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A Mechanistic Study of S-Adenosyl-L-methionine Protection Against Acetaminophen Hepatotoxicity

James Michael Brown
brown287@marshall.edu

A MECHANISTIC STUDY OF S-ADENOSYL-L-METHIONINE PROTECTION AGAINST ACETAMINOPHEN HEPATOTOXICITY

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By James Michael Brown

Approved By:

Monica Valentovic, Ph.D.-Graduate Advisor
Gary Rankin, Ph.D.
Richard Niles, Ph.D.
Nalini Santanam, Ph.D.
Kelley Kiningham, Ph.D.

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Dedication

I dedicate this work to my wonderful wife Kelli. The fact that I have gotten this far is due in no small part to your love and support. You believe in me when I have no faith in myself. I will never be able to pay you back for everything you do for me, but I look forward to trying.

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Finally, thank you to my family and friends. My family has always loved me unconditionally and that has allowed me to grow and take risks in life. My friends, faculty, and advisors from WVWC, Marshall, and UC have also had a profound effect on my life. Thank you all!

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Abbreviations

3-NT…3-Nitrotyrosine

4-HNE…4-Hydroxy-2-nonenal

AAALAC…American Association of Accreditation of Laboratory Animal Care

AIF…Apoptosis Inducing Factor

ALF…Acute Liver Failure

ALT…Alanine Aminotransferase

AM404…*N*-Acylphenolamine

AMAP…3'-Hydroxyacetanilide

ANOVA…Analysis of Variance

APAP….Acetaminophen

APAP-SG…GSH:APAP conjugate

ASK1…Apoptosis Signal-regulating Kinase 1

ATP…Adenosine Triphosphate

AZ…Antizyme

BSA…Bovine Serum Albumin

CBS….Cystathionine β-Synthase

CGL…Cystathionine γ-Lyase

COX…Cyclooxygenase

CPS-1…Carbamoyl Phosphate Synthase-1

CYP…Cytochrome P450

dcSAMe…Decarboxylated S-Adenosyl-L-methionine

DDTC…Sodium Diethyldithiocarbamate

DNA…Deoxyribonucleic Acid

ECL…Enhanced Chemiluminescence

EGTA…Ethylene Glycol Tetraacetic Acid

EIF5A…Eukaryotic Translation Initiation Factor 5A

FAAH…Fatty Acid Amide Hydrolase

GAPDH…Glyceraldehyde 3-Phosphate Dehydrogenase

GPx…Glutathione Peroxidase

GSH…Reduced Glutathione

GSSG…Glutathione Disulfide

GSSG Reductase…Glutathione Reductase

GST…Glutathione S-Transferase

HPLC…High Performance Liquid Chromatography

HRP…Horseradish Peroxidase conjugated antibody

iNOS…Inducible Nitric Oxide Synthase

ip…Intraperitoneal

IV…Intravenous

JNK…C-Jun N-Terminal Kinase

MAT…Methionine Adenosyltransferase

MPT…Mitochondrial Permeability Transition

N + A…N-acetylcysteine given 1 hour after Acetaminophen

NAC…N-acetylcysteine

NADPH…Nicotine Adenine Dinucleotide Phosphate

NAPQI…*N*-Acetyl-p-benzoquinoneimine

NC…Nitrocellulose

NIH…National Institute of Health

NOS…Nitric Oxide Synthase

NSAIDs…Non-Steroidal Anti-Inflammatory Drugs

ODC…Ornithine Decarboxylase

OTC…Over-the-Counter

PAO…Polyamine Oxidase

PAP…p-Aminophenol

PAPS…3'-phosphoadenosine-5'-phosphosulfate

Put…Putrescine

PVDF…Polyvinylidiene Fluoride

RNS…Reactive Nitrogen Species

ROS…Reactive Oxygen Species

S + A…S-Adenosyl-L-methionine given 1 hour after Acetaminophen

SAH…S-Adenosyl-L-homocysteine

SAMC…S-Adenosyl-L-methionine mitochondrial transporter

SAMDC…S-Adenosyl-L-methionine Decarboxylase

SAMe…S-Adenosyl-L-methionine

SOD…Superoxide Dismutase

Spm…Spermine

Spd…Spermidine

SSA…Sulfosalicylic Acid

SSAT… Spermidine/Spermine N^1-Acetyltransferase

SULTs…Sulfotransferases

TBST…Tris Buffered Saline with Tween 20

TNF…Tumor Necrosis Factor

UDPGA…Uridine Diphosphate-Glucuronic Acid

Abstract

Acetaminophen (APAP) toxicity remains the leading cause of drug induced liver failure in the United States. The current therapy for APAP toxicity is N-acetylcysteine (NAC). NAC must be administered within eight hours of APAP overdose for maximum efficacy. That, coupled with the fact that APAP toxicity may not be overtly evident, makes an alternative therapeutic intervention worth exploring. Previous work by our laboratory has demonstrated that S-adenosyl-L-methionine (SAMe) prevents APAP toxicity when given following APAP overdose in C57Bl/6 mice at a level comparable to NAC. The focus of the current work was to examine the mechanistic aspects of this protection in the same mouse model. Male C56Bl/6 mice were randomly allocated into Vehicle (water 15 mL/kg, ip), SAMe (1.25 mmol/kg, ip), APAP (250 mg/kg, ip), and SAMe given one hour after APAP. Mice were fasted the night before experiments and toxicity assessed using liver weight and plasma ALT. Livers were collected from the mice at 2, 4, and 6 hours following APAP administration. Our laboratory then examined levels of SAMe following APAP overdose alone and found that they were depressed by up to 60% and that SAMe given following APAP prevented this drop. Additionally, we demonstrated protection of critical antioxidant pathways by SAMe following APAP overdose. This finding helps explain the previous observation by our laboratory that SAMe prevents an increase in oxidative stress markers following APAP toxicity. We also demonstrated, for the first time, in our model that SAMe prevents oxidative damage in the mitochondria after APAP overdose. However, SAMe was not able to prevent mitochondrial swelling in spite of other signs of mitochondrial protection. Additionally, we also found that SAMe, a known substrate of the polyamine pathway, feeds into this pathway resulting in increased levels of the cytoprotective polyamines spermidine and spermine. Finally, we verified prior findings of an ongoing

collaboration with Dr. Serrine Lau, University of Arizona, about 4-hydroxy-2-nonenal (4-HNE) protein adduction following APAP toxicity. I also identified other potential mitochondrial targets of 4-HNE adduction. My results demonstrate that SAMe is very effective as a therapeutic intervention for APAP toxicity in C57Bl/6 mice, an animal model that is known to metabolize APAP similarly to humans. Our findings suggest a mechanism by which SAMe can prevent APAP toxicity.

Chapter I: Introduction and Literature Review

Acetaminophen

Acetaminophen (APAP) is one of the most widely used analgesic, anti-pyretic over-the-counter (OTC) medications. However, APAP is also the principal cause of drug induced acute liver failure (ALF) in the United States. Between 2000 and 2007, there were an estimated 44,348 cases of APAP intoxication resulting in emergency room visits annually (Manthripragada et al., 2011). The majority of those cases were intentional overdoses with APAP. However, there is also danger to the casual consumer because APAP is present in many OTC medications raising the likelihood of accidental "double-dip" APAP overdose, particularly among children and the elderly. APAP is present in at least 184 currently marketed OTC products, and extra labeling of APAP containing products has only recently been mandated by the FDA (Guggenheimer and Moore, 2011). APAP is also present in at least 70 prescription medication combinations that patients may be taking concurrently with OTC products for pain management (2011).

Despite the widespread use of APAP for over 50 years in the United States, its mechanism of action has only recently been understood. APAP was assumed to act in a similar manner to non-steroidal anti-inflammatory drugs (NSAIDs); however, although APAP displays good analgesic and anti-pyretic effects, it is not anti-inflammatory or anti-platelet (Anderson, 2008). APAP does inhibit cyclooxygenase (COX) activity, although its mechanism is different from NSAIDs.

Both COX-1 and 2 are sensitive to inhibition by APAP (Boutaud et al., 2002). Unlike classical NSAID inhibition of COX, APAP does not appear to act at the active site of the enzyme. Rather, APAP reduces the enzyme to an inactive form (Lucas et al., 2005). This mechanism is supported by the work of Boutaud and Lucas (2002 and 2005), who found that addition of peroxides

reduced the ability of APAP to inhibit COX enzymes, particularly COX-2. APAP also increases COX-2 expression in rat cerebral endothelial cells, consistent with its ability to inhibit central pain (Kis et al., 2005b).

A third COX isozyme, COX-3 is found in canines (Chandrasekharan et al., 2002) and mice (Ayoub et al., 2006). COX-3 is a central nervous system splice variant of COX-1 that retains the first intron. Although COX-3 is inhibited by APAP, the human form of COX-3 is a non-functional protein because of a frame shift brought about by a different length of intron 1 (Kis et al., 2005a). For this reason, COX-3 has been discounted as a major cause of APAP induced analgesia in humans.

Another possible mechanism for APAP analgesia and anti-pyresia is its conversion to an *N*-acylphenolamine (AM404) that acts centrally on the cannabinoid pathway (Hogestatt et al., 2005). Following deacetylation of APAP in the liver, *p*-aminophenol travels to the brain where it is conjugated by fatty acid amide hydrolase (FAAH) to arachidonic acid forming AM404. AM404 then interacts with the capsaicin TRPV1 receptor to mediate reduction of pain and fever. Of note is the finding that the liver did not also produce AM404 in measurable quantities despite a large concentration of FAAH. The authors postulate that AM404 is rapidly metabolized in the liver to another compound whereas it is maintained for a longer period in the brain allowing for it to exert its function. In addition to acting at TRPV1, AM404 inhibits both COX-1 and COX-2 which may be another method of inhibition of these enzymes caused by APAP.

Lending credence to the cannabinoid pathway's importance in APAP pain relief is that antagonism of CB1 receptors prevents APAP associated analgesia in Wistar rats (Ottani et al., 2006). AM404 also activates the CB1 receptor in addition to the TRPV1 receptor which in turn

activates analgesic serotonergic receptors in the spinal column producing analgesia (Mallet et al., 2008). Additionally, AM404 induces significant, dose dependent hypothermia in Sprague-Dawley rats consistent with the anti-pyretic properties of APAP (Rawls et al., 2006). TRPV1 was again noted as the likely receptor for mediating AM404's induction of hypothermia. Another tangential, but confirmatory, piece of evidence in favor of the cannabinoid pathway's involvement in APAPs mechanism of action is the observation that APAP possesses dose-dependent anxiolytic effects in mice thought to be mediated predominantly by CB1 receptors (Umathe et al., 2009).

Although the exact mechanism of action for APAP remains to be discovered, its metabolism is well characterized. APAP is principally metabolized in 1 of 3 manners: glucuronidation, sulfation, or oxidation by cytochrome P450s (CYPs) to the reactive metabolite *N*-acetyl-p-benzoquinoneimine (NAPQI). At gastric pH APAP is neutrally charged and, therefore, predominantly absorbed in the small intestine (Heading et al., 1973). As a result, the rate of gastric emptying is directly proportional to APAP absorption in the body and subsequent excretion of metabolites. APAP undergoes some Phase II metabolism intestinally prior to entering the liver. Glucuronidation is rapidly saturated as APAP levels increase leaving sulfation as the predominant form of metabolism in the intestine (Goon and Klaassen, 1990).

Following absorption, APAP is transported via the portal circulation to the liver before entering the systemic circulation. Metabolism occurs primarily in the liver. Urinary metabolite analysis indicates that approximately 60% of urinary APAP is excreted as a glucuronic acid conjugate, 30% conjugated to sulfate, 8% as either cysteine or mercapturate conjugates, and the remaining 4% as unchanged APAP (Cummings et al., 1967; Levy and Yamada, 1971; Prescott, 1980). Additionally, APAP-glucuronide is excreted in the bile to a greater extent than either APAP-

sulfate or APAP-glutathione (Hjelle and Klaassen, 1984). Taken together, these studies indicate that the vast majority of a therapeutic dose of APAP is safely metabolized by glucuronidation or sulfation, leaving approximately 5-10% of the dose for oxidation via P450 isozymes (Figure 1).

Gender differences play a minor role in APAP metabolism as women exhibit decreased clearance of APAP compared to men, most likely as a consequence of increased glucuronidation of APAP in men (Miners et al., 1983). Pregnant women and women on oral contraceptives clear APAP more rapidly than men because of increased rates of APAP glucuronidation and glutathione conjugation (Miners et al., 1986). Once adulthood is reached, no significant differences are observed in glucuronidation or sulfation based on age (Herd et al., 1991). APAP metabolism is dramatically different in neonates and children under 9 years of age when compared with adolescents and adults. In urinary metabolites, sulfation of APAP predominates over glucuronidation up to 9 years of age (Levy et al., 1975; Miller et al., 1976). The difference in metabolism of APAP in the young is easily explained by the delayed expression of glucuronidation enzymes at early ages (de Wildt et al., 1999).

The purpose of both glucuronidation and sulfation is to make APAP more polar to aid in excretion. Glucuronidation relies on the cofactor uridine diphosphate-glucuronic acid (UDPGA) and UDP-glucuronosyltransferases (UGTs) to attach a glucuronic acid to the para-hydroxyl group of APAP (Parkinson, 2001). Human glucuronidation of APAP is mediated by UGT1A1, 1A6, 1A9, and 2B15 (Court et al., 2001; Mutlib et al., 2006). Isoforms 1A9 and 2B15 are thought to have the highest capacity, although with lower affinity for APAP than the other relevant isotypes. Additionally, 1A6 displays a marked decrease in activity with increasing concentrations of APAP, indicating possible substrate inhibition. The major limiting factor in glucuronidation of APAP appears to be the synthesis of the cofactor UDPGA, which is depleted

by APAP in a dose dependent manner (Price and Jollow, 1984). APAP glucuronidation occurs at highest capacity in the periportal region of the liver away from the primary centrilobular site of necrosis observed during liver toxicity (Mitchell et al., 1989).

Sulfation also serves to increase the polarity of APAP and aid in excretion. Sulfotransferases (SULTs) catalyze sulfation reactions with the cofactor 3'-phosphoadenosine-5'-phosphosulfate (PAPS) acting as the sulfate donor (Parkinson, 2001). SULTs are generally thought to have higher affinity for their substrates than UGTs, but they operate at a slower rate than corresponding UGTs. SULT1A1 and 1A3 are well characterized SULTs that are responsible for the bulk of APAP sulfation *in vivo* (Reiter and Weinshilboum, 1982). Several polymorphic variants of SULT1A1 have been examined with widely varying rates of metabolism. The rate of reaction for SULT1A1*2 is greatly reduced compared to the *1 and *3 variants and could potentially be of importance for inter-individual differences in APAP sulfation (Nagar et al., 2006).

Toxicity caused by APAP is mediated by the toxic metabolite NAPQI, which causes severe, devastating centrilobular necrosis (Lesna et al., 1976). Evidence has existed since the early 1980s that NAPQI was the toxic metabolite of APAP and was formed through the two electron oxidation of APAP to a reactive quinone (Dahlin et al., 1984; Potter and Hinson, 1987). NAPQI has a 4-aminophenoxyl radical intermediate, which could contribute to the oxidative stress observed with APAP toxicity; however, a study by Fisher and others (1985) determined that the intermediate did not react with oxygen to form superoxide.

The formation of NAPQI is catalyzed by the CYP superfamily of enzymes. The four major CYPs in human APAP metabolism are CYP2E1, 3A4, 1A2, and 2D6 (Laine et al., 2009). In

humans, CYP2E1 is the major isozyme responsible for metabolism of APAP *in vivo* (Manyike et al., 2000). CYP2E1 is highly concentrated in the centrilobular regions of the liver, corresponding with the site of APAP induced toxicity (Anundi et al., 1993). Inhibition of CYP3A4, 2E1, and 1A2 with a cocktail of ketoconazole, isoniazid, and caffeine effectively prevents APAP toxicity in rats (Walubo et al., 2004). However, CYP inhibitors have not gained favor for use in humans to prevent APAP toxicity because of their significant side effect profiles. CYP2D6 metabolism of APAP is interesting because of the variable activity of this particular isozyme in humans. Persons possessing a faster metabolizing single nucleotide polymorphism of CYP2D6 may be more likely to experience APAP toxicity at lower doses (Dong et al., 2000).

At therapeutic doses of APAP, NAPQI is rapidly adducted at the 3' position by GSH and excreted primarily as a biliary metabolite (Hinson et al., 1982). The glutathione adduct of APAP is further metabolized to a mercapturic acid conjugate, also commonly found following APAP administration. APAP-GSH conjugation can proceed in either an enzymatic or non-enzymatic pathway. The enzymatic reactions are catalyzed by the glutathione S-transferase enzymes (GST). These enzymes are heavily localized in the centrilobular region of liver lobules, although they are found throughout the lobule (Redick et al., 1982). Additionally, GSTs in the centrilobular region express much higher activity as the concentration of substrate increases, which would be crucial in the APAP detoxification pathway (Kera et al., 1987). A seminal paper by Coles and others in 1988 found that the rate of spontaneous vs. enzymatically catalyzed NAPQI conjugation with GSH depended on levels of NAPQI (Coles et al., 1988). The spontaneous reaction of NAPQI with GSH produced a 2:1:1 ratio of APAP-SG, APAP, and GSSG respectively. Additionally, the spontaneous reaction rate increased dramatically at higher

levels of NAPQI. The enzymatic reaction was catalyzed mainly by GST 2-2, 3-3, and 7-7 and was much more prevalent at lower levels of NAPQI.

At therapeutic doses, GSH is excellent at detoxifying NAPQI before cellular damage occurs. However, GSH becomes depleted with APAP overdose and NAPQI rapidly adducts hepatic proteins causing cell damage. The most common type of protein adduction occurs with free cysteine sulfhydryl groups in proteins (Hoffmann et al., 1985). However, there is also evidence that cysteine alone cannot account for all of the adduction observed with NAPQI (Streeter et al., 1984). Streeter and others observed that bovine serum albumin (BSA), with one free sulfhydryl group, demonstrated excess adduction by NAPQI beyond that which could be explained by cysteine adduction alone. Recent work suggests that the alternate sites of adduction are on lysine and histidine residues (Zhou et al., 1996). An alternative *ipso* adduct, formed by a 1, 2 addition rather than a 1, 4 addition, exists between cysteine and NAPQI (Chen et al., 1999). This adduct seems to occur less frequently than the traditional 1, 4-Michael addition of NAPQI to cysteine; however, it has a longer half-life and reverses with GSH. The reversible nature of the adduct is of interest because it could allow the normally short-lived NAPQI to be transported to more distant portions of the cells.

Site specificity for NAPQI protein adducts is important to the observed toxicity. The APAP regioisomer 3'-hydroxyacetanilide (AMAP) does not manifest the toxicity observed with APAP even though it is also metabolized to a reactive metabolite. APAP preferentially binds mitochondrial proteins whereas AMAP binds primarily microsomal proteins (Nelson et al., 1991). Additionally, APAP causes more oxidation within cells as evidenced by the conversion of xanthine dehydrogenase to xanthine oxidase. Indeed, Nelson's work demonstrates that AMAP can only cause toxicity with depletion of mitochondrial GSH. The issue of binding

specificity is further reinforced by the fact that APAP and AMAP bind plasma membrane proteins equally (Tirmenstein and Nelson, 1989). Also, only APAP selectively depletes mitochondrial GSH even though both AMAP and APAP deplete total cellular GSH. APAP also depletes ATP and the total pool of nucleotides, while AMAP does not (Tirmenstein and Nelson, 1990). All of these observations indicate that the mitochondria are a target of NAPQI and that mitochondrial dysfunction is a hallmark of APAP toxicity.

One of the early events in the progression of APAP toxicity is cellular ATP depletion brought about by mitochondrial damage. NAPQI is capable of inhibiting both Complexes I and II in the mitochondrial respiratory chain, disrupting the proton gradient necessary for ATP production (Burcham and Harman, 1991). Also, NAPQI directly adducts ATP synthase inhibiting production of ATP (Qiu et al., 1998). ATP depletion is critical to the ultimate toxicity of APAP because it interrupts the normal mitochondrial induced death pathways in hepatocytes.

A key event subsequent to NAPQI formation in APAP toxicity is the production of both reactive oxygen species (ROS) and reactive nitrogen species (RNS). GSH depletion in mouse models is rapid, occurring within 20 minutes of administration to mice (Jaeschke, 1990). Like other aspects of APAP toxicity, GSH depletion precedes ROS generation. In fact, mitochondrial damage can lead to the production of ROS. A common indicator of mitochondrial ROS generation is the formation of GSSG, and this compound is found in the mitochondria within 15 minutes of APAP administration (Knight et al., 2001). With the loss of mitochondrial GSH, superoxide produced by the damaged mitochondria is free to react with nitric oxide forming peroxynitrite.

One area of controversy is the source of nitric oxide in the hepatocytes. Some studies involving the inhibition of inducible nitric oxide synthase (iNOS) demonstrate a greatly decreased toxicity indicating the role the protein plays in acetaminophen toxicity (Kamanaka et al., 2003). Recent work appears to contradict prior studies by showing that iNOS is not the primary source of nitric oxide for the formation of peroxynitrite because inhibiting its activity does not alter toxicity or protein nitration (Burke et al., 2010). The same study demonstrated that nonspecific nitric oxide synthase (NOS) inhibition did decrease toxicity suggesting that basal nitric oxide may be sufficient to explain the generation of peroxynitrite associated with APAP toxicity.

The production of peroxynitrite leads to protein nitration and is a precursor to mitochondrial permeability transition (MPT). Protein nitration is predominantly localized to the centrilobular region of the liver consistent with acetaminophen toxicity (Knight et al., 2001). Furthermore, scavenging peroxynitrite with GSH administered 1.5 hours after APAP not only aided survival of mice injected with a toxic dose of APAP, but it also aided cellular regeneration (Bajt et al., 2003).

Peroxynitrite also directly induces mitochondrial damage as indicated by reduction of intact mitochondrial DNA and nitrotyrosine protein adducts localized to the mitochondria following APAP toxicity (Cover et al., 2005a). Mitochondria have a well characterized defense mechanism consisting of several antioxidant enzymes including: catalase, glutathione peroxidase (GPx), glutathione reductase, and manganese superoxide dismutase (SOD) (Figure 2). APAP toxicity decreases the function of all of these antioxidant enzymes (Olaleye and Rocha, 2008; Wu et al., 2009). Without a mechanism to detoxify superoxide, excess peroxynitrite formation can damage the cell leading to the opening of the MPT pore and subsequent necrosis observed with APAP toxicity.

MPT is characterized by loss of mitochondrial membrane potential and permeabilization of the membrane to solutes greater than 1.5 kDa (Aniya and Imaizumi, 2011). Cyclosporin A, a known inhibitor of MPT, reduces hepatotoxicity associated with APAP overdose in mice (Masubuchi et al., 2005). However, even when cyclophilin D, a key component of the MPT pore, is knocked out, elevated levels of peroxynitrite produced with high doses of APAP are sufficient to induce necrosis in hepatocytes indicating, again, the complexity of APAP toxicity (Loguidice and Boelsterli, 2011). Bax is also known to be a component of the MPT pore. Bajt and others (2008) found that Bax knockout mice were still susceptible to APAP toxicity when given 300 mg/kg APAP because oxidative stress eventually overwhelms mitochondrial function. These findings coupled with the cyclophilin D knockout experiments point to a much more complex picture of APAP toxicity where a number of factors can cause cellular necrosis; however, recent work has also demonstrated potential targets for treatment to halt the progression of the MPT component of toxicity following APAP overdose.

Recent efforts to link the massive increase in oxidative stress and MPT in APAP toxicity have focused on C-Jun N-Terminal Kinase (JNK). JNK inhibition protects against APAP toxicity even when the inhibitor is administered up to six hours following APAP (Gunawan et al., 2006). Following activation, JNK facilitates Bax translocation to and permeabilization of the mitochondrial membrane. JNK activation is further linked to mitochondrial dysfunction by the observation that AMAP, which does not decrease mitochondrial GSH, was unable to activate JNK and induce the permeability transition seen with APAP (Hanawa et al., 2008).

Inhibition of JNK by the inhibitor leflunomide (SP600125) reduces mitochondrial cytochrome c release following APAP toxicity (Saito et al., 2010). Another study using the same inhibitor demonstrated prevention of nitrotyrosine formation (Latchoumycandane et al., 2007).

Leflunomide also protected ATP production in hepatocytes by inhibiting JNK activation. Knock-out of apoptosis signal-regulating kinase 1 (ASK1), which regulates the JNK pathway, imparted greater long-term survival to the mice compared with controls, even though indicators of toxicity were initially similar (Nakagawa et al., 2008). All signs point to JNK playing an important role in APAP toxicity.

APAP hepatotoxicity induces DNA fragmentation which may involve JNK activation. APAP overdose resulted in DNA fragmentation that occurred within 2 hours after treatment (Ray et al., 1990). Recent work demonstrated that the formation of the permeability pore lead to release of both apoptosis inducing factor (AIF) and endonuclease G that are thought to be responsible for a portion of the observed DNA fragmentation (Bajt et al., 2006).

Finally, another molecular marker of APAP induced oxidative stress is 4-hydroxy-2-nonenal (4-HNE). This compound is an electrophilic breakdown product of poly-unsaturated fatty acids in cell membranes experiencing oxidative stress (Roede and Jones, 2010). 4-HNE is highly electrophilic and can subsequently bind cellular proteins if not appropriately detoxified. It is known to bind cysteine, histidine, and lysine similar to NAPQI resulting in cellular damage. However, 4-HNE is efficiently metabolized by the cell when produced at normal physiological conditions. In hepatocytes, alcohol dehydrogenase, aldehyde dehydrogenase and GST are responsible for the majority of 4-HNE detoxification (Hartley et al., 1995). Although 4-HNE is clearly produced in response to APAP overdose, it is not clear what role it may have in APAP toxicity.

Early work postulated that lipid peroxidation and subsequent formation of 4-HNE played a significant role in APAP toxicity (Fairhurst et al., 1982). Certainly given the role of GST in

detoxification of 4-HNE it is plausible that the loss of GSH associated with GST activity could contribute to APAP toxicity even in the absence of a direct role for 4-HNE. However, a recent study by Knight and others found that when lipid peroxidation was inhibited, APAP toxicity was not affected (Knight et al., 2003). While this investigation casts doubt on 4-HNE as a causative agent in APAP toxicity, it does not entirely preclude a role for 4-HNE. In fact, mice with metabolic syndrome have a significant increase in toxicity when they received doses of APAP equal to controls (Kon et al., 2010). The authors theorize this is due to increased oxidative stress in the metabolic syndrome mice. The increasing incidence of metabolic syndrome in the United States could increase APAP toxicity.

There is a well-established treatment for APAP overdose, N-acetyl-L-cysteine (NAC). This antidote functions by providing cysteine to the hepatocytes since this is the rate limiting amino acid in GSH synthesis (Lauterburg et al., 1983). Lauterburg and others found that NAC replenishes cellular GSH depleted by APAP toxicity, without adducting APAP directly.

There are currently two NAC protocols in use for the treatment of APAP overdose. The first is a 72 hour oral treatment regimen. The patient is given a loading dose of NAC (140 mg/kg) and then subsequent doses of 70 mg/kg every 4 hours out to 72 hours (2012). However, this is often unpalatable to the patient due to the unpleasant odor of NAC. Therefore, an intravenous (IV) dosing schedule was developed that takes 21 hours. A loading dose of 150 mg/kg of NAC is infused over 1 hour followed by 50 mg/kg infused over 4 hours and finally 100 mg/kg infused over 16 hours until completion. Although effective as a therapy, NAC has some drawbacks. As mentioned above, it is not the most palatable of drugs. Additionally, there is a time sensitive component to NAC administration. For maximum efficacy, the oral dose must be administered within 8-10 hours of APAP ingestion, and the IV regimen must be used when the patient is over

10 hours past APAP ingestion (Kanter, 2006). Therapy that could go beyond this time limitation would be beneficial to patients who delay in seeking treatment.

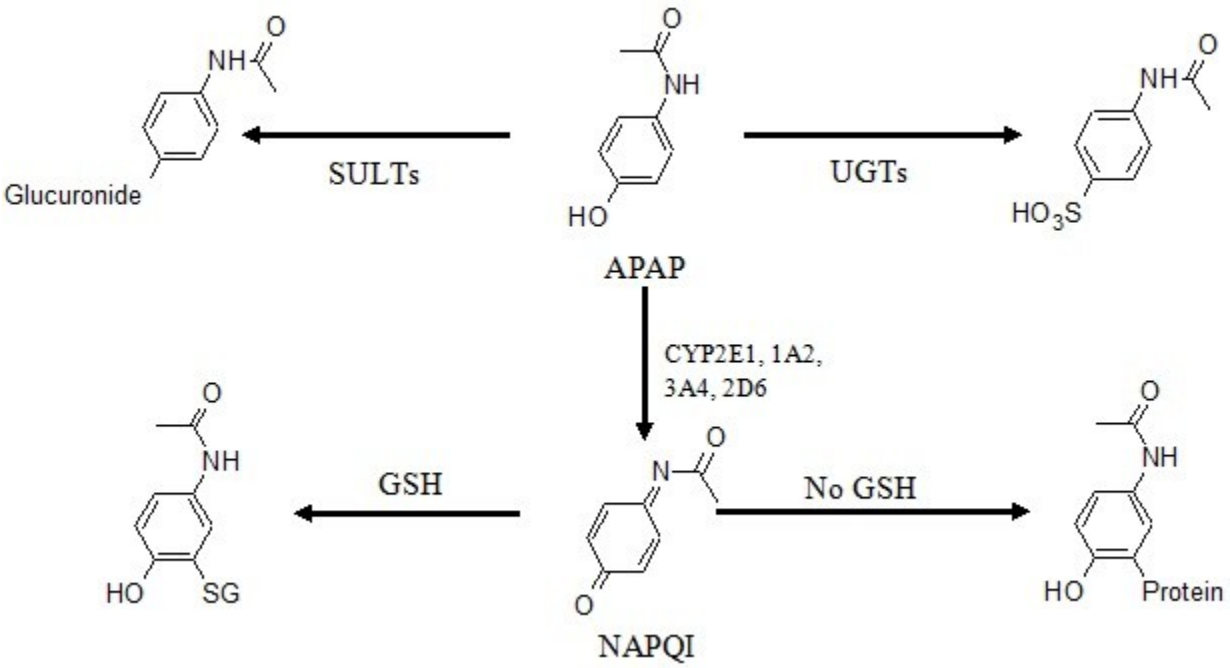

Figure 1. APAP metabolic pathway. The vast majority of a typical APAP dose is metabolized through glucuronidation (60%) and sulfation (30%). The remaining 5-10% of the dose is oxidized by CYP2E1, 3A4, 1A2, and 2D6 to the reactive metabolite NAPQI. Provided GSH stores are sufficient, GSH detoxifies the NAPQI and it is excreted as either a cysteine or mercapturic acid conjugate. However, lack of GSH such as during a toxic dose of APAP results in protein adduction and hepatotoxicity.

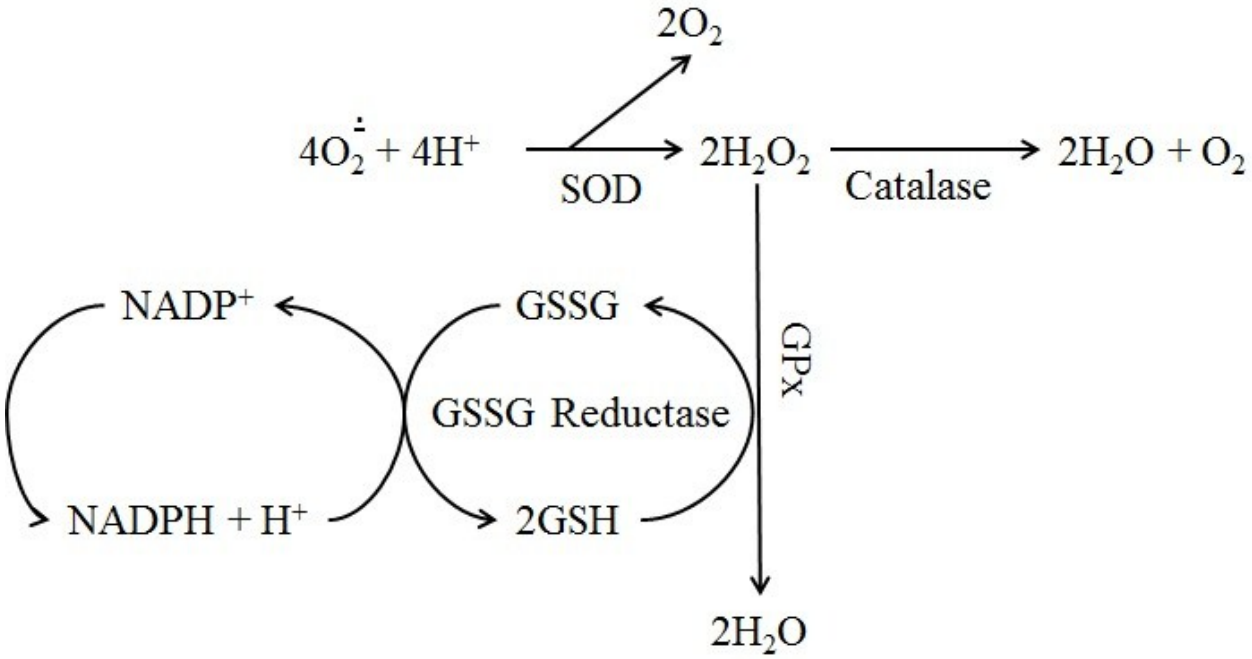

Figure 2. Cellular antioxidant pathway. Superoxide ($O_2^{\cdot-}$) is converted to hydrogen peroxide H_2O_2 by superoxide dismutase (SOD) and then to water by either catalase or glutathione peroxidase (GPx). Glutathione disulfide or oxidized glutathione (GSSG) is converted to reduced glutathione (GSH) through the action of glutathione reductase (GSSG reductase). Glucose 6-phosphate dehydrogenase (G6PD) regenerates NADPH which is a necessary cofactor for GSSG reductase.

S-Adenosyl-L-Methionine

S-Adenosyl-L-methionine (SAMe) was discovered in the 1950s by Giulio Cantoni while conducting research at Case Western University (Kresge et al., 2005). He was the first person to discover that SAMe was a major cofactor in biological methylation reactions. However, SAMe also has many other roles within the cell. SAMe is second only to ATP in the number of reactions for which it acts as a cofactor (Lu, 2000). In fact, 6-8 g of SAMe is produced by the body every day, suggesting that this compound plays vital roles in cellular physiology.

SAMe is the principal biological methyl donor in mammals (Finkelstein, 1990). Its rate of formation is governed by the methionine cycle (Figure 3). SAMe synthesis involves the activation of methionine by combining it with ATP (Mudd, 1963). This reaction is catalyzed by one of three variants of the enzyme methionine adenosyltransferase (MAT). MAT I and III are expressed in the adult liver. The difference between the isozymes is that MAT I is a tetramer and MAT III is a dimer expressed from the same gene (Kotb et al., 1997). Both MAT I and MAT III are constitutively expressed in the liver, correlating with this organ being the primary site of methionine metabolism in the body.

Unlike MAT I/III, MAT II is expressed in every other location in the body, with only a very small amount being found in the liver. MAT II also consists of a different catalytic subunit from MAT I/III, and a Beta subunit (Kotb et al., 1997). Further research found that the Beta subunit serves a regulatory function for the extrahepatic MAT II (De La Rosa et al., 1995). Comparisons of all the isozymes indicate that MAT II has the highest affinity for its substrates followed by MAT I and then MAT III (Sullivan and Hoffman, 1983).

MAT is important to my thesis work because of its differential expression in response to cellular stresses. For instance, MAT II expression is upregulated in liver cancer and is thought to provide the tumor cells with a survival advantage (Cai et al., 1998). While tumors represent a distant connection at best with APAP toxicity, strong evidence documents the critical role of SAMe produced by the MAT II isozyme, in liver growth.

In addition, Huang and others found that both MAT1A and MAT2A genes were upregulated following partial hepatectomy in rats (Huang et al., 1998). MAT1A codes for the subunits found in MAT I/III and MAT2A for MAT II. However, MAT2A mRNA remained significantly elevated out to 3 days post-surgery while at the same time point MAT1A fell below control levels. Another study used hepatocyte growth factor to induce proliferation in the H35 hepatoma cell line. Huang and coworkers (1998) found that when MAT2A expression was decreased by the use of antisense nucleotides growth of the hepatoma cell line was decreased significantly. Multiple studies indicate an important role played by SAMe following liver damage and that MAT II is the predominant isozyme involved in providing this essential factor. MAT II expression is closely tied to proliferation of the hepatocytes and that its promoters are linked with cell cycle progression (Rodriguez et al., 2007).

Following synthesis, SAMe is a substrate for three pathways. SAMe can donate a methyl group to a variety of cellular substrates becoming S-adenosylhomocysteine (SAH) in the transmethylation portion of the methionine pathway (Finkelstein, 1990). Following de-adenylation, SAH can become homocysteine, which can then provide a cellular source of cysteine to replenish GSH stores (Figure 3). SAMe serving as a source of cysteine represents the trans-sulfuration component of SAMe activity. Finally, SAMe can be decarboxylated and serve as a propylamine donor for polyamine synthesis (Finkelstein, 1990).

