously described methods (Chapter 2.2.2). The mass transition used for quantitative LC/MS/MS analysis was MH^+ m/z 531 to 224 for MFA-GSH, MH^+ m/z 991 to 224 for MFA-CoA, MH^+ m/z 418 to 224 for MFA-1-*O*-Gluc, and MH^+ m/z 571 to 224 for MFA-AMP. The retention times for each metabolite were as follows: 7.2 min for MFA-AMP, 7.1 min for MFA-CoA, 11 min for MFA-1-*O*-Gluc, and 9.2 min for MFA-GSH.

3.2.3 Chemical Stability

MFA-AMP, MFA-CoA, MFA-1-*O*-Gluc, MFA-GSH (all 1 µM), and carbamazepine (internal standard) was incubated separately in 0.1 M potassium phosphate buffer (pH 7.4) in 2 ml HPLC vials. Each solution was then placed into the HPLC autosampler warmed to 37°C and injections were taken every 15 minutes for 24 hours for MS/MS analysis to compare the relative chemical stability of each acyl-linked metabolite. Stability comparisons were made by comparing the internal standard peak area to analyte peak area ratio for each sample.

3.2.4 Chemical Reactivity

Chemical reactivity experiments for MFA-AMP, MFA-CoA, and MFA-1-*O*-Gluc were performed by incubating 1 µM of each acyl-linked metabolite separately in 0.1 M potassium phosphate buffer (pH 7.4) containing 10 mM GSH (3 ml total volume) warmed to 37°C in screw-capped glass vials in a shaking incubator (Figure 3.2). Aliquots (100 µl) of the incubation mixture were taken at 0, 2, 5, 10, 30, and 60 minutes and quenched with 100 µl of 1 µM carbamzapine/ACN solution and then immediately injected onto the LC/MS/MS for analysis. Quantitative measurements were made using a MFA-GSH standard curve generated from absolute peak areas. GST-mediated reactivity experiments were carried out by incubating 1 µM of MFA-AMP, MFA-CoA, and MFA-1-*O*-Gluc separately in 0.1 M potassium phosphate buffer (pH 7.4, 37 ^{o}C) containing 10 mM GSH and 6 mg (30 units/ml) (Kalgutkar et al., 2011) of equine liver GST (total volume 3.0 ml) in screw-capped glass vials in a shaking incubator. Aliquots (100 µl) of the incubation mixture were taken at 0, 2, 5, 10, 30, and 60 minutes and quenched with 100 µl of 1 µM carbamzapine/ACN solution and then immediately injected onto the LC/MS/MS for

analysis. Quantitative measurements were made using a MFA-GSH standard curve generated from absolute peak areas.

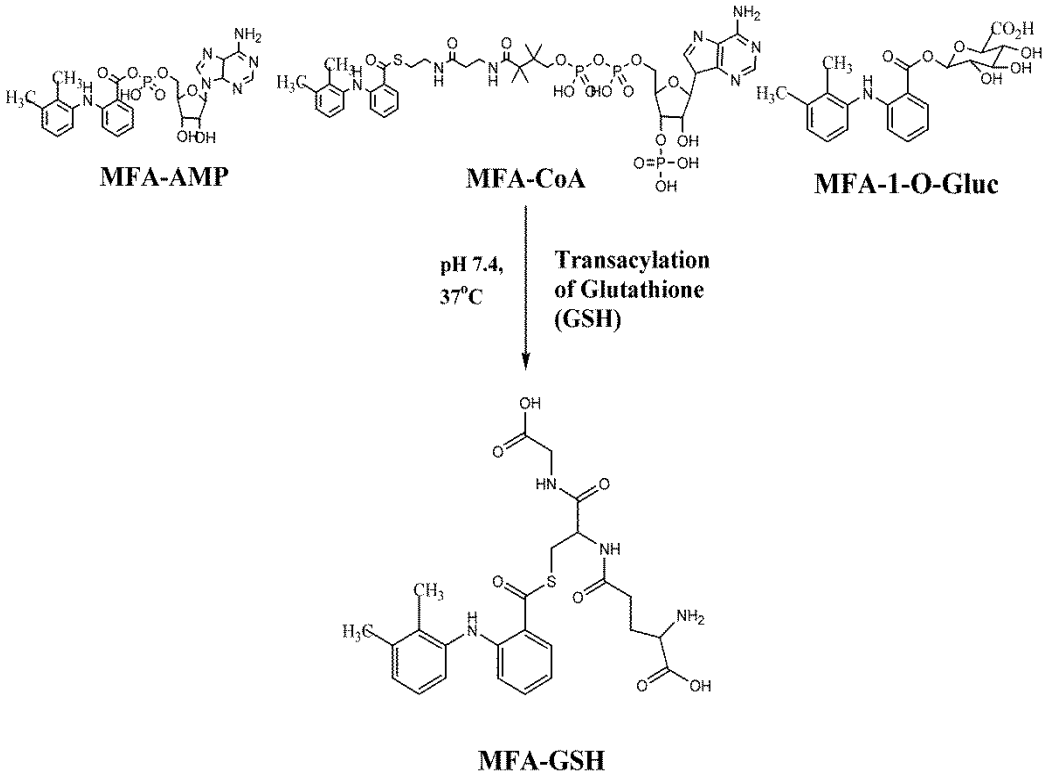


Figure 3.2 Reactivity Assessment of MFA-AMP, MFA-CoA and MFA-1-*O*-Gluc (1 µM) with GSH (10mM).

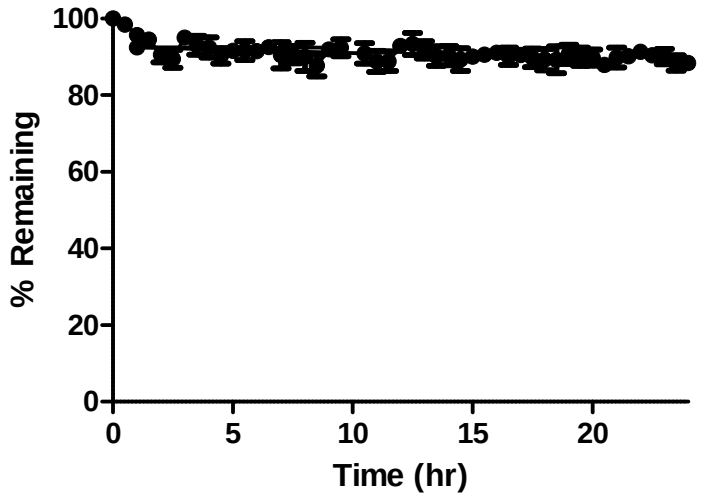
3.3 Results

3.3.1 Chemical Stability Comparisons Between MFA-AMP, MFA-CoA, MFA-1-O-Gluc, and MFA-GSH.

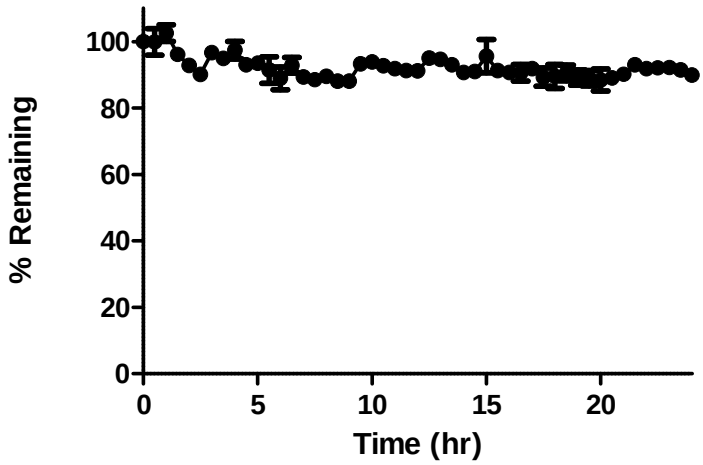
In vitro incubation of the acyl-linked metabolites of mefenamic acid in potassium phosphate buffer at pH 7.4 and 37°C revealed that MFA-AMP, MFA-CoA, and MFA-GSH are chemically

stable with no detectable hydrolysis for at least 24 hours of incubation (Figure 3.3). However, MFA-1-*O*-Gluc showed a half life of degradation of ~16 hours in potassium phosphate buffer at a pH 7.4 and 37°C, which is consistent with previous findings (McGurk et al., 1996).

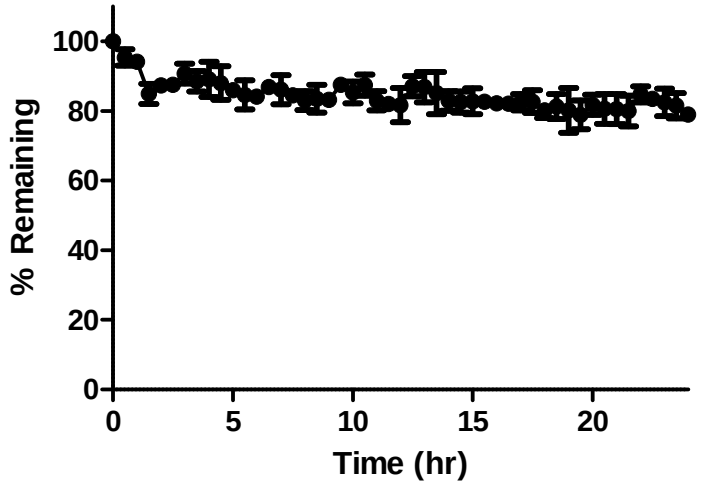
A) MFA-AMP



B) MFA-CoA



C) MFA-GSH



D) MFA-1-*O*-Gluc

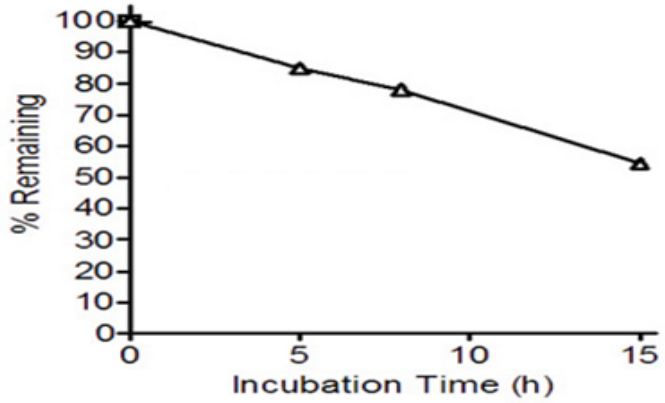


Figure 3.3 Stability of A) MFA-AMP, B) MFA-CoA, C) MFA-GSH and D) MFA-1-*O*-Gluc in 0.1 M potassium phosphate buffer (pH 7.4, 37°C).

3.3.2 Reactivity Assessment of MFA-AMP, MFA-CoA, and MFA-1-*O*-Gluc with GSH

The incubation of 1 µM of MFA-1-*O*-Gluc with GSH (10 mM) in potassium phosphate buffer under physiological conditions resulted in a concentration versus time slope to be 0.02 nM/min and reached a maximum concentration of 1.33 nM after 60 minutes of incubation (Figure 3.4). The slope of MFA-GSH concentration versus time during the reaction of MFA-CoA with GSH was 2.41 nM/min and a maximum concentration of 144.9 nM at 60 minutes (Figure 3.4). The incubation of MFA-AMP with GSH resulted in a MFA-GSH concentration versus time slope of 0.21 nM/min and a maximum concentration of 12.8 nM at 60 minutes (Figure 3.4). Analysis by LC/MS/MS provided chromatograms and mass spectrum of the product MFA-GSH identical to that of the authentic MFA-GSH standard.

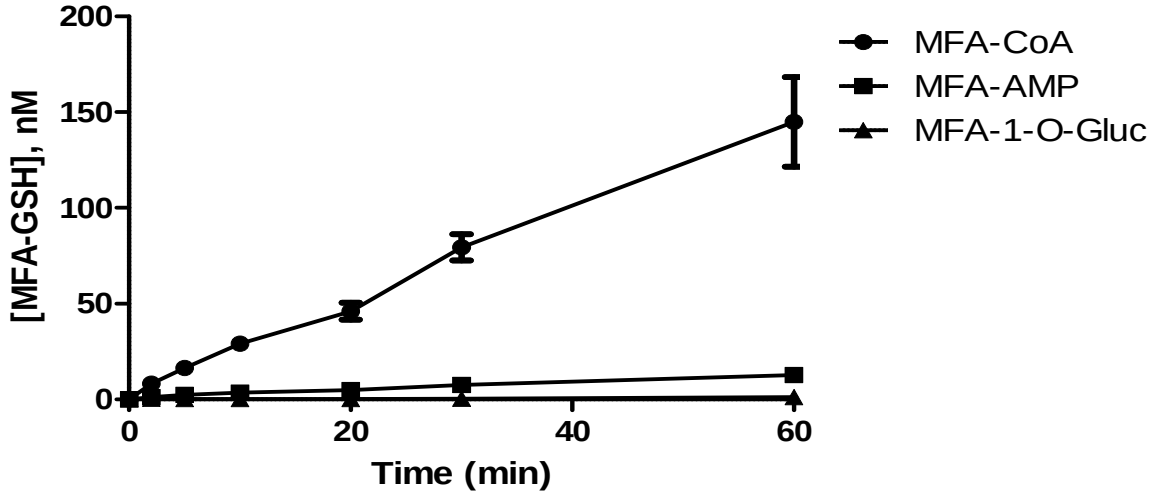
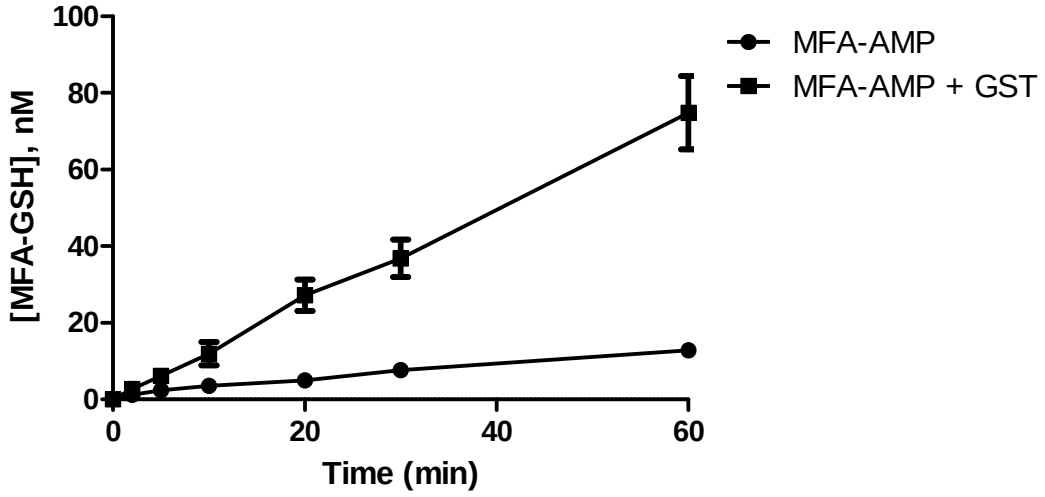


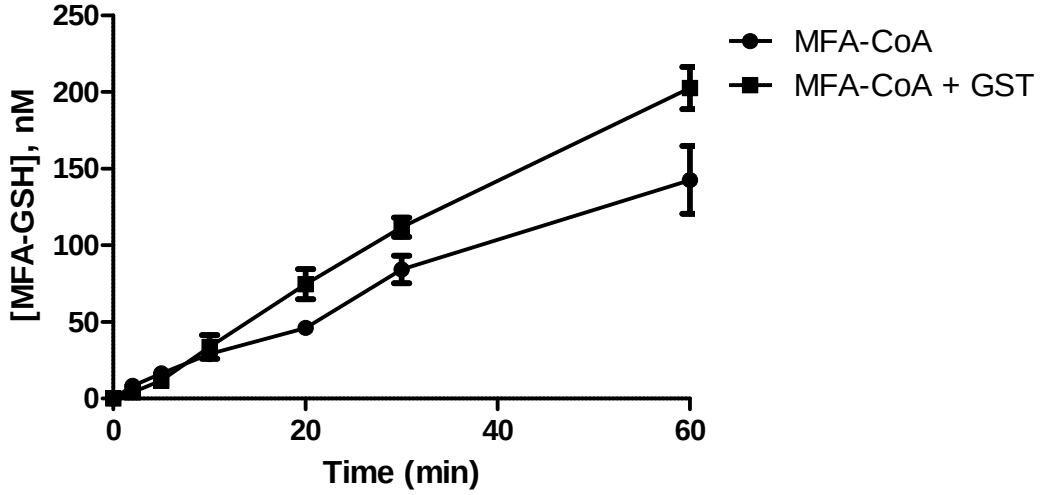
Figure 3.4 Reactivity of MFA-AMP, MFA-CoA, and MFA-1-*O*-Gluc (1 µM) with GSH (10 mM) in 0.1 M potassium phosphate buffer (pH 7.4, 37°C).

GST mediated reactivity assessment of MFA-AMP with GSH revealed a MFA-GSH concentration versus time slope of 1.25 nM/min and a maximum concentration of 74.8 nM after 60 minutes of incubation (Figure 3.5B). The *in vitro* incubation of MFA-CoA with GSH in the presence of equine liver GST revealed a MFA-GSH concentration versus time slope of 3.38 nM/min and a maximum concentration of MFA-GSH of 202.5 nM at 60 minutes (Figure 3.5A), while MFA-1-*O*-Gluc, GST mediated reactivity with GSH yielded a MFA-GSH concentration versus time slope of 0.07 nM/min of MFA-GSH and a maximum concentration of 1.33 nM at 60 minutes (Figure 3.5C).