The importance of the transmethylation pathways within cells are underscored by the fact that all of the five classes of methylase proteins known to exist evolved independently from one another (Schubert et al., 2003). This evolutionary diversity is represented in the wide range of intracellular targets for the methylases. Common targets for the methylases are DNA, proteins, and phospholipids at carbon, sulfur, nitrogen, and oxygen. Transmethylation reactions are governed by the ratio of SAMe:SAH. SAH elevations significantly reduce the methylation of histones, DNA, and phosphatidyl choline (Hoffman et al., 1980). Following donation of a methyl group, SAMe can be recycled back into methionine. S-adenosylhomocysteine hydrolase removes the adenosine from SAH to form homocysteine (Brosnan and Brosnan, 2006). Then, methionine synthase or betaine:homocysteine methyltransferase use tetrahydrofolic acid or betaine respectively to resynthesize methionine. The trans-sulfuration pathway plays a critical role in replenishment of cellular GSH. Almost half of the daily dietary methionine is converted to SAMe which serves as a substrate for trans-sulfuration (Lu, 1999). The enzyme cystathionine β-synthase (CBS) plays a prominent role determining whether homocysteine gets recycled to methionine or ends up as cysteine to replenish cellular GSH or taurine. CBS catalyzes the irreversible conversion of homocysteine to cystathionine through the addition of serine (Lu, 1999). In the presence of adequate cellular cysteine, CBS activity is greatly diminished leading to more creation of methionine and SAMe within the hepatocyte (Finkelstein et al., 1986). Cystathionine is then converted to cysteine through deamination by cystathionine γ-lyase (CGL) where it can become GSH, taurine, or be incorporated into protein (Brosnan and Brosnan, 2006).

The final pathway for SAMe that has relevance to my thesis research is the role of SAMe in the synthesis of polyamines (Figure 4). The first step in polyamine pathway is the *de novo* synthesis of putrescine (Put) by ornithine decarboxylase (ODC) (Coleman et al., 2004). There are other

sources of ornithine in organisms, but the relevant pathway for humans goes through ODC. Arginase was assumed to provide the necessary ornithine for ODC to function, but recent work by Deignan and others casts doubt on this supposition. They found that levels of polyamines in the liver were generally unperturbed in arginase knockout mice (Deignan et al., 2007).

ODC activity also increases during liver regeneration indicating the critical role of polyamines in liver growth and development following injury (Holtta and Janne, 1972). Because it acts as one of the two rate limiting steps in polyamine synthesis, ODC is tightly regulated by levels of polyamines. Antizyme (AZ) inhibits translation of ODC reducing expression when spermidine (Spd) is high within the cell (Kurian et al., 2011).

The next two steps in polyamine synthesis require decarboxylated SAMe (dcSAMe) produced by S-adenosylmethionine decarboxylase (SAMDC). dcSAMe serves as a propylamine donor for the synthesis of Spd and spermine (Spm) from Put (Urdiales et al., 2001). Inhibition of S-adenosylmethionine decarboxylase decreases hepatocyte regeneration following partial hepatectomy in rats (Wiegand and Pegg, 1978). S-adenosylmethionine decarboxylase is stabilized by lack of spermidine in cells (Shirahata and Pegg, 1985). Additionally, high Put and low Spd and Spm are all known to increase the activity of S-Adenosylmethionine decarboxylase. Inhibition of the enzyme greatly increases cellular Put levels indicating that S-adenosylmethionine decarboxylase, like ODC, is a rate limiting step in the synthesis of polyamines (Stjernborg et al., 1993).

When an adequate amount of dcSAMe and Put are available, synthesis of the polyamines Spd and Spm may proceed. Spd and Spm are synthesized by the sequential addition of propylamine groups from dcSAMe by spermidine synthase and spermine synthase respectively (Urdiales et

al., 2001). The entire process of polyamine synthesis can then be reversed through two separate enzymes. First, Spd or Spm are acetylated by spermidine/spermine N^1-acetyltransferase (SSAT) (Casero et al., 1990). Then, Spd and Spm are oxidized back to Put and Spd respectively by polyamine oxidase (PAO), allowing a quick response to cellular needs (Vujcic et al., 2003). The fact that SAMe can participate in polyamine synthesis represents a potential unique advantage over the current treatment for APAP toxicity, NAC. Spm and Spd have many growth promoting and mitochondrial protective properties (Park, 2006). Unlike SAMe, there is no clear path for NAC to create polyamines meaning that SAMe may have a multifaceted protective mechanism against APAP toxicity.

Polyamines have a variety of roles within cells, but perhaps the most pertinent to the current discussion is their ability to induce hepatocyte growth and activate stress response genes. Polyamine gene expression can be driven by ROS generation within the cell (Rhee et al., 2007). Additionally, the polyamine Spd is absolutely necessary for the synthesis of active eukaryotic translation initiation factor 5A (EIF5A). This regulatory factor requires the modified amino acid hypusine for it to be active and is critical in eukaryotic cellular growth and development (Park, 2006). Spd donates the crucial butyl amine group necessary to form hypusine on EIF5A. Recent work further supports the role of Spd in eukaryotic translation. A comparison of Spd and Spm depletion and their effect on EIF5A synthesis, revealed that without Spd, cell growth was arrested (Hyvonen et al., 2007).

Although the effects of APAP toxicity on polyamine levels have not been studied, other toxicants have been found to alter polyamine levels. Carbon tetrachloride leads to an increase in Put within 24 hours of administration followed by an increase in Spd by 48 hours in the mouse liver (Poso and Pegg, 1982). Additionally, administering Put following D-galactosamine in rats

was able to prevent the decrease of Spd and Spm common with toxicity (Daikuhara et al., 1979). To our knowledge, no studies have previously looked at the effect of administering SAMe following APAP toxicity on polyamine levels within the liver.

In addition to being increased long-term in response to a variety of toxicants, polyamines also have mitochondrial protective traits. Particularly, the polyamine Spm appears to play a critical role in mitochondrial health. Spm decreases cytochrome c release in thymocytes exposed to dexamethasone (Hegardt et al., 2003). Although the thymocytes are undergoing apoptosis instead of necrosis in the experiment, APAP clearly increases release of cytochrome c early in its toxicity. Additionally, Spm inhibits mitochondrial swelling analogous to APAP toxicity while protecting oxidative phosphorylation within the mitochondria (Rigobello et al., 1993). Indeed, Spm has quite an affinity for de-energized mitochondria indicating its protective role against MPT (Dalla Via et al., 1998). An additional protective role for Spm is its ability to scavenge free radicals such as those known to cause damage with APAP toxicity (Sava et al., 2006).

Because of the wide diversity of SAMe functions within the human body, it has been thoroughly explored for a variety of medical conditions. For instance, SAMe may reduce pain in those suffering from osteoarthritis, but the data is mixed (Rutjes et al., 2009). Also, the role of SAMe in the synthesis of neurotransmitters led to its investigation for treatment of depression. SAMe appears to be beneficial when it is used to treat major depressive disorder (Nelson, 2010). Additionally, there appears to be a symptomatic and quality of life improvement for fibromyalgia patients treated with SAMe (Jacobsen et al., 1991). Finally, there is mixed to slightly positive evidence according to the Cochrane Review, that SAMe aids people suffering from alcohol induced cirrhosis of the liver (Rambaldi and Gluud, 2006). Cirrhosis disrupts normal methionine

metabolism and supplementing with SAMe provides activated methionine normally not generated by the cirrhotic liver.

Although SAMe holds promise in some of these disease states, the focus of my work is on its ability to protect against APAP induced toxicity and potentially aid in regeneration of the liver following APAP overdose. APAP toxicity significantly depresses ATP levels within cells. A study by Shan and others concluded that loss of ATP significantly decreases the production of GSH in hepatocytes (Shan et al., 1989). SAMe given to people with liver disease is known to increase levels of GSH restoring protective antioxidant activity to the cell (Vendemiale et al., 1989). Also, in spite of different toxicity mechanisms, ethanol abuse also depletes cellular and mitochondrial GSH.

Several studies report the ability of SAMe to protect mitochondrial GSH depletion brought about by ethanol use (Fernandez-Checa et al., 2002; Garcia-Ruiz et al., 1995). Mitochondria are the predominant source of oxidative stress in APAP toxicity, and the maintenance of GSH would aid in absorbing the increase in ROS and RNS caused by APAP. SAMe given before ischemia/reperfusion injury to the liver improves not only cellular and mitochondrial SAMe, but also the ratio of GSH:GSSG (Kaneshiro et al., 1998). SAMe obviously protects against mitochondrial GSH depletion and studies suggest that it is also actively transported into mitochondria.

Recent studies have characterized, at least in humans and yeast, a mitochondrial SAMe transporter. The transporter, SAMC, is found in most major tissues with the liver containing a fairly high expression level (Agrimi et al., 2004). Additionally, SAMe is able to protect

oxidative phosphorylation while also protecting the mitochondrial genome in models of ethanol toxicity in the liver (Bailey et al., 2006).

These properties of SAMe suggest it would be useful to prevent and treat APAP toxicity following overdose. Our laboratory and others have proven the efficacy of SAMe in preventing APAP toxicity when given before and after APAP (Bray et al., 1992; Song et al., 2004; Stramentinoli et al., 1979; Terneus et al., 2008). However, given the myriad of roles SAMe has within the cell, its true mechanism of action is most likely multifaceted and not completely understood. Thus, the rationale for my thesis research was to better characterize how SAMe provides protection against APAP overdose and to determine its therapeutic potential as a new or concomitant treatment with NAC for APAP toxicity.

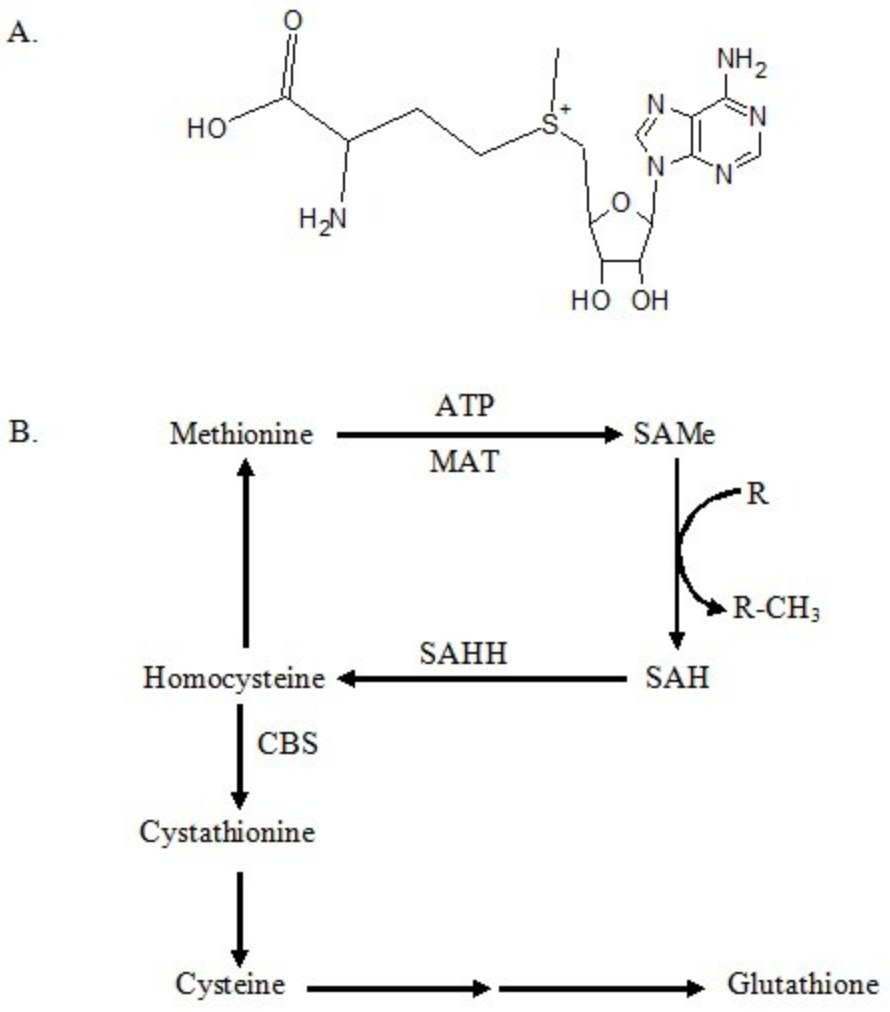

Figure 3. SAMe and the methionine cycle. Panel A represents the chemical structure of SAMe. Note the key activated methyl group attached to the sulfur which is responsible for methyl donation. Panel B is the methionine cycle in which SAMe is a key component. ATP combines with methionine with the help of MAT to create SAMe which can then donate a methyl group as part of the transmethylation pathway. SAH then cycles through the transsulfuration pathway.

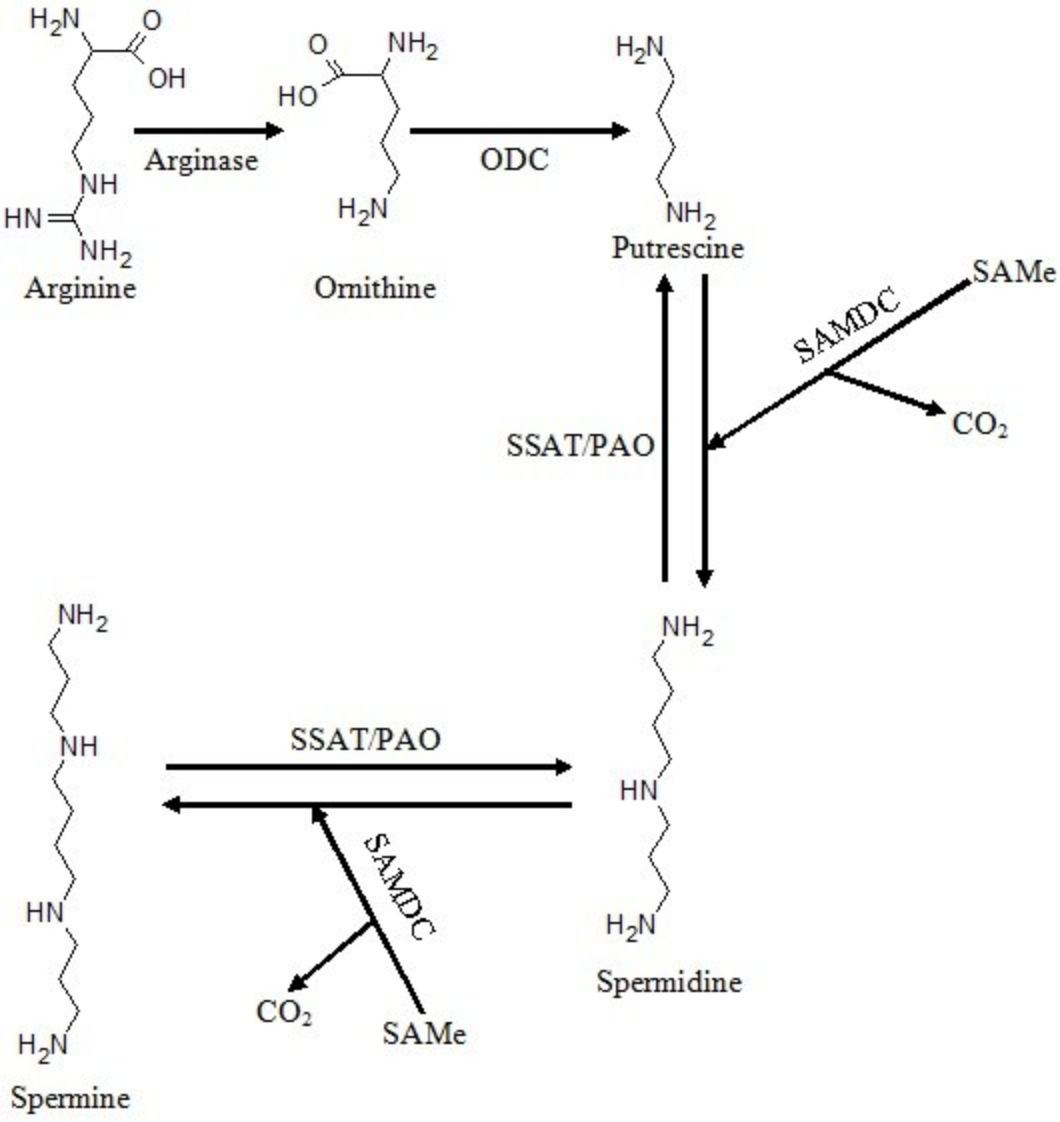

Figure 4. Polyamine Synthesis. The synthesis of putrescine (Put) is catalyzed by the enzyme ornithine decarboxylase (ODC). Then, SAMe is decarboxylated by SAMDC allowing SAMe to act as a propyl amine donor for both spermidine (Spd) and spermine (Spm) synthesis. Spd and Spm can be interconverted by Spermidine/Spermine N^1-Acetyltransferase (SSAT) and Polyamine oxidase (PAO).

Statement of Hypothesis

Our laboratory previously demonstrated equivalent protection from APAP toxicity between SAMe and NAC in the C57BL/6 mouse model. My thesis sought to deepen the mechanistic understanding of the observed protection. To that end, I tested the following hypotheses in the current work. First, SAMe levels will be decreased by APAP toxicity in both liver homogenate and subcellular fractions. Second, the mechanism of SAMe prevention of oxidative stress with APAP overdose is due in part to protection of antioxidant enzyme function. Third, SAMe given following APAP overdose will prevent declines in the protective polyamines Spd and Spm. Finally, SAMe will prevent 4-HNE adduction of proteins following APAP overdose.

Literature Cited

(2011). FDA Drug Safety Communication: Prescription Acetaminophen Products to be Limited to 325 mg Per Dosage Unit; Boxed Warning Will Highlight Potential for Severe Liver Failure (US FDA).

(2012). Acetylcysteine. In Lexi-Drugs.

Agrimi, G., Di Noia, M.A., Marobbio, C.M., Fiermonte, G., Lasorsa, F.M., and Palmieri, F. (2004). Identification of the human mitochondrial S-adenosylmethionine transporter: bacterial expression, reconstitution, functional characterization and tissue distribution. Biochem J *379*, 183-190.

Anderson, B.J. (2008). Paracetamol (Acetaminophen): mechanisms of action. Paediatr Anaesth *18*, 915-921.

Aniya, Y., and Imaizumi, N. (2011). Mitochondrial glutathione transferases involving a new function for membrane permeability transition pore regulation. Drug Metab Rev *43*, 292-299.

Anundi, I., Lahteenmaki, T., Rundgren, M., Moldeus, P., and Lindros, K.O. (1993). Zonation of acetaminophen metabolism and cytochrome P450 2E1-mediated toxicity studied in isolated periportal and perivenous hepatocytes. Biochem Pharmacol *45*, 1251-1259.

Ayoub, S.S., Colville-Nash, P.R., Willoughby, D.A., and Botting, R.M. (2006). The involvement of a cyclooxygenase 1 gene-derived protein in the antinociceptive action of paracetamol in mice. Eur J Pharmacol *538*, 57-65.

Bailey, S.M., Robinson, G., Pinner, A., Chamlee, L., Ulasova, E., Pompilius, M., Page, G.P., Chhieng, D., Jhala, N., Landar, A.*, et al.* (2006). S-adenosylmethionine prevents chronic alcohol-induced mitochondrial dysfunction in the rat liver. Am J Physiol Gastrointest Liver Physiol *291*, G857-867.

Bajt, M.L., Cover, C., Lemasters, J.J., and Jaeschke, H. (2006). Nuclear translocation of endonuclease G and apoptosis-inducing factor during acetaminophen-induced liver cell injury. Toxicol Sci *94*, 217-225.

Bajt, M.L., Farhood, A., Lemasters, J.J., and Jaeschke, H. (2008). Mitochondrial bax translocation accelerates DNA fragmentation and cell necrosis in a murine model of acetaminophen hepatotoxicity. J Pharmacol Exp Ther *324*, 8-14.

Bajt, M.L., Knight, T.R., Farhood, A., and Jaeschke, H. (2003). Scavenging peroxynitrite with glutathione promotes regeneration and enhances survival during acetaminophen-induced liver injury in mice. J Pharmacol Exp Ther *307*, 67-73.

Boutaud, O., Aronoff, D.M., Richardson, J.H., Marnett, L.J., and Oates, J.A. (2002). Determinants of the cellular specificity of acetaminophen as an inhibitor of prostaglandin H(2) synthases. Proc Natl Acad Sci U S A *99*, 7130-7135.

Bray, G.P., Tredger, J.M., and Williams, R. (1992). S-adenosylmethionine protects against acetaminophen hepatotoxicity in two mouse models. Hepatology *15*, 297-301.

Brosnan, J.T., and Brosnan, M.E. (2006). The sulfur-containing amino acids: an overview. J Nutr *136*, 1636S-1640S.

Burcham, P.C., and Harman, A.W. (1991). Acetaminophen toxicity results in site-specific mitochondrial damage in isolated mouse hepatocytes. J Biol Chem *266*, 5049-5054.

Burke, A.S., MacMillan-Crow, L.A., and Hinson, J.A. (2010). Reactive nitrogen species in acetaminophen-induced mitochondrial damage and toxicity in mouse hepatocytes. Chem Res Toxicol *23*, 1286-1292.

Cai, J., Mao, Z., Hwang, J.J., and Lu, S.C. (1998). Differential expression of methionine adenosyltransferase genes influences the rate of growth of human hepatocellular carcinoma cells. Cancer Res *58*, 1444-1450.

Casero, R.A., Jr., Celano, P., Ervin, S.J., Wiest, L., and Pegg, A.E. (1990). High specific induction of spermidine/spermine N1-acetyltransferase in a human large cell lung carcinoma. Biochem J *270*, 615-620.

Chandrasekharan, N.V., Dai, H., Roos, K.L., Evanson, N.K., Tomsik, J., Elton, T.S., and Simmons, D.L. (2002). COX-3, a cyclooxygenase-1 variant inhibited by acetaminophen and other analgesic/antipyretic drugs: cloning, structure, and expression. Proc Natl Acad Sci U S A *99*, 13926-13931.

Chen, W., Shockcor, J.P., Tonge, R., Hunter, A., Gartner, C., and Nelson, S.D. (1999). Protein and nonprotein cysteinyl thiol modification by N-acetyl-p-benzoquinone imine via a novel ipso adduct. Biochemistry *38*, 8159-8166.

Coleman, C.S., Hu, G., and Pegg, A.E. (2004). Putrescine biosynthesis in mammalian tissues. Biochem J *379*, 849-855.

Coles, B., Wilson, I., Wardman, P., Hinson, J.A., Nelson, S.D., and Ketterer, B. (1988). The spontaneous and enzymatic reaction of N-acetyl-p-benzoquinonimine with glutathione: a stopped-flow kinetic study. Arch Biochem Biophys *264*, 253-260.

Court, M.H., Duan, S.X., von Moltke, L.L., Greenblatt, D.J., Patten, C.J., Miners, J.O., and Mackenzie, P.I. (2001). Interindividual variability in acetaminophen glucuronidation by human liver microsomes: identification of relevant acetaminophen UDP-glucuronosyltransferase isoforms. J Pharmacol Exp Ther *299*, 998-1006.

Cover, C., Mansouri, A., Knight, T.R., Bajt, M.L., Lemasters, J.J., Pessayre, D., and Jaeschke, H. (2005a). Peroxynitrite-induced mitochondrial and endonuclease-mediated nuclear DNA damage in acetaminophen hepatotoxicity. J Pharmacol Exp Ther *315*, 879-887.

Cummings, A.J., King, M.L., and Martin, B.K. (1967). A kinetic study of drug elimination: the excretion of paracetamol and its metabolites in man. Br J Pharmacol Chemother *29*, 150-157.

Dahlin, D.C., Miwa, G.T., Lu, A.Y., and Nelson, S.D. (1984). N-acetyl-p-benzoquinone imine: a cytochrome P-450-mediated oxidation product of acetaminophen. Proc Natl Acad Sci U S A *81*, 1327-1331.

Daikuhara, Y., Tamada, F., Takigawa, M., Takeda, Y., and Mori, Y. (1979). Changes in polyamine metabolism of rat liver after administration of D-galactosamine. Favorable effects of putrescine administration on galactosamine-induced hepatic injury. Gastroenterology *77*, 123-132.

Dalla Via, L., Di Noto, V., and Toninello, A. (1998). Spermine binding to liver mitochondria deenergized by ruthenium red plus either FCCP or antimycin A. FEBS Lett *422*, 36-42.

De La Rosa, J., Ostrowski, J., Hryniewicz, M.M., Kredich, N.M., Kotb, M., LeGros, H.L., Jr., Valentine, M., and Geller, A.M. (1995). Chromosomal localization and catalytic properties of the recombinant alpha subunit of human lymphocyte methionine adenosyltransferase. J Biol Chem *270*, 21860-21868.

de Wildt, S.N., Kearns, G.L., Leeder, J.S., and van den Anker, J.N. (1999). Glucuronidation in humans. Pharmacogenetic and developmental aspects. Clin Pharmacokinet *36*, 439-452.

Deignan, J.L., Livesay, J.C., Shantz, L.M., Pegg, A.E., O'Brien, W.E., Iyer, R.K., Cederbaum, S.D., and Grody, W.W. (2007). Polyamine homeostasis in arginase knockout mice. Am J Physiol Cell Physiol *293*, C1296-1301.

Dong, H., Haining, R.L., Thummel, K.E., Rettie, A.E., and Nelson, S.D. (2000). Involvement of human cytochrome P450 2D6 in the bioactivation of acetaminophen. Drug Metab Dispos *28*, 1397-1400.

Fairhurst, S., Barber, D.J., Clark, B., and Horton, A.A. (1982). Studies on paracetamol-induced lipid peroxidation. Toxicology *23*, 249-259.

Fernandez-Checa, J.C., Colell, A., and Garcia-Ruiz, C. (2002). S-Adenosyl-L-methionine and mitochondrial reduced glutathione depletion in alcoholic liver disease. Alcohol *27*, 179-183.

Finkelstein, J.D. (1990). Methionine metabolism in mammals. J Nutr Biochem *1*, 228-237.

Finkelstein, J.D., Martin, J.J., and Harris, B.J. (1986). Effect of dietary cystine on methionine metabolism in rat liver. J Nutr *116*, 985-990.

Fischer, V., West, P.R., Nelson, S.D., Harvison, P.J., and Mason, R.P. (1985). Formation of 4-aminophenoxyl free radical from the acetaminophen metabolite N-acetyl-p-benzoquinone imine. J Biol Chem *260*, 11446-11450.

Garcia-Ruiz, C., Morales, A., Colell, A., Ballesta, A., Rodes, J., Kaplowitz, N., and Fernandez-Checa, J.C. (1995). Feeding S-adenosyl-L-methionine attenuates both ethanol-induced depletion of mitochondrial glutathione and mitochondrial dysfunction in periportal and perivenous rat hepatocytes. Hepatology *21*, 207-214.

Goon, D., and Klaassen, C.D. (1990). Dose-dependent intestinal glucuronidation and sulfation of acetaminophen in the rat in situ. J Pharmacol Exp Ther *252*, 201-207.

Guggenheimer, J., and Moore, P.A. (2011). The therapeutic applications of and risks associated with acetaminophen use: a review and update. J Am Dent Assoc *142*, 38-44.

Gunawan, B.K., Liu, Z.X., Han, D., Hanawa, N., Gaarde, W.A., and Kaplowitz, N. (2006). c-Jun N-terminal kinase plays a major role in murine acetaminophen hepatotoxicity. Gastroenterology *131*, 165-178.

Hanawa, N., Shinohara, M., Saberi, B., Gaarde, W.A., Han, D., and Kaplowitz, N. (2008). Role of JNK translocation to mitochondria leading to inhibition of mitochondria bioenergetics in acetaminophen-induced liver injury. J Biol Chem *283*, 13565-13577.

Hartley, D.P., Ruth, J.A., and Petersen, D.R. (1995). The hepatocellular metabolism of 4-hydroxynonenal by alcohol dehydrogenase, aldehyde dehydrogenase, and glutathione S-transferase. Arch Biochem Biophys *316*, 197-205.

Heading, R.C., Nimmo, J., Prescott, L.F., and Tothill, P. (1973). The dependence of paracetamol absorption on the rate of gastric emptying. Br J Pharmacol *47*, 415-421.

Hegardt, C., Andersson, G., and Oredsson, S.M. (2003). Spermine prevents cytochrome c release in glucocorticoid-induced apoptosis in mouse thymocytes. Cell Biol Int *27*, 115-121.

Herd, B., Wynne, H., Wright, P., James, O., and Woodhouse, K. (1991). The effect of age on glucuronidation and sulphation of paracetamol by human liver fractions. Br J Clin Pharmacol *32*, 768-770.

Hinson, J.A., Monks, T.J., Hong, M., Highet, R.J., and Pohl, L.R. (1982). 3-(glutathion-S-yl)acetaminophen: a biliary metabolite of acetaminophen. Drug Metab Dispos *10*, 47-50.

Hjelle, J.J., and Klaassen, C.D. (1984). Glucuronidation and biliary excretion of acetaminophen in rats. J Pharmacol Exp Ther *228*, 407-413.

Hoffman, D.R., Marion, D.W., Cornatzer, W.E., and Duerre, J.A. (1980). S-Adenosylmethionine and S-adenosylhomocystein metabolism in isolated rat liver. Effects of L-methionine, L-homocystein, and adenosine. J Biol Chem *255*, 10822-10827.

Hoffmann, K.J., Streeter, A.J., Axworthy, D.B., and Baillie, T.A. (1985). Identification of the major covalent adduct formed in vitro and in vivo between acetaminophen and mouse liver proteins. Mol Pharmacol *27*, 566-573.

Hogestatt, E.D., Jonsson, B.A., Ermund, A., Andersson, D.A., Bjork, H., Alexander, J.P., Cravatt, B.F., Basbaum, A.I., and Zygmunt, P.M. (2005). Conversion of acetaminophen to the bioactive N-acylphenolamine AM404 via fatty acid amide hydrolase-dependent arachidonic acid conjugation in the nervous system. J Biol Chem *280*, 31405-31412.

Holtta, E., and Janne, J. (1972). Ornithine decarboxylase activity and the accumulation of putrescine at early stages of liver regeneration. FEBS Lett *23*, 117-121.

Huang, Z.Z., Mao, Z., Cai, J., and Lu, S.C. (1998). Changes in methionine adenosyltransferase during liver regeneration in the rat. Am J Physiol *275*, G14-21.

Hyvonen, M.T., Keinanen, T.A., Cerrada-Gimenez, M., Sinervirta, R., Grigorenko, N., Khomutov, A.R., Vepsalainen, J., Alhonen, L., and Janne, J. (2007). Role of hypusinated eukaryotic translation initiation factor 5A in polyamine depletion-induced cytostasis. J Biol Chem *282*, 34700-34706.

Jacobsen, S., Danneskiold-Samsoe, B., and Andersen, R.B. (1991). Oral S-adenosylmethionine in primary fibromyalgia. Double-blind clinical evaluation. Scand J Rheumatol *20*, 294-302.

Jaeschke, H. (1990). Glutathione disulfide formation and oxidant stress during acetaminophen-induced hepatotoxicity in mice in vivo: the protective effect of allopurinol. J Pharmacol Exp Ther *255*, 935-941.

Kamanaka, Y., Kawabata, A., Matsuya, H., Taga, C., Sekiguchi, F., and Kawao, N. (2003). Effect of a potent iNOS inhibitor (ONO-1714) on acetaminophen-induced hepatotoxicity in the rat. Life Sci *74*, 793-802.

Kaneshiro, Y., Nakano, H., Kumada, K., Boudjema, K., Kitamura, N., Shimura, H., Barama, A., Kigawa, G., Tatsuno, M., Fujiwara, Y.*, et al.* (1998). Augmentation of mitochondrial reduced glutathione by S-adenosyl-L-methionine administration in ischemia-reperfusion injury of the rat steatotic liver induced by choline-methionine-deficient diet. Eur Surg Res *30*, 34-42.

Kanter, M.Z. (2006). Comparison of oral and i.v. acetylcysteine in the treatment of acetaminophen poisoning. Am J Health Syst Pharm *63*, 1821-1827.

Kera, Y., Sippel, H.W., Penttila, K.E., and Lindros, K.O. (1987). Acinar distribution of glutathione-dependent detoxifying enzymes. Low glutathione peroxidase activity in perivenous hepatocytes. Biochem Pharmacol *36*, 2003-2006.

Kis, B., Snipes, J.A., and Busija, D.W. (2005a). Acetaminophen and the cyclooxygenase-3 puzzle: sorting out facts, fictions, and uncertainties. J Pharmacol Exp Ther *315*, 1-7.

Kis, B., Snipes, J.A., Simandle, S.A., and Busija, D.W. (2005b). Acetaminophen-sensitive prostaglandin production in rat cerebral endothelial cells. Am J Physiol Regul Integr Comp Physiol *288*, R897-902.

Knight, T.R., Fariss, M.W., Farhood, A., and Jaeschke, H. (2003). Role of lipid peroxidation as a mechanism of liver injury after acetaminophen overdose in mice. Toxicol Sci *76*, 229-236.

Knight, T.R., Kurtz, A., Bajt, M.L., Hinson, J.A., and Jaeschke, H. (2001). Vascular and hepatocellular peroxynitrite formation during acetaminophen toxicity: role of mitochondrial oxidant stress. Toxicol Sci *62*, 212-220.

Kon, K., Ikejima, K., Okumura, K., Arai, K., Aoyama, T., and Watanabe, S. (2010). Diabetic KK-A(y) mice are highly susceptible to oxidative hepatocellular damage induced by acetaminophen. Am J Physiol Gastrointest Liver Physiol *299*, G329-337.

Kotb, M., Mudd, S.H., Mato, J.M., Geller, A.M., Kredich, N.M., Chou, J.Y., and Cantoni, G.L. (1997). Consensus nomenclature for the mammalian methionine adenosyltransferase genes and gene products. Trends Genet *13*, 51-52.

Kresge, N., Tabor, H., Simoni, R.D., and Hill, R.L. (2005). An escape from Italy, the discovery of S-adenosylmethionine, and the biosynthesis of creatine by Giulio L. Cantoni. 1953. J Biol Chem *280*, e35.

Kurian, L., Palanimurugan, R., Godderz, D., and Dohmen, R.J. (2011). Polyamine sensing by nascent ornithine decarboxylase antizyme stimulates decoding of its mRNA. Nature *477*, 490-494.

Laine, J.E., Auriola, S., Pasanen, M., and Juvonen, R.O. (2009). Acetaminophen bioactivation by human cytochrome P450 enzymes and animal microsomes. Xenobiotica *39*, 11-21.

Latchoumycandane, C., Goh, C.W., Ong, M.M., and Boelsterli, U.A. (2007). Mitochondrial protection by the JNK inhibitor leflunomide rescues mice from acetaminophen-induced liver injury. Hepatology *45*, 412-421.

Lauterburg, B.H., Corcoran, G.B., and Mitchell, J.R. (1983). Mechanism of action of N-acetylcysteine in the protection against the hepatotoxicity of acetaminophen in rats in vivo. J Clin Invest *71*, 980-991.

Lesna, M., Watson, A.J., Douglas, A.P., Hamlyn, A.N., and James, O.F. (1976). Evaluation of paracetamol-induced damage in liver biopsies. Acute changes and follow-up findings. Virchows Arch A Pathol Anat Histol *370*, 333-344.

Levy, G., Khanna, N.N., Soda, D.M., Tsuzuki, O., and Stern, L. (1975). Pharmacokinetics of acetaminophen in the human neonate: formation of acetaminophen glucuronide and sulfate in relation to plasma bilirubin concentration and D-glucaric acid excretion. Pediatrics *55*, 818-825.

Levy, G., and Yamada, H. (1971). Drug biotransformation interactions in man. 3. Acetaminophen and salicylamide. J Pharm Sci *60*, 215-221.

Loguidice, A., and Boelsterli, U.A. (2011). Acetaminophen overdose-induced liver injury in mice is mediated by peroxynitrite independently of the cyclophilin D-regulated permeability transition. Hepatology *54*, 969-978.

Lu, S.C. (1999). Regulation of hepatic glutathione synthesis: current concepts and controversies. FASEB J *13*, 1169-1183.

Lu, S.C. (2000). S-Adenosylmethionine. Int J Biochem Cell Biol *32*, 391-395.

Lucas, R., Warner, T.D., Vojnovic, I., and Mitchell, J.A. (2005). Cellular mechanisms of acetaminophen: role of cyclo-oxygenase. FASEB J *19*, 635-637.

Mallet, C., Daulhac, L., Bonnefont, J., Ledent, C., Etienne, M., Chapuy, E., Libert, F., and Eschalier, A. (2008). Endocannabinoid and serotonergic systems are needed for acetaminophen-induced analgesia. Pain *139*, 190-200.

Manthripragada, A.D., Zhou, E.H., Budnitz, D.S., Lovegrove, M.C., and Willy, M.E. (2011). Characterization of acetaminophen overdose-related emergency department visits and hospitalizations in the United States. Pharmacoepidemiol Drug Saf.

Manyike, P.T., Kharasch, E.D., Kalhorn, T.F., and Slattery, J.T. (2000). Contribution of CYP2E1 and CYP3A to acetaminophen reactive metabolite formation. Clin Pharmacol Ther *67*, 275-282.

Masubuchi, Y., Suda, C., and Horie, T. (2005). Involvement of mitochondrial permeability transition in acetaminophen-induced liver injury in mice. J Hepatol *42*, 110-116.

Miller, R.P., Roberts, R.J., and Fischer, L.J. (1976). Acetaminophen elimination kinetics in neonates, children, and adults. Clin Pharmacol Ther *19*, 284-294.

Miners, J.O., Attwood, J., and Birkett, D.J. (1983). Influence of sex and oral contraceptive steroids on paracetamol metabolism. Br J Clin Pharmacol *16*, 503-509.

Miners, J.O., Robson, R.A., and Birkett, D.J. (1986). Paracetamol metabolism in pregnancy. Br J Clin Pharmacol *22*, 359-362.

Mitchell, M.C., Hamilton, R., Wacker, L., and Branch, R.A. (1989). Zonal distribution of paracetamol glucuronidation in the isolated perfused rat liver. Xenobiotica *19*, 389-400.

Mudd, S.H. (1963). Activation of methionine for transmethylation. VI. Enzyme-bound tripolyphosphate as an intermediate in the reaction catalyzed by the methionine-activating enzyme of Baker's yeast. J Biol Chem *238*, 2156-2163.

Mutlib, A.E., Goosen, T.C., Bauman, J.N., Williams, J.A., Kulkarni, S., and Kostrubsky, S. (2006). Kinetics of acetaminophen glucuronidation by UDP-glucuronosyltransferases 1A1, 1A6, 1A9 and 2B15. Potential implications in acetaminophen-induced hepatotoxicity. Chem Res Toxicol *19*, 701-709.

Nagar, S., Walther, S., and Blanchard, R.L. (2006). Sulfotransferase (SULT) 1A1 polymorphic variants *1, *2, and *3 are associated with altered enzymatic activity, cellular phenotype, and protein degradation. Mol Pharmacol *69*, 2084-2092.

Nakagawa, H., Maeda, S., Hikiba, Y., Ohmae, T., Shibata, W., Yanai, A., Sakamoto, K., Ogura, K., Noguchi, T., Karin, M.*, et al.* (2008). Deletion of apoptosis signal-regulating kinase 1 attenuates acetaminophen-induced liver injury by inhibiting c-Jun N-terminal kinase activation. Gastroenterology *135*, 1311-1321.

Nelson, J.C. (2010). S-adenosyl methionine (SAMe) augmentation in major depressive disorder. Am J Psychiatry *167*, 889-891.

Nelson, S.D., Tirmenstein, M.A., Rashed, M.S., and Myers, T.G. (1991). Acetaminophen and protein thiol modification. Adv Exp Med Biol *283*, 579-588.

Olaleye, M.T., and Rocha, B.T. (2008). Acetaminophen-induced liver damage in mice: effects of some medicinal plants on the oxidative defense system. Exp Toxicol Pathol *59*, 319-327.

Ottani, A., Leone, S., Sandrini, M., Ferrari, A., and Bertolini, A. (2006). The analgesic activity of paracetamol is prevented by the blockade of cannabinoid CB1 receptors. Eur J Pharmacol *531*, 280-281.

Paneda, C., Gorospe, I., Herrera, B., Nakamura, T., Fabregat, I., and Varela-Nieto, I. (2002). Liver cell proliferation requires methionine adenosyltransferase 2A mRNA up-regulation. Hepatology *35*, 1381-1391.

Park, M.H. (2006). The post-translational synthesis of a polyamine-derived amino acid, hypusine, in the eukaryotic translation initiation factor 5A (eIF5A). J Biochem *139*, 161-169.

Parkinson, A. (2001). Casarett & Doull's Toxicology: The Basic Science of Poisons, 6th edn (New York, McGraw-Hill).

Poso, H., and Pegg, A.E. (1982). Effect of carbon tetrachloride on polyamine metabolism in rodent liver. Arch Biochem Biophys *217*, 730-737.

Potter, D.W., and Hinson, J.A. (1987). Mechanisms of acetaminophen oxidation to N-acetyl-P-benzoquinone imine by horseradish peroxidase and cytochrome P-450. J Biol Chem *262*, 966-973.

Prescott, L.F. (1980). Kinetics and metabolism of paracetamol and phenacetin. Br J Clin Pharmacol *10 Suppl 2*, 291S-298S.

Price, V.F., and Jollow, D.J. (1984). Role of UDPGA flux in acetaminophen clearance and hepatotoxicity. Xenobiotica *14*, 553-559.

Qiu, Y., Benet, L.Z., and Burlingame, A.L. (1998). Identification of the hepatic protein targets of reactive metabolites of acetaminophen in vivo in mice using two-dimensional gel electrophoresis and mass spectrometry. J Biol Chem *273*, 17940-17953.

Rambaldi, A., and Gluud, C. (2006). S-adenosyl-L-methionine for alcoholic liver diseases. Cochrane Database Syst Rev, CD002235.

Rawls, S.M., Ding, Z., and Cowan, A. (2006). Role of TRPV1 and cannabinoid CB1 receptors in AM 404-evoked hypothermia in rats. Pharmacol Biochem Behav *83*, 508-516.

Ray, S.D., Sorge, C.L., Raucy, J.L., and Corcoran, G.B. (1990). Early loss of large genomic DNA in vivo with accumulation of Ca2+ in the nucleus during acetaminophen-induced liver injury. Toxicol Appl Pharmacol *106*, 346-351.

Redick, J.A., Jakoby, W.B., and Baron, J. (1982). Immunohistochemical localization of glutathione S-transferases in livers of untreated rats. J Biol Chem *257*, 15200-15203.

Reiter, C., and Weinshilboum, R. (1982). Platelet phenol sulfotransferase activity: correlation with sulfate conjugation of acetaminophen. Clin Pharmacol Ther *32*, 612-621.

Rhee, H.J., Kim, E.J., and Lee, J.K. (2007). Physiological polyamines: simple primordial stress molecules. J Cell Mol Med *11*, 685-703.

Rigobello, M.P., Toninello, A., Siliprandi, D., and Bindoli, A. (1993). Effect of spermine on mitochondrial glutathione release. Biochem Biophys Res Commun *194*, 1276-1281.

Rodriguez, J.L., Boukaba, A., Sandoval, J., Georgieva, E.I., Latasa, M.U., Garcia-Trevijano, E.R., Serviddio, G., Nakamura, T., Avila, M.A., Sastre, J.*, et al.* (2007). Transcription of the MAT2A gene, coding for methionine adenosyltransferase, is up-regulated by E2F and Sp1 at a chromatin level during proliferation of liver cells. Int J Biochem Cell Biol *39*, 842-850.

Roede, J.R., and Jones, D.P. (2010). Reactive species and mitochondrial dysfunction: mechanistic significance of 4-hydroxynonenal. Environ Mol Mutagen *51*, 380-390.

Rutjes, A.W., Nuesch, E., Reichenbach, S., and Juni, P. (2009). S-Adenosylmethionine for osteoarthritis of the knee or hip. Cochrane Database Syst Rev, CD007321.

Saito, C., Lemasters, J.J., and Jaeschke, H. (2010). c-Jun N-terminal kinase modulates oxidant stress and peroxynitrite formation independent of inducible nitric oxide synthase in acetaminophen hepatotoxicity. Toxicol Appl Pharmacol *246*, 8-17.

Sava, I.G., Battaglia, V., Rossi, C.A., Salvi, M., and Toninello, A. (2006). Free radical scavenging action of the natural polyamine spermine in rat liver mitochondria. Free Radic Biol Med *41*, 1272-1281.

Schubert, H.L., Blumenthal, R.M., and Cheng, X. (2003). Many paths to methyltransfer: a chronicle of convergence. Trends Biochem Sci *28*, 329-335.

Shan, X., Aw, T.Y., Shapira, R., and Jones, D.P. (1989). Oxygen dependence of glutathione synthesis in hepatocytes. Toxicol Appl Pharmacol *101*, 261-270.

Shirahata, A., and Pegg, A.E. (1985). Regulation of S-adenosylmethionine decarboxylase activity in rat liver and prostate. J Biol Chem *260*, 9583-9588.

Song, Z., McClain, C.J., and Chen, T. (2004). S-Adenosylmethionine protects against acetaminophen-induced hepatotoxicity in mice. Pharmacology *71*, 199-208.

Stjernborg, L., Heby, O., Mamont, P., and Persson, L. (1993). Polyamine-mediated regulation of S-adenosylmethionine decarboxylase expression in mammalian cells. Studies using 5'-([(Z)-4-amino-2-butenyl]methylamino)-5'-deoxyadenosine, a suicide inhibitor of the enzyme. Eur J Biochem *214*, 671-676.

Stramentinoli, G., Pezzoli, C., and Galli-Kienle, M. (1979). Protective role of S-adenosyl-l-methionine against acetaminophen induced mortality and hepatotoxicity in mice. Biochem Pharmacol *28*, 3567-3571.

Streeter, A.J., Dahlin, D.C., Nelson, S.D., and Baillie, T.A. (1984). The covalent binding of acetaminophen to protein. Evidence for cysteine residues as major sites of arylation in vitro. Chem Biol Interact *48*, 349-366.

Sullivan, D.M., and Hoffman, J.L. (1983). Fractionation and kinetic properties of rat liver and kidney methionine adenosyltransferase isozymes. Biochemistry *22*, 1636-1641.

Terneus, M.V., Brown, J.M., Carpenter, A.B., and Valentovic, M.A. (2008). Comparison of S-adenosyl-L-methionine (SAMe) and N-acetylcysteine (NAC) protective effects on hepatic damage when administered after acetaminophen overdose. Toxicology *244*, 25-34.

Tirmenstein, M.A., and Nelson, S.D. (1989). Subcellular binding and effects on calcium homeostasis produced by acetaminophen and a nonhepatotoxic regioisomer, 3'-hydroxyacetanilide, in mouse liver. J Biol Chem *264*, 9814-9819.

Tirmenstein, M.A., and Nelson, S.D. (1990). Acetaminophen-induced oxidation of protein thiols. Contribution of impaired thiol-metabolizing enzymes and the breakdown of adenine nucleotides. J Biol Chem *265*, 3059-3065.

Umathe, S.N., Manna, S.S., Utturwar, K.S., and Jain, N.S. (2009). Endocannabinoids mediate anxiolytic-like effect of acetaminophen via CB1 receptors. Prog Neuropsychopharmacol Biol Psychiatry *33*, 1191-1199.

Urdiales, J.L., Medina, M.A., and Sanchez-Jimenez, F. (2001). Polyamine metabolism revisited. Eur J Gastroenterol Hepatol *13*, 1015-1019.

Vendemiale, G., Altomare, E., Trizio, T., Le Grazie, C., Di Padova, C., Salerno, M.T., Carrieri, V., and Albano, O. (1989). Effects of oral S-adenosyl-L-methionine on hepatic glutathione in patients with liver disease. Scand J Gastroenterol *24*, 407-415.

Vujcic, S., Liang, P., Diegelman, P., Kramer, D.L., and Porter, C.W. (2003). Genomic identification and biochemical characterization of the mammalian polyamine oxidase involved in polyamine back-conversion. Biochem J *370*, 19-28.

Walubo, A., Barr, S., Abraham, A.M., and Coetsee, C. (2004). The role of cytochrome-P450 inhibitors in the prevention of hepatotoxicity after paracetamol overdose in rats. Hum Exp Toxicol *23*, 49-54.

Wiegand, L., and Pegg, A.E. (1978). Effects of inhibitors of S-adenosylmethionine decarboxylase and ornithine decarboxylase on DNA synthesis in rat liver after partial hepatectomy. Biochim Biophys Acta *517*, 169-180.

Wu, Y.L., Jiang, Y.Z., Jin, X.J., Lian, L.H., Piao, J.Y., Wan, Y., Jin, H.R., Joon Lee, J., and Nan, J.X. (2009). Acanthoic acid, a diterpene in Acanthopanax koreanum, protects acetaminophen-induced hepatic toxicity in mice. Phytomedicine.

Zhou, L., McKenzie, B.A., Eccleston, E.D., Jr., Srivastava, S.P., Chen, N., Erickson, R.R., and Holtzman, J.L. (1996). The covalent binding of [14C]acetaminophen to mouse hepatic microsomal proteins: the specific binding to calreticulin and the two forms of the thiol:protein disulfide oxidoreductases. Chem Res Toxicol *9*, 1176-1182.

Chapter II: General Methods and Materials

The C57BL/6 Mouse Model

Through the work of previous doctoral studies (Cover et al., 2005b; Terneus et al., 2007), we were fortunate to have a well characterized animal model available for my thesis research. C57BL/6 mice have very similar metabolic profiles to humans with CYP2E1 being the predominant P450 responsible for metabolism of APAP in the mice (Bessems and Vermeulen, 2001). Additionally, the rate of sulfation and glucuronidation in the mice is roughly comparable to humans making them a good model to study toxicity due to APAP overdose (Bessems and Vermeulen, 2001).

Male C57BL/6 mice were purchased from Hilltop Lab Animals Incorporated (Scottsdale, PA). Male mice were used to avoid any potential hormonal variations in APAP metabolism. Animals included in the study were between 4-8 weeks of age and weighed 16-24 g. Mice were maintained in a facility in compliance with the guidelines of the American Association for Accreditation of Laboratory Animal Care. Mice were maintained at controlled temperature (21-23°C), humidity (40-55%), and 12 hour light cycles (lights on 6:00 AM to 6:00 PM). An acclimation period of 7 days was observed prior to the beginning of any experiment. The animals received Purina rodent chow and water *ad libitum*. The mice were fasted for 16 hours prior to any experiment to decrease available stores of GSH, but maintained free access to water.

On the days of experiments, the mice were injected in the intraperitoneal (ip) cavity with Veh (water), SAMe, NAC, APAP, and/or a combination of either SAMe or NAC with APAP (S + A or N + A respectively). The main focus of my research was on a recovery scenario whereby SAMe or NAC were given as an antidote following APAP injection. This model most closely approximates someone who has overdosed on APAP and is entering the acute care setting.

Experimental Protocol

Mice were randomly divided into the following groups: Vehicle (Veh; 15 ml/kg water ip injection), SAMe or NAC (1.25 mmol/kg given as a 5 ml/kg injection), APAP (250 mg/kg given as 15 ml/kg ip injection), and SAMe or NAC given one hour following APAP administration. The dose of APAP chosen is equivalent to an average 70 kg individual swallowing 35 Extra Strength Tylenol tablets. A review of the literature puts the dose of APAP used in these studies on the low end of what is currently being used. For instance, some laboratories go as high as 750 mg/kg APAP overdose to induce toxicity (Ramachandran and Raja, 2010). Using a lower dose offers a better approximation of what is physiologically possible in humans.

The collection times were 2, 4, and 6 hours following the initial administration of APAP. Mice were anesthetized with carbon dioxide and blood collected by cardiac puncture with heparinized 1 mL syringes to allow for analysis of plasma ALT activity. Unless subcellular fractioning occurred, livers were placed in ice cold Krebs buffer (126 mM NaCl, 5 mM KCl, 3 mM $MgSO_4$, 3 mM Na_2HPO_4, 1 mM $CaCl_2$; pH 7.4), blotted, and weighed.

Comparison of SAMe-Cl and SAMe-toluene sulfonate Salts

To ensure that SAMe was protective, and not the toluene sulfonate salt we were using in experiments, I conducted experiments with the chloride salt of SAMe. I found comparable ALT and liver weight per 10 g body weight values for both treatment groups (Data not shown). Additionally, there was no observed difference in other parameters measured between the salts. Therefore, I conclude that SAMe is in fact the protective entity, not the toluene sulfonate salt.

Basic Assessment of Toxicity

To normalize comparison among different experiments, certain baseline toxicity parameters were assessed. Plasma alanine aminotransferase (ALT) activity is a common experimental measure of APAP hepatotoxicity. Following necrosis of the centrilobular hepatocytes, ALT is released into the blood. Human liver toxicity in APAP overdose is generally agreed upon as ALT activity over 1,000 IU/L (McClain et al., 1999). Alanine aminotransferase (ALT) was assayed using a method based on the discontinued Sigma 505-P kit and described by the Harvison laboratory (Patel et al., 2011). Additionally, liver weight was determined. Necrosis caused by APAP creates oncotic pressure in the liver drawing water in and increasing the weight of the liver.

General Protocol for Western Blotting

Western blots were performed as follows: Two hundred milligrams of liver was homogenized in 1 mL ice cold Kreb's buffer and protein levels were determined using the Bradford protein assay. A 100 μg protein aliquot was denatured by boiling for 5 minutes. Samples were separated on a 12.5% polyacrylamide gel and transferred to a nitrocellulose membrane (Whatman; Dassel, Germany). Transfer efficiency was verified using MemCode® Reversible Protein Stain Kit (Thermo Scientific; Rockford, IL). MemCode® staining was used to standardize the densitometry to total protein in each of the lanes. The only exceptions to this were the MAT I/III and MAT II proteins that were standardized with GAPDH. Protein staining was used in most cases because of the more reliable and consistent results obtained with staining compared with the typical housekeeping genes.

The membranes were then blocked using a 5% (w/v) milk/TBST solution (10 mM Tris-HCl, 150 mM NaCl, 0.1% Tween-20; pH 8.0) for 1 hour. Membranes were then incubated overnight with constant shaking at 4°C in primary antibody prepared at the appropriate dilution in 5% (w/v)

milk/TBST. The membranes were washed four times with TBST. The appropriate secondary antibody was diluted in 5% (w/v) milk/TBST and incubated with the membrane for 1 hour. All of the antibodies used in the current work can be found in Table 1 below. The membrane was again washed with TBST and developed using Amersham™ ECL™ Western Blotting Detection Reagents (GE Healthcare; Buckinghamnshire, UK). Densitometry was performed on each gel using the Image J software (NIH).

Table 1. Primary and secondary antibodies used in the current work.

	Antibody	Product Number	Manufacturer
Primary Antibodies	MAT I/III[a]	sc-28029	Santa Cruz Biotechnology; Santa Cruz, CA
	MAT II [a]	sc-28031	Santa Cruz Biotechnology; Santa Cruz, CA
	GAPDH[b]	2275-PC-100	Trevigen; Gaithersburg, MD
	Cytochrome c[b]	sc-7159	Santa Cruz Biotechnology; Santa Cruz, CA
	CYP2E1[b]	ab28146	Abcam Inc.; Cambridge, MA
	ODC[c]	ab50269	Abcam Inc.; Cambridge, MA
	3-NT[c]	ab61392	Abcam Inc.; Cambridge, MA
	CPS-1[a]	sc-10515	Santa Cruz Biotechnology; Santa Cruz, CA
	4-HNE[d]	393207	Calbiochem; Merck, Darmstadt, Germany
Secondary Antibodies	[a]Donkey Anti-Goat HRP	sc-2020	Santa Cruz Biotechnology; Santa Cruz, CA
	[b]Goat Anti-Rabbit HRP	sc-2004	Santa Cruz Biotechnology; Santa Cruz, CA
	[c]Goat Anti-Mouse HRP	sc-2005	Santa Cruz Biotechnology; Santa Cruz, CA
	[d]Goat Anti-Rabbit IgG HRP	DC03L	Calbiochem; Merck, Darmstadt, Germany

[a,b,c,d] Superscripts after the primary antibodies correspond with the superscripts found on the secondary antibodies below.
*Selected abbreviations: MAT (Methionine adenosyltransferase), ODC (Ornithine decarboxylase), 3-NT (3-nitro tyrosine), CPS-1 (Carbamoyl phosphate synthase-1).

Statistical Analysis of Data

All values are reported as the mean ± S.E.M. with n = 3-10 animals per group. The final number of animals for each experiment is noted below in the results section. Differences between the

groups were determined using a one-way ANOVA followed by a Tukey's post-hoc test (SigmaStat; SPSS Inc. Chicago, IL). Significant differences were assessed using $\alpha = 0.05$.

Literature Cited

Anderson, M.E. (1985). Determination of glutathione and glutathione disulfide in biological samples. Methods Enzymol *113*, 548-555.

Bessems, J.G., and Vermeulen, N.P. (2001). Paracetamol (acetaminophen)-induced toxicity: molecular and biochemical mechanisms, analogues and protective approaches. Crit Rev Toxicol *31*, 55-138.

Brachet, P., Prevoteau, H., Mathe, V., and Tome, D. (1996). Modulation of putrescine transport in rat intestinal brush-border membrane vesicles by fasting and refeeding. Digestion *57*, 374-381.

Cover, C., Mansouri, A., Knight, T.R., Bajt, M.L., Lemasters, J.J., Pessayre, D., and Jaeschke, H. (2005b). Peroxynitrite-induced mitochondrial and endonuclease-mediated nuclear DNA damage in acetaminophen hepatotoxicity. J Pharmacol Exp Ther *315*, 879-887.

Gogvadze, V., Orrenius, S., and Zhivotovsky, B. (2006). Analysis of Mitochondrial Dysfunction During Cell Death. In Current Protocols in Toxicology, M.D. Maines, L.G. Costa, D.J. Reed, S. Sassa, and I.G. Sipes, eds. (Hoboken, NJ, John Wiley & Sons, Inc.), pp. 2.10.11-12.10.27.

Graham, J.M. (2001). Isolation of nuclei and nuclear membranes from animal tissues. Current Protocols in Cell Biology, Unit 3.10.

LaDu, B.N., Mandel, H.G., and Way, E.L. (1971). Study of Drug Biotransformation. In Fundamentals of Drug Metabolism and Drug Distribution (Baltimore, The Williams & Wilkins Company), pp. 570-571.

Lawrence, R.A., and Burk, R.F. (1976). Glutathione peroxidase activity in selenium-deficient rat liver. Biochem Biophys Res Commun *71*, 952-958.

Mannervik, B. (1999). Measurement of Glutathione Reductase Activity. In Current Protocols in Toxicology, M.D. Maines, L.G. Costa, E. Hodgson, D.J. Reed, and I.G. Sipes, eds. (Hoboken, NJ, John Wiley & Sons, Inc.), pp. 7.2.1-7.2.4.

McClain, C.J., Price, S., Barve, S., Devalarja, R., and Shedlofsky, S. (1999). Acetaminophen hepatotoxicity: An update. Curr Gastroenterol Rep *1*, 42-49.

Patel, N.N., Crincoli, C.M., Frederick, D.M., Tchao, R., and Harvison, P.J. (2011). Effect of structural modifications on 3-(3,5-dichlorophenyl)-2,4-thiazolidinedione-induced hepatotoxicity in Fischer 344 rats. J Appl Toxicol.

Ramachandran, V., and Raja, B. (2010). Protective effects of syringic acid against acetaminophen-induced hepatic damage in albino rats. J Basic Clin Physiol Pharmacol *21*, 369-385.

Terneus, M.V., Kiningham, K.K., Carpenter, A.B., Sullivan, S.B., and Valentovic, M.A. (2007). Comparison of S-Adenosyl-L-methionine and N-acetylcysteine protective effects on acetaminophen hepatic toxicity. J Pharmacol Exp Ther *320*, 99-107.

Wang, S.H., Kuo, S.C., and Chen, S.C. (2003). High-performance liquid chromatography determination of methionine adenosyltransferase activity using catechol-O-methyltransferase-coupled fluorometric detection. Anal Biochem *319*, 13-20.

Wang, W., Kramer, P.M., Yang, S., Pereira, M.A., and Tao, L. (2001). Reversed-phase high-performance liquid chromatography procedure for the simultaneous determination of S-adenosyl-L-methionine and S-adenosyl-L-homocysteine in mouse liver and the effect of methionine on their concentrations. J Chromatogr B Biomed Sci Appl *762*, 59-65.

Zhang, Y.T., Zheng, Q.S., Pan, J., and Zheng, R.L. (2004). Oxidative damage of biomolecules in mouse liver induced by morphine and protected by antioxidants. Basic Clin Pharmacol Toxicol *95*, 53-58.

Chapter III: Temporal study of acetaminophen (APAP) and S-adenosyl-L-methionine (SAMe) effects on subcellular hepatic SAMe levels and methionine adenosyltransferase (MAT) expression and activity.

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J. Michael Brown*, John G. Ball*, Amy Hogsett*, Tierra Williams*, and Monica Valentovic*[1]

*Department of Pharmacology, Physiology, and Toxicology,

Joan C. Edwards School of Medicine, Marshall University, Huntington, WV 25755

[1]Correspondence should be addressed to: Department of Pharmacology, Physiology, and Toxicology; 1 John Marshall Drive, Joan C. Edwards School of Medicine, Marshall University, Huntington, WV 25755. Phone (304-696-7332); Fax (304-696-7391); Email: Valentov@marshall.edu

Abstract

Acetaminophen (APAP) is the leading cause of drug induced liver failure in the United States. Previous studies in our laboratory have shown that S-adenosyl methionine (SAMe) is protective for APAP hepatic toxicity. SAMe is critical for glutathione synthesis and transmethylation of nucleic acids, proteins and phospholipids which would facilitate recovery from APAP toxicity. SAMe is synthesized in cells through the action of methionine adenosyltransferase (MAT). This study tested the hypothesis that total hepatic and subcellular SAMe levels are decreased by APAP toxicity. Studies further examined MAT expression and activity in response to APAP toxicity. Male C57BL/6 mice (16-22 grams) were treated with vehicle (Veh; water 15ml/kg ip injections), SAMe (1.25 mmol/kg), 250 mg/kg APAP (15 ml/kg, ip), or SAMe administered one hour after APAP injection (SAMe and S + A). Hepatic tissue was collected 2, 4, and 6 hours after APAP administration. Levels of SAMe and its metabolite S-adenosylhomocysteine (SAH) were determined by HPLC analysis. MAT expression was examined by Western blot. MAT activity was determined by fluorescence assay. Total liver SAMe levels were depressed at 4 hours by APAP overdose, but not at 2 or 6 hours. APAP depressed mitochondrial SAMe levels at 4 and 6 hours relative to the Veh group. In the nucleus, levels of SAMe were depressed below detectable limits 4 hours following APAP administration. SAMe administration following APAP (S + A) prevented APAP associated decline in mitochondrial and nuclear SAMe levels. In conclusion, the maintenance of SAMe may provide benefit in preventing damage associated with APAP toxicity.

KEY WORDS: S-adenosyl-L-methionine, Acetaminophen, Hepatotoxicity, S-adenosylhomocysteine, Methionine Adenosyltransferase

Introduction

Acetaminophen (APAP) is the leading cause of drug induced liver disease in the United States resulting in over 56,000 emergency room visits and approximately 500 deaths each year (Nourjah et al., 2006). One of the problems associated with APAP toxicity is the wide availability of the drug. APAP is present in over 180 over the counter (OTC) products, which increases the probability of accidental overdose. Acute overdose of APAP leads to severe hepatic centrilobular necrosis (Boyd and Bereczky, 1966; Golden et al., 1981). Rapid treatment with *N*-acetylcysteine (NAC) is currently the clinical treatment for APAP overdose.

The toxicity of APAP is mediated through its biotransformation into *N*-acetyl-p-benzoquinoneimine (NAPQI) by cytochrome P450 2E1, 3A4, and 1A2 (Corcoran et al., 1980; Dahlin et al., 1984; Patten et al., 1993). NAPQI is a strong electrophile that rapidly adducts sulfhydryl groups like those found on reduced glutathione (GSH) (Streeter et al., 1984). GSH depletion by NAPQI precedes APAP toxicity (Larrauri et al., 1987). In addition to adducting proteins, NAPQI also induces mitochondrial dysfunction leading to a severe energy debt and the formation of reactive oxygen species (ROS) that induce further damage in the hepatocytes (Andersson et al., 1990).

The current treatment for APAP overdose is N-acetylcysteine (NAC). NAC functions by replenishing cellular stores of cysteine which is involved in the rate-limiting step in the formation of GSH. NAC has been demonstrated to reduce protein adduction in response to APAP overdose (Corcoran et al., 1985). Also, NAC reduces mitochondrial dysfunction and reactive oxygen generation in hepatocytes following APAP overdose (Reid et al., 2005). In order to be effective as a treatment, however, NAC must be administered within 8-10 hours

following APAP overdose, making the study of alternative therapies attractive (Smilkstein et al., 1988).

Currently, *S*-adenosyl-L-methionine (SAMe) is available over the counter and has gained acceptance as beneficial for depression and alcoholic liver disease (Purohit et al., 2007; Williams et al., 2005). SAMe is a ubiquitous cofactor in a variety of biological reactions. SAMe is found in most tissues and is produced at a rate of 6-8 g per day in the liver. The production of SAMe is catalyzed by methionine adenosyltransferase (MAT) (Lu, 2000). *MAT1A* is expressed constitutively in the adult liver and encodes the α1 subunit which composes MAT I (tetramer) and MAT III (dimer). The gene coding for MAT II is *MAT2,* which is widely distributed throughout the body with the exception of the adult liver (Kotb et al., 1997). However, MAT II is expressed in the adult liver during liver regeneration and hepatic cancer (Martinez-Chantar et al., 2003; Paneda et al., 2002). Furthermore, *MAT1A* expression is increased during liver regeneration following partial hepatectomy (Chen et al., 2004).

The protective action of SAMe upon the liver is hypothesized to be mediated via the transmethylation and transsulfuration pathways. SAMe is the principal biological methyl donor in cells. Following methyl group donation, SAMe becomes *S*-adenosylhomocysteine (SAH) that can enter the transsulfuration pathway leading to replenishment of cellular GSH (Finkelstein, 2000). Alterations in either SAMe or its ratio with SAH have been associated with toxicant exposure. For example, SAMe levels were decreased in humans with alcoholic liver disease (Purohit et al., 2007). Furthermore, any decline in SAMe or decrease in the ratio of SAMe:SAH has been demonstrated to inhibit cellular transmethylation reactions (Purohit et al., 2007) as SAH is a competitive inhibitor of transmethylation reactions (Kharbanda 2007). Previous research by our laboratory and others has demonstrated that SAMe protects against APAP

induced hepatotoxicity when administered just prior to APAP overdose (Bray et al., 1992; Terneus et al., 2007). More recent studies have documented that in a more clinically relevant experimental model, SAMe administered 1 hour after APAP overdose was protective to the liver (Terneus et al., 2008).

In our laboratory, SAMe and NAC displayed a comparable level of protection, when compared on the basis of a mmol/kg dose, for APAP overdose in mice (Terneus et al., 2008). However, the mechanism for SAMe protection of APAP toxicity remains to be elucidated. While decreases in SAMe have been linked to liver damage, little research has been done to examine the effects of APAP toxicity on intracellular SAMe levels. A decrease in intracellular SAMe levels following APAP overdose would have deleterious effects on DNA methylation, phospholipid formation and GSH synthesis. Additionally, given that expression of MAT appears to be required for liver regeneration following damage, the exploration of APAP overdose effects on MAT levels warrants further study. Therefore, the purpose of the current study was to investigate the effect of APAP overdose on hepatic, 15,000 *x g* supernatant, nuclear and mitochondrial SAMe levels, as well as alterations of hepatic MATI/III and MATII. By examining these components of SAMe metabolism, the present study hopes to shed light on the mechanism of SAMe protection against APAP toxicity.

Methods and Materials

Materials. SAMe toluene sulfonate salt as used in all experiments (Sigma Chemical Co., St. Louis, MO). The ALT reagent kit (TR-71021) was purchased from Thermo Electron Corporation (Louisville, CO). All solvents were HPLC grade and other reagents were of comparable quality and purchased from Sigma Chemical Co. (St. Louis, MO) or Fisher Scientific (Pittsburgh, PA).

Animals. Male C57BL/6 mice were obtained from Hilltop Lab Animals Inc. (Scottsdale, PA). Animals included in the study were between 4-8 weeks of age and weighed 16-24 g. Mice were maintained in a facility in compliance with the American Association for Accreditation of Laboratory Animal Care. Mice were maintained at controlled temperature (21-23°C), humidity (40-55%), and 12 hour light cycles (lights on 6:00 AM to 6:00PM). An acclimation period of 7 days was observed prior to the beginning of any experiment. The animals received Purina rodent chow and water *ad libitum*. The mice were fasted for 16 hours prior to any experiment, but maintained free access to water.

SAMe and NAC Treatment Following APAP Overdose. Mice were randomly allocated into the following groups: vehicle (Veh; 15 ml/kg water by intraperitoneal (ip) injection), SAMe (1.25 mmol/kg 5ml/kg ip injection), APAP (250 mg/kg 15ml/kg ip injection, and SAMe administered 1 hour after APAP (S + A; doses same as previously listed). Mice treated with NAC were randomly allocated into Veh, NAC (1.25 mmol/kg with 5 ml/kg ip injection), APAP, and N + A. SAMe and NAC were administered 1 hour following APAP. Mice were anesthetized with carbon dioxide 2, 4, and 6 hours after APAP administration with SAMe treatment, and 4 hours following APAP treatment when NAC was used as a comparison for SAMe. Blood was collected by cardiac puncture in heparin-rinsed 1 ml syringes for determination of serum ALT activity, which serves as an indicator of liver injury. Livers were then isolated and placed in ice cold Krebs buffer (126 mM NaCl, 5 mM KCl, 3 mM $MgSO_4$, 3 mM Na_2HPO_4, 1 mM $CaCl_2$; pH 7.4), blotted, and weighed.

Mitochondrial Isolation. Mitochondria were isolated using a modification of a previously published protocol (Gogvadze et al., 2004). Briefly, the liver was isolated, blotted, weighed and placed in Mitochondrial Isolation Buffer A (225 mM sucrose, 3 mM KH_2PO_4, 5 mM $MgCl_2$, 20

mM KCl, 20 mM triethanolamine, 2 mM EGTA; pH 7.4). The liver was minced and homogenized in a Dounce homogenizer on ice. Following homogenization, the liver was centrifuged at 600 x *g* for 10 minutes. The resultant pellet was discarded and the supernatant was centrifuged at 15,000 x *g* for 5 minutes. After the final centrifugation, the supernatant was retained for analysis of SAMe levels. The pellet containing the mitochondria was resuspended in Mitochondrial Isolation Buffer B (Same as Buffer A except lacking EGTA) for a final concentration of 1 mg tissue weight/1 ml Buffer B. Samples were stored at -80°C until analysis.

Nucleus Isolation. The protocol for isolating the nuclei of liver cells was adapted from Graham (2001). Briefly, liver was homogenized in ice cold Nuclear Isolation Medium (0.25 M sucrose, 25 mM KCl, 5 mM $MgCl_2$, and 10 mM Tris-Cl; pH 7.4) using a Dounce homogenizer and adjusted to a final 3 mL volume. The homogenate was centrifuged at 800 x *g*, the supernatant discarded, new buffer was added and centrifuged at *800 x g* and the supernatant discarded. The pellet was resuspended in 1 mL Nuclear Isolation Medium followed by 2 mL of Sucrose Density Barrier (1.15 M sucrose, 10 mM KCl, 2.5 mM $MgCl_2$, and 5 mM Tris-Cl; pH 7.4) and vortexed. Six mL of Sucrose Density Barrier was then layered under the homogenate and centrifuged for 1 hour at 100,000 x *g*. The pellet was resuspended in 1 mL Nuclear Isolation Medium and stored at -80°C until use.

HPLC Analysis of Hepatic SAMe Levels. A 200 mg aliquot of liver was homogenized on ice in 0.4 mM $HClO_4$ and adjusted to a final 1 ml volume. Mitochondrial and 15,000 *x g* supernatant suspensions were added to an equal volume of 0.4 mM $HClO_4$ to precipitate protein. Nuclear samples were concentrated by lyophilizing the 1 ml of sample (total liver weight 600-900 mg) and reconstituting the sample in 125 µl 0.4 mM $HClO_4$. The samples were then centrifuged at 10,000 x *g* at 4°C and filtered through 0.45 µM Millex®-HV filters (Millipore; Billericia, MD).

A 20 µl sample of the filtrate was analyzed for whole liver, mitochondrial, and 15,000 *x g* supernatant fractions, while 40 µl of sample was required for detection of nuclear SAMe levels. SAMe and SAH levels were detected using a Beckman Coulter HPLC system (Fullerton, CA) with a 126 Solvent Module and a 166 Variable Wavelength Detector. The column was a YMC ODS-AQ 3 µm 120 Å 4.6x150 mm column. The mobile phase was a gradient at a flow of 1 ml/min (Waters Corporation; Milford, MA). The mobile phase gradient program was 8 minutes of 90:10 A:B followed by 12 minutes of 60:40 A:B. Mobile phase A consisted of 8 mM 1-heptane sulfonic acid sodium salt and 50 mM sodium phosphate monobasic (pH 3). Mobile phase B was 100% HPLC grade methanol. The wavelength for detection was 254 nm (Wang et al., 2001).

Analysis of MAT I/II/III Expression in Mouse Liver. Western blot analysis was conducted to examine expression of MAT I/III and MAT II. Approximately 200 mg of liver was homogenized in 1 mL ice cold Krebs buffer and protein levels were determined using the Bradford protein assay. A 100 µg protein aliquot was denatured by boiling for 5 minutes. Samples were separated on a 12.5% polyacrylamide gel and transferred to a PVDF membrane (Pall Corporation; Pensacola, FL). Transfer efficiency was verified using MemCode® Reversible Protein Stain Kit (Thermo Scientific; Rockford, IL). The membrane was then blocked using a 5% (w/v) milk/TBST solution (10 mM Tris-HCl, 150 mM NaCl, 0.1% Tween-20; pH 8.0) for 1 hour. Membranes were next incubated overnight with constant shaking at 4°C in antibody for MAT I/III (sc-28029, Santa Cruz Biotechnology; Santa Cruz, CA) or MAT II (sc-28031, Santa Cruz Biotechnology; Santa Cruz, CA) each at 1:1000 dilution in 5% (w/v) milk/TBST. The membranes were washed four times with TBST. The donkey anti-goat HRP secondary antibody (sc-2020, Santa Cruz Biotechnology; Santa Cruz, CA) was diluted 1:3000

and incubated with the membrane for 1 hour. The membrane was again washed with TBST and developed using Amersham™ ECL™ Western Blotting Detection Reagents (GE Healthcare; Buckinghamnshire, UK). Blots were then reprobed with GAPDH as a loading control (2275-PC-100, Trevigen; Gaithersburg, MD). Goat anti-rabbit HRP secondary antibody was used to develop the blots (sc-2004, Santa Cruz Biotechnology; Santa Cruz, CA). Densitometry was performed on each gel.