A)



B)



C)

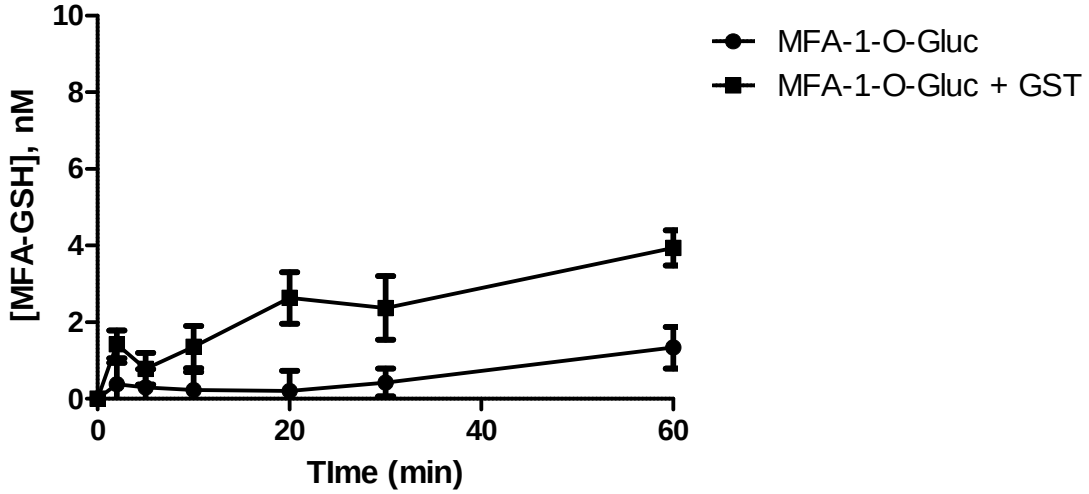


Figure 3.5 Reactivity of A) MFA-AMP, B) MFA-CoA, and C) MFA-1-*O*-G with or without equine liver GST (30 U/ml) in 10 mM GSH/0.1 M Kpi (pH 7.4, 37°C).

3.4 Discussion

Carboxylic-acid containing drugs have been implicated as the cause of idiosyncratic reactions, possibly by way of an immune-based reaction resulting from drug conjugates covalently binding onto macromolecules of the body (Faed, 1984; Spahn-Langguth and Benet, 1992; Boelsterli et al., 1995). Acyl glucuronides are reactive intermediates that can react with proteins by one of two mechanisms. The first route is the transacylation of nucleophilic centers of proteins (-*SH*, -*OH*, -*NH*) leading to the formation of a drug-protein adduct. The second mechanism requires prior acyl migration followed by a ring-chain tautomerism forming a straight chain aldehyde that can form Schiff base type adducts with protein lysines (Ding et al., 1995; Qiu et al., 1998). These resulting adducts can be made irreversible by undergoing an Amadori rearrangement to a stable 1-amino-2-keto product (Ding et al., 1995). *S*-acyl-CoA intermediates have also been shown to be reactive and form covalent bonds with proteins via a direct transacylation mechanism (Sallustio et al., 2000). The *S*-acyl-CoA metabolites of a number of carboxylic acid-containing drugs have been shown to be far more reactive than their corresponding 1-*O*-acyl-glucuronide intermediates. Studies in rats involving the α-fluorination (F-VPA) of the 4-ene valproic acid resulted in no hepatotoxicity (Tang et al. 1995). Coincidentally, further mechanistic studies comparing valproic acid with F-VPA have shown that the α-fluorinated derivative produced no *S*-acyl-CoA intermediates suggesting that the hepatotoxicity associated with valproic acid may in part be due to the transacylating abilities of the *S*-acyl-CoA (Grillo et al., 2001). An emerging topic of interest with regards to carboxylic-acid containing drugs is the formation of the acyl-adenylate metabolite. This mixed anhydride adenosine 5-monophosphate (AMP) is formed as intermediate to the formation of the *S*-acyl-CoA thioester

(Mao et al., 1992; Menzel et al., 1994; Hall and Xiaotao, 1994; Ikegawa et al., 1999). These acyl-AMP intermediates have also been shown to react with proteins of the body and the cysteinyl-thiol of GSH (Goto et al., 2001).

The present studies focus primarily on the determination and comparison of the relative ability of acyl-1-*O*-Gluc, *S*-acyl-CoA, and acyl-AMP metabolites of mefenamic acid to transacylate the nucleophilic cysteinyl-thiol of GSH. We propose that the greater the reactivity of the acyl-linked metabolite with GSH *in vitro*, the higher the probability that the metabolite may acylate nucleophilic centers *in vivo*, and thus the higher probability of a drug-induced toxic reaction.

The relative stabilities of MFA-1-*O*-Gluc, MFA-CoA, and MFA-GSH derivatives *in vitro* in potassium phosphate buffer under physiological conditions (pH 7.4 and 37°C) were assessed. MFA-1-*O*-Gluc was shown to have a half life of degradation of ~16 hours. LC/MS/MS chromatograms for MFA-1-*O*-Gluc incubation suggest that the degradation is due primarily to acyl migration rather than hydrolysis. This long half life for MFA-1-*O*-Gluc, with a fully substituted α-carbon, is consistent with the effects of α-carbon substitution on acyl glucuronide stability. MFA-CoA was shown to be highly stable with no observable hydrolysis for at least 24 hours, which is consistent with previous *S*-acyl-CoA stability data. Clofibric acid-*S*-acyl-CoA has been shown to be highly stable *in vitro* in buffer with a degradation half life of 21 days (~2% hydrolysis/day) (Grillo and Benet, 2000). Likewise, 2-PPA-*S*-acyl-CoA and phenylacetyl-*S*-acyl-CoA has also been shown to be completely stable after one day of incubation (Li et al., 2002). However, 2,4-dichlorophenoxyacetyl-*S*-acyl-CoA has been reported to have a half-life of

degradation of 5.6 hours. MFA-AMP and MFA-GSH were shown to be stable for at least one day in *in vitro* potassium phosphate buffer incubations.

The next set of studies focused on determining and comparing the relative ability of MFA-AMP, MFA-CoA, and MFA-1-*O*-Gluc to transacylate the nucleophilic cysteinyl-thiol of GSH *in vitro* in buffer in the absence and presence of GST. Nonenzymatic reactivity assessment for each metabolite was determined by incubating 1 µM of each acyl-linked metabolite with 10 mM GSH in potassium phosphate buffer at pH 7.4 and 37°C showing the formation of MFA-GSH for each metabolite. MFA-1-*O*-Gluc incubations resulted in the formation of 2.6 nM of MFA-GSH at 60 minutes (0.04 nM/min) (Table 3.1). The formation of MFA-GSH from reactions of GSH with MFA-*S*-CoA resulted in a time-dependent formation of GSH adducts at a rate of 2.41 nM/min (Table 3.1) and a C_{max} of 144.9 nM after 60 minutes; MFA-AMP incubations with GSH revealed a rate of formation of MFA-GSH to be 0.21 nM/min (Table 3.1) and a C_{max} of 12.8 nM at the 60 min time point; and MFA-1-*O*-Gluc reacted with GSH to form MFA-GSH at a rate of 0.02 nM/min (Table 3.1) achieving a maximum concentration of 0.42 nM at 60 minutes. The rank order of nonenzymatic *in vitro* GSH reactivity was as follows: MFA-CoA > MFA-AMP > MFA-1-*O*-Gluc. Overall, these results show a more rapid and extensive spontaneous reaction of MFA-CoA thioesters of mefenamic acid with GSH than its corresponding acyl glucuronide and acyl adenylate derivatives, suggesting that on its own, MFA-CoA may contribute more to the covalent binding and thus an idiosyncratic toxicity than its corresponding MFA-AMP and MFA-1-*O*-Gluc.

Derivative (1 µM)	MFA-GSH Concentration Versus Time Slope (nM/min)
MFA-AMP	0.21
MFA-AMP + GST	1.25
MFA-CoA	2.41
MFA-CoA + GST	3.38
MFA-1-*O*-Gluc	0.02
MFA-1-*O*-Gluc + GST	0.07

Table 3.1 Rates of formation of MFA-GSH.

In the presence of equine liver GST (30 Units/ml) with 10 mM GSH in 0.1 mM potassium phosphate buffer in pH 7.4 and 37°C incubations resulted in an 1.4-fold, 5.95-fold, and 3.5-fold increase in the rate of formation of MFA-GSH for MFA-CoA, MFA-AMP and MFA-1-*O*-Gluc respectively. These results suggest MFA-AMP is a better substrate for GST and may be responsible for more MFA-GSH formed in liver cells than its corresponding MFA-*S*-CoA and MFA-1-*O*-Gluc.

In summary, the results from these studies show that both MFA-*S*-CoA, MFA-AMP, and to a small extent, MFA-1-*O*-Gluc are chemically reactive and capable of transacylating the cysteinyl-thiol of glutathione. The superior ability of MFA-*S*-CoA to react spontaneously with GSH suggests that the acyl-*S*-CoA derivative may play a more important role in the covalent binding of mefenamic acid to proteins *in vivo* and in inducing an idiosyncratic toxicity in patients than

the acyl-AMP and acyl-1-*O*-glucuronide. However, MFA-AMP is a much stronger substrate for GST than its corresponding MFA-*S*-CoA, suggesting that the acyl-adenylate derivative of mefenamic acid may play a more important role in the formation of MFA-GSH in rat hepatocytes. This phenomenon may also result in the formation of an idiosyncratic toxicity, especially if the MFA-GSH is reactive on its own.

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Chapter 4

In vitro Timecourse of Formation of MFA-AMP, MFA-CoA, MFA-1-*O*-Gluc, and MFA-GSH in Rat Hepatocytes.

4.1 Introduction

Recent studies have focused on the use of GSH as a model nucleophile for determining the chemical reactive potential of the acyl-linked metabolites through its formation into *S*-acyl-GSH thioester products. In addition, timecourse of formation studies in hepatocytes are also being used to investigate the relative contribution of each acyl-linked metabolite towards the acylation of GSH. *In vitro* timecourse of formation data can show how certain bioactivation pathways may predominate, which may also be the same pathway predominating *in vivo* and thus responsible for the covalent binding of acidic drugs to proteins that can potentially mediate an immunotoxic reaction in patients. Therefore, acyl-GSH conjugates can serve as a biomarker for mechanistic bioactivation studies of carboxylic acid-containing drugs.

Studies investigating the ability of diclofenac-1-*O*-acyl glucuronide to transacylate GSH *in vitro* in rat and human hepatocyte incubations have shown that when diclofenac (100 µM) was incubated in rat hepatocytes, diclofenac-GSH reached a maximum concentration of 1 and 0.8 nM in rat and human hepatocytes, respectively, after 4 minutes of incubation (Grillo et al., 2003). However, these same incubations revealed that diclofenac-1-*O*-acyl-glucuronide

reached a maximum concentration of 14.6 µM at 30 minutes. The concentration and timecourse of formation of diclofenac-1-*O*-acyl-glucuornide are not consistent with that of diclofenac-GSH suggesting that diclofenac-1-*O*-acyl-glucuronide does not completely account for all of the glutathione conjugate formed from diclofenac hepatocyte incubations. Therefore, it can be concluded that diclofenac-1-*O*-acyl-glucuornide is not the main contributor to the covalent binding of proteins via direct transacylation.

In vitro time-dependent incubation of 2-PPA in rat hepatocytes resulted in a maximum concentration of covalent binding of 230 pmol 2-PPA bound/mg protein in 1 hour after which covalent binding leveled off (Li et al., 2002b). 2-PPA-CoA formation reached a maximum concentration of ~0.93 nmol/million cells in 15 minutes, followed by a slow decrease to ~0.65 nmol/million cells at 3 hours, while 2-PPA-1-*O*-glucuronide formation was roughly linear for 3 hours, reaching a concentration of ~40 nmol/million cells. No 2-PPA-1-*O*-glucuronide was detected prior to 15 minutes of incubation. Previous studies have also shown that 2-PPA-CoA is 70-fold more reactive towards the transacylation of GSH than the 2-PPA-1-*O*-glucuornide (Li et al., 2002a). The timecourse of formation of 2-PPA-CoA better matches that of covalent binding than its corresponding 2-PPA-1-*O*-glucuronide and the greater reactivity of the 2-PPA-CoA compared to the 2-PPA-1-*O*-glucuronide strongly suggests that the acyl-CoA contributes more to covalent binding than its respective acyl-glucuronide.

In vitro bioactivation studies of zompirac (100 µM) in rat hepatocytes (Olsen et al., 2005) showed a rapid formation of zomepirac-CoA (tmax ~10-20 min) achieving a concentration of ~130 nM, which was 20-fold lower in concentration than that of zomepirac-1-*O*-acyl

glucuronide. The concentration of zomepirac-CoA then dropped down to 60 nM at 60 minutes, while zomepirac-1-*O*-glucuronide continued to increase linearly. Previous studies have shown that zomepirac-CoA is ~40-70 fold (Li et al., 2002a; Grillo and Benet, 2002; Olsen et al., 2002) more reactive than its corresponding glucuronide. Furthermore, the time profile of formation of zomepirac-GSH (Grillo and Hua; 2003), which reaches a maximum concentration of ~0.25 nM at 4 minutes followed by a linear decrease, is more consistent with its acyl-CoA derivative than its 1-*O*-glucuronide, suggesting that zomepirac-CoA may contribute more to the transacylation of GSH and covalent binding than the glucuronide.

Previous studies have shown that ibuprofen-CoA formation is enantioselective for the (*R*)-(-)-enantiomer while acyl glucuronidation prefers the (*S*)-(+)-enantiomer (el Mouelhi et al., 1987). When *R*-(-)-ibuprofen (100 µM) was incubated with rat hepatocytes, the formation of ibuprofen-GSH reached a maximum concentration of 1.3 nM after 10 minutes of incubation (Grillo and Hua, 2008). From these same incubations, the maximum concentration of ibuprofen-CoA was 2.6 µM at the 8 minute time point, while ibuprofen-1-*O*-acyl glucuronide concentration increased throughout the 40 minute incubation, supporting the assumption that acyl-CoA formation is more important in the transacylation of GSH than the acyl-glucuronide.

The carboxylic acid-containing drugs ibuprofen, diclofenac, 2-PPA, zomepirac, and phenylacetic acid have all been shown to form acyl glutathione *in vitro* in rat hepatocyte incubations (Figure 4.1). Preliminary studies have shown that mefenamic acid (MFA) forms ~1000-fold more acyl glutathione in *in vitro* in rat hepatocyte incubations (Figure 4.2) than the previously mentioned drugs, suggesting that the MFA acyl-linked metabolite may be very

reactive, especially the acyl-adenylate, whose time profile of formation had not been previously studied in rat hepatocytes. The focus of the present study is to determine the relative contributions of the three metabolic activation pathways, MFA-AMP, MFA-CoA, and MFA-1-*O*-Gluc of mefenamic acid to acylation of GSH in rat hepatocytes *in vitro*.

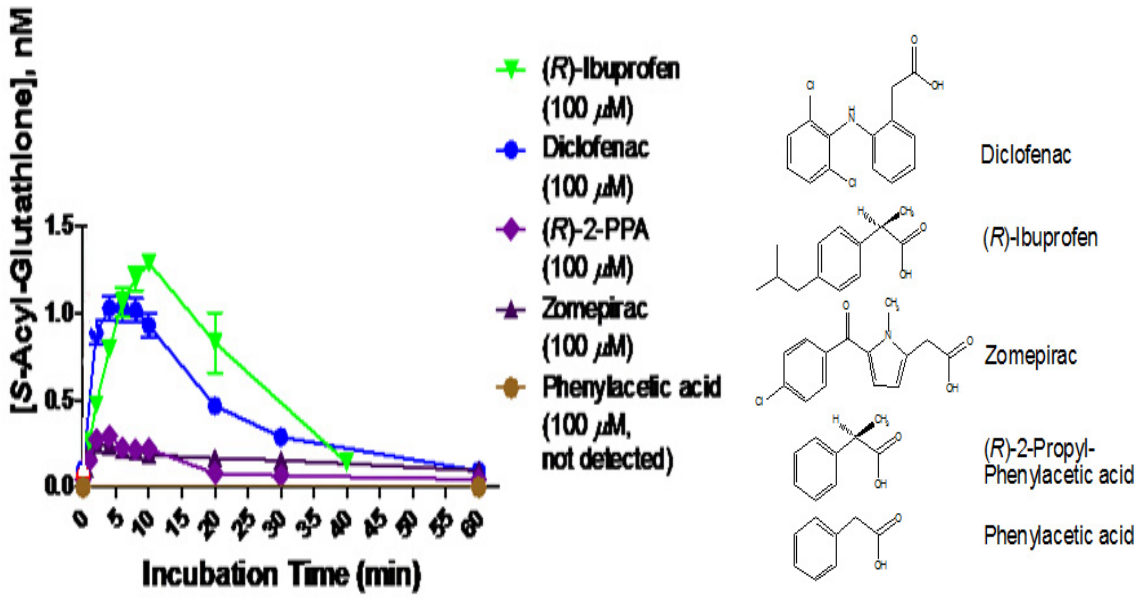

Figure 4.1 Relative amounts of *S*-acyl-glutathione conjugates formed in incubations with rat hepatocytes.

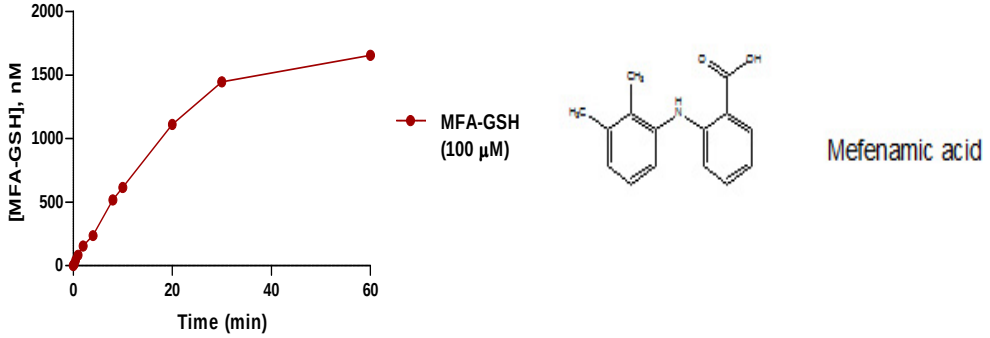

Figure 4.2 Preliminary time-dependent formation of MFA-GSH in rat hepatocytes.

4.2 Experimental Section

4.2.1 Chemicals

Williams Media E and L-glutamine were purchased from Gibco (Grand Island, NY). Sprague-Dawley rats were purchased from Charles River (Wilmington, MA).

4.2.2 Instrumentation and Analytical Methods

All analytical LC/MS/MS analyses were performed from methods previously described (Chapter 2).

4.2.3 *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 assessed by trypan blue exclusion testing. Incubations of hepatocytes (2 million viable cells/mL) with mefenamic acid (100 µM) were performed in Williams Media E fortified with 4 mM glutamine in a 50 ml round bottom flask. Incubations were performed with continuous rotation and gassed with 95% O_2/5% CO_2 at 37°C. Aliquots were taken at 0, 0.2, 0.5, 1, 2, 4, 8, 10, 20, 30, and 60 minutes and analyzed for MFA-AMP, MFA-CoA, MFA-GSH, and MFA-1-*O*-Gluc by LC/MS/MS.

For the analyse of MFA-AMP, MFA-GSH, and MFA-1-*O*-Gluc, aliquots (200 µl) of the incubation mixture were taken and added directly into microcentrifuge tubes (2 ml) and quenched with a solution (200 µl) of acetonitrile, 3% formic acid, and 2 µM carbamazepine internal standard.

Samples were then centrifuged (14,000 rpm, 5 min) and the supernatant fraction (200 µl) was transferred to HPLC autosampler vials for LC/MS/MS analysis.

For the analyse of MFA-CoA formation, aliquots (200 µl) from the same incubations were taken and transferred directly into microcentrifuge tubes (2 ml) and quenched with a solution (400 µl) of acetonitrile containing 2 µM carbamazepine 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 hour evaporation of residual hexane under the fume hood. The samples were then analyzed for MFA-CoA by LC/MS/MS.

4.3 Results

4.3.1 Stability of S-Acyl-Glutathione Conjugates in Incubations with Rat Hepatocytes

Incubation of MFA-GSH (100 µM) with rat hepatocytes, under physiological conditions, revealed that it is metabolically unstable, possessing a degradation half life of ~8 minutes. Stability results in buffer (pH 7.4, 37°C) (Figure 3.3C) showed that MFA-GSH is stable for the 24 hour incubation period suggesting that the degradation of MFA-GSH, as well as diclofenac and ibuprofen glutathione conjugates, are enzyme mediated. MFA-1-*O*-Gluc was shown to be stable during the 1 hour incubation in rat hepatocytes.

4.3.2 Timecourse of Formation of MFA-GSH, MFA-AMP, MFA-CoA, and MFA-1-*O*-Gluc in Rat Hepatocyte Incubations

When mefenamic acid (100 µM) was incubated in rat hepatocytes, the formation of MFA-GSH was rapid and reached a concentration of 16.5 µM at 60 minutes, while MFA-1-*O*-Gluc formation increased linearly achieving a concentration of 42.2 µM at 60 minutes (Figure 4.3). From the same incubations, MFA-AMP rapidly reached a maximum concentration at ~ 20 seconds of ~90.2 nM and plateaued for the remaining 60 minute incubation, while MFA-CoA was not detectable until the 4 minute time point and reached a maximum concentration of 45.6 nM at 60 minutes (Figure 4.3 and Figure 4.4).

Rat hepatocyte incubations using lower doses of mefenamic acid (31 µM) revealed an increase of MFA-GSH to a maximum concentration of 884.7 nM at 20 minutes followed by a steady decrease in concentration to 155.7 nM at 60 minutes. MFA-AMP and MFA-CoA achieved a maximum concentrations of 108 nM at 12 seconds and 65.5 nM at 30 minutes respectively followed by a decrease in concentration as well. MFA-1-*O*-Gluc reached a maxiumum concentration of 6.68 µM at 60 minutes (Figure 4.5).

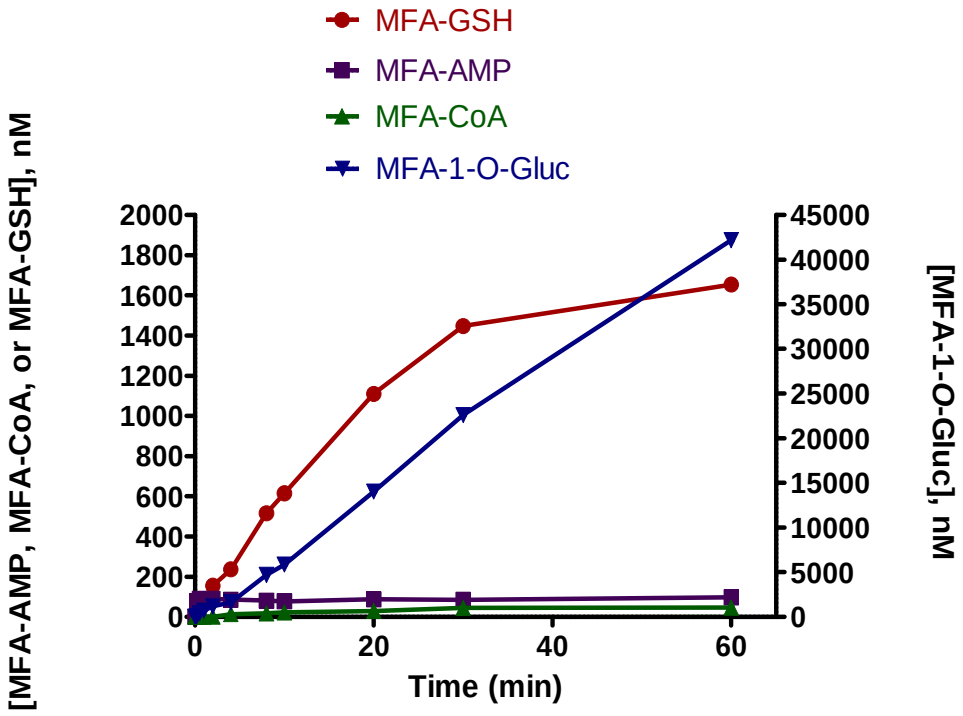

Figure 4.3 Time-dependent formation of MFA-conjugates in rat hepatocytes

(MFA dose 100 µM).

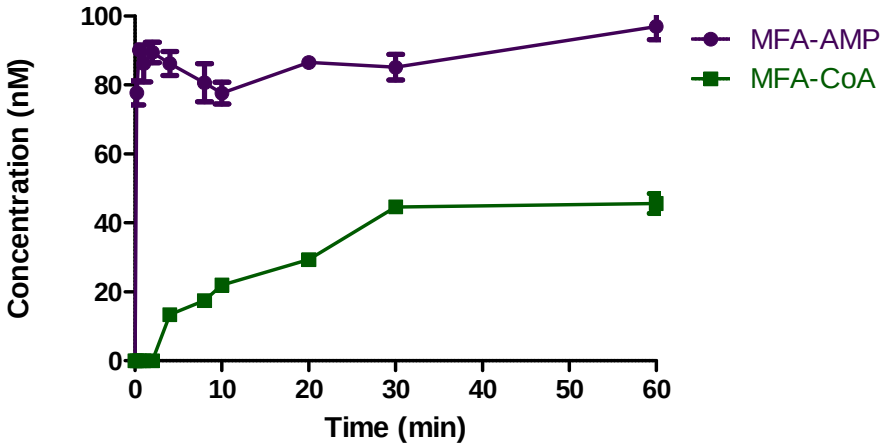

Figure 4.4 Time-dependent formation of MFA-AMP and MFA-CoA from MFA (100 µM) in rat hepatocytes.

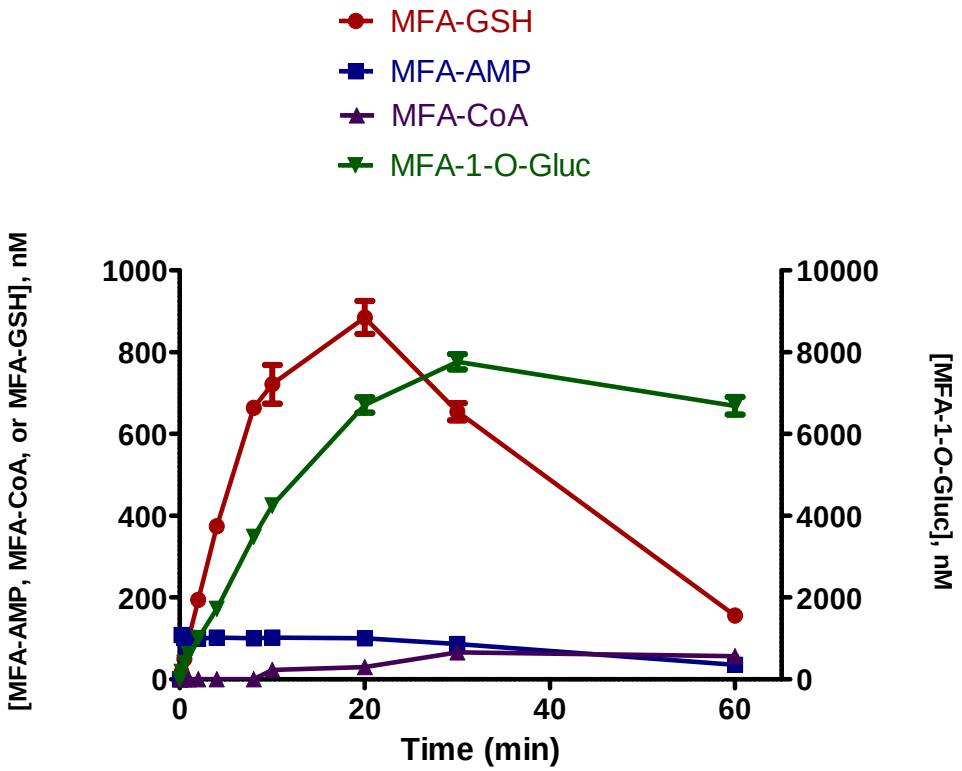

Figure 4.5 Time-dependent formation of MFA-conjugates from MFA (31 µm) in rat hepatocytes.

4.4 Discussion

Carboxylic acid containing NSAIDs are a class of drugs that have an unusual high incidence of withdrawal from the market (Bakke et al., 1984). During the years of 1964 through 1993, 47 drugs were withdrawn from the U.S., British, and Spanish markets due to severe toxicity and 10 of those withdrawn were carboxylic acid containing drugs (Bakke et al., 1984; Bakke et al., 1995). The most frequent types of adverse reactions associated with carboxylic acid-containing drugs are hepatotoxicity, allergic skin reactions, and renal toxicity. Although the exact mechanism of toxicity is not fully understood, the covalent modification of cellular proteins by chemically reactive metabolites formed from the bioactivation of carboxylic acid-containing

drugs may be the cause of these idiosyncratic reactions. The two major phase II metabolic bioactivation pathways of carboxylic acid-containing drugs are the formation of the acyl-CoA and acyl-glucuronidation intermediates. However, the acyl-AMP, intermediate of the acyl-CoA pathway, is also believed to play a role in the formation of idiosyncratic toxicities. Many studies have shown that all three metabolites are reactive and are capable of transacylating GSH and proteins, resulting in the formation of thioester-, ester-, or amide-linked covalent adducts (Benet et al., 1993, Grillo and Benet, 2002; Grillo et al., 2003, Sidenius et al., 2004; Mitamura et al., 2011).

The present study reveals that mefenamic acid undergoes bioactivation in rat hepatoctye incubations leading to the transacylation of GSH. The incubation of 100 µM of mefenamic acid in rat hepatocyte preparations revealed the formation of MFA-AMP, MFA-CoA, MFA-1-*O*-Gluc, and MFA-GSH. The time to maximum MFA-AMP concentration was extremely rapid, ~20 seconds, leveling off at 90.2 nM for the remaining 60 minute incubation. MFA-1-*O*-Gluc formation was linear for the whole 60 minute incubation reaching a concentration of 42.2 µM. MFA-CoA was not detected until 4 minutes and achieved a concentration of 45.6 nM at 60 minutes. MFA-GSH formation was high compared to other studied NSAIDs and increased to a concentration of 16.5 µM at 60 minutes. Rat hepatocytes were still fully viable after 60 minutes of incubation.

Previous rat hepatocyte incubations using lower doses of mefenamic acid (31 µM) revealed an increase of MFA-GSH to a maximum concentration of 885 nM at 20 minutes followed by a steady decrease in concentration to 156 nM at 60 minutes. MFA-AMP, MFA-CoA, and MFA-1-

O-Gluc all exhibited similar decrease as well. Stability studies in rat hepatocytes have revealed that MFA-GSH is metabolically unstable, resulting in a rapid hydrolysis ($t_{1/2}$ ~ 8 min). Further investigation have shown no γ-glutamyltranspeptidase (γ-GT) products (*N*-acetylcysteinylglycine), which is consistent with previous results showing the lack of sufficient γ-GT activity in rat liver tissue (Hinchman and Ballatori, 1990). Stability studies in buffer have also revealed that MFA-GSH is chemically stable for at least 24 hours. Therefore the degradation of MFA-GSH in rat hepatocytes is believed to be the result of intracellular thioesterase or peptidase activity, suggesting that MFA-GSH is freely permeable to cellular membranes and able to access cellular enzymes. Another potential route of degradation is by glutathione *S*-transferase (GST)-mediated hydrolysis (Ibarra et al., 2003), which was not investigated in the present study. We also hypothesized that in addition to intracellular thioesterase activity, the decrease of MFA-GSH, as well as MFA-AMP, MFA-CoA, and MFA-1-*O*-Gluc, in rat hepatocyte incubations may also result from the rapid consumption of mefenamic acid by phase I and acyl glucuronidation metabolism. Further investigation into this phenomenon will be performed in the following chapters.

The time course of formation of MFA-GSH appears to be consistent with the MFA-1-*O*-Gluc. However, previous reactivity studies have shown that MFA-1-*O*-Gluc is not reactive towards the transacylation of GSH in the presence and absence of GST. Therefore, it is unlikely that the acyl glucuronide of mefenamic acid is responsible for the unusually high concentration of MFA-GSH formed in these incubations. MFA-CoA is known to be highly reactive towards GSH, but its concentration in rat hepatocytes is insufficient to account for all of the MFA-GSH formed in rat hepatocytes. MFA-AMP concentration in rat hepatocytes is low compared to that of MFA-GSH

(~6%) and was shown to be mildly reactive toward the transacylation of GSH. However, in the presence of GST, MFA-AMP forms ~6-fold more MFA-GSH than on its own, suggesting that the acyl-adenylate intermediate is a good substrate for GST. MFA-GSH formation also begins to level off at 30 minutes suggesting enzyme (GST) saturation. MFA-AMP time to maximum concentration was also shown to be extremely rapid (~20 sec) to the point that any MFA-AMP converted into MFA-GSH or MFA-CoA is quickly replenished within a matter of seconds. Therefore, we hypothesized that MFA-AMP is responsible for the high amount of MFA-GSH formed in rat hepatocyte incubations due to its rapid rate of formation and its high specificity towards GST. These ideas will be tested in subsequent chapters.

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Chapter 5

Mechanistic Inhibition Studies of MFA-AMP, MFA-CoA, and MFA-1-*O*-Glucuronide in Rat Hepatocytes

5.1 Introduction

A major concern in drug development today is the metabolism of therapeutic compounds to chemically reactive intermediates. The unrealized potential of reactive metabolites to covalently bind to macromolecules of the body and DNA to elicit an idiosyncratic toxicity is still not fully understood. In the majority of cases, phase II conjugative metabolism results in the detoxification and clearance of xenobiotics. However, there are a number of drugs that undergo phase II bioactivation into reactive metabolites that are associated with a toxicological event. The glucuronidation of lipophilic compounds occurs through the addition of a highly hydrophilic and ionized glucuronic acid moiety that usually results in the formation of chemically stable metabolites with increased water solubility suited for transport into the bile and urine via efflux transporters located in the hepatic bile canalicular and renal tubular brush border membranes (Schaub et al., 1997; Sabordo et al., 2000; Westley et al., 2006). However, glucuronidation of carboxylic acid-containing drugs generates unstable electrophilic acyl-linked glucuronides that have the potential to react with nucleophilic centers of macromolecules. There are a number of cases of chemically reactive acyl-glucuronides believed to mediate an immune-mediated toxicity. Conjugation of these reactive metabolites with liver GSH results in

glutathione conjugates that can continue to undergo further bioactivation following their transport to the kidney in the form of mercapturic acid derivatives. As a result, acyl-glucuronides and acyl-glutathione conjugates can distribute from their site of formation in the liver to elicit toxicity in non-hepatic tissues. In addition to acyl-glucuronidation, carboxylic acid-containing drugs can also be metabolized into acyl-CoA and acyl-adenylate derivatives. Both intermediates have been shown to be reactive and capable of transacylating tissue proteins. The exact mechanism of toxicity elicited by acyl-CoA and acyl-adenylate conjugates is still under investigation, but a number of studies utilizing inhibitors and inducers have shown a stronger reactivity correlation with the acyl-CoA intermediates than that of the corresponding acyl-glucuronides.