Determination of MAT Activity. An endpoint fluorometric assay for determination of MAT activity developed by Wang, et al. (2003) was modified for a high throughput assay using a fluorescent plate reader. The assay measures the formation of scopoletin through MAT activity. A standard curve was constructed from scopoletin with a lower detection limit of 50 pmol. The protocol of Wang was followed except at the end when the extracted scopoletin was transferred to a 96 well plate and read using a BioTeck Synergy 2 (BioTek Instruments; Winooski, VT) with excitation and emission at 347 and 415 nm, respectively.

Statistical Analysis. Values represent Mean ± S.E.M. with n=3-10 animals/group. Differences in the groups were analyzed using a one-way ANOVA followed by a Tukey's post-hoc test (SigmaStat; SPSS Inc. Chicago, IL). All statistical analyses were conducted using a 95% confidence interval.

Results

The body weights of the animals were not significantly different between groups (Table 2). In addition, liver to body weight ratios were diminished in the S + A group 4 hours after APAP overdose when compared to APAP treated mice (Table 2). However, the same reduction in the liver to body weight ratio was not observed when the livers were collected at 6 hours following

APAP overdose (Table 2). To provide a comparison for the current standard of treatment for APAP overdose, NAC (1.25 mmol/kg) was administered 1 hour following APAP overdose and livers collected 4 hours following APAP overdose. When adjusted for body weight, NAC prevented the rise in liver weight that typically serves as an indicator of APAP toxicity (Table 3). These liver weight results indicate that equimolar doses of SAMe and NAC (1.25 mmol/kg) are able to reduce the liver to body weight ratio when administered following APAP overdose.

Table 2. Body weight and liver weight per 10 g body weight changes following APAP and SAMe.

	Group	Body Weight (g)	Liver weight /10 g body weight
SAMe 2 h	**Veh**	24.00 ± 0.00[a]	0.461 ± 0.00552[a]
	SAMe	23.60 ± 0.40[a]	0.400 ± 0.0256[a]
	APAP	24.40 ± 0.40[a]	0.456 ± 0.0231[a]
	S + A	22.80 ± 0.80[a]	0.467 ± 0.0108[a]
SAMe 4 h	**Veh**	20.80 ± 0.49[a]	0.395 ± 0.0164[a]
	SAMe	20.40 ± 0.75[a]	0.415 ± 0.00650[ac]
	APAP	20.80 ± 0.49[a]	0.477 ± 0.0102[b]
	S + A	20.80 ± 0.49[a]	0.464 ± 0.0177[c]
SAMe 6 h	**Veh**	18.40 ± 0.40[a]	0.363 ± 0.00475[a]
	SAMe	19.20 ± 0.49[a]	0.385 ± 0.00858[a]
	APAP	18.40 ± 0.40[a]	0.486 ± 0.0192[b]
	S + A	19.00 ± 0.58[a]	0.470 ± 0.0187[b]

Mice were randomly allocated into the following groups: Veh (water), SAMe (1.25 mmol/kg), APAP (250 mg/kg), SAMe 1 hour after APAP (S + A). SAMe and APAP were administered by ip injection. Values represent Mean ± S.E.M. (n=5-10). Values were measured 2, 4 or 6 hours post APAP or Veh injection.

[abc] Different letters denote statistical differences ($p<0.05$), while groups with the same letters indicate no difference in the results.

Table 3. The effect of NAC and APAP on body and liver weight per 10 g body weight

	Group	Body Weight (g)	Liver weight/10g body weight
NAC 4 h	Veh	20.00 ± 0.63[a]	0.416 ± 0.0296[a]
	NAC	19.60 ± 0.75[a]	0.443 ± 0.00492[a]
	APAP	18.00 ± 0.00[a]	0.547 ± 0.0150[b]
	N + A	20.00 ± 0.89[a]	0.466 ± 0.0101[a]

Mice were randomly allocated into the following groups: Veh (water), NAC (1.25 mmol/kg), APAP (250 mg/kg), NAC 1 hour after APAP (N + A). NAC and APAP were administered by ip injection. Values represent Mean ± S.E.M. (n=5-10). Values were measured 4 hours post APAP or Veh.

[ab] Different letters denote statistical differences ($p<0.05$), while groups with the same letters indicate no difference in the results.

Plasma ALT is a common measure of liver toxicity as ALT is released into the serum following damage to the liver. Prior experiments on APAP toxicity have indicated that toxicity induces a steady increase in plasma ALT between 2 and 6 hours. Plasma ALT levels typically range between 2000 and 6000 IU/L for the time periods studied in mice (Andringa et al., 2008; Hinson et al., 2002; Knight et al., 2003). Toxicity from APAP overdose was demonstrated two hours following APAP administration (Figure 5). At 4 hours following APAP administration, equivalent doses of SAMe and NAC protect the liver as indicated by a significant decrease in serum ALT when compared to the APAP treatment group (Figure 5). When the livers were collected 6 hours following APAP overdose, SAMe treatment was unable to reduce serum ALT levels back to that of the Veh treatment group, although SAMe was able to significantly lower serum ALT levels compared with the APAP treatment group (Figure 5). NAC significantly reduced ALT when administered 1h following APAP (Figure 5).

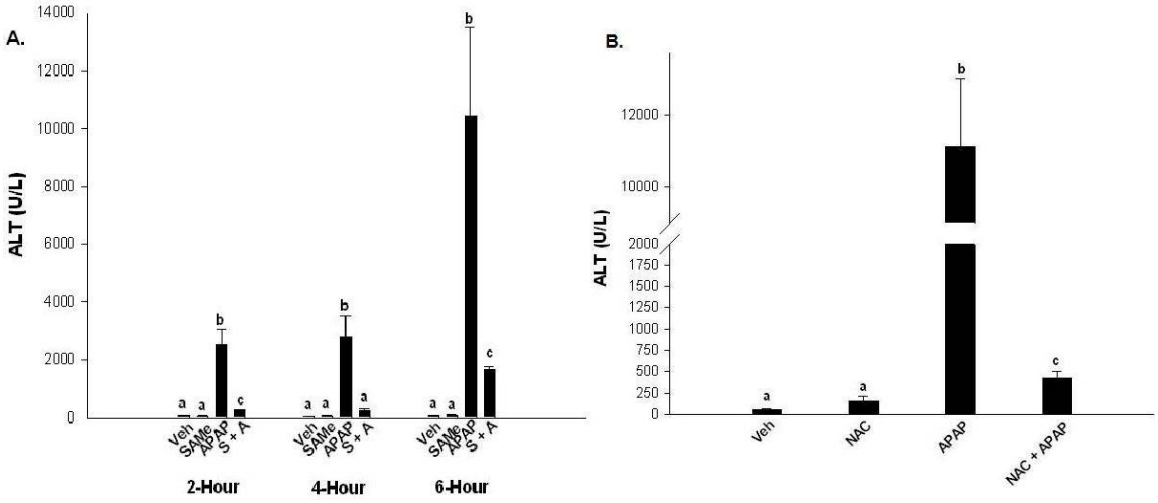

Figure 5. The effect of SAMe and NAC on serum ALT levels following APAP overdose in C57BL/6 mice. In panel A of the figure, mice were randomly allocated into the following groups: Veh (water), SAMe (1.25 mmol/kg), APAP (250 mg/kg), SAMe 1 hour after APAP (S + A). Plasma was collected 2, 4 and 6 hours following the initial APAP overdose. Panel B of the figure consisted of mice allocated into groups of Veh (water), NAC (1.25 mmol/kg), APAP (250 mg/kg), and NAC administered 1 hour following APAP (N + A). Serum was collected 4 hours following APAP administration. Values represent mean ± S.E.M. with n=5-10 mice/group. Different letters denote statistical differences ($p<0.05$), while groups with the same letters indicate no difference in the results.

Hepatic SAMe levels were measured following APAP overdose because perturbations in SAMe can inhibit transmethylation and transsulfuration reactions (Lu, 2000). At 4 and 6 hours following APAP administration SAMe levels were significantly ($p<0.05$) decreased compared to the Veh groups (Figure 6). Administration of SAMe 1 hour following APAP prevented the decrease in liver homogenate SAMe levels (Figure 6). Peak SAMe levels were observed in the SAMe and S + A groups 1 hour following SAMe administration (Figure 6). Mice were collected at 1 hour after SAMe administration but 2 hours following APAP administration (Figure 6). In Table 4 it can be observed that levels of SAH significantly decreased 2 hours following APAP administration when compared to the Veh group. In addition, the ratio of SAMe:SAH increases in the APAP treatment group at 2 hours (Figure 6). Also, by 6 hours the ratio of SAMe:SAH in

the SAMe group decreases because of the drop in SAMe levels as the levels of SAH remain constant (Figure 6; Table 4).

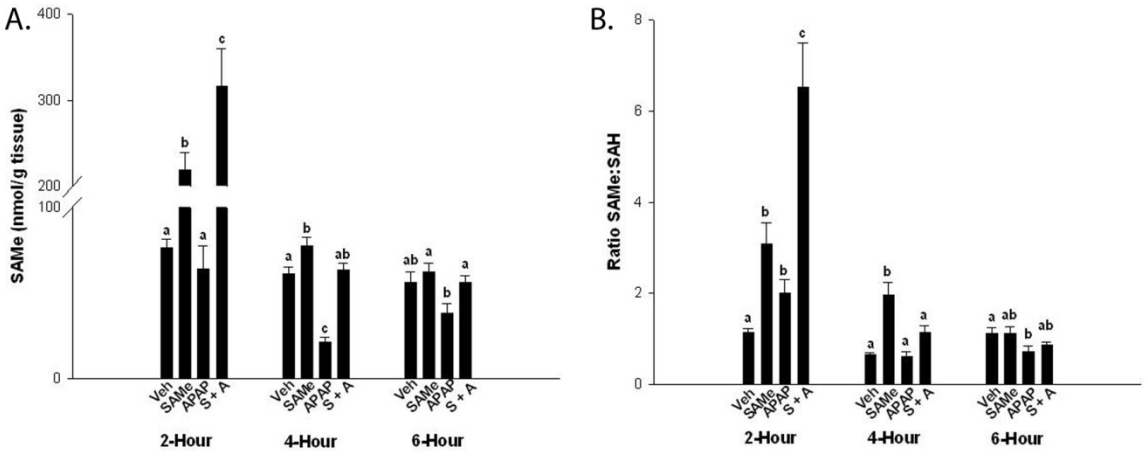

Figure 6. Alterations of SAMe levels following APAP overdose. C57BL/6 mice were randomly allocated into Veh (water), SAMe (1.25 mmol/kg), APAP (250 mg/kg), and SAMe administered 1 hour after APAP (S + A). Livers were collected at 2, 4 and 6 hours following APAP overdose and processed for analysis of SAMe levels by HPLC. Values represent mean ± S.E.M. Panel A represents SAMe levels (nmol/g tissue) following APAP administration, while panel B represents the ratio of SAMe:SAH. Different letters denote statistical differences ($p<0.05$), while groups with the same letters indicate no difference in the results.

Table 4. The Effect of SAMe on Total Hepatic SAH levels following APAP overdose.

	Group	Liver SAH Levels (nmol/g tissue)
SAMe 2 h	Veh	67.176 ± 1.540[a]
	SAMe	75.404 ± 9.352[a]
	APAP	34.630 ± 9.046[b]
	S + A	49.118 ± 2.549[ab]
SAMe 4 h	Veh	44.130 ± 2.110[a]
	SAMe	61.770 ± 4.310[b]
	APAP	42.310 ± 4.410[a]
	S + A	40.580 ± 2.100[a]
SAMe 6 h	Veh	51.290 ± 5.470[ab]
	SAMe	57.810 ± 4.400[ab]
	APAP	54.630 ± 2.940[a]
	S + A	64.250 ± 0.840[b]

Mice were randomly allocated into the following groups: Veh (water), SAMe (1.25 mmol/kg), APAP (250 mg/kg), SAMe 1 hour after APAP (S + A). SAMe and APAP were administered by ip injection. Values represent Mean ± S.E.M. (n=5-10). SAH levels were measured in liver homogenate 2, 4 or 6 hours post APAP or Veh.

[ab] Different letters denote statistical differences ($p<0.05$), while groups with the same letters indicate no difference in the results.

As would be expected, administration of NAC did not increase SAMe levels in the NAC group (Figure 7). Perhaps the most interesting finding in the NAC study was that NAC administration following APAP prevented a significant decrease in the levels of SAMe in the N + A group (Figure 7). No changes were observed in SAH levels following APAP administration (Table 5). The ratio of SAMe:SAH in the APAP treatment groups is decreased when compared to the Veh group (Figure 7). Administration of NAC following APAP was not able to significantly increase the ratio of SAMe:SAH when compared with the APAP at 4 hours (Figure 7). SAMe was also unable to increase the ratio of SAMe:SAH at the same time period (Figure 6).

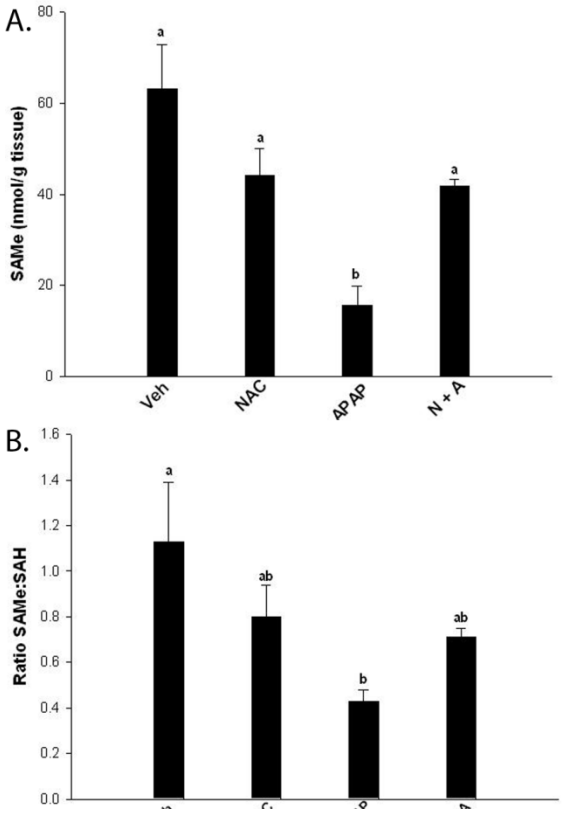

Figure 7. The observed effects of NAC on SAMe levels in the liver after APAP overdose. C57BL/6 mice were randomly allocated into Veh (water), NAC (1.25 mmol/kg), APAP (250 mg/kg), and NAC administered 1 hour following APAP (N + A). Livers were collected 4 hours following administration of APAP and processed for determination of SAMe levels via HPLC. Panel A represents SAMe levels when NAC was given following APAP overdose, and panel B represents the ratio of SAMe:SAH under the same experimental conditions. All values represent mean ± S.E.M. with n=5 mice/group. Different letters denote statistical differences ($p<0.05$), while groups with the same letters indicate no difference in the results.

Table 5. The Effect of NAC and APAP on Hepatic SAH levels.

	Groups	Liver Homogenate SAH Levels (nmol/g tissue)
NAC 4 h	Veh	59.230 ± 4.070[a]
	NAC	60.090 ± 8.500[a]
	APAP	39.420 ± 11.020[a]
	N + A	59.710 ± 3.140[a]

Mice were randomly allocated into the following groups: Veh (water), NAC (1.25 mmol/kg), APAP (250 mg/kg), NAC 1 hour after APAP (N + A). NAC and APAP were administered by ip injection. Values represent Mean ± S.E.M. (n=5-10). SAH levels were measured in liver homogenate 4 hours post APAP or Veh.

[a] Different letters denote statistical differences ($p<0.05$), while groups with the same letters indicate no difference in the results.

After analyzing whole liver levels of SAMe, the experiment next proceeded to measuring subcellular levels of SAMe. First, the effect of APAP overdose on hepatic mitochondrial SAMe levels was analyzed. Mitochondria are known to have SAMe transporters (Aqrimi et al., 2004). Levels of SAMe were decreased at both 4 and 6 hours following APAP overdose in the APAP treatment groups but not at 2 hours following APAP administration (Figure 8). At 2, 4, and 6 hours following APAP overdose, administration of SAMe after APAP causes a significant increase in levels of mitochondrial SAMe when compared to the Veh group (Figure 8). Mitochondrial SAH levels are decreased significantly 4 hours following APAP overdose, but remain unchanged for all other groups and time periods analyzed (Table 6). Additionally, the ratio of SAMe:SAH is increased in the S + A group for the mitochondria at all time periods (Figure 8). One other aspect of note, is that the ratio of SAMe:SAH in the mitochondria is not decreased in the APAP treatment group (Figure 8).

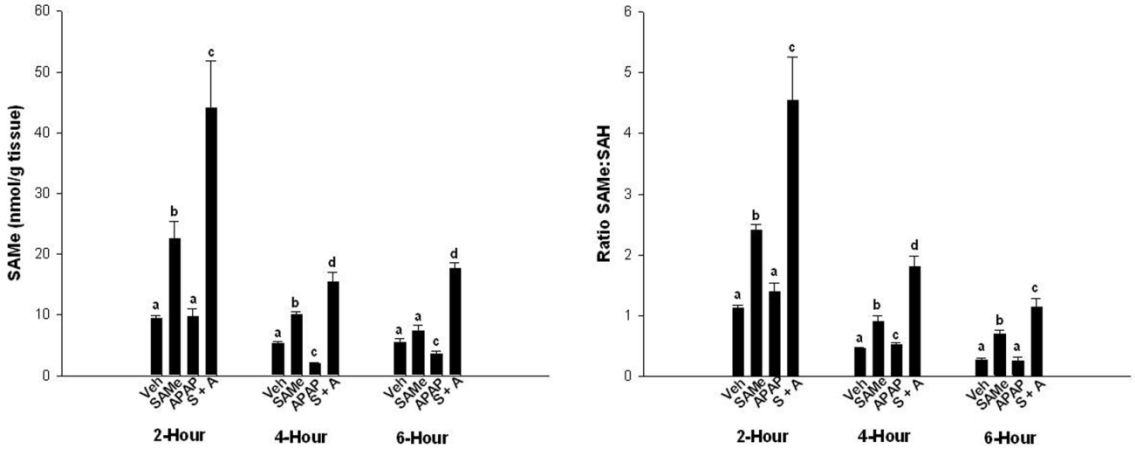

Figure 8. Examination of the effect of exogenous SAMe on levels of SAMe in liver mitochondria of C57BL/6 mice. Mice were randomly divided in to Veh (water), SAMe (1.25 mmol/kg), APAP (250 mg/kg), and SAMe administered 1 hour following APAP (S + A). Livers were collected and mitochondria isolated at 2, 4 and 6 hours following APAP overdose. Levels of SAMe and SAH were determined by HPLC. Panel A represents levels of mitochondrial SAMe when SAMe was administered following APAP overdose. Panel B is the ratio of SAMe:SAH in the same treatment groups. All values represent mean ± S.E.M. with n=5 mice/group. Different letters denote statistical differences ($p<0.05$), while groups with the same letters indicate no difference in the results.

Table 6. Mitochondrial and 15,000 *x g* supernatant SAH levels in animals treated with APAP followed 1 hour later by SAMe

	Group	Mitochondria SAH Levels (nmol/g tissue)	15,000 *x g* SAH Levels (nmol/g tissue)
SAMe 2 h	Veh	8.329 ± 0.250[a]	20.603 ± 1.146[a]
	SAMe	9.237 ± 0.810[a]	7.446 ±0.212[b]
	APAP	7.488 ± 1.642[a]	16.856 ± 3.736[ac]
	S + A	9.667 ± 0.855[a]	5.315 ± 0.656[d]
SAMe 4 h	Veh	11.359 ± 0.747[a]	23.871 ± 1.804[a]
	SAMe	11.609 ± 1.342[a]	22.147 ± 2.184[a]
	APAP	3.633 ± 0.389[b]	3.911 ± 0.334[b]
	S + A	8.544 ± 0.361[a]	8.991 ± 0.481[c]
SAMe 6 h	Veh	7.427 ± 0.743[a]	26.267 ± 1.717[a]
	SAMe	6.013 ± 0.618[a]	29.343 ± 2.493[a]
	APAP	3.006 ± 0.311[a]	8.218 ± 3.484[b]
	S + A	6.377 ± 1.379[a]	13.700 ± 3.719[c]

Mice were randomly allocated into the following groups: Veh (water), SAMe (1.25 mmol/kg), APAP (250 mg/kg), SAMe 1 hour after APAP (S + A). SAMe and APAP were administered by ip injection. Values represent Mean ± S.E.M. (n=5-10). Mitochondrial and 15,000 *x g* supernatant SAH levels were measured 2, 4 or 6 hours post APAP or Veh.

[abc] Different letters denote statistical differences ($p<0.05$), while groups with the same letters indicate no difference in the results.

Levels of 15,000 *x g* supernatant SAMe were determined in addition to the levels of mitochondrial SAMe. Hepatic 15,000 *x g* supernatant SAMe levels were significantly decreased at 4 and 6 hours following APAP overdose (Figure 9). The drop in SAMe observed in the APAP treatment group at 4 and 6 hours was prevented by SAMe administration following APAP overdose (Figure 9). 15,000 *x g* supernatant SAH levels were decreased in the SAMe and S + A groups at two hours (Table 6). SAH levels were also significantly depressed at 4 and 6 hours in the 15,000 *x g* supernatant APAP treatment groups and this was reversed by administration of SAMe following APAP overdose (Table 6). In the S + A group at both 2 and 4 hours the ratio of SAMe:SAH was significantly increased (Figure 9). In the APAP treatment group at 6 hours, there was no observed alteration of the ratio of SAMe:SAH (Figure 9).

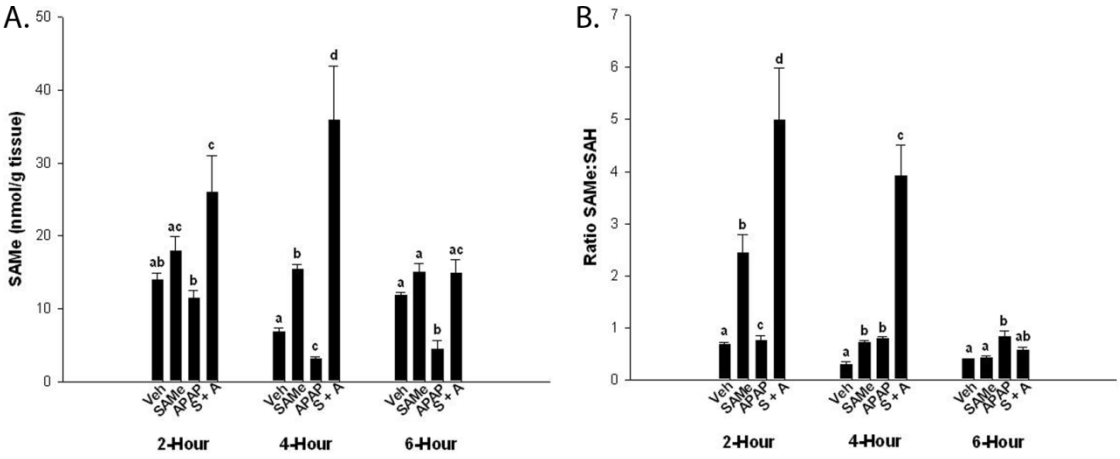

Figure 9. Alterations of 15,000 x g supernatant fraction SAMe levels following APAP overdose in C57BL/6 mice. The mice were randomly allocated into Veh (water), SAMe (1.25 mmol/kg), APAP (250 mg/kg), and SAMe administered 1 hour following APAP (S + A). Livers were collected at 2, 4 and 6 hours following APAP administration and processed for collection of the 15,000 *x g* supernatant. Levels of SAMe were determined by HPLC. Panel A represents SAMe levels in the 15,000 *x* g supernatant fraction, while panel B is the ratio of SAMe:SAH in the same samples. All values represent mean ± S.E.M. with n=5 mice/group. Different letters denote statistical differences ($p<0.05$), while groups with the same letters indicate no difference in the results.

APAP treatment altered subcellular hepatic nuclear levels of SAMe. No change was observed in nuclear SAMe levels 4 hours following APAP administration between Veh, SAMe and S + A groups. The detection limit was 12.5 pmol and the APAP treated group all had nuclear SAMe levels below the detection limit of 12.5 pmol (Figure 10). Levels of SAH could be determined 4 hours following APAP overdose, but there was no significant change observed in any of the groups (Table 7). The ratio of SAMe:SAH in the nuclei of the liver cells was also unchanged when observed at 4 hours (Figure 10). The ratio for the APAP group could not be determined because of the lack of SAMe data.

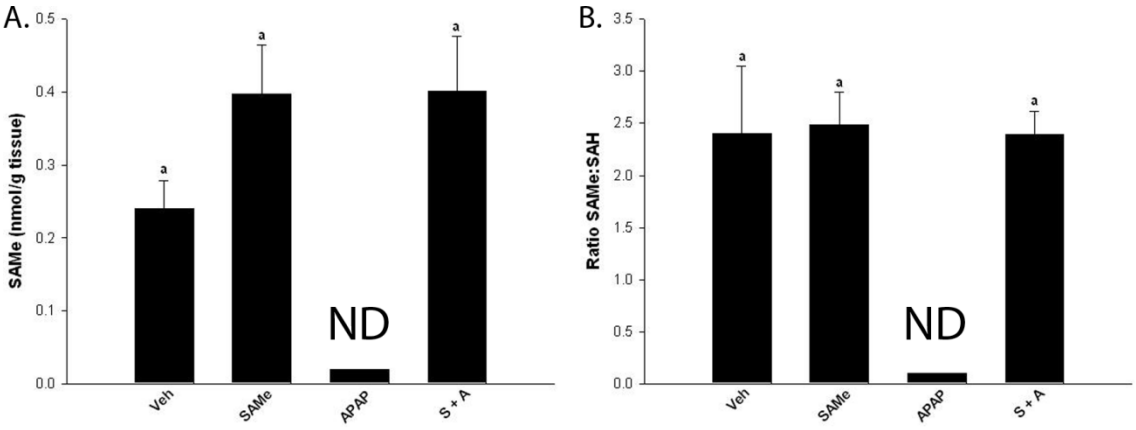

Figure 10. Alterations of nucleus SAMe levels following APAP overdose in C57BL/6 mice. Mice were randomly put into Veh (water), SAMe (1.25 mmol/kg), APAP (250 mg/kg), or SAMe administered 1 hour following APAP groups. Livers were collected at 4 hours and nuclei isolated. SAMe levels were determined by HPLC analysis. All groups had n=3-5 and values are listed as mean ± S.E.M. Panel A represents levels of SAMe in the nucleus of cells following APAP overdose, and panel B represents the ratio of SAMe:SAH in the same groups. SAMe levels in the APAP treatment group were below the current detection limit of 12.5 pmol (ND group). Different letters denote statistical differences ($p<0.05$), while groups with the same letters indicate no difference in the results.

Table 7. Levels of SAH in the nucleus following APAP overdose.

	Group	Nucleus SAH Levels (nmol/g tissue)
SAMe 4 h	Veh	0.109 ± 0.0163^a
	APAP	0.159 ± 0.0122^a
	SAMe	0.143 ± 0.0279^a
	S + A	0.167 ± 0.0272^a

Mice were randomly allocated into the following groups: Veh (water), SAMe (1.25 mmol/kg), APAP (250 mg/kg), SAMe 1 hour after APAP (S + A). SAMe and APAP were administered by ip injection. Values represent Mean ± S.E.M. (n=5-10). Nuclear SAH levels were measured 4 and 6 hours post APAP or Veh treatment.

[a] Different letters denote statistical differences ($p<0.05$), while groups with the same letters indicate no difference in the results.

Analysis of MAT I/III expression in the liver indicates that there is a significant decrease in the levels of MAT I/III in the APAP group when compared to the Veh group 2 and 4 hours following APAP administration (Figure 11). The administration of SAMe 1 hour after APAP

increased levels of MAT I/III back to Veh level at 2 and 4 hours following APAP overdose (Figure 11). The presence of MAT II was not detected in hepatic tissue 2 and 4 hours following APAP overdose (Figure 11). Protein transfer was confirmed by staining as described in *Methods and Materials* and recombinant MAT II protein was used to confirm the viability of the MAT II antibody (Figure 11).

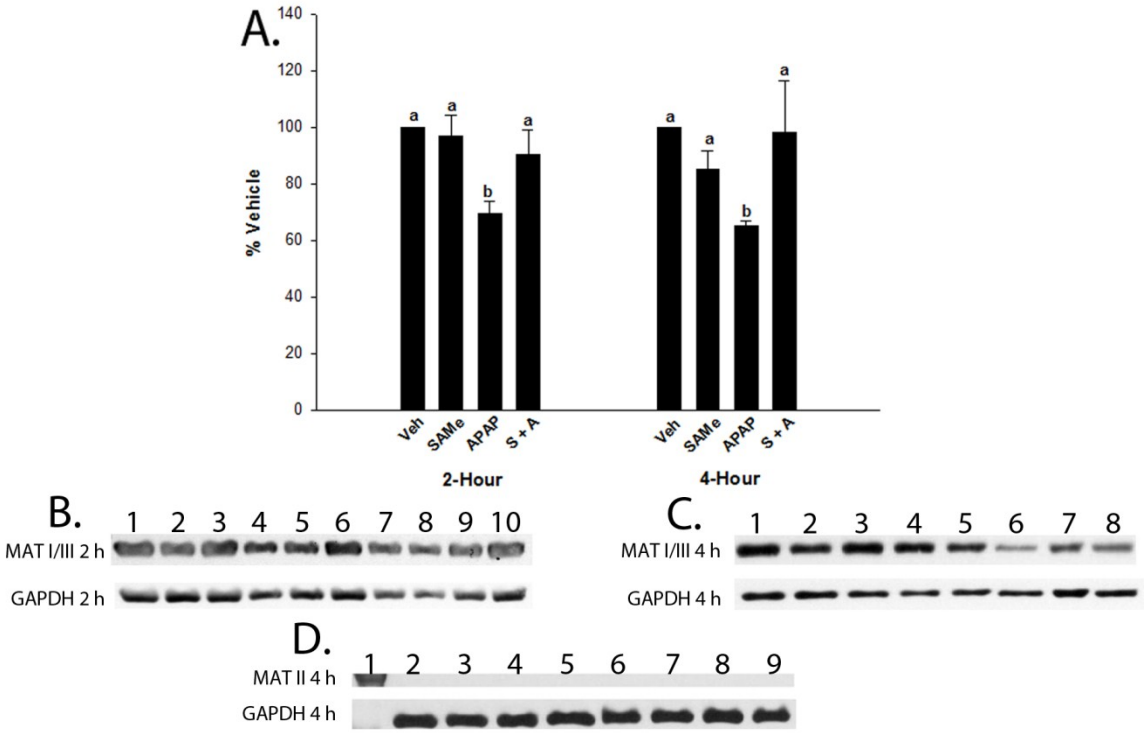

Figure 11. MAT I/II/III expression in hepatic tissue 2 hours and 4 hours following APAP overdose in C57BL/6 mice. Hepatic tissue was collected after administration of Veh (water), 1.25 mmol/kg SAMe, 250 mg/kg APAP, and SAMe 1 hour following APAP. Each lane was loaded with 100 µg protein and detection was accomplished with chemiluminescence. Panel A of the figure represents the density of the individual MAT I/III bands expressed as % Veh (n=3-5 animals/group). Values represent mean ± S.E.M. Different letters denote statistical differences ($p<0.05$), while groups with the same letters indicate no difference in the results. Panel B is a representative blot 2 hours following APAP administration. Lanes 1, 2, and 3 represent Veh samples. Lanes 4, 5, and 6 represent SAMe samples. Lanes 7 and 8 represent APAP samples, while 9 and 10 represent S + A treatment groups. Panel C is a representative blot 4 hours following APAP administration. Lanes depicted are as follows: Veh (1 and 2), SAMe (3 and 4), APAP (5 and 6), and S + A (7 and 8). Panel D is a representative example of MAT II expression 4 hours following APAP administration. Lane assignments include: recombinant MAT II (1), Veh (2 and 3), SAMe (4 and 5), APAP (6 and 7), and S + A (8 and 9). Similar blots were conducted for MAT II expression 2 hours following APAP administration with lack of expression also observed (data not shown).

MAT activity was significantly increased in the APAP treatment group when compared to the SAMe and S + A groups 2 hours following administration of APAP (Figure 12). Although the trend is there, the Veh group was not significantly elevated compared to the SAMe and S + A groups at 2 hours (Figure 12). No differences were observed in MAT activity 4 hours following

APAP overdose (Figure 12). When taken together with the 2 hour MAT expression data, it is observed that MAT activity in the SAMe and S + A groups decrease compared to the APAP group, while expression of MAT in the APAP group is decreased 2 hours following APAP administration.

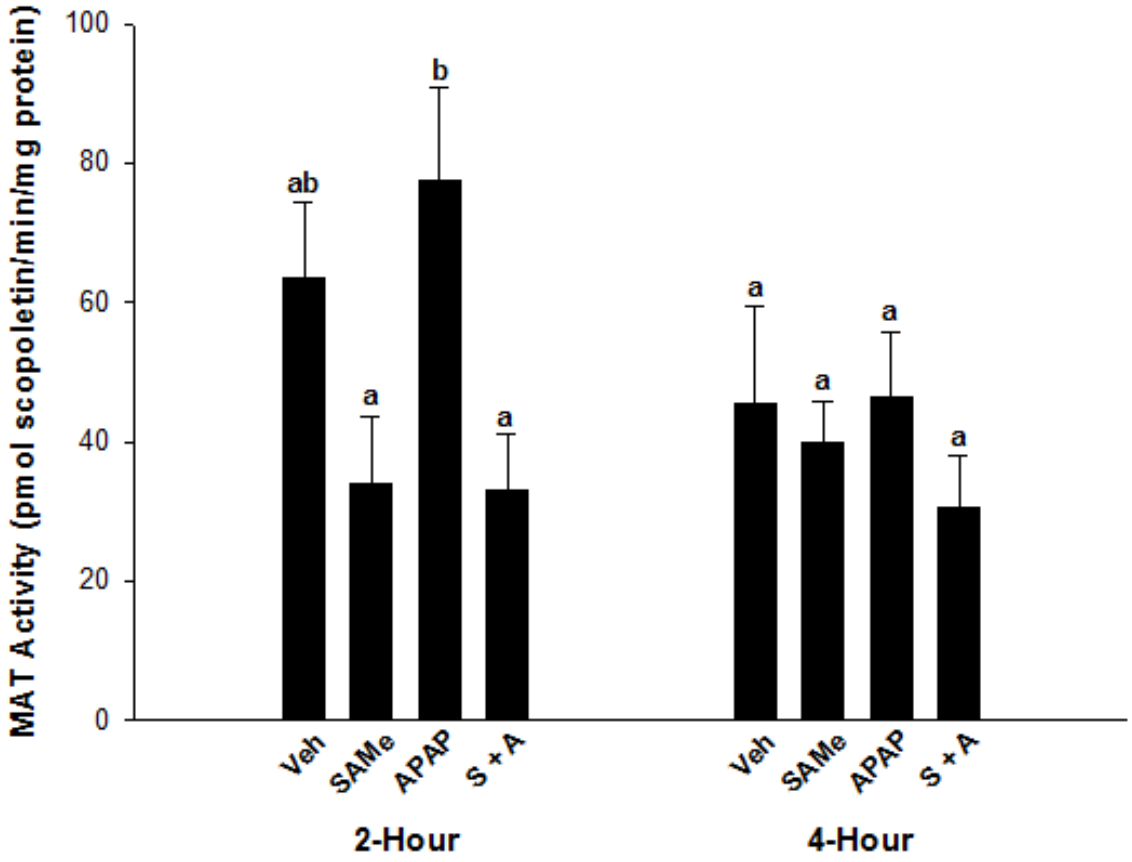

Figure 12. MAT activity alterations following APAP overdose. Mice were allocated at random into Veh (water), SAMe (1.25 mmol/kg), APAP (250 mg/kg), or SAMe administered 1 hour following APAP groups. Livers were collected 2 and 4 hours following APAP injection and MAT activity was determined by an endpoint fluorescence assay measuring pmol of scopoletin formed per minute per mg protein. Values represent mean ± S.E.M. (n=5 mice per group). Different letters denote statistical differences ($p<0.05$), while groups with the same letters indicate no difference in the results.

Discussion

SAMe has been demonstrated to be an effective treatment for APAP overdose in mouse animal models (Stramentinoli et al., 1979; Valentovic, et al., 2004). Although it is known that SAMe can participate in the replenishment of GSH through the transsulfuration pathway (Lu, 1998), other mechanisms of protection remain to be explored. In order to gain an understanding of SAMe metabolism in the liver following APAP overdose, the purpose of the current study was to analyze hepatic SAMe and SAH levels. The observed decrease in SAMe levels following APAP overdose could prevent any protection that SAMe could afford, and also could act as a precursor to APAP toxicity, much as the depletion of GSH precedes APAP toxicity (Moore et al., 1985). In addition, the observed decrease in MAT I/III expression in the liver at 4 hours would lead to SAMe depletion following APAP overdose.

SAMe is the primary methyl donor in cells and is capable of methylating phospholipids, proteins, and nucleic acids. Polyamine synthesis is dependent on adequate cellular SAMe levels (Lieber and Packer, 2002). Studies of the effects of toxicity on SAMe levels in the cell are lacking, especially in the area of subcellular analysis of SAMe levels. Perturbations in the ratio of SAMe:SAH can inhibit transmethylation reactions and conceivably the ability of SAMe to protect against APAP toxicity (Finkelstein et al., 1974). In the current study of total liver SAMe, the ratio of SAMe:SAH was increased in the S + A group at two hours following APAP administration (Figure 6). The levels of SAMe were decreased in whole liver 4 and 6 hours after APAP treatment, which would inhibit crucial transmethylation reactions. When the ratios of SAMe:SAH in liver homogenate was calculated there was an observed increase in the ratio at 2 hours when comparing the Veh and APAP groups, no change at 4 hours, and a decrease in the APAP treatment group when compared to Veh at 6 hours. The trend observed in the ratios was

due to the SAH levels decreasing 2 hours following APAP administration and steadily increasing thereafter. Decrease of the ratio of SAMe:SAH could inhibit crucial transmethylation reactions. Of interest for clinical situations was that the administration of SAMe following APAP overdose lead to an increase in liver homogenate SAMe back to control levels.