Time-dependent *in vitro* rat hepatocyte incubations with 2-phenylpropionic acid (2-PPA) reveal that both 2-PPA-acyl-CoA (2-PPA-CoA) and 2-PPA-acyl-glucuronide (2PPA-1-*O*-Gluc) are involved in their covalent binding to hepatic proteins. Incubations with trimethylacetic acid (TMA), an inhibitor of acyl-CoA formation, resulted in a 66% decrease in acyl-CoA formation and a 53% decrease in covalent binding. However, the complete inhibition of 2-PPA-1-*O*-Gluc in the presence of borneol, an inhibitor of glucuronidation, only resulted in a 18.7% decrease in protein covalent binding (Li et al., 2002b). These results suggest that 2-PPA-CoA plays a more important role than 2PPA-1-*O*-Gluc in covalent adduct formation in rat hepatocytes even though 2-PPA-1-*O*-Gluc concentration was ~40-fold greater than its corresponding acyl-CoA derivative. This result was also consistent with previous studies showing the reactivity of 2-PPA-CoA with glutathione to be 70-fold greater than 2-PPA-1-*O*-Gluc (Li et al., 2002a).

The metabolic activation of tolmetin to the chemically reactive tolmetin-acyl-CoA (Tol-CoA) in rats following a seven day preincubation with clofibric acid, an inducer of acyl-CoA formation, revealed that tolmetin-1-*O*-acyl-glucuronide was 40-fold higher in concentration than Tol-CoA in control rats and was not affected by the pretreatment with clofibric acid. Clofibric acid induced rats resulted in a 7.3-fold increase in Tol-CoA concentration and a 4.4-fold increase in liver protein covalent binding versus controls (Olsen et al., 2007). Tolmetin-carnitine conjugates also experienced an 8-fold increase in concentration in clofibric acid treated rats, which is consistent with previous studies showing the induction of carnitine acyl transferases (Katsutani et al., 2000) and increases in free carnitine and CoA levels after pretreatment with fibrates (Gregus et al., 1998). Since the formation of tolmetin-carnitine intermediates is dependent on the formation of Tol-CoA, mechanistically, the identification of tolmetin-carnitine derivatives in these rat liver homogenates is significant. Tolmetin taurine and glycine conjugate concentrations were unaffected in the clofibric acid treated compared to control rats. These data show that changes induced by clofibric acid on Tol-CoA appear to have a stronger correlation with the amount of covalent binding than the corresponding 1-*O*-acyl-glucuornide, further supporting the hypothesis that acyl-CoA intermediates contribute more to protein covalent binding than acyl-glucuronides.

Ibuprofen is metabolized to chemically reactive ibuprofen-1-*O*-acyl-glucuronide (Ibu-1-*O*-Gluc) and ibuprofen-*S*-acyl-CoA (Ibu-CoA), both of which are involved in the transacylation of nucleophilic sites on proteins (Grillo and Hua, 2008). When a pseudoracemic mixture of (*R*)-(-) and (*S*)-(+)-ibuprofen was incubated in rat hepatocytes, >99% of the ibuprofen-GSH formed (Ibu-GSH) was derived from the bioactivation of the (*R*)-(-)-ibuprofen, which is enantioselective

for acyl-CoA formation. (-)-Borneol mediated inhibition of (*R*)-(-)-ibuprofen glucuronidation resulted in a 98% decrease in Ibu-1-*O*-Gluc formation but no decrease in Ibu-GSH was observed. Inhibition of acyl-glucuronidation with (-)-borneol is consistent with other experiments involving the NSAID diclofenac, in which complete inhibition of diclofenac acyl glucuronidation led to no effect on the formation of diclofenac-GSH (Grillo et al., 2003). Inhibition of the acyl-CoA synthetase (ACS) by known inhibitors: pivalic acid (Xiaotao and Hall, 1993), valproic acid, and lauric acid (Li et al., 2003) resulted in a 47%, 54%, and 93% inhibition, respectively, of Ibu-CoA formation and a subsequent decrease of 53%, 66%, and 93% inhibition of Ibu-GSH respectively in rat hepatocyte incubations. The results from these experiments strongly suggest that the transacylation of GSH by ibuprofen is mediated by Ibu-CoA rather than the Ibu-1-*O*-Gluc in hepatocyte incubations.

A number of studies have shown a causative association between drug acyl-CoA and/or acyl-1-*O*-glucuronide metabolites and idiosyncratic toxicity. The use of specific inhibitors helps determine the relative importance of acyl-CoA and acyl-1-*O*-glucuronide metabolites in terms of glutathione conjugation, covalent binding, and potentially the onset of an idiosyncratic reaction. Currently, no studies have investigated the reactive potential of the acyl-linked metabolites of mefenamic acid. In addition, very little is known about the chemical reactivity and disposition of a drug derived acyl-adenylate intermediate and its role in the elicitation of a toxic reaction. The focus of the present studies is to determine the importance of MFA-AMP in the transacylation of GSH in relation to MFA-CoA and MFA-1-*O*-Gluc with the use of specific inhibitors for acyl glucuronidation [(-)-borneol)], acyl-CoA synthetase (lauric acid), phase-I metabolism [(1-aminobenzotriazole (ABT)], and temperature to modulate metabolism.

5.2 Experimental Section

5.2.1 *In Vitro* Studies with Rat Hepatocytes

Freshly isolated rat (250-300 g, male Sprague-Dawley) hepatocytes were prepared according to the previously described methods (Chapter4). Inhibition experiments were performed in the presence or absence of (-)-borneol (100 µM) for mefenamic acid acyl-glucuronidation, in the presence or absence of lauric acid (1 mM) for acyl-CoA formation, with the coincubation of mefenamic acid (31 µM) in rat hepatocytes ($2x10^6$ cells/ml). Incubations (n=3) were performed as previously described for 10 minutes and terminated with the appropriate quenching solution. ABT Phase-I metabolism inhibition was performed with a 30 minute preincubation of ABT (500 µM) followed by the addition of mefenamic acid (100 µM). Incubations (n=3) were performed as described above and aliquots were taken at 0, 0.2, 0.5, 1, 2, 4, 8, 10, 20, 30, and 60 minutes and quenched for analysis. Temperature sensitive incubations (n=3) were carried out at 37°C (physiological temperature), 25°C (room temperature) and 0°C (on ice) followed by the addition of mefenamic acid (100 µM) and aliquots were taken at 0, 0.2, 0.5, 1, 2, 4, 8, 10, 20, 30, and 60 minutes and quenched for analysis.

5.2.2 Chemical Reactivity

Chemical reactivity experiments for MFA-AMP, MFA-CoA, and MFA-1-*O*-Gluc were performed as previously described (chapter 3) at 25°C and 0°C.

5.3 Results

5.3.1 Acyl-Glucuronidation and Acyl-CoA Synthetase Inhibition in Rat Hepatocytes

In order to determine the importance of acyl glucuronidation or acyl-CoA formation of mefenamic acid towards the formation of MFA-GSH in rat hepatocytes, the effects of (-)-borneol and lauric acid inhibition on glucuronidation and acyl-CoA synthetase, respectively were examined. Inhibition experiments were performed with the coincubation of mefenamic acid (100 μM) with rat hepatocytes (2 x 10^6 cells/ml, 10 min) in the presence or absence of (-)-borneol (100 μM) or lauric acid (1 mM). In the presence of (-)-borneol, MFA-1-*O*-Gluc and MFA-CoA formation were inhibited by 91.1% and 3.3%, respectively, compared to controls. However, the concentration of MFA-GSH increased 16% when compared to control incubations. In the presence of lauric acid, MFA-1-*O*-Gluc and MFA-CoA concentrations decreased by 68.0% and 58.4%, respectively, while MFA-GSH formation decreased by 66.1% (Table 5.1).

Inhibitor	MFA-1-*O*-Gluc		MFA-CoA		MFA-GSH	
	Conc. (nM)	% Control	Conc. (nM)	% Control	Conc. (nM)	% Control
Control	1060 ± 137	-	14.6 ± 1.36	-	344 ± 53.4	-
(-)-Borneol (100 μM)	94.4 ± 12.6	8.9 ± 1.2	14.1 ± 1.60	96.7 ± 10.9	398 ± 23.3	116 ± 6.78
Lauric Acid (1000 μM)	341 ± 25.2	32.0 ± 2.4	6.06 ± 0.25	41.6 ± 1.71	117 ± 12.0	33.9 ± 3.48

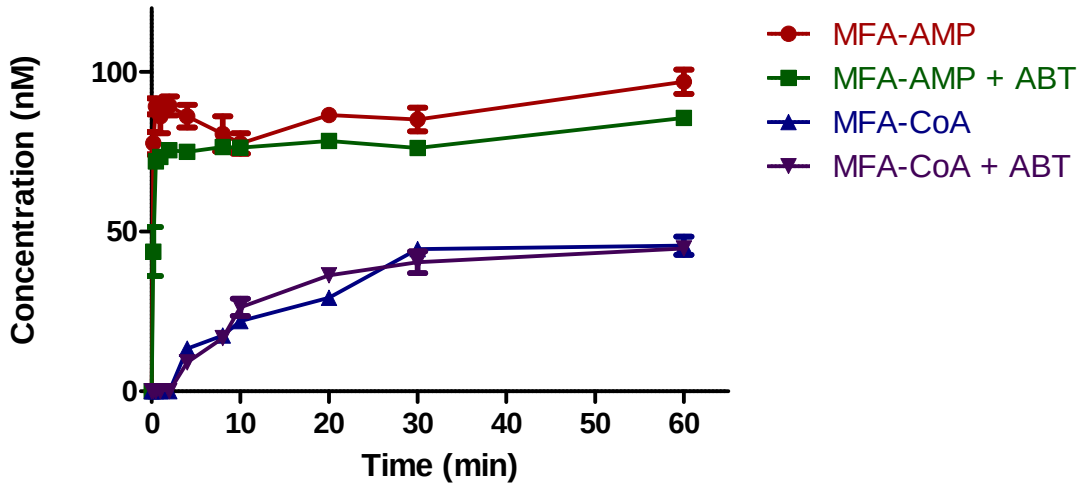
Table 5.1 Inhibition of acyl-glucuronidation by (-)-borneol) and of acyl-CoA synthetase by lauric acid in rat hepatocytes incubations (10 min, 37°C, pH 7.4).

5.3.2 ABT Phase-I Metabolism Mediated Inhibition in Rat Hepatocytes

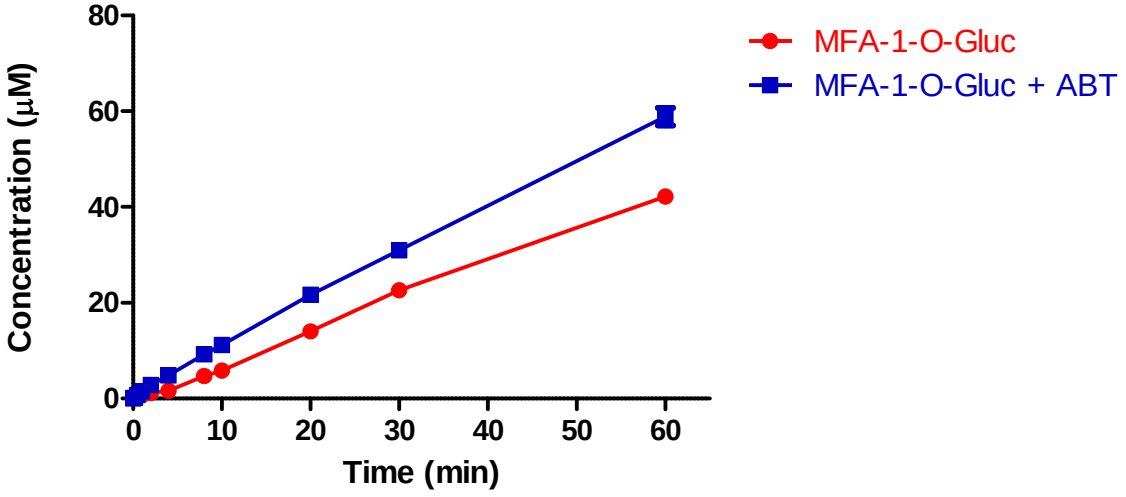
In order to determine the relative importance of MFA-AMP and MFA-CoA compared to MFA-1-*O*-Gluc in the formation of MFA-GSH in rat hepatocytes, phase-I CYP450 metabolism was inhibited by ABT. For hepatocyte incubations in the absence and presence of ABT (500 µM), MFA-AMP concentrations reached a relative maximum concentration of 90.2 nM and 72.1 nM, respectively, at 30 seconds and the concentrations remained relatively constant through 60 minutes. MFA-CoA was undetectable until the 4 minute time point and reached a

concentration of 45.6 nM and 44.8 nM at the 60 minute time point in the absence and presence of ABT, respectively, (Figure 5.1A). By contrast, MFA-1-*O*-Gluc formation increased linearly and reached a concentration of 42.2 µM and 58.9 µM (40% increase) at the 60 minute time point with and without ABT, respectively (Figure 5.1B). However, MFA-GSH formation achieved a concentration of 1.5 µM and 1.4 µM at 30 minutes followed by a concentration of 1.7 µM and 2.0 µM at 60 minutes of incubation in the presence and absence of ABT (Figure 5.1C).

A)



B)



C)

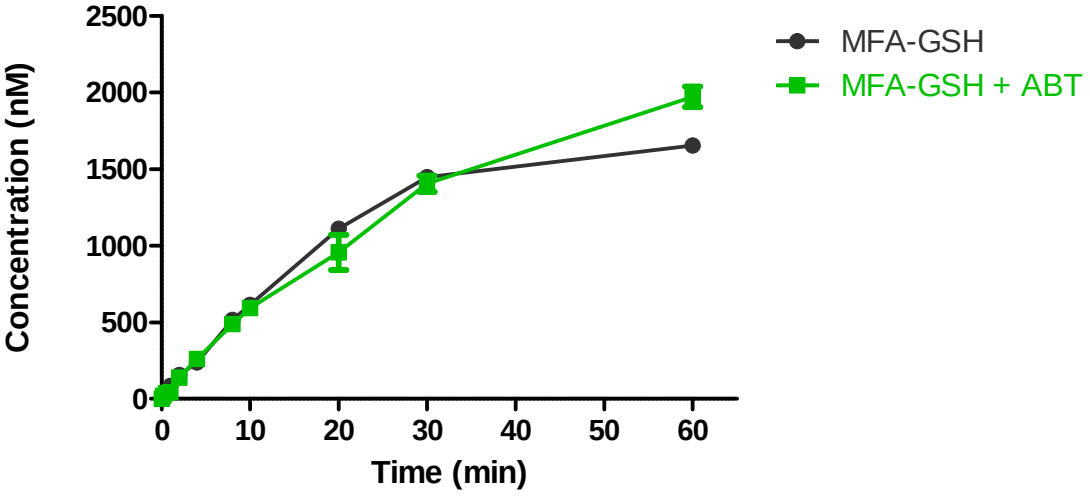


Figure 5.1 Time-dependent formation of MFA-GSH in the absence and presence of ABT in rat hepatocytes.

5.3.3 Temperature Dependent Inhibition in Rat Hepatocytes

When mefenamic acid (100 µM) was incubated in rat hepatocytes at physiological temperature (37°C), the formation of MFA-GSH was rapid and reached a concentration of 1.7 µM at 60 minutes, while MFA-1-*O*-Gluc formation increased linearly achieving a concentration of 42.2 µM at 60 minutes. Under the same incubation conditions, MFA-AMP rapidly reached a maximum concentration at ~ 20 seconds of ~90.2 nM and plateaued for the remaining 60 minute incubation, while MFA-CoA was undetectable until the 4 minute time point and reached a maximum concentration of 45.6 nM at 60 minutes (Figure 5.2).

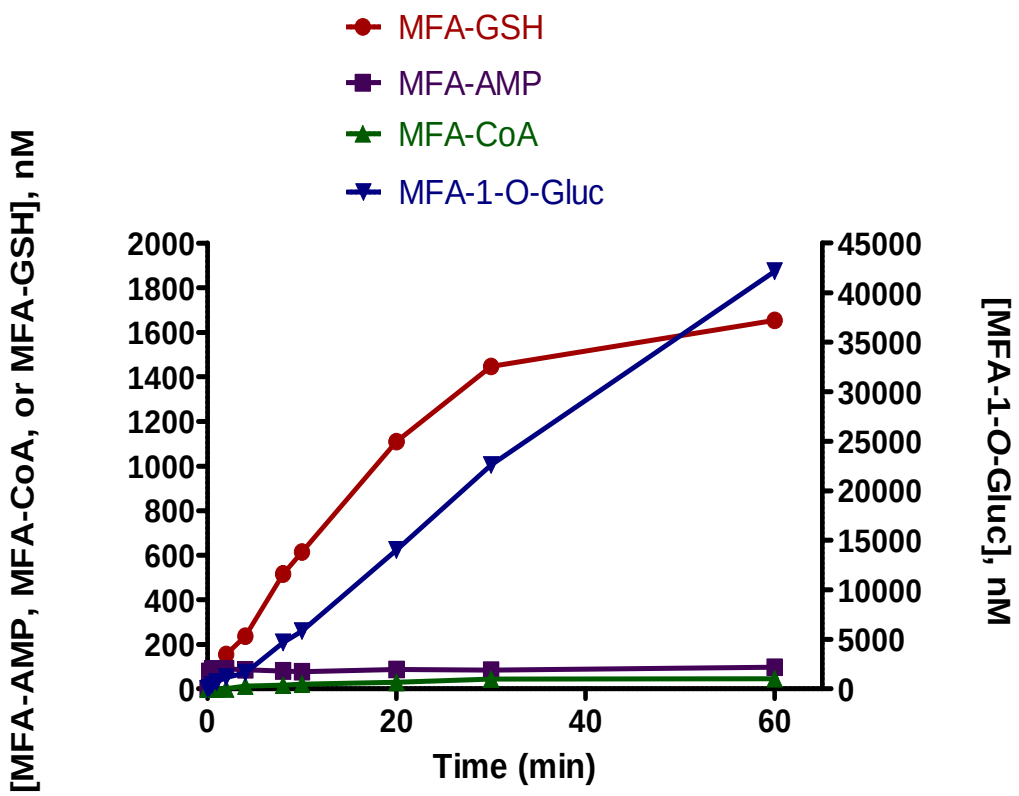


Figure 5.2 Time-dependent formation of MFA-conjugates in rat hepatocytes incubations (pH 7.4, 37°C).

The incubation of mefenamic acid in rat hepatocytes at 25°C (room temperature) resulted in decreased rate of formation for MFA-AMP, reaching a maximum concentration of 115 nM at 1 minute of incubation. MFA-CoA was undetectable until 10 minutes and reached a concentration of 26.9 nM at 60 minutes of incubation. Compared to the 37°C incubations MFA-1-*O*-Gluc concentrations dropped considerably to 118 nM at 60 minutes while MFA-GSH levels decreased to 413.3 nM at 60 minutes (Figure 5.3).

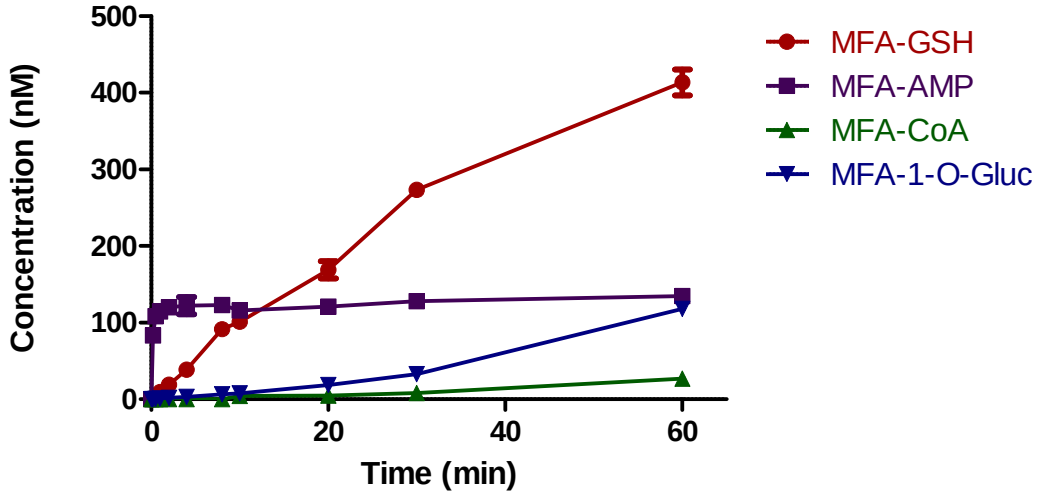


Figure 5.3 Time-dependent formation of MFA-conjugates in rat hepatocyte incubations (pH 7.4, 25°C).

The concentrations of MFA-CoA, MFA-1-*O*-Gluc, and MFA-GSH showed a further decline during the incubation of mefenamic acid (100 µM) in rat hepatocytes at 0°C (ice). The time to maximum concentration for MFA-AMP remained at 1 minute reaching a concentration of 94.1 nM. MFA-CoA levels remained undetectable until 20 minutes reaching a maximum

concentration of 14.1 nM at 60 minutes. MFA-1-*O*-Gluc concentrations showed markedly less formation to 2.4 nM at 60 minutes and MFA-GSH concentrations were significantly decreased to 83.9 nM at the 60 minute time point (Figure 5.4).

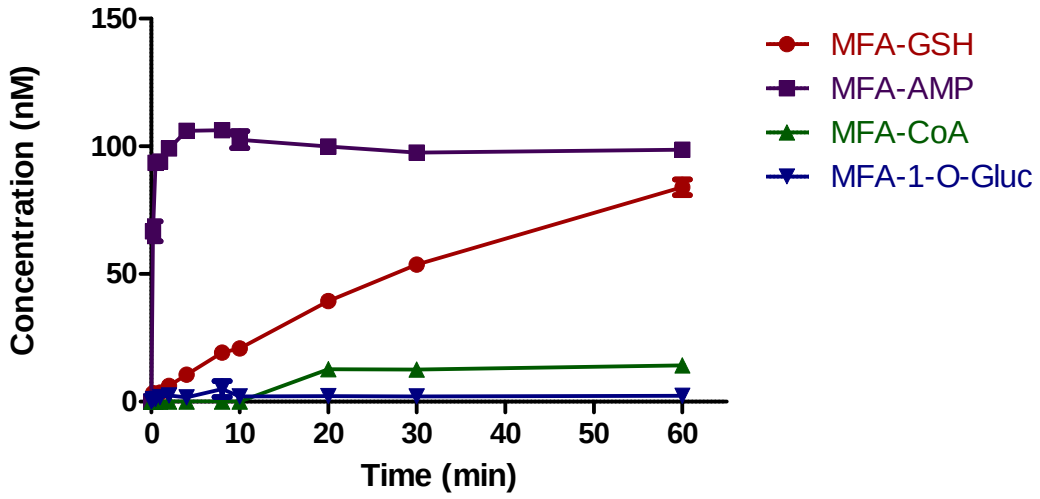


Figure 5.4 Time-dependent formation of MFA-conjugates in rat hepatocyte incubations (pH 7.4, 0°C).

5.3.4 Temperature Dependent Inhibition of GST in Buffer

The incubation of 1 µM of MFA-AMP with GSH (10 mM) in potassium phosphate buffer at room temperature (25°C, pH 7.4) resulted in a MFA-GSH concentration versus time slope of 0.08 nM/min, reaching a maximum concentration of 4.5 nM after 60 minutes of incubation. The MFA-GSH concentration versus time slope of MFA-CoA incubated with GSH was 0.5 nM/min and a maximum concentration of 28.4 nM at 60 minutes. In the presence of equine liver GST, reactivity assessment of MFA-AMP with GSH revealed a MFA-GSH concentration

versus time slope of 1.0 nM/min and a maximum concentration of 47.7 nM after 60 minutes of incubation. The *in vitro* incubation of MFA-CoA with GSH in the presence of GST revealed a MFA-GSH concentration versus time slope of 1.2 nM/min and a maximum MFA-GSH concentration of 72.3 nM at 60 minutes (Figure 5.5, Table 5.2).

The incubation of 1 µM of MFA-AMP with GSH (10 mM) in potassium phosphate buffer on ice (0°C, pH 7.4) resulted in a MFA-GSH concentration versus time slope of 0.05 nM/min, reaching a maximum concentration of 2.9 nM after 60 minutes of incubation. The MFA-GSH concentration versus time slope resulting from MFA-CoA with GSH was 0.29 nM/min and a maximum concentration of 17.1 nM at 60 minutes. In the presence of GST, reactivity assessment of MFA-AMP with GSH revealed a MFA-GSH concentration versus time slope of 0.40 nM/min and a maximum concentration of 23.8 nM after 60 minutes of incubation. The *in vitro* incubation of MFA-CoA with GSH in the presence of GST revealed a MFA-GSH concentration versus time slope MFA-GSH of 0.24 nM/min and a maximum concentration of MFA-GSH of 14.3 nM at 60 minutes (Figure 5.6, Table 5.2).

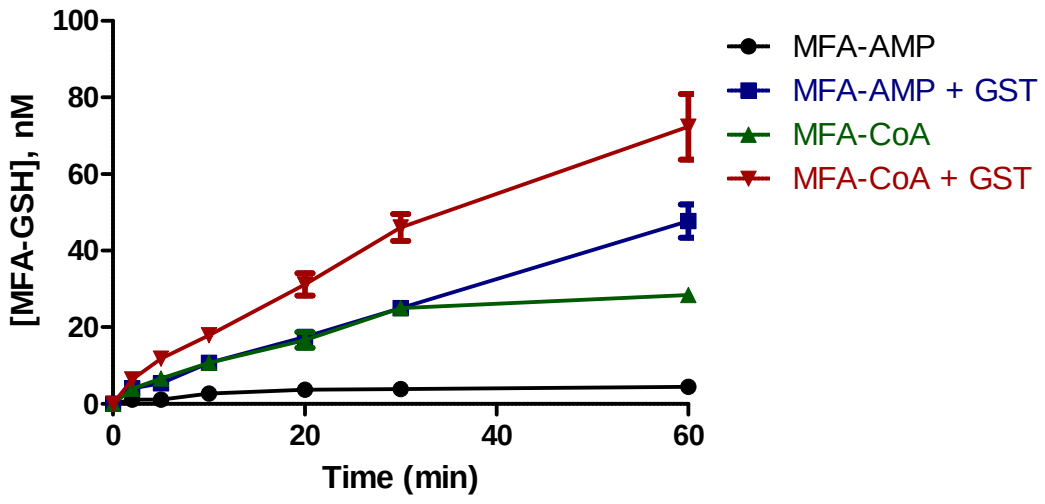


Figure 5.5 Reactivity with GST (+ or - equine liver GST); 1 µM derivatives in 10 mM GSH/0.1 M Kpi (pH 7.4, 25° C).

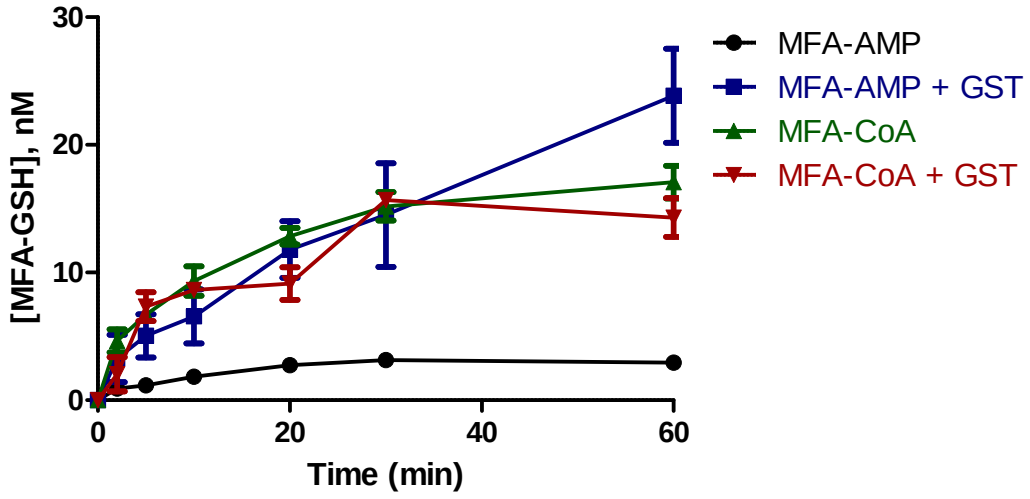


Figure 5.6 Reactivity with GST (+ or - equine liver GST); 1 µM derivatives in 10 mM GSH/0.1 M Kpi (pH 7.4, 0° C).

Derivative (1 µM)	MFA-GSH Conc. Versus Time Slope (nM/min) 37°C	MFA-GSH Conc. Versus Time Slope (nM/min) 25°C	MFA-GSH Conc. Versus Time Slope (nM/min) 0°C
MFA-AMP	0.21	0.08	0.05
MFA-AMP + GST	1.25	1.0	0.4
MFA-CoA	2.41	0.48	0.29
MFA-CoA + GST	3.38	1.2	0.24

Table 5.2 MFA-GSH concentration versus time slope under varying conditions of temperature and GST enzymes.

5.4 Discussion

Here we carried out a number of studies aimed at gaining a better understanding of the phase II-type bioactivation of carboxylic acid-containing drugs into chemically reactive metabolites. The acyl-linked metabolites of acidic drugs, namely acyl-glucuronides, acyl-CoAs, and acyl-adenylate intermediates, are proposed to be potentially toxic through the covalent modification of macromolecules of the body. A majority of previously published experiments focused primarily on the unstable and chemically reactive 1-β-*O*-acyl glucuronides and their interactions with proteins. More recently, studies elucidating the toxic potential of *S*-acyl-CoA thioesters have established their ability to transacylate nucleophilic sites on proteins and GSH. However, few experiments exist characterizing the stability, reactivity, and the relationship to the formation of acyl-glutathione formation of a drug derived acyl-adenylate intermediate. The

focus of the present work is to determine the importance of MFA-AMP, relative to MFA-1-*O*-Gluc and MFA-CoA, in the transacylation of GSH in rat hepatocytes *in vitro* with the use of specific inhibitors and temperature.

Mefenamic acid has previously been shown to undergo bioactivation into MFA-1-*O*-Gluc, MFA-CoA, and MFA-AMP in rat hepatocytes (chapter 4). MFA-CoA formation occurs through the acyl-adenylate intermediate, MFA-AMP via the acyl coenzyme A synthetase (ACS), in the same fashion as fatty acyl-CoA synthetase enzymes function in the metabolism of long-chain fatty acids primarily located in microsomal, mitochondrial, and peroxisomal subcellular fractions (Bruggera et al., 2001). Both the acyl-glucuronidation and the acyl-adenylate/acyl-CoA pathways of mefenamic acid are potentially involved in the formation of MFA-GSH in rat hepatocytes, however the relative contribution from each acyl-linked metabolite has not been elucidated. Experiments performed with lauric acid (CoASH inhibitor) coincubated with mefenamic acid in rat hepatocytes resulted in a decrease of 58.4% and 66.1% in MFA-CoA and MFA-GSH concentrations, respectively. By contrast, the coincubation of (-)-borneol (inhibitor of glucuronidation) with mefenamic acid resulted in a decrease of 91.1% of MFA-1-*O*-Gluc concentrations in rat hepatocytes. However, no decrease in MFA-GSH occurred, suggesting that MFA-CoA and/or MFA-AMP are primarily involved in the transacylation of GSH to form MFA-GSH (Table 5.1). These results are consistent with similar inhibition experiments performed on other NSAIDs (Li et al., 2002b; Grillo and Hua, 2003; Grillo and Hua, 2008).

1-Aminobenzotriazole (ABT) is a suicide substrate of both hepatic and pulmonary cytochrome P450 (Mugford et al., 1991). ABT is considered to be a nonselective mechanism-based

inactivator of both human and non-human P450 enzymes. ABT mediated destruction of P450 occurs through the alkylation of the prosthetic heme group, which requires NADPH and oxygen, and is inhibited by carbon monoxide but not by reduced glutathione (Ortiz de Montellano and Mathews, 1981; Ortiz de Montellano and Correia, 1983). ABT effectively destroys several P450 isozymes, including P450 2B, 2C, 3A (phenobarbital-inducible), 1A (3-methylcholanthrene-inducible), and 4A subfamilies (Ortiz de Montellano and Correia, 1983; Ortiz de Montellano and Reich, 1984). However, ABT does not damage the endoplasmic reticular mixed function oxidase system, which includes the NADPH cytochrome c reductase or cytochrome b5 (Mathews et al., 1985), and produces little cellular toxicity (Mico et al., 1988). In addition, ABT does not alter Phase II metabolizing enzymes including glucuronidation and glutathione conjugation (Mugford et al., 1992). Therefore, ABT's selective inhibition towards P450 allows for the modulation of Phase II metabolism by directing more drug for conjugation by UGT, CoASH, and GST, thus making mechanistic drug metabolism and toxicity studies possible. The preincubation of ABT prior to mefenamic acid in rat hepatocytes resulted in an increase of MFA-1-*O*-Gluc concentrations by ~40% (Figure 5.1B). However, the overall concentrations of MFA-AMP, MFA-CoA, and MFA-GSH remained relatively the same (Figure 5.1 A ,C). These results further support the idea that MFA-AMP and MFA-CoA are more important than MFA-1-*O*-Gluc in the formation of MFA-GSH in rat hepatocytes. However, the relatively low concentrations of MFA-AMP and MFA-CoA is still insufficient to account for the high amount of MFA-GSH formed in rat hepatocytes.

Rates of an enzyme catalyzed reaction tend to double for every ten degree increase in temperature until the optimal temperature (37°C) is reached. The rate of formation of MFA-

AMP to a maximum concentration in rat hepatocytes is extremely rapid (< 20 seconds) under physiological conditions (Figure 5.2). Therefore, in an attempt to slow down the rate of MFA-AMP formation for further inhibition studies, the temperature of incubation was decreased to 25°C and 0°C. When mefenamic acid was incubated in rat hepatocytes at room temperature (25°C), MFA-AMP time to maximum concentration increased to ~1 min and an increase in maximum concentration was observed (115 nM) compared to those incubations performed at physiological conditions (control). However, MFA-CoA time to detection increased from 4 minutes to 10 minutes and a 41% decrease in concentration at the 60 minute time point. MFA-1-*O*-Gluc and MFA-GSH concentrations also decreased by ~99% and 97% compared to control, respectively (Figure 5.3). Incubations performed at 0°C (ice) resulted in a time to maximum concentration of ~ 1 min for MFA-AMP with a slight increase in concentration (94.1 nM) compared to controls. MFA-CoA was not detected until 20 minutes of incubation and its concentration decreased by 69% compared to control. MFA-1-*O*-Gluc and MFA-GSH concentrations experienced near complete inhibition in comparison to control incubations (Figure 5.4).