Mitochondria are dependent on the presence of SAMe for proper function. Inhibition of SAMe transport into hepatic mitochondria has been demonstrated to sensitize the cells to tumor necrosis factor (TNF) hepatotoxicity (Song et al., 2007). TNF has been shown to be unregulated in response to APAP overdose (Dambach et al., 2006). Also, SAMe has been demonstrated to aid in the maintenance of mitochondrial membrane potential in a cell culture model (Lotkova et al., 2005). One of the hallmarks of APAP toxicity is mitochondrial dysfunction (Bajt et al., 2008). We present here, for the first time, analysis of mitochondrial and 15,000 *x g* supernatant levels of SAMe following APAP overdose.

The observed decrease in mitochondrial SAMe levels could make mitochondria more susceptible to APAP toxicity. We were also able to establish a time course for the depression of mitochondrial SAMe levels occurring between 2 and 4 hours following APAP administration. A spike in cellular SAMe levels was observed in liver homogenate, mitochondria, and 15,000 *x g* supernatant at 2 hours following SAMe administration. When SAMe was administered following APAP, it was able to dramatically increase levels of mitochondrial SAMe at 2, 4, and 6 hours. SAMe increased to levels surpassing the control mice indicating that SAMe transport into mitochondria is not inhibited by APAP toxicity. Further studies may provide better insight into mitochondrial SAMe transport regulation in response to APAP toxicity.

The purpose of SAMe in the cytosol is to function as a methylator and in the synthesis of polyamines as well as participating in the transsulfuration pathway to replenish cellular reduced glutathione. At 4 and 6 hours following APAP overdose, the levels of SAMe in the 15,000 *x g* supernatant are depressed when compared to the control mice. When SAMe was administered following APAP it was able to aid in the recovery of SAMe levels to amounts surpassing controls at 2 and 4 hours.

Given the role of SAMe in methylation of DNA, we examined the amount of SAMe present in the nuclei of cells treated with APAP. Caudill et al. (2001) noted that, in cases of SAH elevation, DNA trended toward hypomethylation regardless of SAMe levels. We found that levels of SAH were not elevated in the APAP treatment group compared to any other group indicating that it would be unlikely for the hepatocytes to experience DNA hypomethylation as a result of APAP toxicity even though SAMe levels dropped below the detectable limit of 12.5 pmol in the APAP treatment groups.

Expression of MAT I/III is important for the protection of the liver, and its absence can lead to unwanted proliferation of hepatocytes (Lu et al., 2001). These findings have significance because the presence of MAT I/III appears to be necessary for the normal function of hepatocytes (Lu and Mato, 2008). Furthermore, SAMe has been found to prevent dysfunction of MAT I/III following administration of carbon tetrachloride, a prototypical liver toxicant (Corrales et al., 1992). MAT levels were significantly depressed at 2 and 4 hours following APAP administration when compared with the Veh groups. SAMe levels have been found to fall initially following partial hepatectomy; however, expression of *MAT1A* increases as soon as 3 hours following partial hepatectomy (Huang et al., 1998). Our data did not demonstrate the

increase in expression of MAT I/III proteins, but we did not look at mRNA expression as did Huang and associates.

Unfortunately, the rise in MAT II expression that typically accompanies liver regeneration was not observed by our laboratory at 2 or 4 hours following APAP overdose (Latasa et al., 2001). Perhaps future studies monitoring time periods beyond 4 hours following APAP administration will yield an appreciable expression of MAT II in APAP treated mice. In a prior study looking at MAT II following partial hepatectomy, a rise in the mRNA of *MAT2A* was observed as soon as 6 hours following the surgery (Horikawa et al., 1996). Future studies would also examine mRNA levels for MAT II which could yield earlier indications that MAT II was being upregulated.

Work done on MAT activity concluded that MAT I and MAT II activity are inhibited by SAMe, while MAT III is actually activated by SAMe (Sullivan and Hoffman, 1983). Given that there was no expression of MAT II observed at the time periods studied, the activity present comes from MAT I/III. When SAMe was administered either alone or after APAP overdose, activity of MAT was significantly decreased when compared with the APAP group at 2 hours. MAT activity was not different between groups 4 hours post APAP treatment. Given the propensity of SAMe to inhibit MAT, the data at 2 hours following APAP administration is easily understood. A spike in SAMe levels occurs 2 hours following APAP overdose in liver homogenate which could lead to the observed MAT inhibition without a significant change in expression for the SAMe or the SAMe administered following APAP group. By 4 hours the increase in SAMe brought about by SAMe administration in liver homogenate samples has largely dissipated leading to the return of MAT activity to Veh levels in the SAMe and S + A groups. Previous research found a link between reduced glutathione depletion and decreased MAT activity (Corrales et al., 1991). The same group found that cysteine modification was most likely

responsible for the observed decrease in MAT activity (Pajares et al., 1991). Cellular reduced glutathione depletion is a hallmark of APAP toxicity making the current findings interesting in the sense that APAP administration did not reduce activity of MAT at 2 or 4 hours (Mitchell et al., 1973). The observed decrease in MAT I/III expression could be because of covalent modifications at the antibody binding site and, if so, does not translate to a decrease in MAT activity for the time periods studied.

In conclusion, this is the first study to report a time analysis of SAMe levels in mitochondria, 15,000 *x g* supernatant, and the nucleus following APAP overdose. APAP overdose decreased SAMe in whole liver, mitochondria, and 15,000 *x g* supernatant at 4 and 6 hours, while there is no depression of SAMe levels observed at 2 hours. When SAMe was administered following APAP, the levels of SAMe did not decline. SAMe was not detectable in the nucleus at 2 hours following APAP treatment even though our level of sensitivity was 12.5 pmol of SAMe. We have also demonstrated here for the first time that administration of NAC following APAP aided in the maintenance of liver homogenate SAMe levels 4 hours after APAP overdose. At both 2 and 4 hours following APAP administration, levels of MAT were significantly decreased with respect to the Veh group in the APAP treatment group. MAT activity tended to decrease when SAMe was administered and livers collected at 2 hours following APAP overdose, but no changes were observed at 4 hours. These results provide important insight into the effects of APAP overdose on SAMe metabolism in C57BL/6 mice and chart a path for future study.

Acknowledgements

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References

Andersson, B.S., Rundgren, M., Nelson, S.D., and Harder, S. 1990. N-acetyl-p-benzoquinone imine-induced changes in the energy metabolism in hepatcytes. Chem Biol Interact. 75, 201-211.

Andringa, K.K., Bajt, M.L., Jaeschke, H., and Bailey, S.M. 2008. Mitochondrial protein thiol modifications in acetaminophen hepatotoxicity: effect on HMG-CoA synthase. Toxicol Lett. 117, 188-197.

Aqrimi, G., Di Noia, M.A., Marobbio, C.M., Fiermonte, G., Lasorsa, F.M., and Palmieri, F. 2004. Identification of the human mitochondrial S-adenosylmethionine transporter: bacterial expression, reconstitution, functional characterization and tissue distribution. Biochem J. 379, 183-190.

Bajt, M.L., Farhood, A., Lemasters, J.J, and Jaeschke, H. 2008. Mitochondrial bax translocation accelerates DNA fragmentation and cell necrosis in a murine model of acetaminophen hepatotoxicity. J Pharmacol Exp Ther. 324, 8-14.

Boyd, E.M., and Bereczky, G.M. 1966. Liver necrosis from paracetamol. Br J Pharmacol Chemother. 26, 606-614.

Bray, G.P., Tredger, J.M., and Williams, R. 1992. S-adenosylmethionine protects against acetaminophen hepatotoxicity in two mouse models. Hepatology. 15, 297-301.

Caudill, M.A., Wang, J.C., Melnyk, S., Pogribny, I.P., Jernigan, S., Collins, M.D., Santos-Guzman, J., Swendseid, M.E., Coqqer, E.A., and James, S.J. 2001. Intracellular S-adenosylhomocysteine concentrations predict global DNA hypomethylation in tissues of methyl-deficient cystathionine beta-synthase heterozygous mice. J Nutr. 131, 2811-2818.

Chen, L., Zeng, Y., Yang, H., Lee, T.D., French, S.W., Corrales, F.J., Garcia-Trevijano, E.R., Avila, M.A., Mato, J.M., and Lu, S.C. 2004. Impaired liver regeneration in mice lacking methionine adenosyltransferase 1A. FASEB J. 18, 914-916.

Corcoran, G.B., Mitchell, J.R., Vaishnav, Y.N., and Horning, E.C. 1980. Evidence that acetaminophen and N-hydroxyacetaminophen form a common arylating intermediate, N-acetyl-p-benzoquinoneimine. Mol Pharmacol. 18, 536-542.

Corcoran, G.B., Racz, W.J., Smith, C.V., and Mitchell, J.R. 1985. Effects of N-acetylcysteine on acetaminophen covalent binding and hepatic necrosis in mice. J Pharmacol Exp Ther. 232, 864-872.

Corrales, F., Gimenez, A., Alvarez, L., Caballeria, J., Pajares, M.A., Andreu, H., Pares, A., Mato, J.M., and Rodes, J. 1992. S-adenosylmethionine treatment prevents carbon tetrachloride-induced S-adenosylmethionine synthetase inactivation and attenuates liver injury. Hepatology. 16, 1022-1027.

Corrales, F., Ochoa, P., Rivas, C., Martin-Lomas, M., Mato, J.M., and Pajares, M.A. (1991). Inhibition of glutathione synthesis in the liver leads to S-adenosyl-L-methionine synthetase reduction. Hepatology. 14, 528-533.

Dahlin, D.C., Miwa, G.T., Lu, A.Y., and Nelson, S.D. 1984. N-acetyl-p-benzoquinone imine: A cytochrome P-450-mediated oxidation product of acetaminophen. Proc Natl Acad Sci U.S.A. 81, 1327-1331.

Dambach, D.M., Durham, S.K., Laskin, J.D., and Laskin, D.L. 2006. Distinct roles of NF-kappa B p50 in the regulation of acetaminophen-induced inflammatory mediator production and hepatotoxicity. Toxicol Appl Pharmacol. 211, 157-165.

Finkelstein, J.D. 1990. Methionine metabolism in mammals. J Nutr Biochem. 1, 228-237.

Finkelstein, J.D., Kyle, W.E., and Harris, B.J. 1974. Methionine metabolism in mammals: regulatory effects of S-adenosylhomocysteine. Arch Biochem Biophys. 165, 774-779.

Gogvadze, V., Orrenius, S., and Zhivotovsky, B. 2004. "Analysis of Mitochondrial Dysfunction During Cell Death." Current Protocols in Toxicology. John Wiley & Sons, Inc. Hoboken, NJ. pp. 2.10.1-2.10.27.

Golden, D.P., Mosby, E.L., Smith, D.J., and Mackercher, P. 1981. Acetaminophen toxicity. Report of two cases. Oral Surg Oral Med Oral Pathol. 51, 385-389.

Graham, J.M. 2001. "Isolation of nuclei and nuclear membranes from animal tissues." Current Protocols in Cell Biology. John Wiley & Sons, Inc. Hoboken, NJ. Chapter 3: Unit 3.10.

Hinson, J.A., Bucci, T.J., Irwin, L.K., Michael, S.L., and Mayeux, P.R. 2002. Effect of inhibitors of nitric oxide synthase on acetaminophen-induced hepatotoxicity in mice. Nitric Oxide. 6: 160-167.

Horikawa, S., Ozasa, H., Ito, K., Katsuyama, I., Tsukada, K., and Sugiyama, T. 1996. Expression of S-adenosylmethionine synthetase isozyme genes in regenerating rat liver after partial hepatectomy. Biochem Mol Biol Int. 40, 807-814.

Huang, Z.Z., Mao, Z., Cai, J., and Lu, S.C. 1998. Changes in methionine adenosyltransferase during liver regeneration in the rat. Am J Physiol. 275, G14-G21.

Kharbanda, K.K. 2007. Role of transmethylation reactions in alcoholic liver disease. World J Gastroenterol. 13, 4947-4954.

Knight, T.R., Fariss, M.W., Farhood, A., and Jaeschke, H. 2003. Role of lipid peroxidation as a mechanism of liver injury after acetaminophen overdose in mice. Toxicol Sci. 76: 229-236.

Kotb, M., Mudd, S.H., Mato, J.M., Geller, A.M., Kredich, N.M., Chou, J.Y., and Cantoni, G.L. 1997. Consensus nomenclature for the mammalian methionine adenosyltransferase genes and gene products. Trends Genet. 13, 51-52.

Larrauri, A., Fabra, R., Gomez-Lechon, M.J., Trullenque, R., and Castell, J.V. 1987-1988. Toxicity of paracetamol in human hepatocytes. Comparison of the protective effects of sulfhydryl compounds acting as glutathione precursors. Mol Toxicol. 1, 301-311.

Latasa, M.U., Boukaba, A., Garcia-Trevijano, E.R., Torres, L., Rodriquez, J.L., Caballeria, J., Lu, S.C., Lopez-Rodas, G., Franco, L., Mato, J.M., and Avila, M.A. 2001. Hepatocyte growth factor induces MAT2A expression and histone acetylation in rat hepatocytes: role in liver regeneration. FASEB J. 15, 1248-1250.

Lieber, C.S., and Packer, L. 2002. S-adenosylmethionine: molecular, biological, and clinical aspects—an introduction. Am J Clin Nutr. 76, 1148S-1150S.

Lotkova, H., Cervinkova, Z., Kucera, O., Krivakova, P., and Kand'ar, R. 2005. Protective effect of S-adenosylmethionine on cellular and mitochondrial membranes of rat hepatocytes against tert-butylhydroperoxide-induced injury in primary culture. Chem Bio Interact. 156, 13-23.

Lu, S.C., Alvarez, L., Huang, Z.Z., Chen, L., An, W., Corrales, F.J., Avila, M.A., Kanel, G., and Mato, J.M. 2001. Methionine adenosyltransferase 1A knockout mice are predisposed to liver injury and exhibit increased expression of genes involved in proliferation. Proc Natl Acad Sci U S A. 98, 5560-5565.

Lu, S.C., and Mato, J.M. 2008. S-Adenosylmethionine in cell growth, apoptosis, and liver cancer. J Gastroenterol Hepatol. Suppl 1, S73-S77.

Lu, S.C. 2000. S-Adenosylmethionine. Int J Biochem Cell Biol. 32, 391-395.

Lu, S.C. 1998. Regulation of hepatic glutathione synthesis. Semin Liver Dis. 18, 331-343.

Martinez-Chantar, M.L., Garcia-Trevijano, E.R., Latasa, M.U., Martin-Duce, A., Fortes, P., Caballeria, J., Avila M.A., and Mato, J.M. 2003. Methionine adenosyltransferase II beta subunit gene expression provides a proliferative advantage in human hepatoma. Gastroenterology. 124, 940-948.

Mitchell, J.R., Jollow, D.J., Potter, W.Z., Gillette, J.R., and Brodie, B.B. 1973. Acetaminophen-induced hepatic necrosis. IV. Protective role of glutathione. J. Pharmacol. Exp. Ther. 187: 211-217.

Moore, M., Thor, H., Moore, G., Nelson, S., Moldeus, P., and Orrenius, S., 1985. The toxicity of acetaminophen and N-acetyl-p-benzoquinone imine in isolated hepatocytes is associated with thiol depletion and increased cytosolic Ca2+. J Biol Chem. 260, 13035-13040.

Nourjah, P., Ahmad, S.R., Karwoski, C., and Willy, M. 2006. Estimates of acetaminophen (Paracetomol)-associated overdoses in the United States. Pharmacoepidemiol Drug Saf. 15, 398-405.

Pajares, M.A., Corrales, F.J., Ochoa, P., and Mato, J.M. 1991. The role of cysteine-150 in the structure and activity of rat liver S0adenosyl-L-methionine synthetase. Biochem J. 275 (Pt 1): 225-229.

Paneda, C., Gorospe, I., Herrera, B., Nakamura, T., Fabregat, I., and Varela-Nieto, I. 2002. Liver cell proliferation requires methionine adenosyltransferase 2A mRNA up-regulation. Hepatology. 35, 1381-1391.

Patten, C.J., Thomas, P.E., Guy, R.L., Lee, M., Gonzalez, F.J., Guengerich, F.P., and Yang, C.S. 1993. Cytochrome P450 enzymes involved in acetaminophen activation by rat and human liver microsomes and their kinetics. Chem Res Toxicol. 6, 511-518.

Purohit, V., Abdelmalek, M.F., Barve, S., Benevenga, N.J., Halsted, C.H., Kaploqitz, N., Kharbanda, K.K., Liu, Q.Y., Lu, S.C., McClain, C.J., Swanson, C., and Zakhari, S. 2007. Role

of S-adenosylmethionine, folate, and betaine in the treatment of alcoholic liver disease: summary of a symposium. Am J Clin Nutr. 86, 12-24.

Reid, A.B., Kurten, R.C., McCullough, S.S., Brock, R.W., and Hinson, J.A. 2005. Mechanisms of acetaminophen-induced hepatotoxicity: role of oxidative stress and mitochondrial permeability transition in freshly isolated mouse hepatocytes. J Pharmacol Exp Ther. 312, 509-516.

Smilkstein, M.J., Knapp, G.L., Kulig, K.W., and Rumack, B.H. 1988. Efficacy of oral N-acetylcysteine in the treatment of acetaminophen overdose. Analysis of the national multicenter study (1976 to 1985). New Engl J Med. 319, 1557-1562.

Song, Z., Zhou, Z., Song, M., Uriarte, S., Chen, T., Deaciuc, I., and McClain, C.J. 2007. Alcohol-induced S-adenosylhomocysteine accumulation in the liver sensitizes to TNF hepatotoxicity: possible involvement of mitochondrial S-adenosylmethionine transport. Biochem Pharmacol. 74, 521-531.

Stramentinoli, G., Pezzoli, C., and Galli-Kienle, M. 1979. Protective role of S-adenosyl-L-methionine against acetaminophen induced mortality and hepatotoxicity in mice. Biochem Pharmacol. 28, 3567-3571.

Streeter, A.J., Dahlin, D.C., Nelson, S.D., and Baillie, T.A. 1984. The covalent binding of acetaminophen to protein. Evidence for cysteine residues as major sites of arylation in vitro. Chem Biol Interact. 48, 349-366.

Sullivan, D.M., and Hoffman, J.L. 1983. Fractionation and kinetic properties of rat liver and kidney methionine adenosyltranferase isozymes. Biochemistry. 22, 1636-1641.

Terneus, M.V., Brown, J.M., Carpenter, A.B., and Valentovic, M.A. 2008. Comparison of S-adenosyl-L-methionine (SAMe) and N-acetylcysteine (NAC) protective effects on hepatic damage when administered after acetaminophen overdose. Toxicology. 244, 25-34.

Terneus, M.V., Kiningham, K.K., Carpenter, A.B., Sullivan, S.B., and Valentovic, M.A. 2007. Comparison of S-adenosyl-L-methionine and N-acetylcysteine protective effects on acetaminophen hepatic toxicity. J Pharmacol Exp Ther. 320, 99-107.

Valentovic, M., Terneus, M., Harmon, R.C., and Carpenter, A.B. 2004. S-adenosylmethionine (SAMe) attenuates acetaminophen hepatotoxicity in C57BL/6 mice. Toxicol Lett. 154, 165-174.

Wang, S.H., Kuo, S.C., and Chen, S.C. 2003. High-performance liquid chromatography determination of methionine adenosyltransferase activity using catechol-*O*-methyltransferase-coupled fluorometric detection. Anal Biochem. 319, 13-20.

Wang, W., Kramer, P.M., Yang, S., Pereira, M.A., and Tao, L. 2001. Reversed-phase high-performance liquid chromatography procedure for the simultaneous determination of S-adenosyl-L-methionine and S-adenosyl-L-homocysteine in mouse liver and the effect of methionine on their concentrations. J Chromatogr B Biomed Sci Appl. 762, 59-65.

Williams, A.L., Girard, C., Jui, D., Sabina, A., and Katz, D.L. 2005. S-adenosylmethionine (SAMe) as treatment for depression: a systematic review. Clin Invest Med. 28, 132-139.

Chapter IV: Novel Protective Mechanisms for S-Adenosyl-L-methionine against Acetaminophen Hepatotoxicity: Improvement of Key Antioxidant Enzymatic Function

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J. Michael Brown*, John G. Ball*, Michael Scott Wright*, Stephanie Van Meter*, and Monica A. Valentovic*[1]

*Department of Pharmacology, Physiology, and Toxicology,

Joan C. Edwards School of Medicine, Marshall University, Huntington, WV 25755

Email addresses: JMB (mike.brown555@gmail.com); JGB (BallJ@marshall.edu); MSW (Wright172@live.marshall.edu); SVM (Vanmete4@marshall.edu); MAV (Valentov@marshall.edu)

[1]To whom correspondence should be addressed: Department of Pharmacology, Physiology, and Toxicology; 1 John Marshall Drive, Joan C. Edwards School of Medicine, Marshall University, Huntington, WV 25755. Phone (304-696-7332); Fax (304-696-7391); Email: Valentov@marshall.edu

Abstract

Acetaminophen (APAP) overdose leads to severe hepatotoxicity, increased oxidative stress and mitochondrial dysfunction. S-adenosyl-L-methionine (SAMe) protects against APAP toxicity at a mmol/kg equivalent dose to N-acetylcysteine (NAC). SAMe acts as a principal biological methyl donor and participates in polyamine synthesis which increase cell growth and has a role in mitochondrial protection. The purpose of the current study tested the hypothesis that SAMe protects against APAP toxicity by maintaining critical antioxidant enzymes and markers of oxidative stress. Male C57Bl/6 mice were treated with vehicle (Veh; water 15 ml/kg, ip), SAMe (1.25 mmol/kg, ip), APAP (250 mg/kg, ip), and S + A (SAMe given 1 hour following APAP). Liver was collected 2 and 4 hours following APAP administration; mitochondrial swelling as well as hepatic catalase, glutathione peroxidase (GPx), glutathione reductase, and both Mn- and Cu/Zn-superoxide dismutase (SOD) enzyme activity were evaluated. Mitochondrial protein carbonyl, 3-nitrotyrosine cytochrome c leakage were analyzed by Western blot. SAMe significantly increased SOD, GPx, and glutathione reductase activity at 4 hours following APAP overdose. SAMe greatly reduced markers of oxidative stress and cytochrome C leakage following APAP overdose. Our studies also demonstrate that a 1.25 mmol/kg dose of SAMe does not inhibit CYP 2E1 enzyme activity. The current study identifies a plausible mechanism for the decreased oxidative stress observed when SAMe is given following APAP.

KEY WORDS: S-adenosyl-L-methionine, Acetaminophen, Hepatotoxicity, Oxidative stress, Antioxidant, Nitrotyrosine

1. Introduction

Acetaminophen (APAP) is the leading cause of drug induced liver injury. APAP overdose is a serious clinical problem resulting in over 26,000 hospitalizations per year in the United States (Nourjah et al., 2006). APAP toxicity results in severe hepatic centrilobular necrosis (Anundi et al., 1993). Currently, N-acetylcysteine (NAC) is the accepted treatment for APAP overdose in humans.

APAP toxicity requires biotransformation by cytochrome P450 isozymes 2E1, 1A2, and 3A4 of APAP to N-acetyl-p-benzoquinoneimine (NAPQI), the ultimate hepatotoxic metabolite (Dahlin et al., 1984; Patten et al., 1993; Chen et al., 1998). Excess NAPQI rapidly depletes cellular stores of reduced glutathione (GSH) and subsequently adducts proteins in the liver precipitating cellular dysfunction (Hinson et al., 1995). The current treatment for APAP toxicity, NAC, works by replenishing cellular GSH to prevent the cell from being overwhelmed by NAPQI (Smilkstein et al., 1988). Because of the rapid conversion of APAP to NAPQI, prompt administration of NAC is critical to improving the clinical outcome; therefore, therapeutic interventions that work longer term to combat APAP toxicity are desirable.

APAP hepatotoxicity is characterized by greatly increased oxidative stress due to mitochondrial dysfunction and the generation of superoxide (Andersson et al., 1990). A common indicator of oxidative stress is lipid peroxidation, which has been found to be greatly increased in response to APAP toxicity (Wendel et al., 1979). In addition to the generation of reactive oxygen species (ROS), APAP also causes the production of reactive nitrogen species (RNS) leading to nitrotyrosine adduction of proteins (Hinson et al., 2000). Histology indicates that nitrotyrosine adduction is localized to the centrilobular region where APAP toxicity is most prevalent.

A key defense modulating the severity of APAP hepatotoxicity are the antioxidant enzymes glutathione reductase (GSSG reductase), glutathione peroxidase (GPx), catalase, and superoxide dismutase (SOD). APAP toxicity has previously been demonstrated to reduce antioxidant enzyme activities and a few exogenous treatments have been tested to restore enzymatic function (Olaleye et al., 2008; Wu et al., 2010). NAC has also been demonstrated to protect the function of GPx and SOD when administered 2 hours prior to APAP overdose (300 mg/kg body weight) (Wang et al., 2010). An endogenously produced molecule, such as S-adenosyl-L-methionine (SAMe), with the same protective benefits could prove a beneficial therapeutic compound.

Mitochondrial dysfunction is another hallmark of APAP toxicity and a contributing factor to the greatly increased oxidative stress observed with APAP overdose. Masubuchi and others determined that APAP injury induces mitochondrial permeability transition (MPT) in mice which is characterized by the loss of membrane potential and cellular ATP depletion (Masubuchi et al., 2005). MPT is also associated with the exodus of proteins from the mitochondrial inner membrane such as cytochrome c which has a role in initiating apoptosis (El-Hassan et al., 2003). ATP depletion occurs with APAP toxicity and acts as a precursor to the necrotic damage by inhibiting completion of apoptosis (Jaeschke and Bajt, 2006).

Prior research by our laboratory has demonstrated the ability of S-adenosyl-L-methionine (SAMe) to protect against APAP toxicity at a mmol/kg dose comparable to NAC (Terneus et al., 2007 and 2008). Humans produce 6-8 g per day of SAMe which is necessary for methylation of cellular proteins, DNA, and phospholipids. In addition to transmethylation reactions, SAMe also participates in the transsulfuration pathway to aid in the replenishment of GSH (Lu, 2000). SAMe is known to participate in polyamine synthesis which has a critical role in enhancing liver regeneration following partial hepatectomy (Fernandez et al., 2003; Brosnan and Brosnan, 2006).

Polyamine synthesis and the ability to aid in liver regeneration are both unique properties that SAMe possesses over NAC as a potential therapeutic intervention. SAMe has already been recognized to protect mitochondria from alcohol-induced dysfunction (Bailey et al., 2006; Song et al., 2007). Additionally, our laboratory has previously demonstrated SAMe's ability to reduce oxidative stress markers such as the formation of protein carbonyls associated with APAP hepatic toxicity (Terneus et al., 2008).

Given SAMe's proven ability to protect against APAP toxicity, the current study sought to deepen the understanding of the mechanism of SAMe protection. First, subcellular protection by SAMe for APAP hepatic damage within the mitochondria and cytosol is not known. Second, information is lacking on the temporal protection of antioxidant enzyme activity by SAMe following exposure to APAP overdose. If protection of antioxidant enzyme activity by SAMe is minimal or only occurs after hepatic damage is manifested, then maintaining antioxidant enzyme activity is not a primary mechanism for SAMe protection of APAP hepatic toxicity. Additionally, protein carbonyl formation and nitrotyrosine adduction were analyzed on mitochondrial samples to determine levels of oxidative stress at a mitochondrial level with SAMe treatment after APAP overdose.

2. Methods and Materials

2.1 Reagents. SAMe toluene sulfonate salt was used for the experiments and was purchased from Sigma-Aldrich Chemical Company (St. Louis, MO). All other chemicals were purchased from Sigma-Aldrich Chemical Company (St. Louis, MO) or Fisher Scientific (Pittsburgh, PA). The SOD Assay kit was purchased from Sigma Chemical Company (19160; St. Louis, MO). The OxyBlot™ Protein Oxidation Detection Kit purchased from Millipore (S7150; Temecula,

CA). Alanine aminotransferase (ALT) was assayed using a method based on the discontinued Sigma 505-P kit as described by Patel and associates (2011).

2.2 Animals. Male C57BL/6 mice were obtained from Hilltop Lab Animals Inc. (Scottsdale, PA). Animals included in the study were between 4-8 weeks of age and weighed 16-24 g. Mice were maintained in an American Association for Accreditation of Laboratory Animal Care (AAALAC) accredited facility. Mice were maintained at controlled temperature (21-23°C), humidity (40-55%), and 12 hour light cycles (lights on 6:00 AM to 6:00PM). An acclimation period of 7 days was observed prior to the beginning of any experiment. The animals received Purina rodent chow and water *ad libitum*. The mice were fasted for 16 hours prior to any experiment, but were provided free access to water.

2.3 SAMe Treatment Following APAP Overdose. Mice were randomly allocated into the following groups: Vehicle (Veh; 15 mL/kg water by intraperitoneal (ip) injection), SAMe (1.25 mmol/kg 5mL/kg ip injection), APAP (250 mg/kg 15mL/kg ip injection), and SAMe administered 1 hour after APAP (S + A; doses same as previously listed). SAMe was administered 1 hour following APAP. Mice were anesthetized with carbon dioxide 2 and 4 hour after APAP administration. Blood was collected by cardiac puncture in heparin-rinsed 1 mL syringes for determination of plasma ALT activity, which serves as an indicator of liver injury. Livers were then isolated and placed in ice cold Kreb's buffer (126 mM NaCl, 5 mM KCl, 3 mM $MgSO_4$, 3 mM Na_2HPO_4, 1 mM $CaCl_2$; pH 7.4), blotted, and weighed.

2.4 Mitochondrial Isolation. Mitochondria were isolated using a modification of a previously published protocol by Gogvadze and colleagues (2006). Briefly, the liver was isolated, blotted, weighed and placed in Mitochondrial Isolation Buffer A (225 mM sucrose, 3 mM KH_2PO_4, 5

mM $MgCl_2$, 20 mM KCl, 20 mM triethanolamine, 2 mM EGTA; pH 7.4). The liver was minced and homogenized in a Dounce homogenizer on ice. Following homogenization, the liver was centrifuged at 600 x *g* for 10 minutes. The resultant pellet was discarded and the supernatant was centrifuged at 15,000 x *g* for 5 minutes. After the final centrifugation, the supernatant was retained for analysis of cytosolic SAMe levels. The pellet containing the mitochondria was resuspended in Mitochondrial Isolation Buffer B (Same as Buffer A except lacking EGTA) for a final concentration of 1 mg tissue weight/µL Buffer B. Samples were stored at -80°C until analysis.

2.5 Catalase Activity Assay. The protocol for determination of catalase activity was based on a paper by Zhang and coworkers (2004). Briefly, tissue was homogenized in Kreb's buffer (10 mL/g tissue) and centrifuged at 1000 *x g* for 15 minutes retaining the supernatant. For the assay, the disappearance of 15 mM H_2O_2 was measured at 240 nm and the change in absorbance was recorded over 1 minute. The level of catalase was calculated with the extinction coefficient (43.6 M^{-1} cm^{-1}).

2.6 GPx Activity Assay. Lawrence and Burk (1976) developed a protocol which was employed to determine hepatic GPx activity. Liver tissue (100 mg) was homogenized in 1 mL of phosphate buffer (50 mM KH_2PO_4, 1 mM sodium azide, 1 mM EDTA; pH 7.2). The reaction mixture consisted of 0.5 mL phosphate buffer, 0.1 mL 2 mM NADPH, 0.1 mL glutathione reductase (1 Unit), 0.1 mL 10 mM reduced glutathione, and 0.1 mL sample. The reaction was initiated with the addition of H_2O_2 at a final concentration of 0.25 mM and disappearance of NADPH was measured for 1 minute at 340 nm. Activity was calculated using the extinction coefficient for NADPH (6.22 x 10^3 M^{-1} cm^{-1}).

2.7 GSSG Reductase Activity Assay. GSSG reductase activity was determined based on a protocol developed by Mannervik with modifications (1999). Briefly, 100 mg of liver was homogenized in Kreb's buffer (10 mL/g tissue) and centrifuged 15 minutes at 1000 *x g*. 50 µL of sample was added to a test tube containing 2.7 mL phosphate buffer (120 mM KH_2PO_4; pH 7.2), 0.1 mL 15 mM EDTA, and 0.05 mL 65.3 mM glutathione disulfide. The reaction was initiated by the addition of 0.05 mL 10 mM NADPH. Consumption of NADPH was monitored for 1 minute at 340 nm. A standard curve of known quantities of GSSG reductase was constructed for calculations.

2.8 SOD Activity Assay. SOD activity was determined using a Fluka designed spectrophotometric kit purchased from Sigma Chemical Company (19160; St. Louis, MO). Cu/Zn-SOD was inhibited by incubating the sample at room temperature sodium diethyldithiocarbamate (DDTC) at a final concentration of 25 mM. The assay was completed according to manufacturer's recommendations.

2.9 Mitochondrial Swelling Assay. Mitochondrial swelling was determined by a turbidometric technique (Gogvadze et al., 2006). Mitochondrial suspension containing 1 mg protein was set to constant spinning in 2 mL incubation buffer (150 mM KCl, 0.5 mM KH_2PO_4, 5 mM Tris Base, 100 mM succinic acid; pH 7.4). Subsequently, 2 µL 2.5 mM rotenone, 5.5 µL 10 mM $CaCl_2$, and 20 µL 0.5 M KH_2PO_4 were added at one minute intervals to initiate the reaction which was monitored at 540 nm for 5 minutes recording absorbance every 15 seconds.

2.10 Western Blotting. Western blot analysis was conducted to examine expression of mitochondrial and cytosolic cytochrome c and mitochondrial 3-nitrotyrosine (3-NT) protein adduction. A 100 µg protein aliquot was denatured by boiling for 5 minutes. Samples were

separated on a 12.5% polyacrylamide gel and transferred to a NC membrane (Whatman; Dassel, Germany). Transfer efficiency was verified using MemCode® Reversible Protein Stain Kit (Thermo Scientific; Rockford, IL). The membrane was then blocked using a 5% (w/v) milk/TBST solution (10 mM Tris-HCl, 150 mM NaCl, 0.1% Tween-20; pH 8.0) for 1 hour. Membranes were next incubated overnight with constant shaking at 4°C in antibody for cytochrome c (sc-7159, Santa Cruz Biotechnology; Santa Cruz, CA) or 3-NT (ab61392, Abcam; Cambridge, MA) in 5% (w/v) milk/TBST. The membranes were washed four times with TBST. Appropriate secondary antibodies were incubated with the membranes for 1 hour. The membrane was again washed with TBST and developed using Amersham™ ECL™ Western Blotting Detection Reagents (GE Healthcare; Buckinghamnshire, UK). Densitometry was performed on each gel (n = 3-5 mice/group).

2.11 OxyBlot Analysis. Mitochondrial protein oxidation was assessed using an OxyBlot™ kit. The manufacturer's instructions were followed and 20 µg of protein was derivatized for each sample. Protein loaded was confirmed by MemCode® Reversible Protein Stain staining as in the Western blot protocol and results were quantified by densitometry (n = 3-5 mice/group).

2.12 Cyp 2E1Enzyme Activity and expression. In vitro hepatic biotransformation of aniline to p-aminophenol (PAP) was used as an indicator of CYP2E1 hepatic enzyme activity. These experiments were conducted in order to evaluate whether SAMe inhibited P450 mediated APAP metabolism. Microsomes were isolated from livers 2 and 4 hour post APAP treatment using the methods of Schlenkman and Jansson (1999). Aniline para-hydroxylation to PAP was measured as described previously (Valentovic et al., 1988). Values were reported as nmol/mg protein/20 minute incubation.

2.13 Statistical Analysis. Values represent Mean ± S.E.M. with n=3-5 animals/group. Differences in the groups were analyzed using a one-way ANOVA followed by a Tukey's post-hoc test (SigmaStat; SPSS Inc. Chicago, IL). All statistical analyses were conducted using a 95% confidence interval.

3. Results

3.1 SAMe Attenuation of Hepatic APAP toxicity. Body weights were similar between all treatment groups (Table 8). Liver weight/10 g body weight were increased 2 and 4 hours after APAP treatment relative to the Veh mice. Plasma ALT levels were significantly increased 2 and 4 hours ($p<0.05$) in the APAP group confirming APAP overdose (Figure 13). SAMe partially protected the liver from APAP toxicity as indicated by a less marked increase in ALT levels compared to the APAP group. ALT values were 80% lower in the S + A group compared to the APAP mice. SAMe afforded partial correction as ALT values were still higher than Veh ALT at either the 2 or 4 hour time period. The ALT measurement indicates that SAMe administered 1 hour after APAP reduced hepatic toxicity.

Table 8. Comparison of liver and body weight following APAP administration in C57Bl/6 mice

	Group	Body wt (g)	Liver wt / 10 g body wt
2 h	Veh	25.20 ± 1.02[a]	0.424 ± 0.011[a]
	SAMe	24.80 ± 0.80[a]	0.416 ± 0.013[a]
	APAP	24.40 ± 0.75[a]	0.505 ± 0.042[b]
	S + A	23.60 ± 0.75[a]	0.515 ± 0.029[b]
4 h	Veh	23.60 ± 0.40[a]	0.421 ± 0.007[a]
	SAMe	23.60 ± 0.40[a]	0.407 ± 0.025[ab]
	APAP	23.20 ± 0.49[a]	0.543 ± 0.021[c]
	S + A	22.40 ± 0.98[a]	0.493 ± 0.026[b]

Value are mean ± S.E.M. with n=5 animals per group.