These temperature dependent incubations all show significant decreases in concentration for MFA-1-*O*-Gluc and MFA-GSH through the inhibition of UGTs and GSTs. MFA-CoA concentrations also decreased but were not completely inhibited. Although the rate of formation of MFA-AMP increased, the time to maximum concentration was still rapid and the achieved maximum concentration increased, suggesting that the formation of MFA-AMP is minimally affected. This phenomenon was not observed for MFA-CoA. If GST is actively involved in the conversion of MFA-AMP to MFA-GSH, then the Arrhenius equation would

predict a time to maximum concentration of MFA-AMP to be ~4 seconds under physiologic conditions only if GST activity was completely inhibited. Once the CoASH becomes saturated, any MFA-AMP converted into MFA-GSH via GST would be replenished in a matter of seconds and thus no change in concentration would be observed thereafter under physiologic conditions. As a result, we believe that a constant supply of MFA-AMP is available for glutathione conjugation by way of GST and this would account for the large differences in concentration between MFA-AMP and MFA-GSH. The significant drop in MFA-GSH concentrations due to GST inhibition at the lower temperature suggests that GSTs are important in mefenamic acid mediated conjugation with GSH. Previous studies in buffer have shown that MFA-AMP is a very good substrate of GSTs (Figure 3.5A). In fact, the slight increase in maximum concentration of MFA-AMP at lower temperatures in rat hepatocytes suggests the impairment of GST mediated conversion of MFA-AMP into MFA-GSH, resulting in higher acyl-adenylate concentrations compared to controls. These results are consistent with the lauric acid/(-)-borneol and ABT-induced inhibition experiments in suggesting that the transacylation of GSH is mediated by MFA-AMP/MFA-CoA and not the MFA-1-*O*-Gluc pathway. Therefore, we propose that the formation of MFA-GSH occurs through a process similar to a zero order infusion of MFA-AMP into the GST, which would account for the significant concentration differences between the two metabolites.

Further *in vitro* experiments with MFA-AMP and MFA-CoA in the presence of GST incubated at 25°C and 0°C in buffer continue to show the effects of temperature on the activity of GSTs. MFA-AMP catalyzed glutathione conjugation via GST decreased by 20% and 68% at 25°C and 0°C, respectively, compared to controls. GST mediated conjugation of MFA-CoA with GSH

decreased by 65% and 92% at 25°C and 0°C, respectively, compared to those incubations performed at 37°C. These results are consistent with the previous observations in rat hepatocyte incubations suggesting that GSTs are inhibited at lower temperatures and that MFA-AMP is a stronger substrate for GST than its corresponding acyl-CoA derivative. Therefore, we propose that the high concentrations of MFA-GSH in rat hepatocytes are due to some extent to the chemically reactive MFA-CoA, but primarily due to MFA-AMP via GST mediated glutathione conjugation.

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Chapter 6

Determination of the Relative Reactivity of MFA-AMP, MFA-CoA, MFA-1-*O*-Gluc, and MFA-GSH Towards Glycine, Taurine, and *N*-acetyl-L-cysteine

6.1 Introduction

The model nucleophile glutathione is a commonly used biomarker of chemical reactivity for bioactivation studies. *S*-acyl-GSH thioesters are metabolic products of reactive intermediates derived from carboxylic acid-containing drugs, in which the cysteinyl-thiol of GSH is covalently bound to the carbonyl-carbon of the carboxylic acid functional group. The rationale is that if a reactive metabolite is involved in glutathione conjugation, then that metabolite is also likely to react with other nucleophilic groups found in proteins (-SH, $-NH_2$, -OH). It is assumed that the pathway that predominates in *S*-acyl-GSH formation *in vitro* may also be the pathway that predominates *in vivo* and is responsible for the covalent binding to nucleophilic sites on proteins potentially leading to an idiosyncratic reaction in patients. However, the electrophilic acyl-1-*O*-glucuronide, acyl-CoA, and acyl-adenylate metabolites derived from carboxylic acid-containing drugs have also been shown to be involved in the transacylation of amino nucleophilic functional groups, such as taurine and glycine, located on acceptor molecules such as amino acids, peptides, and proteins.

Studies involving the administration of tolmetin (Tol) to rats resulted in the detection of tolmetin-taurine (Tol-Tau) and tolmetin-glycine (Tol-Gly) conjugates (Olsen et al., 2003) in urine. Taurine and glycine conjugation occurs through the formation of an acyl-CoA intermediate followed by *N*-acylation of the amino group catalyzed by an amino acid N-acyltransferase (Hutt and Caldwell, 1990). *N*-acyltransferases are located in the mitochondrial matrix alongside medium-chain acyl-CoA synthetase (Hutt and Caldwell, 1990) suggesting that Tol-acyl-CoA (Tol-CoA) formation may also occur via medium-chain acyl-CoA synthetase. However, formation of xenobiotic acyl-CoA can also occur through long-chain acyl-CoA synthetase located in microsomes, peroxisomes, and outer mitochondrial membranes (Knights, 1998), and thus it is possible that Tol-CoA is formed outside the mitochondria and subsequently transported into the mitochondrial matrix where glycine and taurine conjugation may take place. The formation of glycine and taurine conjugates in tolmetin administered rats may thus be indicative of other acyl-CoA processes such as covalent binding to amino functional groups located on proteins to form tolmetin-protein adducts.

A number of studies have shown that acyl-CoAs are 40-70-fold more reactive towards glutathione than their corresponding acyl glucuronides (Shore et al., 1995a; Grillo and Benet, 2002; Li et al., 2002; Olsen et al., 2002). However, acyl-CoAs are also known to play an important role in amino acid conjugation (Hutt and Caldwell, 1990), taurine conjugation (Hutt and Caldwell, 1990), acyl carnitine ester formation (Brass, 1995), and lipid synthesis (Dodds, 1995). Studies involving the incubation of zomepirac (ZP) in rat hepatocytes and *in vivo* rat livers, resulted in the formation of zomepirac-1-*O*-glucuronide(ZP-*O*-Gluc), zomepirac-CoA (ZP-CoA), zomepirac-glycine (ZP-Gly) and zomepirac-taurine (ZP-Tau) conjugates (Olsen et al.,

2005). Incubation of zomepirac in rat hepatocytes revealed a rapid formation of ZP-CoA, reaching a maximum concentration of 0.12 µM at 10 minutes. However, ZP-*O*-Gluc concentrations increased linearly over 2 hours to 35 µM. ZP-Gly and ZP-Tau formation also increased for 2 hours but their concentrations were considerably lower than ZP-*O*-Gluc. Analysis of rat liver homogenates showed ZP-*O*-Gluc to be the most abundant acyl-linked metabolite formed *in vivo*, however ZP-CoA concentration was approximately 200-fold lower than its corresponding glucuronide while ZP-Gly concentrations were similar to that of ZP-CoA. ZP-Tau levels (45 nM) were 10-fold higher than that of ZP-Gly. A correlation between the concentrations of ZP-Tau and ZP-Gly with ZP-CoA may not exist due to the fact that amino acid conjugations are high affinity-low capacity metabolic pathways (Hutt and Caldwell, 1990). However, the detection of ZP-Gly and ZP-Tau in rat livers may mirror of the covalent binding of acyl-CoA metabolites to nucleophilic sites on proteins.

The bile acid, cholic acid, has been shown to form *N*-acyl amino acid conjugates in hepatocytes. *N*-acyl-amidation of cholic acid with amino acids such as glycine and taurine occurs through a two-step enzymatic mechanism. The first step involves the bile acid:CoA ligase, which involves the conversion of cholic acid into its acyl-adenylate intermediate followed by reaction with coenzyme A to form cholic acid-CoA (Polokoff et al., 1979, Lim and Jordan, 1981; Simion et al., 1983; Kase et al., 1986; Vessey et al., 1987). The second step is catalyzed by the bile acid CoA: amino acid *N*-acyltransferase that facilitates the transfer of cholic acid (CA) from its acyl-CoA conjugate to the amino group of taurine or glycine (Kase and Bjorkhem, 1989; Steinber et al., 2000). Studies involving the non-enzymatic reactivity of cholic acid-adenylates (CA-AMP) and cholic acid-CoAs (CA-CoA) with the bionucleophiles glycine, taurine, GSH, and *N*-

acetylcysteine (NAC) showed CA-AMP to be 5.5-fold and 8.2-fold more reactive than CA-CoA towards the amino groups found in glycine and taurine, respectively. However, CA-CoA was 3.3-fold and 4.9-fold more reactive than CA-AMP with the thiol functional groups found in GSH and NAC, respectively (Mitamura et al., 2011). These results suggest that acyl-CoAs are likely to be involved in the non-enzymatic formation of glutathione and NAC adducts, while acyl-adenylates have a greater tendency to transacylate the amino groups on glycine and taurine. The covalent modification of –SH and $–NH_2$ functional groups located on tissue proteins by the chemically reactive acyl-adenylates and acyl-CoA metabolites could potentially lead to the formation of immunogenic drug-protein adducts leading to a hypersensitivity reaction in patients.

One goal of the present study was to investigate the chemical, non-enzymatic, reactivity of MFA-1-*O*-Gluc, MFA-AMP, MFA-CoA, and MFA-GSH towards four biologically relevant bionucleophiles, glycine, taurine, GSH, and NAC *in vitro* in buffer under physiological conditions (pH 7.4, 37°C). Authentic mefenamyl-glycine (MFA-Gly), mefenamyl-taurine (MFA-Tau), and mefenamyl-NAC (MFA-NAC) standards had to be synthesized and characterized by liquid chromatography/electrospray ionization-mass spectrometry (LC/MS/MS).

6.2 Experimental Section

6.2.1 Chemicals

Glycine, taurine, *N*-acetyl-L-cysteine were all purchased from Sigma-Aldrich Chemical Co (St. Louis, MO).

6.2.2 Synthesis of MFA-Gly and MFA-Tau

The synthesis of mefenamyl-glycine (MFA-Gly) and mefenamyl-taurine (MFA-Tau) conjugates was carried out with a solution consisting of 110 mg *N*,*N*'-dicyclohexylcarbodiimide in 0.4 ml pyridine (Ikegawa et al., 1999). The *N*,*N*'-dicylcohexylcarbodiimide solution was added to a solution consisting of 0.489 mmol mefenamic acid and 0.467 mmol of taurine (Tau) or 0.467 mmol glycine (Gly) in 50% pyridine (1.2 ml pyridine/1.2 ml water). The reaction mixture was stirred at 4°C for 7 hr. The reaction mixture was then centrifuged at 3000 rpm for 5 min to remove *N*-acylurea derivatives. The supernatant was transferred to another culture tube followed by the addition of 10 ml acetone. The resulting precipitate was isolated by centrifugation at 3000 rpm for 5 min and then blown to dryness under a stream of nitrogen gas and dissolved in DMSO to make a crude solution. Both crude MFA-Tau and MFA-Gly solutions underwent HPLC/UV purification using a reverse phase gradient system of 10 mM aqueous ammonium acetate to acetonitrile over 12 minutes at a flow rate of 0.5 ml/min using an XTerra C-18 (5.0 µm, 4.6 x 150 mm) column at 245 nm wavelength. MFA-Gly eluted at a retention time of 11.51 minutes (Figure 6.1) and MFA-Tau eluted at a retention time of 9.08 minutes (Figure 6.2). Both peaks were collected at their respective retention times and blown down to dryness under a stream of nitrogen gas. The percent yields of MFA-Tau and MFA-Gly were both ~ 5% Pure MFA-Tau and MFA-Gly solids were then weighed out to make 1 mM solutions in DMSO, analyzed for homogeneity by HPLC/UC at three wavelengths: 220 nm, 254 nm, and 262 nm, and the solutions were shown to contain no additional peaks at all three wavelengths. LC/MS/MS analysis was accomplished using the same liquid chromatography parameters followed by positive ionization mass spectrometry. Full ion positive mode scan revealed only MFA-Tau or

MFA-Gly and no other contaminants. 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%). The mass transition used for quantitative LC/MS/MS analysis of MFA-Gly was MH^+ m/z 299 to 224. 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]$^+$, 18%), m/z 224([M + H - Tau]$^+$, 99%), m/z 209 ([M + H –140]$_+$, 22%), m/z 180([M + H –169]$^+$, 20%). The mass transition used for quantitative LC/MS/MS analysis of MFA-Tau was MH^+ m/z 349 to 224.

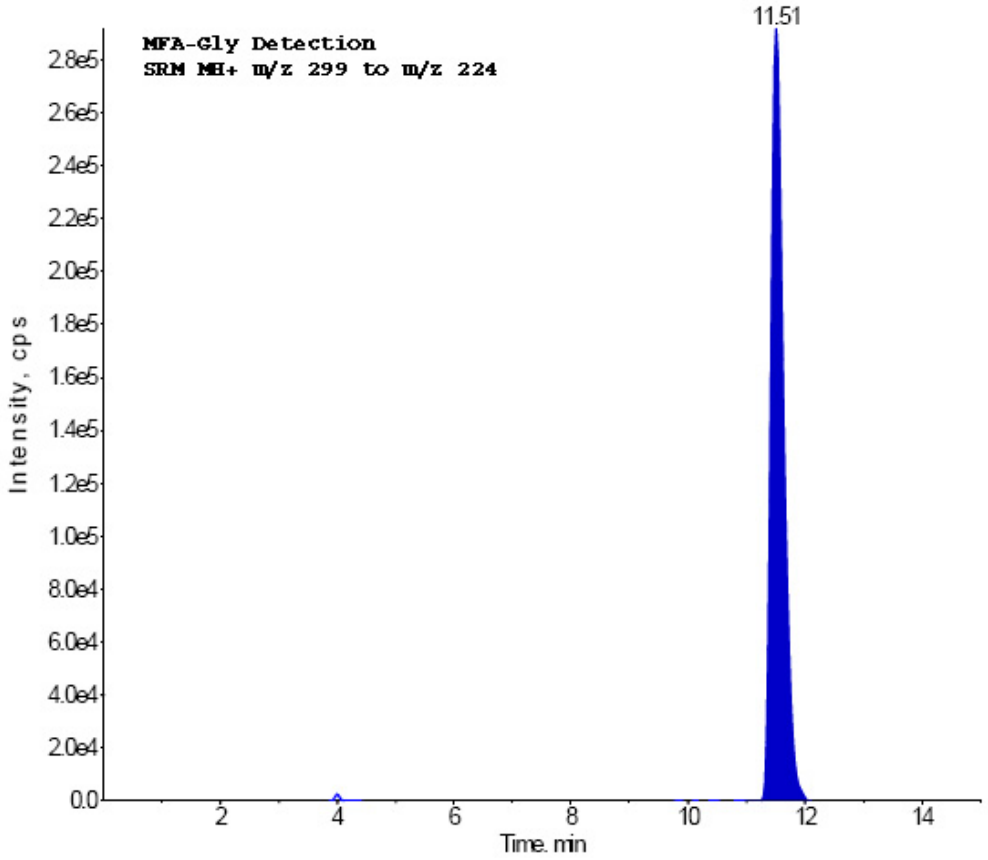


Figure 6.1 Representative reverse-phase gradient LC/MS/MS Single Reaction Monitoring (SRM) of MFA-Gly authentic standard.

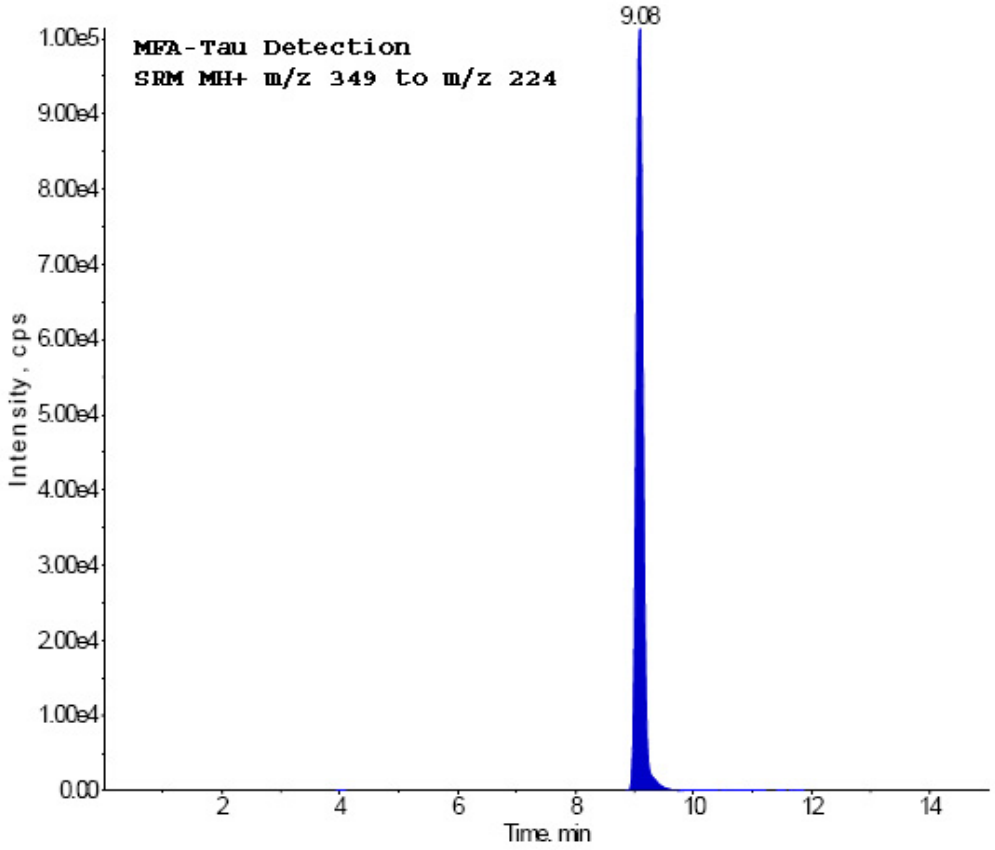


Figure 6.2 Representative reverse-phase gradient LC/MS/MS Single Reaction Monitoring (SRM) of MFA-Tau authentic standard.

6.2.3 Synthesis of MFA-NAC

MFA-NAC conjugates were obtained by reacting MFA-GSH (1 mM) (Chapter 2) with NAC (20 mM) in 0.1 M potassium phosphate buffer (pH 7.4, 37°C) in a volume of 10 ml until the reaction was complete, as indicated by HPLC analysis of the reaction mixture (Grillo and Benet, 2002). The incubation was then acidified with 1 M HCl, followed by a 1:1 extraction of MFA-NAC/buffer with ethyl acetate. The ethyl acetate layer was dried with ~ 8 mg MgSO4 and

evaporated to dryness followed by analysis by HPLC/UV and LC/MS/MS. HPLC/UV analysis was accomplished using a reverse phase gradient system of 10 mM aqueous ammonium acetate to acetonitrile over 12 minutes at a flow rate of 0.5 ml/min using an XTerra C-18 (5.0 µm, 4.6 x 150 mm) column. Samples were analyzed at three wavelengths: 220 nm, 254 nm, and 262 nm. MFA-NAC eluted at a retention time of 9.14 minutes (Figure 6.3) and was shown to contain no additional peaks at all three wavelengths. LC/MS/MS analysis was accomplished using the same liquid chromatography parameters followed by positive ionization mass spectrometry. Full ion positive mode scan revealed only MFA-NAC and no other contaminants. 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%). The mass transition used for quantitative LC/MS/MS analysis of MFA-NAC was MH^+ m/z 387 to 224.

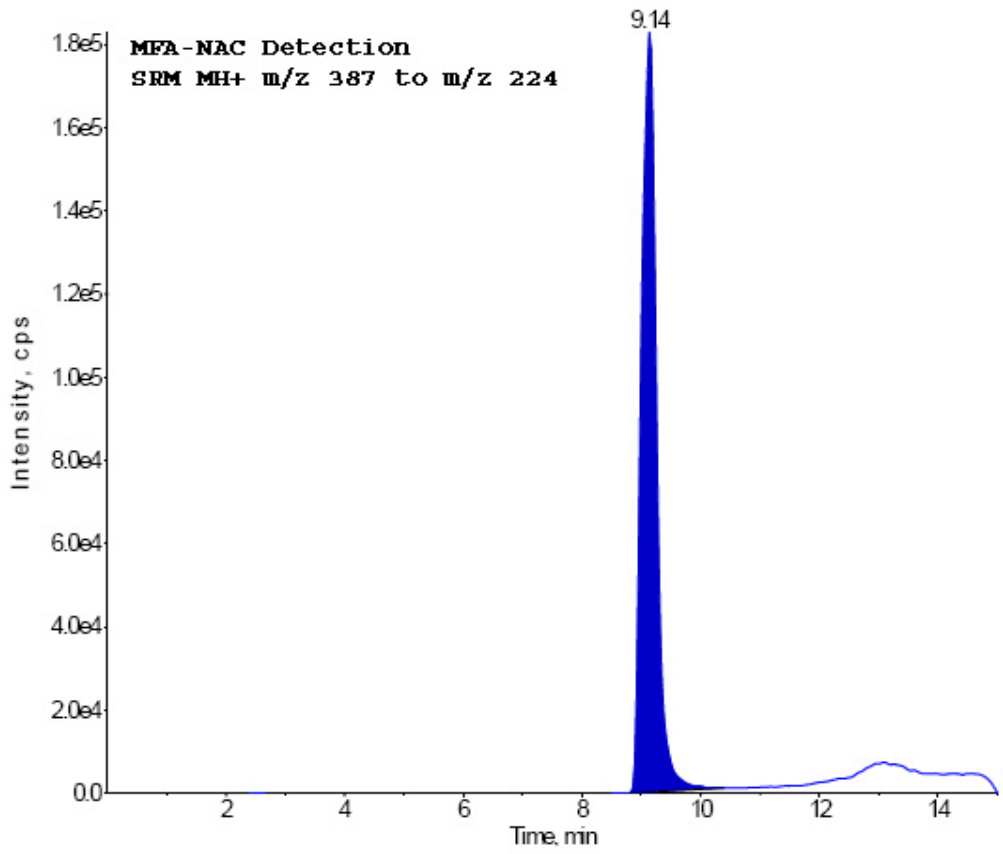


Figure 6.3 Representative reverse-phase gradient LC/MS/MS Single Reaction Monitoring (SRM) of MFA-NAC authentic standard.

6.2.4 Chemical Stability

1 µM of MFA-Tau, MFA-Gly, MFA-NAC and carbamazepine (internal standard) were incubated in 0.1 M potassium phosphate buffer (pH 7.4) in 2 ml HPLC vials. Each solution was then placed into the HPLC autosampler warmed to 37°C and injections were taken every 15 minutes for 3 hours for MS/MS analysis for the comparison of the relative chemical stability of each acyl-linked metabolite. Stability comparisons were made by comparing the internal standard peak area to the analyte peak area ratio for each sample.

6.2.5 Chemical Reactivity

Chemical reactivity experiments for MFA-AMP, MFA-CoA, MFA-1-*O*-Gluc, and/or MFA-GSH (Figure 6.4) were performed by incubating 1 µM of each acyl-linked metabolite separately in 0.1 M potassium phosphate buffer (pH 7.4) containing 10 mM GSH, 10 mM taurine, 10 mM glycine, or 10 mM NAC (3 ml total volume) warmed to 37°C in screw-capped glass vials in a shaking incubator. Aliquots (100 µl) of the incubation mixture were taken at 0, 2, 5, 10, 30, and 60 minutes and quenched with 100 µl of 1 µM carbamazepine/ACN solution and then immediately injected onto the LC/MS/MS for analysis. Quantitative measurements were made using a MFA-Tau, MFA-Gly, or MFA-NAC standard curve generated from absolute peak areas.

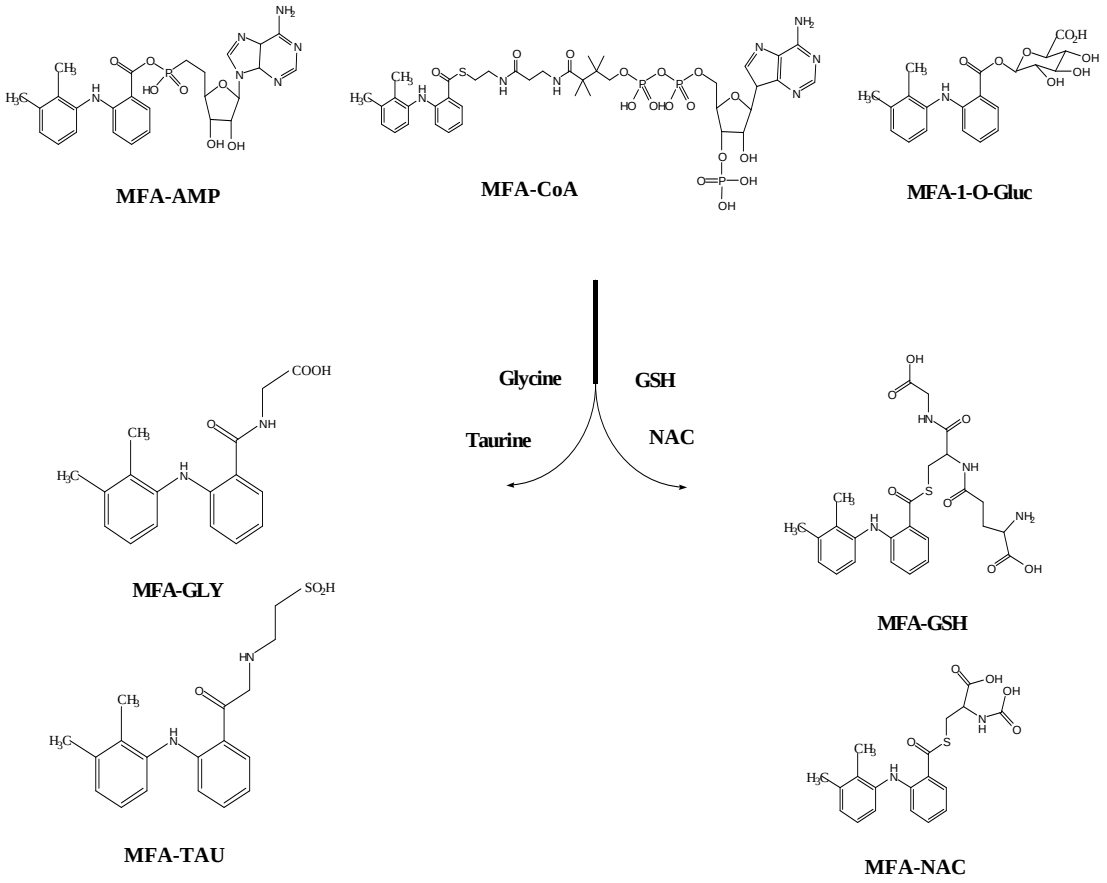


Figure 6.4 Reactivity assessment of MFA-AMP, MFA-CoA and MFA-1-*O*-Gluc (1 µM) with Gly, Tau, GSH, and NAC (10mM).

6.2.*6 In Vitro* Studies with Rat Hepatocytes

Hepatocytes were prepared and incubated with mefenamic acid (100 µM) as descrived earlier. Aliquots were taken at 0, 0.2, 0.5, 1, 2, 4, 8, 10, 20, 30, and 60 minutes and quenched for analysis of MFA-AMP, MFA-Gly, and MFA-Tau by LC/MS/MS. For the analysis of MFA-AMP, MFA-Gly, and MFA-Tau, aliquots (200 µl) of the incubation mixture were taken and added directly into microcentrifuge tubes (2 ml) and quenched with a solution (200 µl) of acetonitrile, 3% formic acid, and 2 µM carbamazepine internal standard. Samples were then centrifuged

(14,000 rpm, 5 min) and the supernatant fraction (200 µl) transferred to HPLC autosampler vials for LC/MS/MS analysis.

6.3 Results

6.3.1 Chemical Stability Comparisons Between MFA-Gly, MFA-Tau, and MFA-NAC

In vitro incubation of MFA-Gly, MFA-Tau, and MFA-NAC (1 mM) in potassium phosphate buffer at pH 7.4 and 37°C revealed that MFA-Gly, MFA-Tau, and MFA-NAC are chemically stable with no detectable hydrolysis for at least 3 hours of incubation (Figure 6.5).

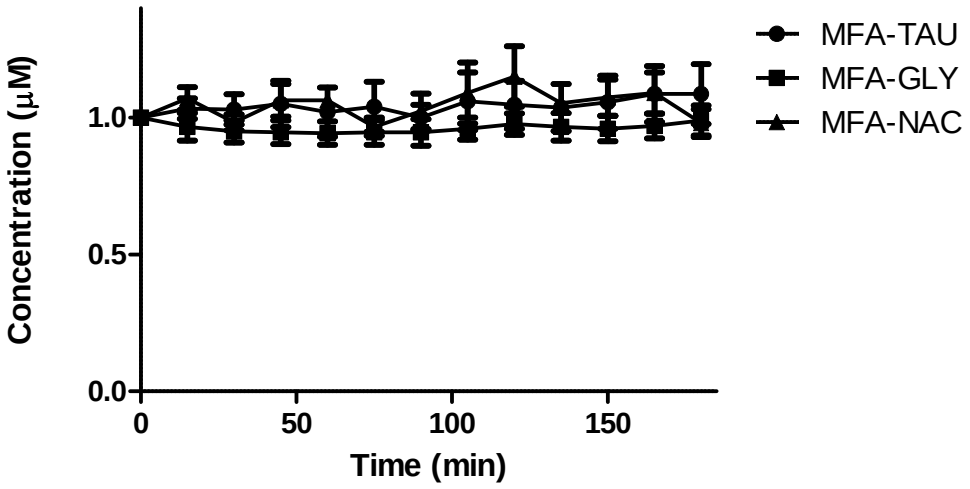
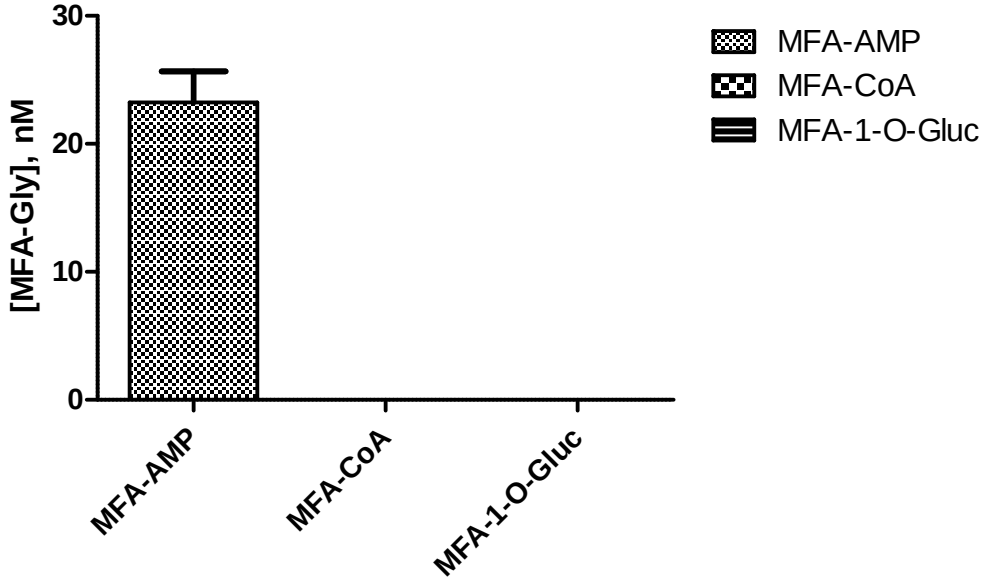


Figure 6.5 Stability assessment of MFA-Gly, MFA-Tau, and MFA-NAC in 0.1 M potassium phosphate buffer (pH 7.4, 37°C).

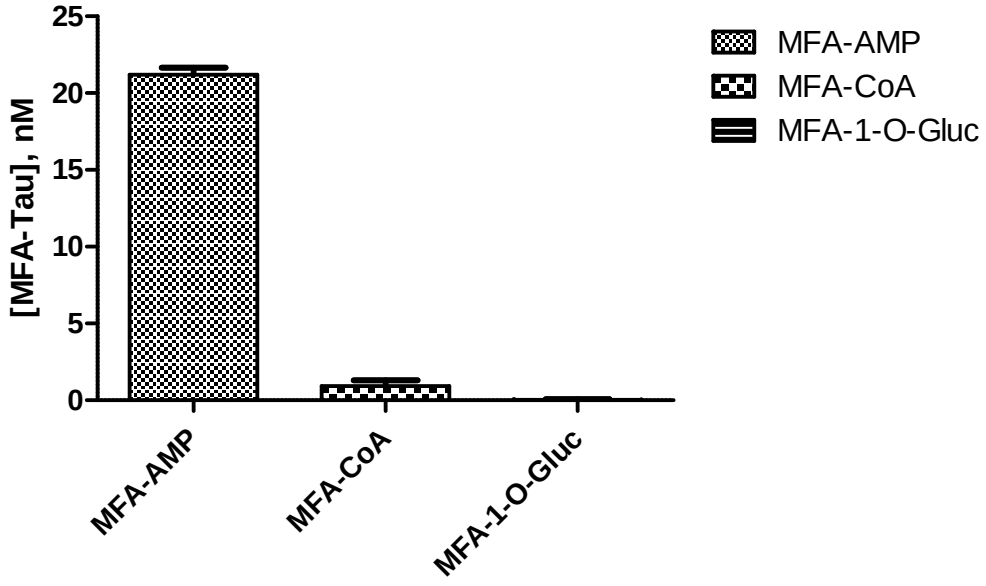
6.3.2 Reactivity Assessment of MFA-AMP, MFA-CoA, MFA-1-O-Gluc or MFA-GSH with Glycine, Taurine, and N-Acetyl-L-cysteine

The incubation of 1 µM of MFA-AMP with glycine (10 mM) in potassium phosphate buffer under physiological conditions resulted in the formation of 23.2 nM of MFA-Gly conjugate after 60 minutes of incubation (Figure 6.6A). The incubation of 1 µM of MFA-CoA and MFA-1-*O*-Gluc did not result in any MFA-Gly formation. The incubation of MFA-AMP and MFA-CoA with taurine (10 mM) in potassium phosphate buffer under physiological conditions revealed a concentration of 21.2 nM and 0.93 nM of MFA-Tau, respectively (Figure 6.6B). MFA-1-O-Gluc reactivity with taurine did not produce taurine conjugates. Previous GSH reactivity studies with MFA-AMP, MFA-CoA, and MFA-1-*O*-Gluc revealed a concentration of MFA-GSH of 12.8 nM, 145 nM, and 1.3 nM respectively at the 60 minute time point (Figure 6.6C). The dosing of NAC in buffer with MFA-AMP, MFA-CoA, MFA-1-*O*-Gluc, and MFA-GSH under physiological conditions resulted in the formation of MFA-NAC conjugates at 7.5 nM, 140.6 nM, 0 nM, and 779.6 nM, respectively (Figure 6.6D).