[a, b, c, ab] Different letters denote statistical differences ($p<0.05$), while groups with the same letters indicate no difference in the results.

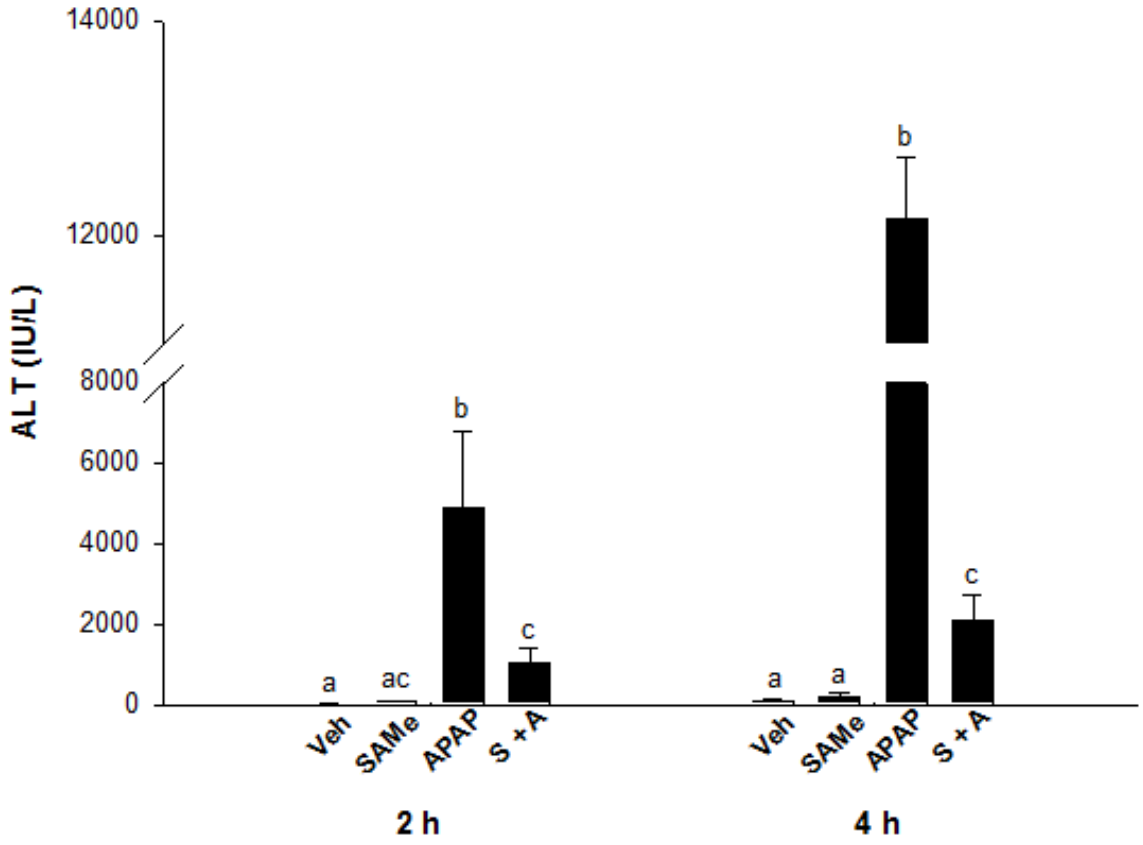

Figure 13. ALT levels in blood plasma 2 and 4 hours following APAP administration to C57Bl/6 mice. Mice were randomly allocated into Veh (water), SAMe (1.25 mmol/kg), APAP (250 mg/kg), and SAMe administered 1 hour following APAP (S + A). Values represent mean ± S.E.M. with n=5 mice per group. Different letters denote statistical differences ($p<0.05$), while groups with the same letters indicate no difference in the results.

3.2 Antioxidant Enzyme Activity protection by SAMe. Relative to the Veh group, SAMe did not alter catalase or GPx enzyme activity (Figures 14 & 15). Catalase activity was similar for all groups at the 2 hour time period but at 4 hours, APAP diminished catalase enzyme activity over 30% when compared to Veh and SAMe groups ($p<0.05$). The S + A group did not show significant elevations in catalase activity compared with the APAP group, but there was a trend toward improvement. APAP depressed GPx activity at both the 2 and 4 hour ($p<0.05$) time periods when compared to the Veh and SAMe groups (Figure 15). GPx was diminished at 2

hours in the S + A group compared to the Veh and SAMe groups but activity was partially restored at 4 hours in the S + A group. SAMe did not totally reverse the effects of APAP as GPx activity in the S + A group were higher than the APAP group but still lower than the Veh and SAMe groups at 4 hours ($p<0.05$).

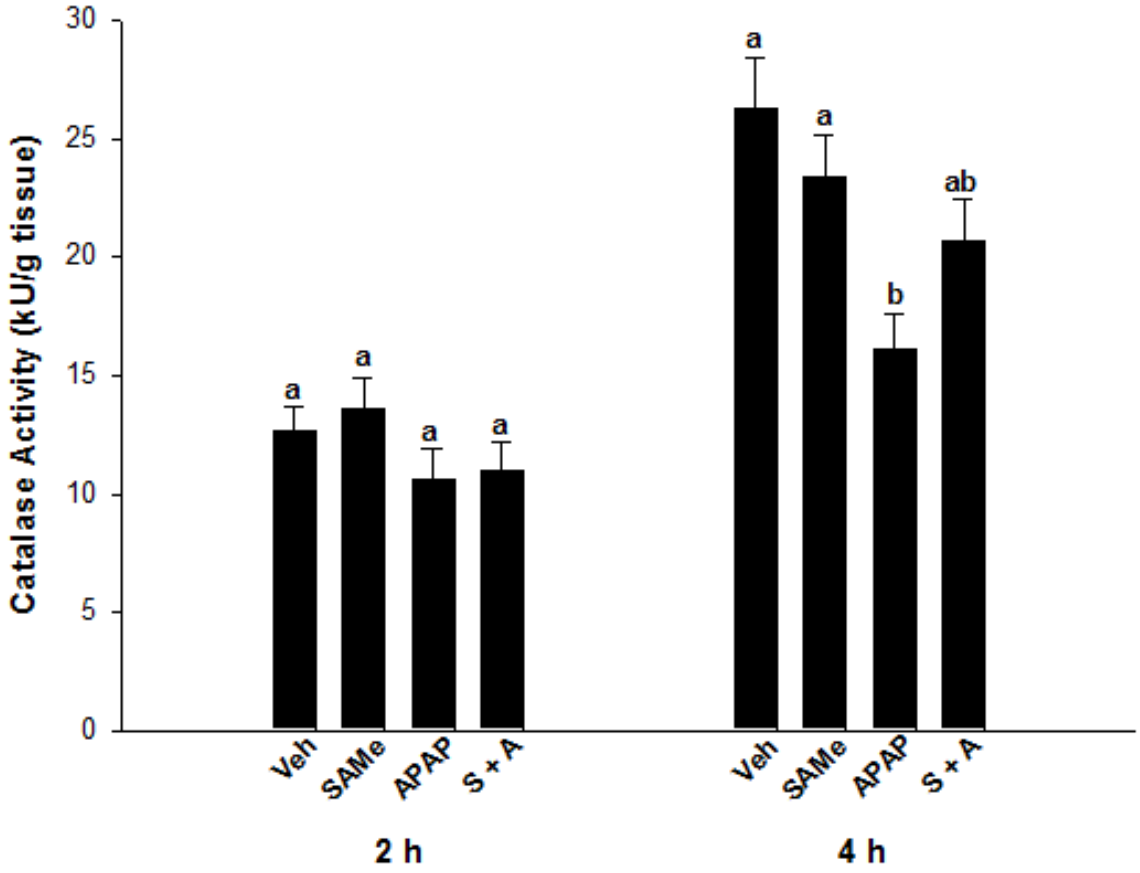

Figure 14. Catalase enzymatic activity in the liver 2 and 4 hours following APAP overdose in C57Bl/6 mice was assessed with mice randomly divided into Veh (water), SAMe (1.25 mmol/kg), APAP (250 mg/kg), and SAMe administered 1 hour post-APAP (S + A). Values represent mean ± S.E.M. with n=5 mice per group. Different letters denote statistical differences ($p<0.05$), while groups with the same letters indicate no difference in the results.

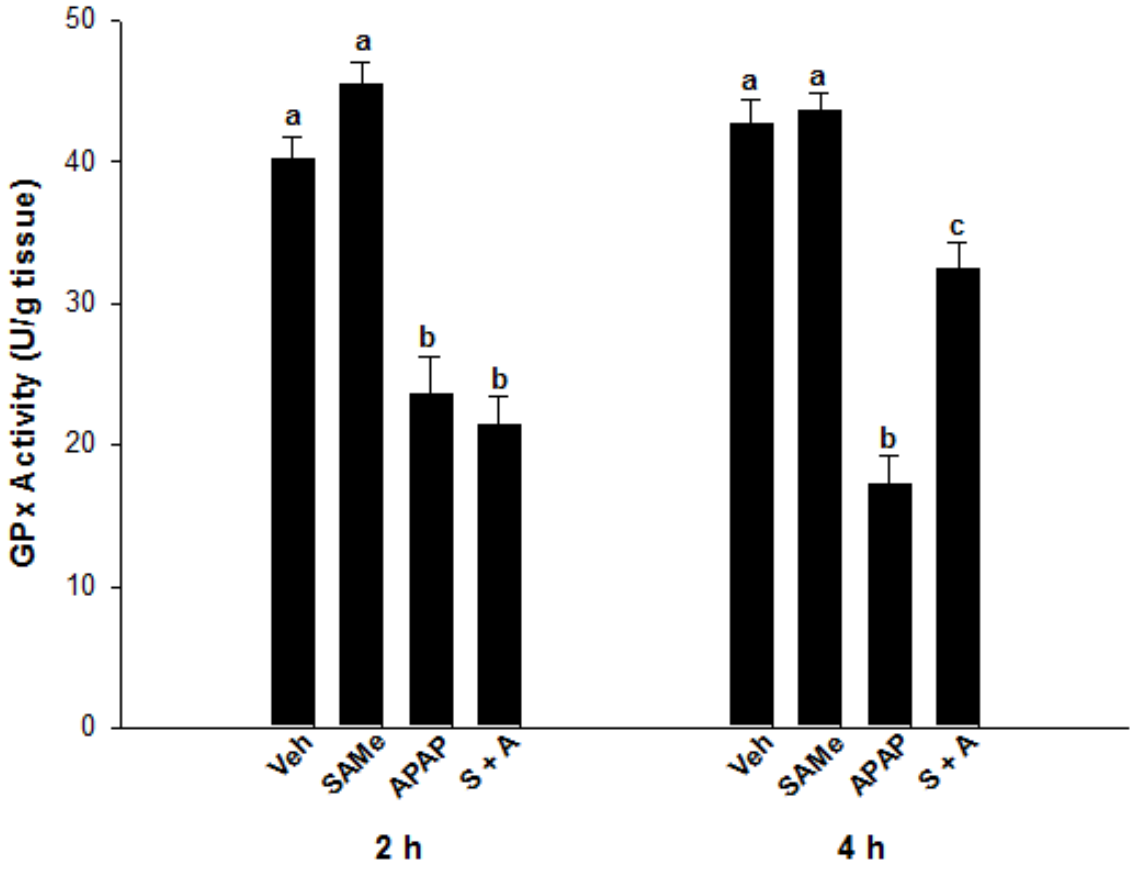

Figure 15. Liver GPx activity alterations when SAMe was administered 1 hour after APAP overdose. C57Bl/6 mice were randomly divided into Veh (water), SAMe (1.25 mmol/kg), APAP (250 mg/kg), and SAMe administered 1 hour following APAP (S + A). Livers were collected 2 and 4 hours following APAP overdose. All values represent mean ± S.E.M. Different letters denote statistical differences ($p<0.05$), while groups with the same letters indicate no difference in the results.

SAMe treatment did not alter GSSG reductase enzyme activity at either the 2 or 4 hour time periods when compared to Veh (Figure 16). APAP treatment depressed GSSG reductase activity ($p<0.05$) by 36% and 42% of Veh levels, at the 2 and 4 hour time periods, respectively. GSSG reductase activity at 2 hours in the S + A treatment group was similar to the APAP and SAMe levels. However, at 4 hours S + A had returned GSSG reductase activity to levels comparable to Veh and SAMe activity.

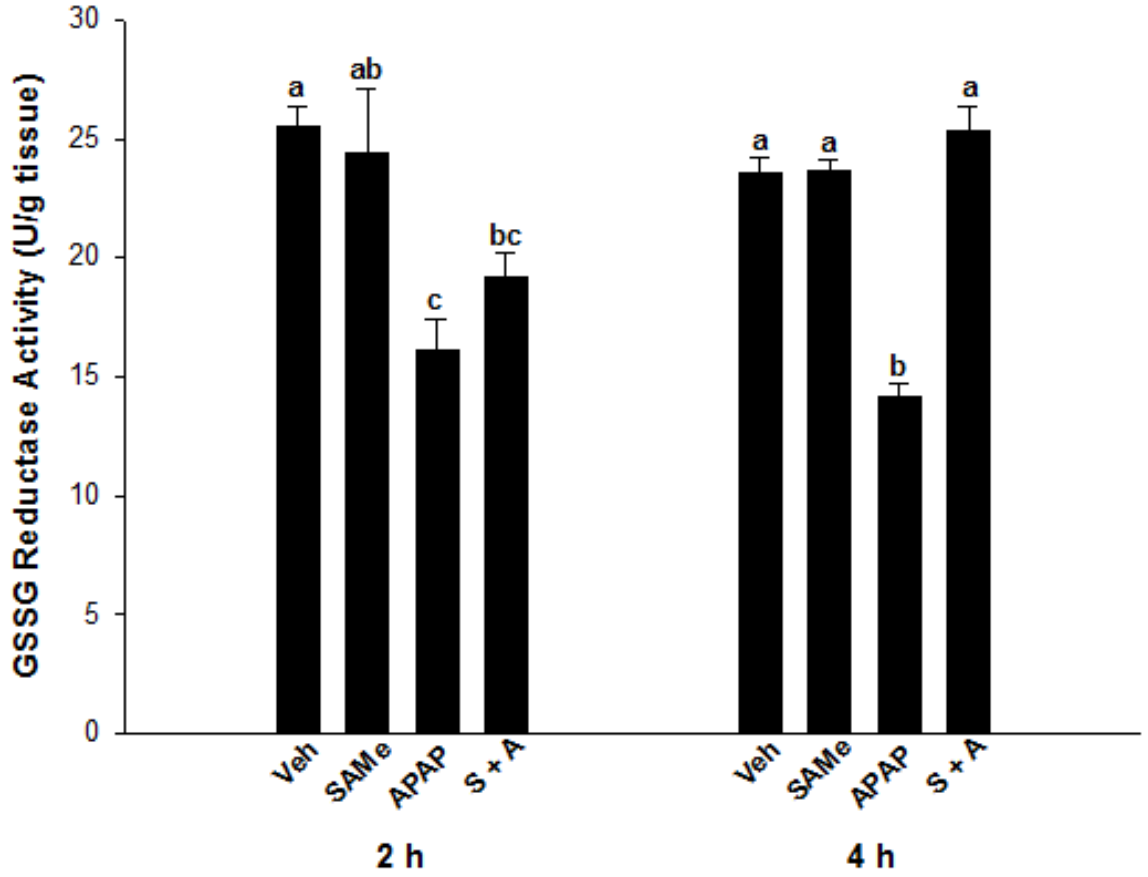

Figure 16. GSSG reductase activity in the liver following APAP overdose in C57Bl/6 mice was determined by enzymatic assay. Mice were randomly allocated into Veh (water), SAMe (1.25 mmol/kg), APAP (250 mg/kg), and SAMe administered 1 hour following APAP (S + A) and livers collected 2 and 4 hours following APAP overdose. Each group represents 5 experiments with different mice. Different letters denote statistical differences ($p<0.05$), while groups with the same letters indicate no difference in the results. SOD activity was measured for 2 and 4 hour treatment groups and expressed as total, cytosolic (Cu/Zn-SOD), and mitochondrial (Mn-SOD). SAMe treatment did not alter total, Cu/Zn, and Mn-SOD at any time period examined (Figure 17). In the 2 hour treatment group, total SOD and Mn-SOD activity were lower ($p<0.05$) in the S + A group compared to the Veh and SAMe mice. The Veh, SAMe, and APAP groups showed no significant difference (Figure 17, Panel A) for any form of SOD at 2 hours. The Veh group did not differ significantly from the S + A group for Cu/Zn-SOD. Mn-SOD was significantly decreased ($p<0.05$) in the S + A group when compared to the Veh and SAMe groups at 2 hours, and no difference was observed when compared to the APAP treatment group.

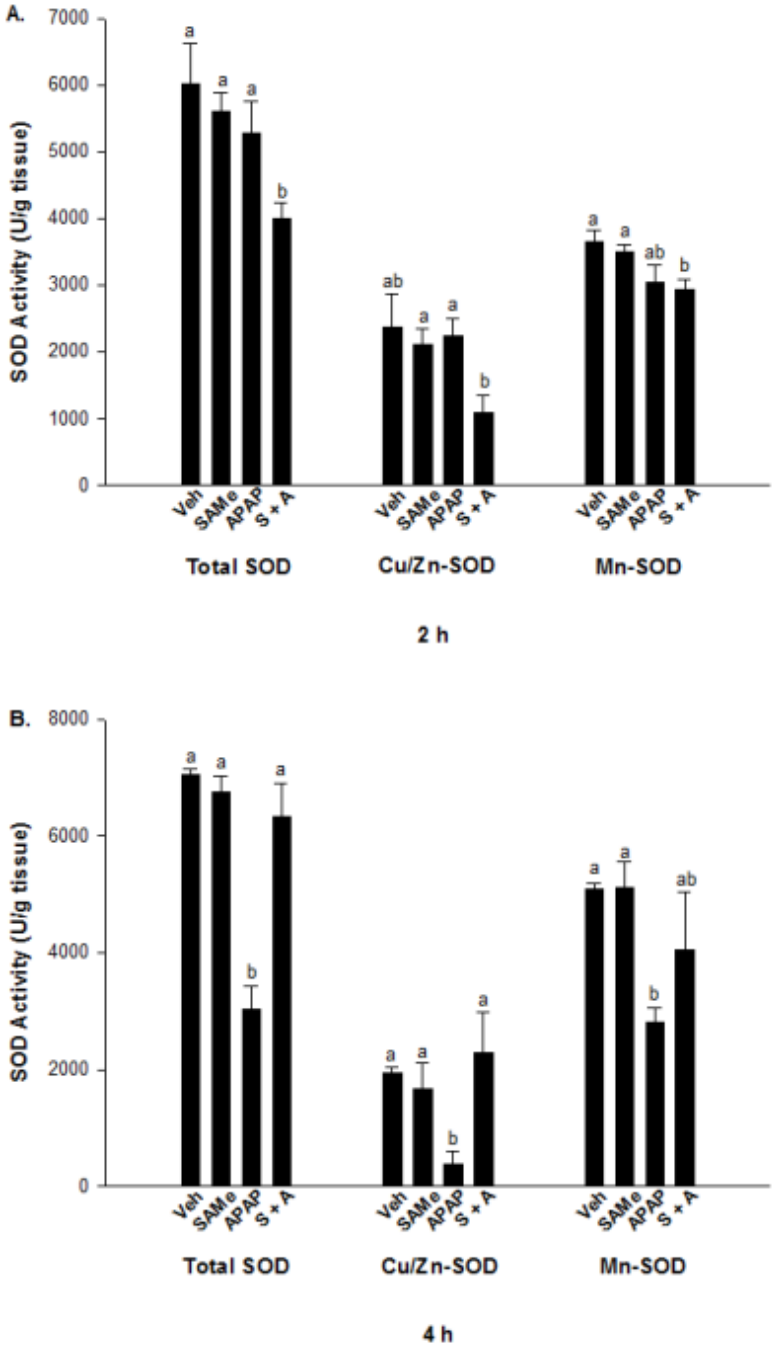

Figure 17. SOD activity following APAP overdose in C57Bl/6 mice. Cu/Zn-SOD was inhibited with DDTC for determination of Mn-SOD. Panel A represents SOD levels 2 hours following APAP overdose, while panel B represents SOD levels 4 hours following APAP overdose. Different letters denote statistical differences ($p<0.05$), while groups with the same letters indicate no difference in the results.

In the 4 hour treatment groups, total, Cu/Zn, and Mn-SOD activities were decreased ($p<0.05$) in the APAP group when compared with the Veh and SAMe groups (Figure 17, Panel B). It appeared that a longer time period of 4 hours was needed to produce a decline in SOD activity. Treatment with SAMe (S + A) increased total and Cu/Zn-SOD activity to levels similar to Veh and SAMe. The 4 hour Mn-SOD activity in the S + A group was not significantly different from the APAP group.

3.3 SAMe attenuates mitochondrial APAP induced Protein Carbonyl and 3-Nitrotyrosine adduction. As a global measure of the effect of SAMe's ability to reduce mitochondrial oxidative stress, OxyBlots were performed on mitochondrial samples to detect protein carbonyl formation. In both 2 and 4 hour samples, densitometry was significantly increased ($p<0.05$) in the APAP treatment groups when compared to the Veh and SAMe groups (Figure 18). When SAMe was administered following APAP, the density returned to be comparable to the Veh and SAMe treatment groups indicating that SAMe treatment lessens the formation of protein carbonyls in the mitochondria of APAP treated C57Bl/6 mice. These results are consistent with the protection observed by S + A for the antioxidant enzyme activities (Figures 14-17).

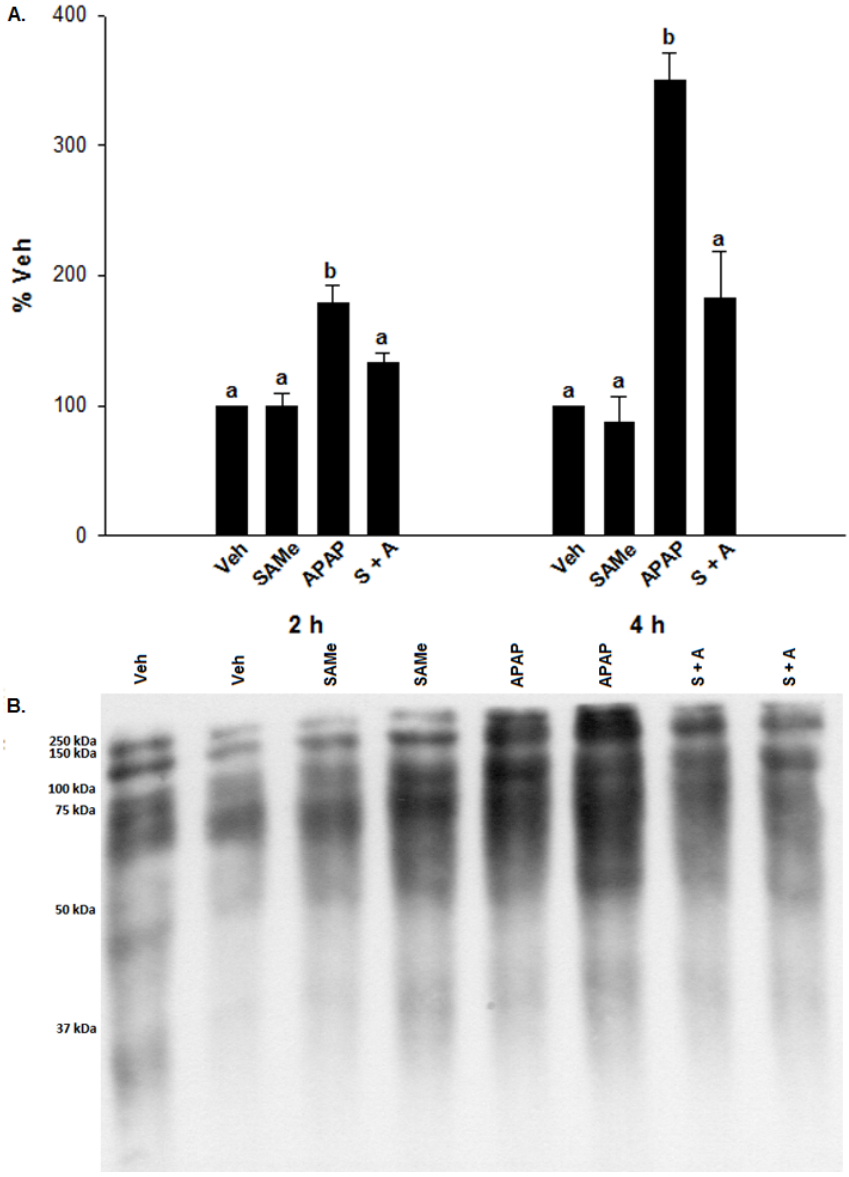

Figure 18. Protein carbonyl formation in C57Bl/6 mouse mitochondria 2 and 4 hours following APAP overdose. Protein carbonyls were assessed using an OxyBlot™ kit. Panel A contains densitometry from 2 and 4 hour OxyBlots™ following APAP overdose. Densitometry was normalized to total protein staining (not shown) and is expressed as % Veh. All values represent mean ± S.E.M. with n=4 mice per group. Different letters denote statistical differences ($p<0.05$), while groups with the same letters indicate no difference in the results. Panel B is a representative 4 hour blot with lanes 1-2 corresponding to Veh, 3-4 SAMe, 5-6 APAP, and 7-8 SAMe administered 1 hour following APAP (S + A).

APAP significantly increased mitochondrial 3-NT protein adduction 4 hours following overdose when compared to Veh and SAMe treatment groups (Figure 19). SAMe decreased the amount of mitochondrial 3-NT adduction when administered following APAP. Even though there was a similar increase in mitochondrial 3-NT adduction in the 2 hour group, SAMe was less effective at decreasing the amount of adduction observed at this time period (data not shown).

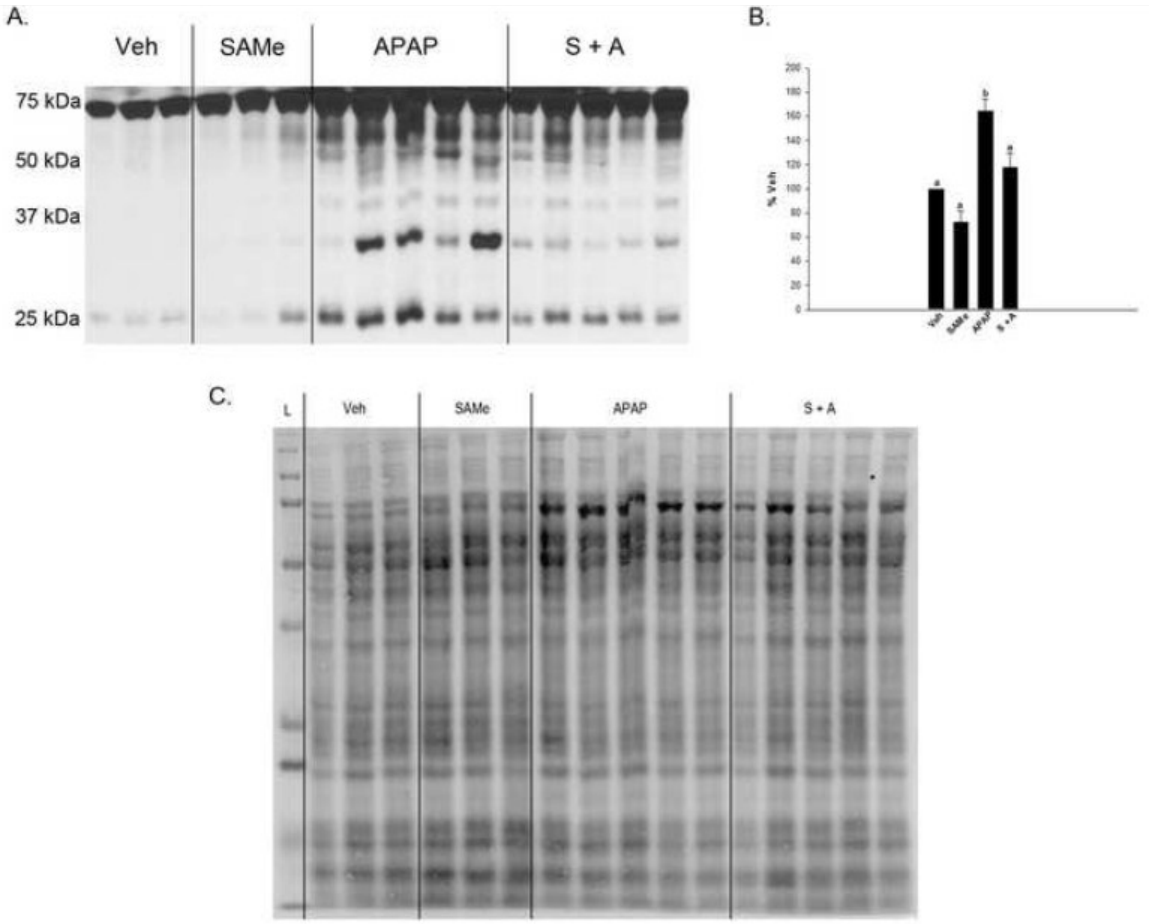

Figure 19. Mitochondrial 3-NT protein formation 4 hours following APAP overdose. Panel A is a representative 4 hour blot containing mitochondrial samples from the indicated groups. Panel B is the densitometry from panel A for whole lane 3-NT adduction normalized to total lane protein staining. Protein staining such as that presented in Panel C was used to normalize protein levels. Different letters denote statistical differences ($p<0.05$), while groups with the same letters indicate no difference in the results. Values are mean ± S.E.M. with n=3-5 mice per group.

3.4 Cytochrome c release. Mitochondrial function was also analyzed by examining mitochondrial swelling following APAP overdose. There was a significant increase in mitochondrial swelling at both 2 and 4 hours following APAP overdose (Figure 20, Panels A and B). SAMe was not able to prevent the increased swelling observed during these time periods, and there was not a significant difference observed between the APAP and S + A groups except in the 4 hour treatment group 1 minute after swelling was induced when the S + A group was significantly lower than the APAP group.

Cytochrome c leakage was used to assess mitochondrial function associated with APAP toxicity. Cytochrome c leakage into the cytosol was 20% greater in the APAP group when compared with the Veh group 2 and 4 hours following APAP overdose, although the results were not significant at the 2 hour time period (Figure 20, Panel C). SAMe was able to prevent the release of cytochrome c into the cytosol at 4 hours when compared to the APAP group. The increased cytosolic cytochrome c is also evident in a representative blot included in Figure 20, Panel D.

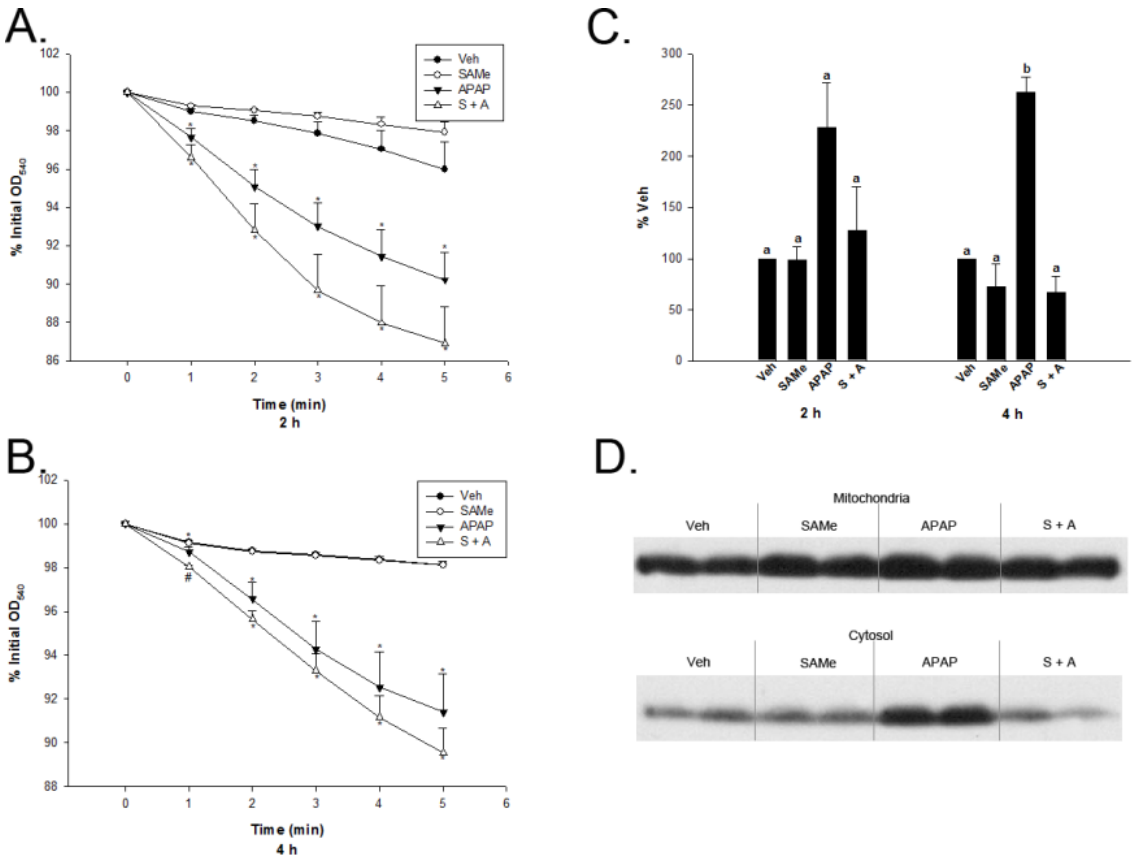

Figure 20. Mitochondrial swelling and cytochrome c leakage were used to assess mitochondrial function following APAP overdose. Mice were randomly allocated into Veh (water), SAMe (1.25 mmol/kg), APAP (250 mg/kg), and SAMe administered 1 hour following APAP. Livers were collected 2 and 4 hours following APAP administration and analyzed for mitochondrial swelling (Panel A and B). Panel C represents densitometry conducted on cytosolic cytochrome c levels following APAP overdose when analyzed at 2 and 4 hours. Panel D is a representative 4 hour cytosol cytochrome c blot depicting Veh (Lanes 1 and 2), SAMe (Lanes 3 and 4), APAP (Lanes 5 and 6), and S + A (Lanes 7 and 8). All values represent mean ± S.E.M. with at least 4 mice represented in each group. Different letters denote statistical differences ($p<0.05$), while groups with the same letters indicate no difference in the results.

3.5 CYP 2E1 enzyme activity and expression. Aniline para-hydroxylation to PAP was used as an indicator of CYP2E1 enzyme activity. SAMe treatment, at the dose used in this study, did not alter aniline hydroxylation. APAP diminished CYP2E1 enzyme activity at 2 and 4 hours relative to the Veh and SAMe groups (Figure 21). The S + A group had higher enzyme activity than the APAP group at 2 hours but PAP formation was slower than the Veh and SAMe groups at 2 hours. Enzyme activity was similar to Veh and SAMe at 4 hours. These results indicate that the mechanism for protection by SAMe is not due to inhibition of P450 metabolism at the dose used in our studies. Western analysis of protein expression also showed that SAMe does not alter CYP2E1 enzyme expression at 2 or 4 hours. APAP diminished CYP 2E1 expression at 2 and 4 hours compared to all other groups.

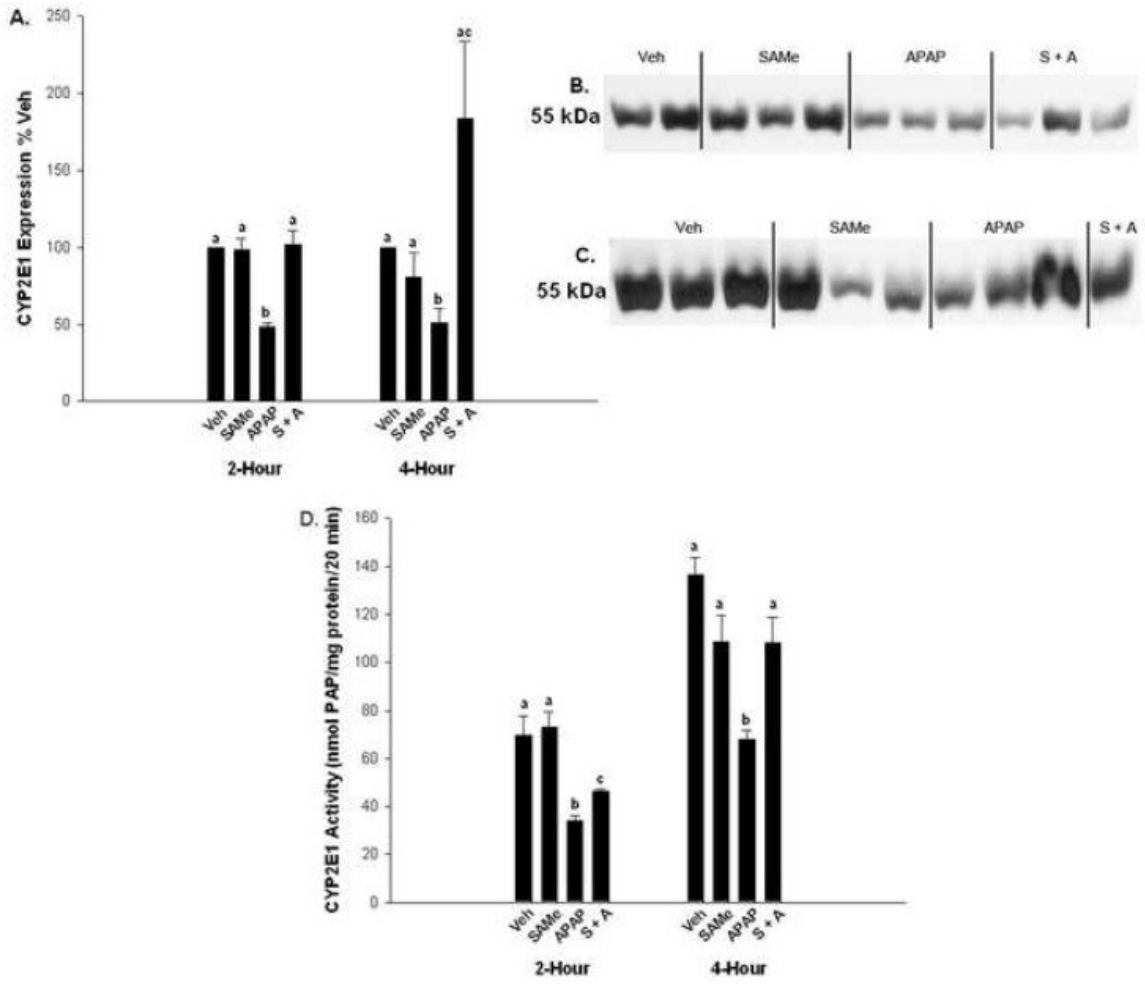

Figure 21. CYP2E1 enzyme activity and expression in APAP treated mice. Panel A represent densitometry for CYP2E1 enzyme expression measured at 2 hours (Panel B) and 4 hours (Panel C) using a Western blot. APAP inhibited CYP2E1 expression while SAMe did not inhibit CYP2E1 expression. Panel D represent para-hydroxylation of aniline to p-aminophenol (PAP) measured 2 and 4 hours after APAP treatment in groups as described in Fig. 1. Enzyme activity was expressed as nmol PAP/mg protein/20 minutes. All values represent mean ± S.E.M. with at least 4 mice represented in each group. Superscripts denote statistical differences between groups ($p<0.05$).

4. Discussion

Our laboratory has previously reported that SAMe reduced APAP toxicity in C57Bl/6 mice when administered 0 or 1 hours after APAP (Terneus et al., 2007 & 2008). SAMe can reduce protein carbonylation and lipid peroxidation when administered after a toxic dose of APAP. Previous work established the importance of ROS and RNS generation in APAP toxicity (Cover et al., 2005). Damage caused by ROS and RNS is thought to be preceded by NAPQI induced GSH depletion, which we have previously demonstrated the ability of SAMe to prevent in APAP treated mice. However, the current study shows that SAMe also has the potential to protect the function of critical antioxidant enzymes normally altered by APAP toxicity.

SOD has been extensively studied with APAP toxicity. Decreased levels of Mn-SOD have been shown to significantly increase APAP toxicity, which is consistent with the generation of superoxide occurring primarily in the mitochondria with APAP toxicity (Yoshikawa et al., 2009; Ramachandran et al., 2011). Furthermore, Mn-SOD activity has recently been demonstrated to be decreased with nitration secondary to APAP toxicity (Agarwal et al., 2011). At 4 hours, SAMe given 1 hour after APAP (S + A group) decreased 3-NT adducts globally in mitochondrial protein which is consistent with the observed correction in Mn-SOD activity observed for the S + A group compared to APAP. The exact mechanism for SAMe decreasing APAP mediated nitration of tyrosine residues in proteins is not known. However, these results suggest SAMe interferes with APAP induced mitochondrial dysfunction and oxidative stress.

In contrast to decreased expression of Mn-SOD, Cu/Zn-SOD knockout mice show almost complete protection against APAP overdose (Zhu et al., 2006). SAMe given as an antidote to APAP toxicity clearly protects total SOD activity 4 hours following APAP with significant increases in Cu/Zn-SOD activity and improvement. We clearly observed protection when SAMe

was given following APAP indicating that, in our model, the modest increase in Mn-SOD may be of greater importance than the increase in Cu/Zn-SOD. In addition, full function of all antioxidant enzymes is important in reducing oxidative stress as hydrogen peroxide formed from Mn-SOD and Cu/Zn-SOD is also toxic and is detoxified by catalase and glutathione peroxidase.

Evidence has existed for some time that the GPx-GSSG reductase system was protective in cases of APAP toxicity (Adamson et al., 1989). However, it is also apparent that over expression of intracellular GPx can increase APAP toxicity most likely as a result of enhanced GSH depletion (Mirochnitchenko et al., 1999). Our results clearly demonstrated a depression of GPx activity 2 and 4 hours following APAP overdose, which was reversed at 4 hours by SAMe administration. The fact that GPx activity was not returned to Veh level may be beneficial in light of the study on GPx over expression increasing APAP toxicity. Additionally, the increased activity of GSSG reductase at 4 hours in the S + A group would serve to increase cellular levels of GSH and decrease glutathione disulfide levels, which is consistent with prior results from our laboratory (Terneus et al., 2007 and 2008).

Catalase represents an important link in the detoxification of ROS generated as a result of APAP toxicity because it does not require GSH to function. Clear evidence exists that APAP can depress catalase activity at 6 and 24 hours following APAP administration (Yan et al., 2009; Chandrasekaran et al., 2011). Additionally, antioxidant administration prevents the observed decrease in catalase activity. SAMe does not have antioxidant properties and was still able to improve catalase function, although not significantly, 4 hours following APAP administration.

Formation of the reactive metabolite NAPQI has long been known to be a prerequisite for mitochondrial dysfunction associated with APAP toxicity (Weis et al., 1992). More recent work

suggests that the observed changes in mitochondrial function are caused by JNK activation following both GSH depletion and resultant oxidative stress (Saito et al., 2010). Additionally, peroxynitrite formation has been demonstrated to increase fragmentation of mitochondrial DNA, which could be a cause of some of the observed mitochondrial dysfunction (Cover et al., 2005). We demonstrated a clear reduction in markers of oxidative stress when SAMe was given after APAP in the 4 hour treatment group. Additionally, the reduction in mitochondrial 3-NT adduct formation at 4 hours with SAMe administration could be beneficial to function following APAP overdose.

Cytochrome c has previously been used as a marker for APAP toxicity (Song et al., 2004). However, it is clear that APAP toxicity is a necrotic event and caspase activation associated with apoptosis is abrogated by ATP depletion associated with toxicity (Kon et al., 2004). SAMe prevents release of cytochrome c into cell cytosol at both 2 and 4 hours following APAP toxicity indicating mitochondrial protection. The dose of APAP used in our studies induced necrosis as the percent release of cytochrome c into cytosol was less than 30% of total cytochrome c.

One finding of interest was that SAMe did not prevent mitochondrial swelling when administered following APAP overdose. This finding contradicts the work of Song and associates (2004) which clearly demonstrated SAMe decreased mitochondrial swelling when given as an antidote to APAP toxicity. Three factors may account for the difference in findings between the current and previous study. First, our laboratory used a much higher concentration of calcium to initiate swelling in combination with additional phosphate and rotenone, which were not used in the Song research. Waldmeier and others demonstrated that even a high concentration of cyclosporine A cannot indefinitely inhibit permeability transition when isolated mitochondria are exposed to high concentrations of both calcium and phosphate (Waldmeier et

al, 2009). Second, the concentration of SAMe used by Song et al. (2004) was double that used by our laboratory in the current experiment. Perhaps SAMe needs to be present at a higher level than was used in the current experiment to provide protection against APAP induced mitochondrial swelling. Additionally, APAP (200 mg/kg, ip) has been demonstrated to undergo rapid conversion to NAPQI depleting cellular glutathione within 20 minutes of administration (Bajt et al., 2011). The current protocol administered SAMe 1 hour after APAP, allowing more than enough time for NAPQI to form and cause some damage that could weaken the mitochondria, rendering them more susceptible to swelling. However, given the bulk of the data presented in this paper, SAMe is providing clear protection following APAP overdose.

Biotransformation of APAP by cytochrome P450 is critical for formation of NAPQI and development of hepatotoxicity (Patten et al., 1993; Chen et al., 1998). Caro and Cederbaum (2005) reported that SAMe inhibited in vitro CYP2E1 metabolism in rat microsomes and in HepG2 cells. The IC50 was reported as 1.5 mM for CYP2E1. In our studies, inhibition of CYP2E1 by SAMe is not the mechanism for reduced hepatic APAP toxicity in vivo when we administered SAMe 1 hour after APAP. In our study we administer a dose of 1.25 mmol/kg SAMe which corresponds to approximately 30 μmoles distributed within the entire animal. The low amount administered to the animal is not sufficient to inhibit P450 enzyme activity. However, we also measured in vitro CYP2E1 metabolism and expression (Figure 21) which confirms P450 metabolism is not inhibited by SAMe.

In conclusion, we present here a clear line of evidence that the observed decrease in oxidative stress mediated by SAMe is in part due to the maintenance of antioxidant enzyme function following APAP overdose. Antioxidant enzyme function is greatly improved 4 hours following APAP overdose when SAMe is given as an antidote. Also, cytochrome c release is inhibited by

SAMe 4 hours following APAP, indicating a protection of mitochondrial function. Mitochondrial dysfunction due to oxidative stress and resultant ATP depletion are critical factors in APAP toxicity. By protecting against mitochondrial oxidative stress, SAMe provides a potentially useful therapeutic intervention following APAP toxicity.

Acknowledgements

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References

Adamson, G.M, Harman, A.W., 1989. A role for the glutathione peroxidase/reductase enzyme system in the protection from paracetamol toxicity in isolated mouse hepatocytes. Biochem Pharmacol. **38,** 3323-3330.

Agarwal, R., MacMillan-Crow, L.A., Rafferty, T.M., Saba, H., Roberts, D.W., Fifer, E.K., James, L.P., Hinson, J.A., 2011. Acetaminophen-induced hepatotoxicity in mice occurs with inhibition of activity and nitration of mitochondrial manganese superoxide dismutase. J Pharmacol Exp Ther. 337, 110-116.

Andersson, B.S., Rundgren, M., Nelson, S.D., Harder, S., 1990. N-acetyl-p-benzoquinone imine-induced changes in the energy metabolism in hepatocytes. Chem Biol Interact. 75**,** 201-211.

Anundi, I., Lahteenmaki, T., Rundgren, M., Moldeus, P., Lindros, K.O.,1993. Zonation of acetaminophen metabolism and cytochrome P450 2E1-mediated toxicity studied in isolated periportal and perivenous hepatocytes. Biochem Pharmacol. 45, 1251-1259.

Bailey, S.M., Robinson, G., Pinner, A., Chamlee, L., Ulasova, E., Pompilius, M., Page, G.P., Chhieng, D., Jhala, N., Landar, A., Kharbanda, K.K., Ballinger, S., Darley-Usmar, V., 2006. S-adenosylmethionine prevents chronic alcohol-induced mitochondrial dysfunction in the rat liver. Am J Physiol Gastrointest Liver Physiol. 291, G857-867.

Bajt, M.L., Ramachandran, A., Yan, H.M., Lebofsky, M., Farhood, A., Lemasters, J.J., , Jaeschke, H., 2011. Apoptosis-Inducing Factor Modulates Mitochondrial Oxidant Stress in Acetaminophen Hepatotoxicity. Toxicol Sci. 122, 598-605.

Brosnan, J.T., Brosnan, M.E., 2006. The sulfur-containing amino acids: an overview. J Nutr. 136, 1636S-1640S.

Chandrasekaran, V.R., Periasamy, S., Liu, L.L., Liu, M.Y., 2011. 17beta-Estradiol protects against acetaminophen-overdose-induced acute oxidative hepatic damage and increases the survival rate in mice. Steroids. 76**,**118-124.

Caro, A.A., Cederbaum, A.I. 2005. Inhibition of CYP2E1 catalytic activity in vitro by S-adenosyl-L-methionine. Biochem Pharmacol. 69, 1081-1093.

Chen, W., Koenigs, L.L., Thompson, S.J., Peter, R.M., Rettie, A.E., Trager, W.F., Nelson, S.D., 1998. Oxidation of acetaminophen to its toxic quinone imine and nontoxic catechol metabolites

by baculovirus-expressed and purified human cytochromes P450 2E1 and 2A6. Chem Res Toxicol. 11**,** 295-301.

Cover, C., Mansouri, A., Knight, T.R., Bajt, M.L., Lemasters, J.J., Pessayre, D., Jaeschke, H., 2005. Peroxynitrite-induced mitochondrial and endonuclease-mediated nuclear DNA damage in acetaminophen hepatotoxicity. J Pharmacol Exp Ther. 315**,** 879-887.

Dahlin, D.C., Miwa, G.T, Lu, A.Y., Nelson, S.D., 1984. N-acetyl-p-benzoquinone imine: a cytochrome P-450-mediated oxidation product of acetaminophen. *Proc Natl Acad Sci U S A.* 8**,** 327-1331.

El-Hassan, H., Anwar, K., Macanas-Pirard, P., Crabtree, M., Chow, S.C., Johnson, V.L., Lee, P.C., Hinton, R.H., Price, S.C., Kass, G.E., 2003. Involvement of mitochondria in acetaminophen-induced apoptosis and hepatic injury: roles of cytochrome c, Bax, Bid, and caspases. Toxicol Appl Pharmacol. 191, 118-129.

Fernandez, L., Perez, V, Munoz, M., Corpa, J.M., Abad, M., Carbajo, M.T., 2003. Effects of S-adenosylmethionine on hepatic regeneration after partial hepatectomy in the rat. J Physiol Biochem. 59**,** 63-64.

Gogvadze, V., Orrenius, S., Zhivotovsky, B., 2006. Analysis of Mitochondrial Dysfunction during cell death. In, *Current Protocols in Toxicology*, *volume 1*. (Maines, M. D.; Costa, L. G.; Reed, D. J.; Sassa, S.; Sipes, I. G., eds.), pp 2.10.11-12.10.27. John Wiley & Sons, Inc., Hoboken, NJ.

Hinson, J.A., Michael, S.L., Ault, S.G., Pumford, N.R., 2000. Western blot analysis for nitrotyrosine protein adducts in livers of saline-treated and acetaminophen-treated mice. Toxicol Sci. 53, 467-473.

Hinson, J.A., Pumford, N.R., Roberts, D.W., 1995. Mechanisms of acetaminophen toxicity: immunochemical detection of drug-protein adducts. Drug Metab Rev. 27**,** 73-92.

Jaeschke, H., Bajt, M.L., 2006. Intracellular signaling mechanisms of acetaminophen-induced liver cell death. Toxicol Sci. 89, 31-41.

Kon, K., Kim, J.S., Jaeschke, H., Lemasters, J.J., 2004. Mitochondrial permeability transition in acetaminophen-induced necrosis and apoptosis of cultured mouse hepatocytes. Hepatology. 40, 1170-1179.

Latchoumycandane, C., Goh, C.W., Ong, M.M., Boelsterli, U.A., 2007. Mitochondrial protection by the JNK inhibitor leflunomide rescues mice from acetaminophen-induced liver injury. Hepatology. 45**,** 412-421.

Lawrence, R.A., Burk, R.F., 1976. Glutathione peroxidase activity in selenium-deficient rat liver. Biochem Biophys Res Commun. 71, 952-958.

Lu, S.C., 2000. S-Adenosylmethionine. Int J Biochem Cell Biol. 32, 391-395.

Mannervik, B., 1999. Measurement of Glutathione Reductase. In, *Current Protocols in Toxicology*, *volume 2*.(Maines, M. D.; Costa, L. G.; Hodgson, E.; Reed, D. J.; Sipes, I. G., eds.) pp 7.2.1-7.2.4. John Wiley & Sons, Inc., Hoboken, NJ.

Masubuchi, Y., Suda, C., Horie, T., 2005. Involvement of mitochondrial permeability transition in acetaminophen-induced liver injury in mice. J Hepatol. 42, 110-116.

Mirochnitchenko, O., Weisbrot-Lefkowitz, M., Reuhl, K., Chen, L., Yang, C., Inouye, M., 1999. Acetaminophen toxicity. Opposite effects of two forms of glutathione peroxidase. J Biol Chem. 274, 10349-10355.

Nourjah, P., Ahmad, S.R., Karwoski, C., Willy, M., 2006. Estimates of acetaminophen (Paracetomal)-associated overdoses in the United States. Pharmacoepidemiol Drug Saf. 15, 398-405.

Olaleye, M.T., Rocha, B.T., 2008. Acetaminophen-induced liver damage in mice: effects of some medicinal plants on the oxidative defense system. Exp Toxicol Pathol. 59, 319-327.

Patel, N.N., Crincoli, C.M., Frederick, D.M., Tchao, R., Harvison, P.J., 2012. Effect of structural modifications on 3-(3,5-dichlorophenyl)-2,4-thiazolidinedione-induced hepatotoxicity in Fischer 344 rats. J Appl Toxicol. 32,108-1710.

Patten, C.J., Thomas, P.E., Guy, R.L., Lee, M., Gonzalez, F.J., Guengerich, F.P., Yang, C.S.,1993. Cytochrome P450 enzymes involved in acetaminophen activation by rat and human liver microsomes and their kinetics. Chem Res Toxicol. 6, 511-518.

Ramachandran, A., Lebofsky, M., Weinman, S.A., Jaeschke, H., 2011. The impact of partial manganese superoxide dismutase (SOD2)-deficiency on mitochondrial oxidant stress, DNA fragmentation and liver injury during acetaminophen hepatotoxicity. Toxicol Appl Pharmacol. 251**,** 226-233.

Saito, C.,Lemasters, J.J., Jaeschke, H.,2010. c-Jun N-terminal kinase modulates oxidant stress and peroxynitrite formation independent of inducible nitric oxide synthase in acetaminophen hepatotoxicity. Toxicol Appl Pharmacol. 246**,** 8-17.

Schenkman, J.B., Jansson, I., 1999. Measurement of Cytochorme P450. In, *Current Protocols in Toxicology, volume 1.* (Maines, M. D.; Costa, L. G.; Reed, D. J.; Sassa, S.; Sipes, I. G., eds.), pp 4.1.1-4.1.14. John Wiley & Sons, Inc., Hoboken, NJ.

Smilkstein, M.J., Knapp, G.L., Kulig, K.W., Rumack, B.H.,1988. Efficacy of oral N-acetylcysteine in the treatment of acetaminophen overdose. Analysis of the national multicenter study (1976 to 1985). N Engl J Med. 319, 1557-1562.

Song, Z., McClain, C.J., Chen, T., 2004. S-Adenosylmethionine protects against acetaminophen-induced hepatotoxicity in mice. Pharmacology. 71**,** 199-208.

Song, Z., Zhou, Z., Song, M., Uriarte, S., Chen, T., Deaciuc, I., McClain, C.J., 2007. Alcohol-induced S-adenosylhomocysteine accumulation in the liver sensitizes to TNF hepatotoxicity: possible involvement of mitochondrial S-adenosylmethionine transport. Biochem Pharmacol. 74, 521-531.

Terneus, M.V., Brown, J.M., Carpenter, A.B., Valentovic, M.A., 2008. Comparison of S-adenosyl-L-methionine (SAMe) and N-acetylcysteine (NAC) protective effects on hepatic damage when administered after acetaminophen overdose. Toxicology. 244**,** 25-34.

Terneus, M.V., Kiningham, K.K., Carpenter, A.B., Sullivan, S.B., Valentovic, M.A., 2007. Comparison of S-Adenosyl-L-methionine and N-acetylcysteine protective effects on acetaminophen hepatic toxicity. J Pharmacol Exp Ther. 320**,** 99-107.

Valentovic, M., Elliott, C., Teets, V.J., Brown, P.I., Yang, D., Rankin, G.O. 1988. Enzyme induction produced by N-(3,5-dichlorophenyl)succinimide (NDPS) in rats. Biochem Pharmacol. 37, 768-770.

Waldmeier, P.C., Feldtrauer, J.J., Qian, T., Lemasters, J.J., 2009. Inhibition of the mitochondrial permeability transition by the nonimmunosuppressive cyclosporin derivative NIM811. Mol Pharmacol. 62, 22-29.

Wang, A.Y., Lian, L.H., Jiang, Y.Z., Wu, Y.L., Nan, J.X., 2010. Gentiana manshurica Kitagawa prevents acetaminophen-induced acute hepatic injury in mice via inhibiting JNK/ERK MAPK pathway. World J Gastroenterol. 16, 384-391.

Weis, M., Kass, G.E., Orrenius, S., Moldeus, P., 1992. N-acetyl-p-benzoquinone imine induces Ca2+ release from mitochondria by stimulating pyridine nucleotide hydrolysis. J Biol Chem. 267**,** 804-809.

Wendel, A., Feuerstein, S., Konz, K.H., 1979. Acute paracetamol intoxication of starved mice leads to lipid peroxidation in vivo. Biochem Pharmacol. 28, 2051-2055.

Wu, Y.L., Jiang, Y.Z., Jin, X.J., Lian, L.H., Piao, J.Y., Wan, Y., Jin, H.R., Joon Lee, J., Nan, J.X., 2010. Acanthoic acid, a diterpene in Acanthopanax koreanum, protects acetaminophen-induced hepatic toxicity in mice. Phytomedicine. 17, 475-479.

Yan, S.L., Wu, S.T., Yin, M.C., Chen, H.T., Chen, H.C., 2009. Protective effects from carnosine and histidine on acetaminophen-induced liver injury. J Food Sci. 74, H259-265.

Yoshikawa, Y.,Morita, M., Hosomi, H., Tsuneyama, K., Fukami, T., Nakajima, M., Yokoi, T., 2009. Knockdown of superoxide dismutase 2 enhances acetaminophen-induced hepatotoxicity in rat. Toxicology. 264, 89-95.

Zhang, Y.T., Zheng, Q.S., Pan, J., Zheng, R.L., 2004. Oxidative damage of biomolecules in mouse liver induced by morphine and protected by antioxidants. Basic Clin Pharmacol Toxicol. 95, 53-58.

Zhu, J.H., Zhang, X., McClung, J.P., Lei, X.G., 2006. Impact of Cu, Zn-superoxide dismutase and Se-dependent glutathione peroxidase-1 knockouts on acetaminophen-induced cell death and related signaling in murine liver. Exp Biol Med (Maywood). 231, 1726-1732.

Chapter V: S-Adenosyl-L-methionine protection of acetaminophen mediated oxidative stress, 4-Hydroxy-2-nonenal (4-HNE) protein adduction and alterations in polyamine pathway

J. Michael Brown*, Christopher Kuhlman[#], Marcus Terneus*, Matthew Labinsky[#], Andre Benja Lamyaithong*, Serrine S. Lau[#] and Monica A. Valentovic*

*Department of Pharmacology, Physiology and Toxicology, Joan C. Edwards School of Medicine, Huntington, WV

[#]Department Southwest Environmental Health Sciences Center, Department of Pharmacology and Toxicology, University of Arizona Health Sciences Center, Tucson, Arizona

Abstract

Acetaminophen (APAP) causes severe centrilobular necrosis in cases of overdose leading to about 45,000 emergency room visits annually. Our laboratory has previously found good protection against APAP overdose by S-adenosyl-L-methionine (SAMe). In order to gain a better mechanistic understanding of how SAMe protects against APAP toxicity, we analyzed mitochondrial protein adduction by the reactive lipid intermediate 4-hydroxy-2-nonenal (4-HNE) as well as methionine S-oxidation. Additionally, we analyzed levels of the polyamines spermidine (Spd) and spermine (Spm) following APAP overdose since SAMe is essential for their synthesis. Each experiment consisted of 20 mice divided into: Veh (15 ml/kg water, ip), SAMe (1.25 mmol/kg, ip), APAP (250 mg/kg), and SAMe given one hour after APAP. We found 4-HNE adduction of several proteins including aldehyde dehydrogenase and carbamoyl phosphate synthase-1 (CPS-1) using proteomic analysis. We confirmed CPS-1 adduction with Western blotting. SAMe also protected levels of the polyamines Spd and Spm following overdose with APAP. The current work leads to a better understanding of the mechanism by which SAMe is able to prevent APAP toxicity.

Introduction

Acetaminophen (APAP) is a well-recognized and widely studied hepatotoxicant. APAP toxicity is clinically relevant as it is the number one cause of drug induced liver failure (Manthripragada et al., 2011). Post-translational modifications of proteins have been identified with many toxins including APAP. This drug can induce covalent binding by generation of a toxic P450-derived intermediate, NAPQI (Zhou et al., 1996). APAP can further induce oxidative stress resulting in a series of events that can generate reactive oxygen and nitrogen species, products recognized to induce covalent modifications of proteins (Knight et al., 2001).

The purpose of this study was to determine post-translational modification of proteins following APAP treatment in a mouse model. We first evaluated protein carbonyl generation in liver following APAP treatment and the reduction in protein carbonyl formation by treatment with SAMe. Studies conducted by the University of Arizona research group under the direction of Dr. Serrine Lau then evaluated 4-hydroxynonenal (4-HNE) adduction of liver proteins. Further studies by our laboratory then examined leakage of mitochondrial proteins into the cytosol as markers of damage and the protection afforded by SAMe. Finally, we examined whether APAP alters hepatic polyamine pathway products spermine and spermidine and whether SAMe preserves the polyamine pathway in APAP treated mice.

Methods

Materials*:* SAMe toluene sulfonate salt was used for the experiment and was purchased from Sigma Chemical Company (St. Louis, MO). All other chemicals were purchased from Sigma Chemical Company or Fisher Scientific (Pittsburgh, PA). Alanine aminotransferase (ALT) was

assayed using a method based on the discontinued Sigma 505-P kit described by the Harvison laboratory (Patel et al., 2011).

***Animals**:* Male C57Bl/6 mice were obtained from Hilltop Lab Animals Inc. (Scottsdale, PA) and were between 4-8 weeks of age weighing 18-24 g at the time of the experiment. Mice were fasted for 16 hours prior to the experiment.

***Treatment Groups**:* Mice were randomly allocated into the following groups: vehicle (Veh; 15 ml/kg water, ip inj), SAMe (1.25 mmol/kg 5 ml/kg, ip inj), APAP (250 mg/kg 15 ml/kg, ip inj), and SAMe given 1 hour following APAP (S+A; S + A). Mice were anesthetized with carbon dioxide 4 hours after APAP administration. Blood was collected by cardiac puncture in heparin-rinsed 1 mL syringes for determination of plasma ALT activity. Mitochondria and cytosolic fractions were then obtained for Western blotting.

***Mitochondrial Isolation**:* Mitochondria were isolated using a modification of a previously published protocol by Gogvadze and colleagues (2006). Briefly, the liver was isolated, blotted, weighed and placed in Mitochondrial Isolation Buffer A (225 mM sucrose, 3 mM KH_2PO_4, 5 mM $MgCl_2$, 20 mM KCl, 20 mM triethanolamine, 2 mM EGTA; pH 7.4). The liver was minced and homogenized in a Dounce homogenizer on ice. Following homogenization, the liver was centrifuged at 600 x *g* for 10 minutes. The resultant pellet was discarded and the supernatant was centrifuged at 15,000 x *g* for 5 minutes. After the final centrifugation, the supernatant was retained for analysis of cytosolic SAMe levels. The pellet containing the mitochondria was resuspended in Mitochondrial Isolation Buffer B (Same as Buffer A except lacking EGTA) for a final concentration of 1 mg tissue weight/1 ml Buffer B. Samples were stored at -80°C until analysis.

***Western Blotting**:* Western blot analysis was conducted to examine expression of CPS-1 and then stripped and reprobed with 4-HNE to confirm adduction. A 100 µg cytosolic protein aliquot was denatured by boiling for 5 minutes. Samples were separated on a 12.5% polyacrylamide gel and transferred to a nitrocellulose (NC) membrane (Whatman; Dassel, Germany). Transfer efficiency was verified using MemCode® Reversible Protein Stain Kit (Thermo Scientific; Rockford, IL). The membrane was then blocked using a 5% (w/v) milk/TBST solution (10 mM Tris-HCl, 150 mM NaCl, 0.1% Tween-20; pH 8.0) for 1 hour. Membranes were next incubated overnight with constant shaking at 4°C in antibody for CPS-1 (sc-10515; Santa Cruz Biotechnology; Santa Cruz, CA) at a 1:200 dilution in 5% (w/v) milk/TBST. The membranes were washed four times with TBST. The donkey anti-goat HRP secondary antibody (sc-2020, Santa Cruz Biotechnology; Santa Cruz, CA) was diluted 1:2000 and incubated with the membrane for 1 hour. They were washed with TBST and developed using Amersham^{TM} ECL^{TM} Western Blotting Detection Reagents (GE Healthcare; Buckinghamnshire, UK). The membranes were then stripped and re-exposed to ECL to confirm stripping. Next, they were blocked and reprobed with 4-HNE primary antibody (393207; Calbiochem; Merck, Darmstadt, Germany). After appropriate washing, membranes were exposed to goat anti-rabbit IgG HRP for 1 hour (DC03L; Calbiochem), washed and developed as previously indicated.

Analysis of Polyamine Levels. Polyamines were detected by the use of a fluorescent HPLC protocol (Brachet et al., 1996). Although the assay was designed to detect Put, Spd, and Spm, I was only able to reliably detect Spd and Spm levels. The mouse liver was homogenized in 250 µl of 0.4 M $HClO_4$ and centrifuged at 1,500 *x g* for 15 minutes. The derivatization required dansylation of the polyamines for detection by fluorescence. The reaction mixture consisted of:

100 µL supernatant, 400 µL of 10 mg/mL dansyl-Cl, and 200 µL of saturated sodium carbonate. The reaction proceeded in the dark for 90 minutes at 50°C. The reaction was halted with 100 µL L-proline (100 g/L) for 30 minutes at 50°C. Spd and Spm were separated on a Luna 5u C18 250 x 4.6 mm ID column from Phenomenex. The program was a linear gradient from 100% solvent A: 0% solvent B to 0% solvent A:100% solvent B over a 28 minute period followed by 10 minutes at 100% solvent B. The flow rate was 1.5 mL/min. The excitation wavelength was 338 nm and the emission wavelength was 425 nm.

Determination of Mitochondrial Glutathione. Mitochondrial GSH was determined using a spectrophotometric assay (Anderson, 1985). For the assay, 200 mg of liver was homogenized in 5% sulfosalicylic acid (SSA) for a final volume of 1 mL. GSH was assessed using glutathione reductase with 5,5'-dithiobis(2-nitrobenzoic acid) and NADPH. GSSG was measured by derivitizing the samples with 2-vinylpyridine first allowing determination of a percentage of oxidized glutathione within the cell.

Post-translational modifications*:* Samples were run on a 2D SDS-PAGE gel and selected spots were isolated. Spots that exhibited increased 4-HNE adduction on the 2D gel in the APAP group compared with the S + A group were selected. Selected spots of interest were analyzed at the University of Arizona Proteomics center using a LTQ-Orbitrap Mass Spectrometer (financial support provided by National Institute of Health National Center for Research Resources 1 S10 RR028868-01). The data was analyzed using SEQUEST and Scaffold software.

Statistical Analysis: All values are reported as mean ± S.E.M. with n = 5 animals per group. The final number of animals for each experiment is noted below in the results section. Differences between the groups were determined using a One-Way ANOVA followed by a

Tukey's post-hoc test (SigmaStat; SPSS Inc. Chicago, IL). Significant differences were assessed using $\alpha = 0.05$.

Results:

ALT. APAP administration significantly elevated plasma ALT at 4 hours (Figure 22). SAMe administration following APAP significantly decreased plasma ALT at the same time period.

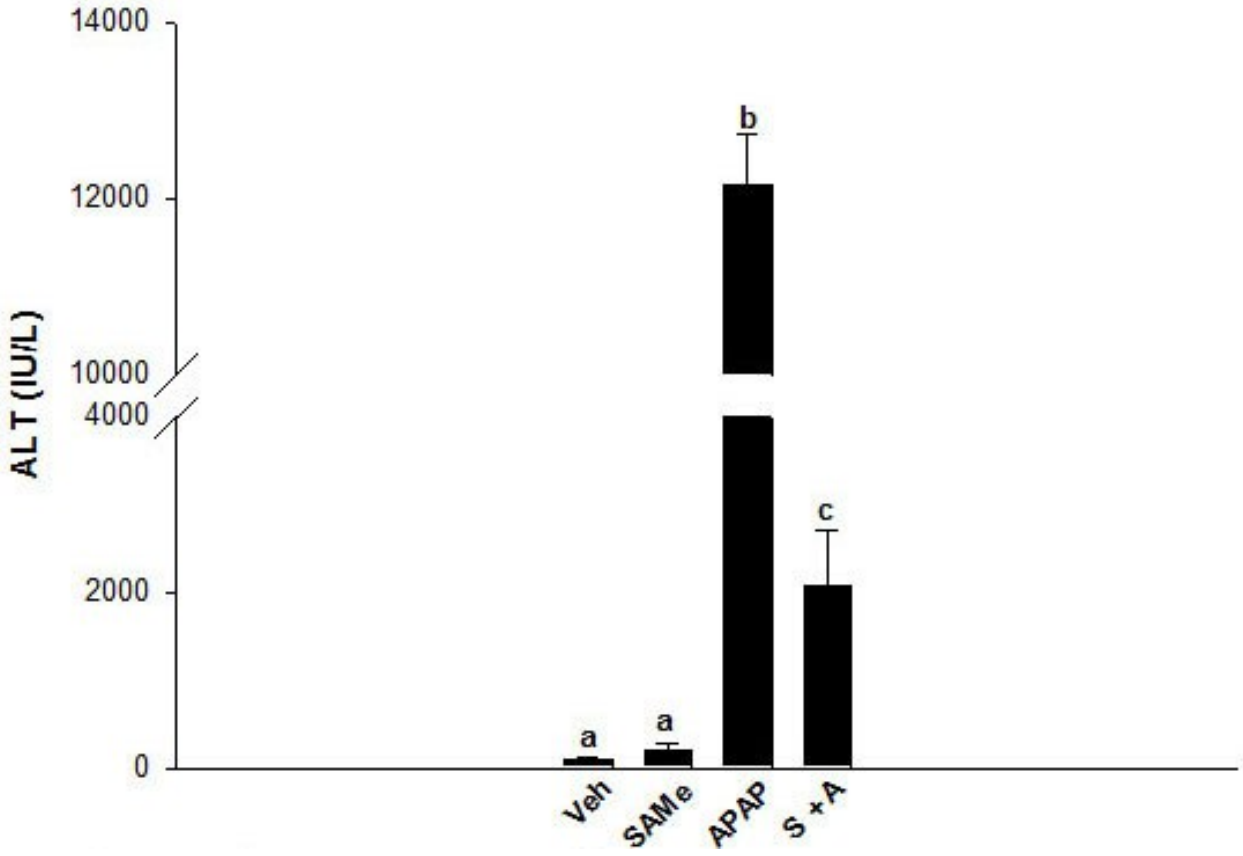

Figure 22. Plasma ALT levels in C57Bl/6 mice 4 h after APAP overdose. Mice were randomly allocated into the above listed groups and blood collected by cardiac puncture for ALT analysis. Values represent mean ± S.E.M. with n = 5 mice per group. Different letters denote statistical differences ($p<0.05$) between the groups.

Proteomics. A 2D gel was run in the Dr. Lau's laboratory and 10 spots were identified for analysis in both the mitochondrial and cytosolic fractions that were positive for 4-HNE binding. Figure 23 is an image of the 2D gel with the 10 spots identified. These spots were then analyzed for 4-HNE adduction (Table 9) and methionine oxidation (Table 10).

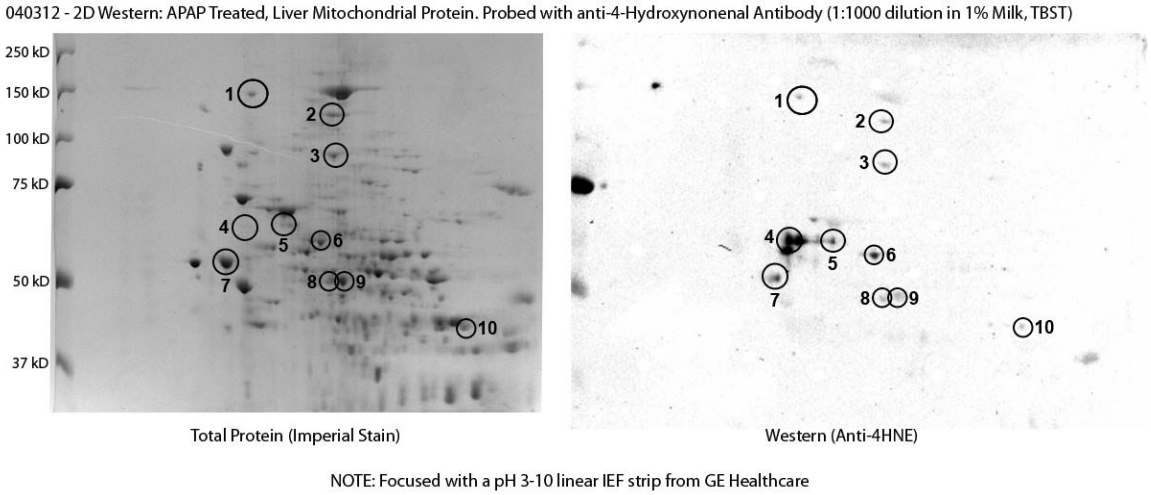

Figure 23. This is a representative 2D gel of mitochondrial samples 4 hour post APAP treatment. The left panel is protein staining and the right panel is 4-HNE adducted proteins.