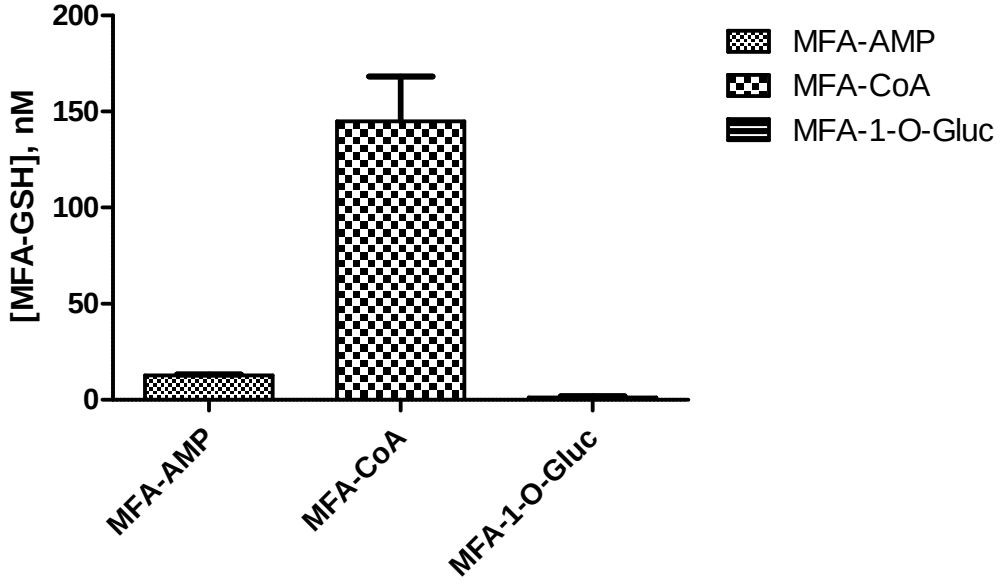
A)


30
20
10
0
[MFA-Gly], nM
MFA-AMP
MFA-CoA
MFA-1-O-Gluc
MFA-AMP
MFA-CoA
MFA-1-O-Gluc

B)


25
20
15
10
5
0
[MFA-Tau], nM
MFA-AMP
MFA-CoA
MFA-1-O-Gluc
MFA-AMP
MFA-CoA
MFA-1-O-Gluc

C)



D)

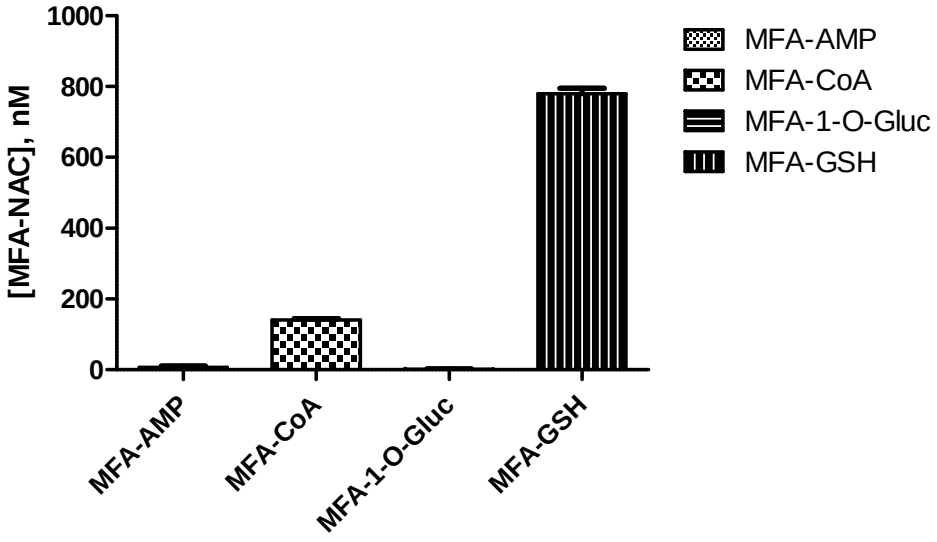


Figure 6.6 Reactivity of MFA-AMP, MFA-CoA, MFA-1-O-Gluc or MFA-GSH (1 µM) with A) Gly, B) Tau, C) GSH and D) NAC (10 mM) in 0.1 M potassium phosphate buffer (pH 7.4, 37°C).

6.3.3 Timecourse of Formation of MFA-AMP, MFA-Tau, and MFA-Gly in Rat Hepatocyte Incubations

The incubation of mefenamic acid (100 µM) in rat hepatocytes under physiological conditions (pH 7.4, 37°C) resulted in the continued rapid formation of MFA-AMP, reaching a concentration of 121 nM at 30 seconds, while MFA-Tau levels were undetectable up until 4 minutes and then reached a concentration of 15.7 nM at 60 minutes (Figure 6.7). MFA-Gly levels were undetectable during the 60 minute incubation period.

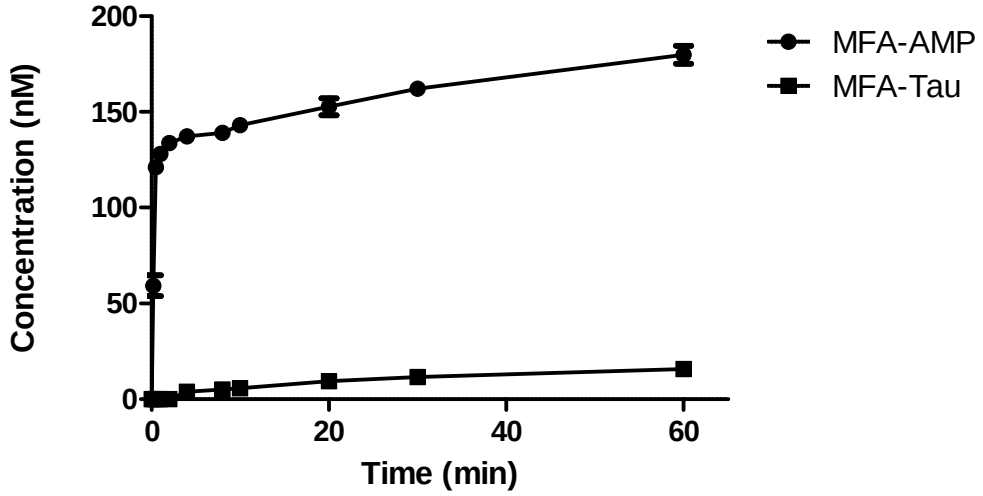


Figure 6.7 Time-dependent Formation of MFA-AMP and MFA-Tau in rat hepatocytes (MFA Dose 100 µM).

6.4 Discussion

Many studies have shown that carboxylic acid-containing drugs are metabolized into the reactive electrophilic acyl-linked metabolites acyl-CoA, acyl-adenylates, and acyl-1-*O*-glucuronides. These reactive metabolites can bind irreversibly with proteins and it has been

suggested that these drug-protein adducts can cause an allergic reaction in hypersensitive individuals (Stogniew and Fenselau, 1982) and may be hepatotoxic (Shore et al., 1995b). Due to the current inability to predict an idiosyncratic toxicity potentially caused by the covalent binding of a reactive metabolite to proteins, early assessment of electrophilicity for reactive metabolites in drug development involves screens using nucleophilic trapping agents such as glutathione, cyanide, and/or amines. Acyl-CoAs, acyl-1-*O*-glucuronides, and acyl-adenylates derived from acidic drugs have been shown to react with glutathione in buffer as well as to form thioester-linked glutathione conjugates *in vitro* and *in vivo* in rat and human hepatocyte incubations via transacylation reactions (Li et al., 2002; Grillo and Hua; 2003; Grillo et al., 2003; Mitamura et al., 2007). In addition to the acyl-CoA and acyl-1-*O*-glucuronide conjugates, we have also isolated the acyl-adenylate intermediate derived from mefenamic acid in rat hepatocytes and assessed its ability to react with glutathione; however, MFA-CoA has been shown to be 11.5-fold nonenzymatically more reactive toward GSH than MFA-AMP. Previous studies involving tolmetin have also identified Tol-CoA dependent metabolites, that is, Tol-Gly and Tol-Tau conjugates in rat urine. (Olsen et al., 2003) These conjugates are believed to be formed enzymatically (N-acyltransferases) via an acyl-CoA metabolite suggesting a covalent binding potential for the acyl-CoA metabolite with amino groups located on proteins. The aim of the present study is dedicated to the synthesis of MFA-Gly, MFA-Tau, and MFA-NAC conjugates for use as authentic standards to determine the ability of MFA-AMP, MFA-CoA, and MFA-1-*O*-Gluc to transacylate the amino-groups of glycine and taurine and the thiol-groups of GSH and NAC.

Stability assessments of MFA-Gly, MFA-Tau, and MFA-NAC in potassium phosphate buffer under physiological conditions (pH 7.4 and 37°C) showed all three metabolites to be completely stable for at least three hours, which is consistent with MFA-AMP and MFA-CoA stability (Figure 6.5 and Figure 3.3). The incubation of MFA-AMP with glycine revealed a rate of MFA-Gly concentration versus time slope of 0.39 nM/min (Figure 6.6A). Neither MFA-CoA nor MFA-1-*O*-Gluc produced any detectable MFA-Gly during the 60 minute incubation. MFA-AMP and MFA-CoA, both reacted with taurine producing a MFA-Tau concentration versus time slope of 0.35 nM/min and 0.02 nM/min, respectively (Figure 6.6B). Previously, MFA-AMP, MFA-CoA, and MFA-1-*O*-Gluc reaction with GSH produced a MFA-GSH concentration versus time slope of 0.21 nM/min, 2.41 nM/min, and 0.02 nM/min, respectively (Table 3.1). MFA-AMP, MFA-CoA, and MFA-GSH incubation with NAC revealed a MFA-NAC concentration versus time slope of MFA-NAC formation of 0.12 nM/min, 2.3 nM/min, and 13 nM/min, respectively (Figure 6.6D). MFA-1-*O*-Gluc mediated formation of MFA-NAC was below the limit of detection.

The results from these studies demonstrate the superior ability of MFA-CoA to transacylate the –SH functional groups of GSH and NAC compared to MFA-AMP. It was found that MFA-CoA was 11.5 and 17.7-fold more reactive than MFA-AMP towards the acylation of cysteine-sulfyhdryl groups of GSH and NAC, respectively. However, MFA-AMP was 17.5-fold more reactive toward the acylation of N-acyl-amidation of glycine and taurine than its corresponding acyl-CoA derivative. This preference in reactivity of MFA-CoA towards the nucleophilic cysteine-sulfhydryl groups of GSH and NAC and MFA-AMP towards the amino groups of glycine and taurine is likely due to different reaction mechanisms. The strong nucleophilic nature of the amino functional groups and the ability of the phospho-ester bond of the free AMP leaving

group to stabilize a negative charge via resonance suggest that MFA-AMP preferred reactivity towards glycine and taurine occurs via a SN1-type reaction. Conversely, MFA-CoA reactivity preference towards the thiol groups of GSH and NAC likely occurs via a SN2-type reaction because the free coenzyme A moiety cannot stabilize a negative charge as well as the free AMP.

Reactivity assessments with NAC have also shown that MFA-GSH is highly reactive in itself. MFA-GSH is 5.7-fold and 100-fold more reactive towards NAC than MFA-CoA and MFA-AMP, respectively. The distinguishing feature that separates mefenamic acid from other studied NSAIDs such as diclofenac and zomepirac (Figures 4.1 and 4.2), is that mefenamic acid produces ~100-fold more glutathione adducts in rat hepatocyte incubations compared to previously studied carboxylic acid-containing drugs. If the toxicity associated with mefenamic acid indeed results from the covalent binding of reactive metabolites to tissue proteins, then it is conceivable that the unusually high formation of MFA-GSH is responsible.

During the incubation of mefenamic acid (100 µM) in rat hepatocytes under physiological conditions, MFA-Tau was undetectable until 4 minutes but reached a concentration of 15.7 nM at 60 minutes. However, it was not determined if the MFA-Tau formed primarily occurs nonenzymatically from MFA-AMP reactivity or enzymatically via the MFA-CoA metabolite.

Mefenamic acid can undergo bioactivation into the reactive intermediates, MFA-AMP, MFA-CoA, MFA-1-*O*-Gluc, and MFA-GSH all of which possess the ability to react with nucleophilic sites on proteins. One hypothesis used to explain the toxicity mediated by mefenamic acid is the covalent modification of tissue proteins by its acyl-linked metabolites leading to the generation of immunogenic protein adducts. Our studies provide corroborating evidence to

previous studies that acyl-AMP, acyl-CoA, acyl-1-*O*-glucuronides, and acyl-GSH derivatives can acylate proteins leading to amide-linked or thiol-ester linked covalent adducts. However, further studies are required to determine the exact role of such adduct induced tissue injury.

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Chapter 7

Conclusions

Animal toxicity and human adverse reactions are significant contributors to the overall attrition of new molecular entities in drug discovery. Approximately 50% of all new compounds entering preclinical animal toxicological studies fail to continue to human clinical trials and 30% of those compounds in clinical trials are terminated because of safety concerns (Kramer et al., 2007). There have also been a number of instances in which therapeutic compounds are shown to be associated with rare, but serious adverse reactions following their FDA approval. These events are referred to as idiosyncratic reactions because they are not related to the known drug pharmacology, they can occur at any dose within the therapeutic range, and the latency period can vary from a week up to a year (Uetrecht, 2000). Idiosyncratic adverse drug reactions also occur at a very low frequency (1 in 10,000 to 1 in 100,000 patients) and thus even a large clinical study consisting of 10,000 patients may not suffice in detecting such an event. As a result, this phenomenon has become a major concern clinically because the available animal models of toxicity are poor predictors of idiosyncratic reactions in humans.

Although there are several possible biochemical mechanisms that could lead to the onset of an idiosyncratic toxicity, much attention has been focused on the bioactivation of drugs to electrophilic, reactive metabolites. The belief that chemically reactive intermediates covalently forming adducts with essential tissue macromolecules resulting in the disruption of cellular processes or initiating an immune response in hypersensitive individuals has been a

toxicological hallmark since the Millers' proposal of dependent carcinogenicity/hepatotoxicity of aminoazo dyes (Miller and Miller, 1947). Further studies relating bioactivation and the covalent binding of structurally diverse drugs, including acetaminophen (Mitchell et al., 1973; Nelson et al., 1976) to proteins continue to provide supportive evidence of this idea. A list of structural alerts, which includes carboxylic acid functional groups, prone to undergo bioactivation by drug metabolizing enzymes to chemically reactive intermediates, has also been developed to aid in drug design (Kalgutkar et al., 2005). However, the consequences associated with bioactivation remain poorly understood and the identities of the macromolecular targets to which bioactivated intermediates bind and for which binding results in toxicity still remain an area of active research. Therefore, the assessment of electrophilic reactive metabolite formation to potentially eliminate or reduce the formation of reactive intermediates by structural modifications is the current approach taken early in drug development. Thus, *in vitro* screens for reactive metabolites using nucleophilic trapping agents (glutathione, cyanide, and/or amines) are performed early in drug metabolism assessment studies. In addition, covalent binding studies using radiolabeled drug candidates to quantitatively measure the formation of drug-protein adducts in the liver along with other qualifying considerations are also being utilized to some extent to predict potential drug induced toxicity. However, currently no experimental evidence has provided a direct link between the quantitative relationship of covalent binding from its trapped electrophile and the onset of toxicity.

Mefenamic acid is a carboxylic acid-containing nonsteroidal anti-inflammatory drug (NSAID) prescribed for its analgesic and antipyretic effects. Commonly used for the treatment of pain, mefenamic acid has been implicated in several cases of hematologic disturbances including

agranulocytosis, hepatic and renal disturbances, nephritis, renal failure, renal papillary necrosis and severe intolerance reactions, as well as hypersensitivity reactions of the skin and mucous membranes. One potential mechanism of these toxicities is the bioactivation of mefenamic acid into reactive acyl-linked metabolites, acyl-1-*O*-glucuronides, acyl-CoA, and acyl-adenylates, that can covalently bind onto macromolecules resulting in the direct impairment of the protein's normal function or initiate an immune response in hypersensitive individuals.

The *in vitro* incubation of mefenamic acid in rat hepatocytes revealed ~1000-fold more GSH conjugate formation than previously studied NSAIDs. Presumably, the more GSH adducts formed, the greater the reactivity of its acylating metabolites, and hence the higher probability that those metabolites will covalently bind onto a protein and stimulate an idiosyncratic reaction. MFA-CoA, MFA-AMP, and MFA-1-*O*-Gluc metabolites were all shown to be formed in these same incubations. Only the concentration and time course of formation of the MFA-1-*O*-Gluc was consistent with MFA-GSH formation. However, our reactivity assessments and inhibition studies have shown that MFA-1-*O*-Gluc is not involved in the unusually high acylation of GSH. MFA-CoA proved to be highly reactive but its concentration, time course of formation, as well as its inhibition do not account for all of the MFA-GSH formed. MFA-AMP was shown to be less reactive than its acyl-CoA derivative and although its time course of formation was consistent with that of MFA-GSH, the concentration of MFA-AMP still does not appear to account for the high amount of GSH acylation. Further investigation revealed that MFA-AMP acylation of GSH is markedly mediated by GST. In addition, the time to maximum concentration of MFA-AMP was rapid (~20 seconds) while the concentration then levels off for the duration of the incubation. Therefore, it is conceivable that the acylation of GSH continuously occurs

through MFA-AMP via GST and that any MFA-AMP converted into MFA-GSH is quickly replenished within a matter of seconds. We propose that this process occurs in a fashion similar to a zero-order infusion of MFA-AMP directly into the GST enzyme, which may account for the unusually high concentration of MFA-GSH formed in rat hepatocytes and the large differences in concentration between MFA-AMP and MFA-GSH. We also showed that MFA-AMP is selective in terms of nonenzymatic covalent binding onto nucleophilic amino functional groups, while MFA-CoA is selective for thiol functional groups. MFA-GSH in itself is also highly reactive towards NAC, and thus potentially involved in the covalent binding to endogenous proteins as well. Therefore, if covalent binding is indeed the cause of drug induced idiosyncratic reactions, then we propose that the toxicity associated with mefenamic acid may occur through the high concentration of MFA-GSH, which is mediated to some extent by MFA-CoA but primarily through MFA-AMP via GST, covalently binding onto nucleophilic sites located on tissue macromolecules whose modification of proteins may elicit an idiosyncratic toxicity. However, further studies are still necessary to determine the exact mechanism of toxicity in patients.

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