Table 9. 4-HNE Protein modifications 4 hours Post APAP in Mitochondrial and Cytosolic proteins

Protein	Accession number	MW (kDa)	Subcellular location	Number of peptides	% Coverage
Aldehyde dehydrogenase, mitochondria	IPI00111218	57	Mitochondria	6-Spot 5 35-Spot 6 33-Spot 9	22-Spot 5 55-Spot 6 55-Spot 9
Carbamoyl phosphate synthetase (CPS-1)	IPI00111908	164	Mitochondria	49-Spot 3 35-Spot 6 34-Spot 8	36-Spot 3 30-Spot 6 29-Spot 8
Hypoxia up-regulated protein	IPI00123342	111	Mitochondria	46	51.25
Acyl-CoA dehydrogenase family member 9, mitochondria	IPI00331710	69	Mitochondria	13	26.24
Protein disulfide isomerase	IPI00133522	57	Mitochondria	50	70.33
Alanine aminotransferase	IPI00319992	55	Mitochondria	23-Spot 8 18-Spot 9	56-Spot 8 47-Spot 9
78 kDa glucose-regulated protein	IPI00319992	72	Mitochondria	24	38.02
Plastin-3	IPI00115528	71	Mitochondria	14	23.49

*The number of peptides represents how many individual peptides were adducted with 4-HNE. In cases where a spot is listed, the protein was observed at multiple locations corresponding with the spots identified in Figure 23. In the % coverage column, 4-HNE adduction contributes to the percent of global protein modification caused by APAP overdose.

Table 10. Methionine S-Oxidation modifications in Mitochondrial and Cytosolic Proteins 4 hours Post APAP

Protein	Accession number	MW (kDa)	Subcellular location	Number of peptides	% Average Coverage
Alanine aminotransferase	IPI00154045	55	Mitochondria	15	23 %
Aldehyde dehydrogenase	IP100111218	57	Mitochondria	12	6%
ATP Synthase subunit beta	IPI00468481	56	Mitochondria	8	8%
Betaine-homocysteine S methyltransferase	IPI00130950	45	Mitochondria	3	9%
Carbamoyl phosphate synthetase (CPS-1)	IPI00111908	16	Mitochondria	62	4%
Hypoxia up-regulated protein	IPI00123342	11	Mitochondria	12	16%
Glucose regulated protein 78kDa	IPI00319992	72	Mitochondria	71	11%
Mitochondrial Stress protein	IPI00880839	73	Mitochondrial	5	11%
S-adenosylmethionine synthase	IPI00454016	44	Mitochondria	1	3%
Sarcosine dehydrogenase	IPI00136213	102	Mitochondria	1	1%

*The number of peptides represents how many individual peptides were modified with methionine S-oxidation. In the % coverage column, methionine S-oxidation modification contributes to the percent of global protein modification caused by APAP overdose.

4-HNE Western Blots. Our previous collaboration with Dr. Serrine Lau from the University of Arizona on 4-HNE adduction lead to a possible mitochondrial target of carbamoyl phosphate synthase-1 (CPS1). We decided to confirm their mass spectrophotometry results to see if we have a novel target for 4-HNE adduction in cases of APAP overdose. First, we sought to find if CPS-1 leaks from the mitochondria as cytochrome c does following APAP overdose. CPS-1 appeared in the cytosol in a significantly higher amount following APAP overdose (Figure 24). SAMe given following APAP decreased leaking of CPS-1 into the cytosolic fraction (Figure 24). SAMe alone did not alter CPS-1 in either the mitochondria or cytosol.

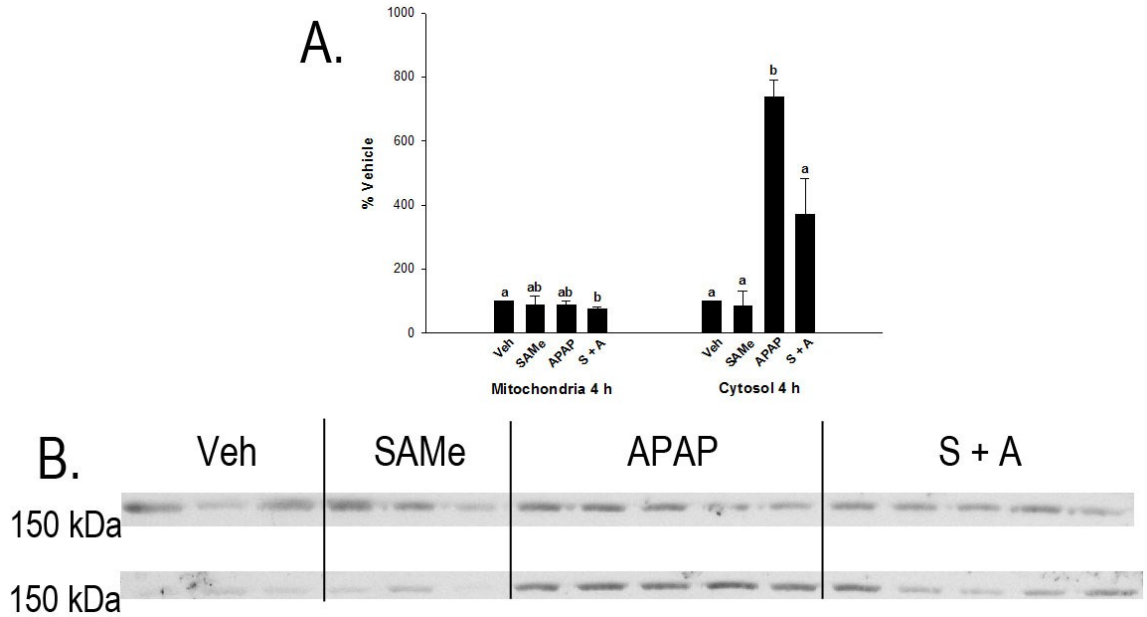

Figure 24. Mitochondrial and cytosolic fraction CPS-1 expression 4 hours following APAP overdose. Panel A contains the results for the CPS-1 Western blots. Protein expression was normalized to total protein lane staining. Values represent mean ± S.E.M. with n=3-5 mice per group. Superscripts denote statistical differences between the groups. Panel B contains representative mitochondrial (top) and cytosolic (bottom) CPS-1 Western blots 4 hours following APAP administration. Lanes marked with the appropriate treatment. Different letters denote statistical differences ($p<0.05$), while groups with the same letters indicate no difference in the results.

We also demonstrated 4-HNE adduction of CPS-1 confirming Dr. Lau's previous work. CPS-1 in the cytosol immunostained with 4-HNE antibody exhibited greater 4-HNE adduction in the APAP group than the S+A group (Figure 25). Although some CPS-1 leakage was also observed when SAMe was given following APAP, the CPS-1 bands were not positive for 4-HNE adduction after the blots were stripped and re-probed for 4-HNE. No significant adduction of CPS-1 by 4-HNE was observed in either the Veh or SAMe treatment groups.

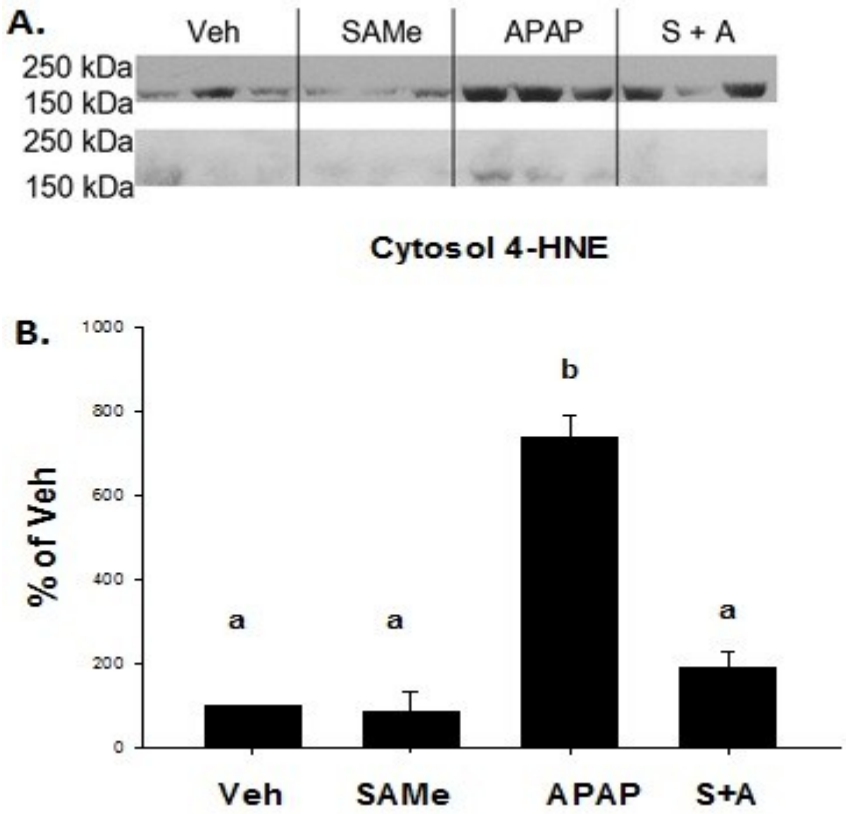

Figure 25. 4-HNE adduction of cytosolic CPS-1 4 hours following APAP overdose. Panel A(top) is a representative CPS-1 Western blot 4 hours following APAP administration. The blot was stripped and re-probed with 4-HNE antibody to confirm adduction. Groups are listed above the respective lanes. Relative protein expression is depicted in Panel B. APAP increased 4-HNE adduction of CPS-1. Different letters denote statistical differences ($p<0.05$), while groups with the same letters indicate no difference in the results.

Next, we examined mitochondrial and cytosolic fractions for oxidative stress. Figure 26 shows a Western analysis for cytosolic 4-HNE adduction 4 hours after APAP treatment. Figure 27 is a representative gel for the mitochondrial fraction. APAP increased mitochondrial and cytosolic 4-HNE adduction relative to VEH, SAMe and S+A groups. The relative amount of mitochondrial 4-HNE protein adduction at 2 and 4 hours post APAP are shown in Figure 28.

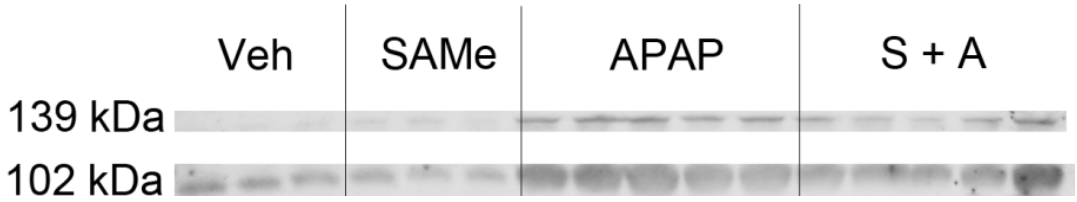

Figure 26. 4-HNE cytosolic protein adduction following APAP toxicity. Representative 4 hour Western blot used to examine 4-HNE adduction levels in Figure 28.

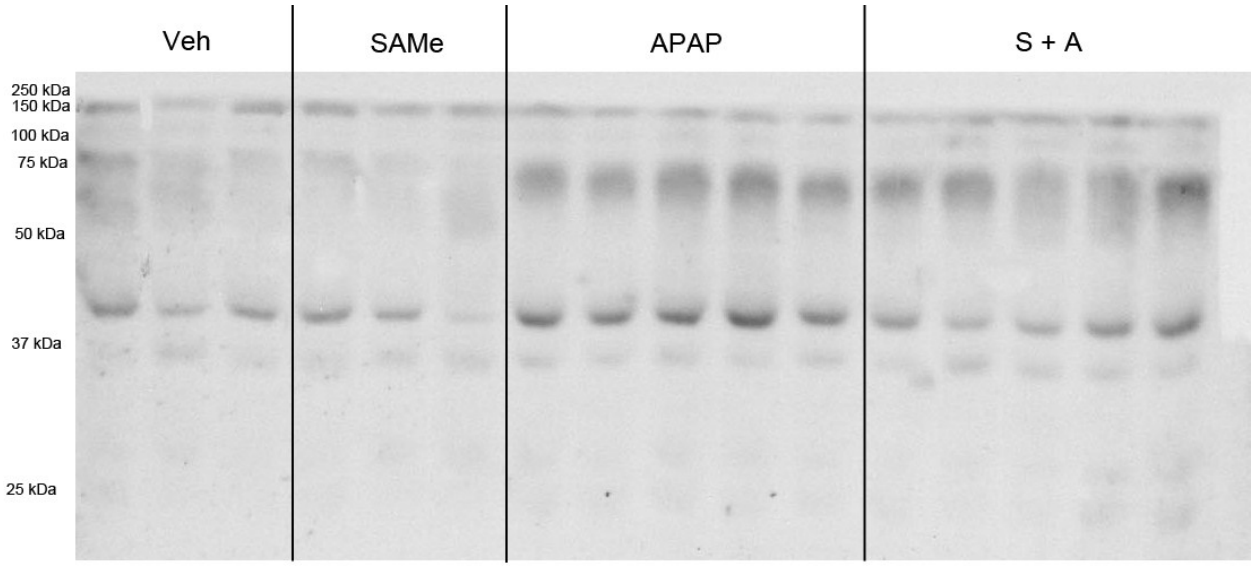

Figure 27. 4-HNE Adducted Proteins in Mitochondrial Fraction. Representative 4-HNE blot of Veh, SAME, APAP and SAMe treatment 1 hour after APAP (S+A). Mitochondrial samples depicted are 4 hours post APAP treatment. Densitometry analysis is shown in Figure 28.

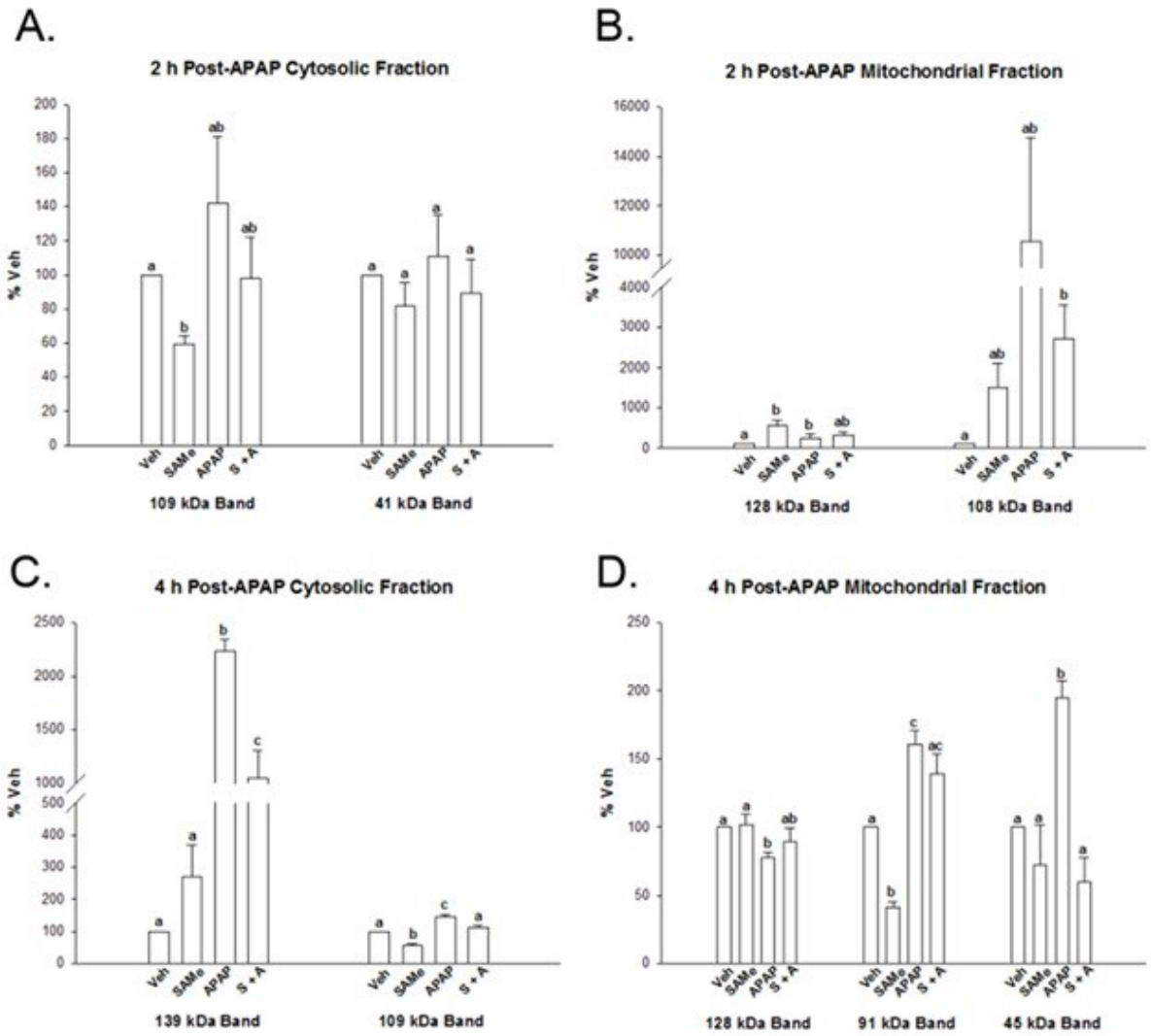

Figure 28. 4-HNE adduction of mitochondrial and cytosolic proteins 2 and 4 hours following overdose. Western blot analysis was conducted on mouse liver homogenate 2 and 4 hours following APAP overdose and 1 and 3 hours following SAMe administration respectively. Panel A and B represent cytosol and mitochondria 2 hours following APAP overdose. Panel C and D are the same fractions examined 4 hours following APAP overdose. The most dramatic differences were observed in the 4 hour treatment groups where the cytosolic 139 and 109 kDa bands were significantly reduced ($p<0.05$) when SAMe was given after APAP. Additionally, in the mitochondrial samples 4 hours following APAP administration, SAMe was able to reduce the signal on the 45 kDa band back to that of Veh ($p<0.05$). Different letters denote statistical differences ($p<0.05$), while groups with the same letters indicate no difference in the results.

Cytosolic fractions had a greater 4-HNE adduction of bands at MW 109 and 139 kDa in the APAP group compared to the VEH, SAMe and S+A APAP groups at 4 hour post APAP. The S+A group had less 4-HNE adduction at the 139 kDa cytosolic band than APAP but SAMe did not totally prevent 4-HNE adduction as the band intensity was higher than the VEH and SAMe groups. Mitochondria VEH, SAMe, and S+A groups showed a similar amount of 4-HNE adduction for the 45, 91 and 128 kDa bands. The APAP group had a higher intensity for 4-HNE adduction for the 45 kDa band compared to all other groups at 4 hour post APAP. The 91 kDa band was increased in the APAP group relative to the Veh and SAMe groups at 4 hour. The identity of the proteins comprising these bands is not known. The results indicate that 4-HNE adduction occurs at a higher level in the APAP group and this is prevented by SAMe administration (S+A group).

The Effect of SAMe on Mitochondrial GSH Following APAP overdose. Our laboratory previously demonstrated the ability of SAMe to protect against APAP toxicity induced GSH depletion. We expanded upon that finding by examining at 4 hour mitochondrial GSH levels following APAP overdose. The percentage of oxidized GSH was significantly increased 4 hours following APAP administration (Figure 29). SAMe decreased the amount of oxidized GSH to Veh levels.

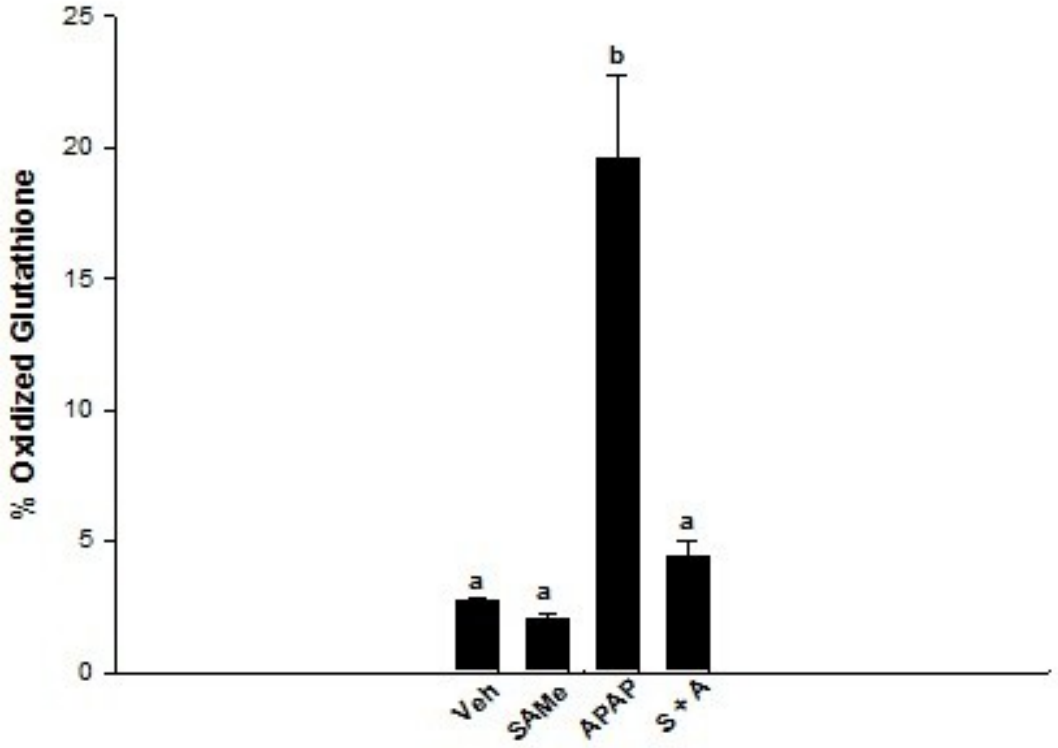

Figure 29. Mitochondrial percent oxidized GSH 4 hours after APAP administration. Mice were randomly allocated into Veh (water), SAMe (1.25 mmol/kg), APAP (250 mg/kg), and SAMe administered 1 hour following APAP. Livers were collected 4 hours following APAP administration and analyzed for total and oxidized glutathione to calculate the percent oxidized glutathione. All values represent mean ± S.E.M. with at least 4 mice represented in each group. Different letters denote statistical differences ($p<0.05$), while groups with the same letters indicate no difference in the results.

Polyamine Levels Following APAP Toxicity. SAMe has an integral role in the synthesis of Spd and Spm by acting as a propylamine donor in their synthesis. We therefore examined levels of those polyamines with APAP toxicity to determine if SAMe has a role in their synthesis during a toxic event. We also sought to examine Put levels; however, we were never able to reliably ascertain a Put peak (data not shown). At 2 hours following APAP administration, there was no difference in either Spd or Spm levels in any group compared with Veh (Table 11). However, at 4 hours, APAP treatment significantly increased Spd levels compared with Veh possibly

indicating increased flux through the polyamine pathway. Furthermore, when SAMe was given following APAP, the increase in Spd was significantly higher than the APAP group (Table 11). SAMe alone had no effect on Spd levels.

In contrast to Spd, Spm levels were decreased at 4 hours in the APAP treatment group (Table 11). SAMe alone had no significant effect on Spm levels even though there was an increase compared with Veh. Additionally, although SAMe increased Spm levels back to where they were not different than Veh, this did not represent a significant increase over the APAP only group (Table 11). By 6 hours, levels of Spd had returned to Veh levels in every treatment group and there were no significant differences. Spm, however, was still significantly decreased 6 hours following APAP administration compared with Veh (Table 11). Although SAMe was able to significantly increase polyamine levels at 6 hours when given following APAP, it did not succeed in returning levels to that of Veh mice. SAMe alone did not alter Spm levels at 6 hours.

Table 11. Polyamine levels following APAP overdose in C57BL/6 mice.

	Group	Spd (µmol/g tissue)	Spm (µmol/g tissue)
2 h	Veh	1.075 ± 0.0639[a]	6.182 ± 0.516[a]
	SAMe	0.893 ± 0.119[a]	5.835 ± 0.456[a]
	APAP	1.006 ± 0.120[a]	5.153 ± 0.828[a]
	S + A	0.799 ± 0.100[a]	5.213 ± 0.340[a]
4 h	Veh	1.262 ± 0.445[a]	6.312 ± 0.793[ab]
	SAMe	1.555 ± 0.395[a]	9.251 ± 1.138[a]
	APAP	10.716 ± 3.730[b]	2.354 ± 0.773[c]
	S + A	16.272 ± 0.612[c]	4.327 ± 0.543[bc]
6 h	Veh	1.641 ± 0.295[a]	9.454 ± 1.365[a]
	SAMe	2.492 ± 01.067[a]	7.933 ± 0.989[ab]
	APAP	1.948 ± 1.682[a]	4.708 ± 1.153[c]
	S + A	1.262 ± 0.182[a]	7.171 ± 0.384[b]

*Mice were randomly allocated into the following groups: Veh (water), SAMe (1.25 mmol/kg), APAP (250 mg/kg), SAMe 1 hour after APAP (S + A). SAMe and APAP were administered by ip injection. Values represent Mean ± S.E.M. (n=5). Spd and Spm levels were measured 2, 4 or 6 hour post APAP or Veh.

[abc] Different letters denote statistical differences ($p<0.05$), while groups with the same letters indicate no difference in the results.

Conclusion

Mitochondria have been recognized as an early target for APAP overdose. Generation of oxygen radicals during APAP overdose can lead to post-translational modifications of proteins by 4-HNE. Proteins can also be adducted by NAPQI, the toxic metabolite of APAP. The effect of these adductions is not known and future studies need to evaluate the long term effects of adduction on protein and enzyme function. The results of our study show that 4-HNE adducts proteins in the mitochondria and cytosol. SAMe administered 1 hour after APAP reduces APAP hepatic toxicity when evaluated 4 hours after APAP overdose. SAMe also prevents 4-HNE adduction of proteins in the mitochondria and cytosol. Proteomic analysis indicated 2 mitochondrial proteins (CPS-1 and sarcosine dehydrogenase) are adducted by 4-HNE. Further

studies indicated that CPS-1 leaks from the mitochondria into the cytosol following APAP administration and that SAMe reduced this leakage.

Acknowledgements

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Literature Cited

Gogvadze, V.; Orrenius, S.; Zhivotovsky, B. In: Maines, M. D.; Costa, L. G.; Reed, D. J.; Sassa, S.; Sipes, I. G., eds. *Current Protocols in Toxicology*, *volume 1*. Hoboken, NJ: John Wiley & Sons, Inc., 2006: 2.10.11-12.10.27.

Knight, T.R., Kurtz, A., Bajt, M.L., Hinson, J.A., and Jaeschke, H. (2001). Vascular and hepatocellular peroxynitrite formation during acetaminophen toxicity: role of mitochondrial oxidant stress. Toxicol Sci *62*, 212-220.

Manthripragada, A.D., Zhou, E.H., Budnitz, D.S., Lovegrove, M.C., and Willy, M.E. (2011). Characterization of acetaminophen overdose-related emergency department visits and hospitalizations in the United States. Pharmacoepidemiol Drug Saf.

Patel, N.N., Crincoli, C.M., Frederick, D.M., Tchao, R., Harvison, P.J., 2012. Effect of structural modifications on 3-(3,5-dichlorophenyl)-2,4-thiazolidinedione-induced hepatotoxicity in Fischer 344 rats. J Appl Toxicol. 32,108-1710.

Zhou, L., McKenzie, B.A., Eccleston, E.D., Jr., Srivastava, S.P., Chen, N., Erickson, R.R., and Holtzman, J.L. (1996). The covalent binding of [14C]acetaminophen to mouse hepatic microsomal proteins: the specific binding to calreticulin and the two forms of the thiol:protein disulfide oxidoreductases. Chem Res Toxicol *9*, 1176-1182.

Chapter VI: Summary and Conclusion

At the end of this thesis research, my project is as fascinating to me today as it was when I began the study over six years ago. In the field of toxicology, APAP is without doubt one of the most studied chemical entities. APAP even has an entire section devoted to its toxicity annually at the Society of Toxicology meeting. Additionally, SAMe represents an intriguing treatment option for APAP toxicity. Therefore, the goal of my work was to move SAMe forward as a potential therapeutic alternative to treat the high rate of APAP overdose observed in the United States. My work provides evidence for the ability of SAMe to protect against major causes of APAP toxicity, as well as highlighting the novel features that SAMe possesses over NAC that warrant further study and potential consideration of SAMe to treat APAP overdose in humans.

Cross-translation Between C57Bl/6 Mice and Humans

Perhaps the most pressing concern with any study using an animal model is the translation to humans. As stated in the methods and materials in Chapter II, the C57Bl/6 mouse model is an excellent analog for human metabolism of APAP. As for SAMe, its use as a drug in both humans and animals is also very well characterized. One of the attractive characteristics of SAMe as a potential therapeutic intervention is its oral bioavailability. Oral SAMe is absorbed primarily in the duodenum (Stramentinoli, 1987). Humans administered enteric coated ^{14}C-SAMe tablets demonstrated elevated plasma levels out to 48 hours following administration. In contrast, IV SAMe levels rapidly decline in human subjects over the same time period. Therefore, oral SAMe may actually be the more favorable treatment. This could prove advantageous for two reasons. First, SAMe would be directly delivered to the liver through portal blood system to a person suffering from APAP overdose. Second, there would not be metabolism and deposit of SAMe in other areas of the body such as the cerebrospinal fluid.

Approximately 60% of an oral dose in humans was maintained in stable pools (Stramentinoli, 1987). However, these experiments were conducted in otherwise healthy humans, not ones suffering from toxicity. There has been one study on oral SAMe in mice with APAP toxicity. BALB/c mice were protected by SAMe given orally (Oz et al., 2004). However, we chose not to use oral SAMe due to the prohibitive cost required to feed mice a steady diet of SAMe. An additional shortcoming of oral administration is the imprecise nature of the amount of SAMe ingested by the mice due to differences in feeding habits (Oz et al., 2004). Therefore, intraperitoneal injections of SAMe were used prior to my arrival in my thesis advisor's laboratory.

Further evidence of portal transport of SAMe being critical to absorption in the liver is found in a study using isolated perfused rat livers (Zappia et al., 1978). The researchers found that the uptake of SAMe by the liver was around 20% of the administered dose. However, of the SAMe absorbed, only 1% ended up in the bile, indicating the trapping of SAMe within hepatocytes. Another interesting finding from this study was decreased SAMe absorption in fatty liver induced by a choline deficient diet. SAMe absorption in the damaged fatty liver was less than the healthy liver (Zappia et al., 1978). This finding is of interest in context of my research. Examination of the 2 hour SAMe and S + A groups demonstrates that SAMe given after APAP is incorporated into mouse liver at a higher rate than the healthy SAMe only treatment group. Although fatty liver and APAP toxicity are different conditions, the difference in absorption is interesting.

All of these findings indicate that SAMe ip injection is a plausible analog for oral SAMe administration. Intraperitoneal injections are second only to IV in their rate of absorbance (Gad, 2007). Additionally, drugs given intraperitoneal are absorbed through the portal circulation of

the animal, which is a valid approximation of an oral dose of SAMe. Therefore, I believe that our model is well suited as an animal model, allowing inference to oral dosing in humans.

Liver Homogenate SAMe and SAH Levels Following APAP Overdose

My work clearly demonstrates intraperitoneal absorption of SAMe particularly at the early time points. An interesting additional experiment would be to examine SAMe levels in the plasma to determine if the spike observed in the 2 hour group where SAMe was given following APAP corresponds with decreased plasma availability of SAMe. In essence, do we see higher than 20% absorption of SAMe in cases of APAP toxicity?

Certainly, an interesting finding was that administration of NAC was able to maintain SAMe levels comparable to control at 4 hours following APAP administration. However, compared with the 4 hour SAMe group, the ratio was not significantly different in the group where NAC was given after APAP. The ratio of SAMe:SAH is critical for transmethylation reactions and, therefore, this could represent a subtle, but important, difference between SAMe and NAC treatment. One interesting unexplored variable in liver toxicology is what happens to SAH levels following APAP overdose.

Based on research discussed in the literature review, it is clear that elevated SAH levels inhibit critical cellular transmethylation reactions that could prove detrimental to hepatocytes. Therefore, an interesting question is the disposition of SAH in response to APAP toxicity. One possible answer is flux through the transsulfuration pathway to synthesize cysteine leading to enhanced GSH synthesis in the manner discussed earlier. Prudova et al. (2006) determined that cystathionine β-synthase, the enzyme responsible for converting homocysteine to cystathionine, was stabilized by the presence of SAMe. At the earliest time point studied, 2 hours, we noted a

significant increase in the ratio of SAMe:SAH (Figure 6). Examination of this data indicates that SAMe is not significantly decreased at that time point, but SAH is significantly depressed by 2 hours.

SAMe is a finite commodity within the cell. Therefore, once SAMe levels are depleted by 4 and 6 hours, it is unable to stabilize cystathionine β-synthase allowing accumulation of extra SAH decreasing the ratio of SAMe:SAH. However, this is a largely academic argument as there is clear evidence of GSH depletion within 15 minutes of APAP administration (Knight et al., 2001). Administration of SAMe following APAP not only provides substrate for additional GSH, but prior research from our laboratory demonstrates significant increases in GSH when SAMe is given as an antidote to APAP administration (Terneus et al., 2008). Thus, even though SAH levels increase in the S + A groups at 4 and 6 hours (Figure 6), there is a plausible outlet for the extra SAH generated due to the observed increase in SAMe at the same time period. Indeed, examining the N + A group in comparison to the S + A group at 4 hours (Tables 4 and 5), one finds that SAMe did lower SAH levels compared with Veh when given after APAP, albeit not significantly. However, NAC under the same conditions had slightly higher SAH levels when given as an antidote. An interesting future study would be to examine cystathionine β-synthase following APAP toxicity and determine the effect of SAMe on its expression and activity when given as an antidote to APAP overdose.

There is little evidence for the recycling of homocysteine to SAMe through the methionine cycle. A review of the literature yields no information on the important cofactor Vitamin B12 during APAP toxicity. This vitamin is necessary for the function of methylfolate-homocysteine methyltransferase (Finkelstein, 1990). Folate, which is also essential in homocysteine recycling,

appears unaffected by long-term administration of APAP (Varela-Moreiras et al., 1994). Recycling of SAH to SAMe seems unlikely; it would be far easier for a cell to simply use the SAH to replenish the rapidly diminishing stores of GSH.

Mitochondrial and Nuclear SAMe Following APAP Overdose

The overwhelming majority of work on SAMe and mitochondrial function in instances of toxicity has focused on ethanol toxicity in hepatocytes. In those studies, there is convincing evidence that SAMe can protect mitochondrial protein synthesis (Sykora et al., 2009) and also protects against oxidative stress damage to the oxidative phosphorylation pathway by stimulating synthesis of protective proteins (Andringa et al., 2010). Certainly, protection against oxidative stress induced mitochondrial damage is an attractive prospect. Additionally, Song and others (2007) used 3-deza-adenosine to inhibit S-adenosylhomocysteine hydrolase that is responsible for breaking down SAH into homocysteine and adenosine. Inhibition of this critical methionine cycle enzyme lead to greatly reduced mitochondrial. We demonstrated that giving SAMe after APAP overdose protects mitochondrial SAMe levels that are key to mitochondrial function (Figure 8). As will be discussed later, this retention of SAMe also protects mitochondrial GSH levels.

APAP decreased SAMe levels below detection. There is sparse information relating to nuclear SAMe levels in instances of toxicity. SAMe was able to prevent nuclear blebbing in arsenic treated lymphocytes (Ramirez et al., 2003). Whether this is at all applicable to the current research is unknown. However, there is the potential for an interesting follow-up study looking at the effect of APAP, a short-term toxicant, on long-term DNA methylation levels following overdose. A bacterial study demonstrates that APAP does adduct DNA, most likely through NAPQI (Dybing et al., 1984). It is interesting to speculate about the consequences of APAP

adduction to DNA in survivors of APAP overdose. Of particular concern is the liver since it has significant regeneration following APAP overdose in survivors.

Methionine Adenosyltransferase

We decided to examine MAT expression and activity in order to ascertain whether it might play a role in SAMe depletion following APAP overdose. APAP has been demonstrated to reduce MAT activity in mice (Shirota et al., 2002). However, we did not find changes in MAT activity at either 2 or 4 hours post APAP administration. This represents one of the more vexing findings in the research, but I have a few theories to explain these results. First, we used 250 mg/kg APAP and the study showing inhibition used 400 mg/kg APAP. Second, we did not see any expression of MAT II which would be anticipated to be induced when SAMe is given after APAP. I suggest that longer term studies may prove beneficial in determining MAT II activity if present. Despite these vexing findings, we demonstrated for the first time that expression of MAT I/III is decreased both 2 and 4 hours after APAP overdose and that SAMe protects MAT expression (Figure 11).

Improvement in Antioxidant Enzyme Function by SAMe

Our work found improvement in all liver homogenate antioxidant enzyme function in the S + A group at the 4 hour time point (Figures 13-17). We are proposing SAMe as an alternative treatment to NAC for APAP toxicity and a key question is how the two treatments compare in regards to antioxidant enzyme function. In a perfect world, we would have had time to conduct the NAC studies in our model for a valid comparison; however, that is one of a few experiments left to be done by a future graduate student.

Several studies have also looked at NAC protection of antioxidant enzyme function with APAP overdose. One study looked at NAC given 30 minutes before a 400 mg/kg APAP dose. Livers were collected at 6 hours. GSSG reductase was increased, but not back to levels of control animals in the study (Acharya and Lau-Cam, 2010). SAMe, by comparison, returned GSSG reductase to a level similar to Veh by 4 hours indicating, in this case, SAMe provides better protection than NAC which by 6 hours had not yet returned GSSG reductase activity to control levels. Another study in Wistar rats given NAC daily for 15 days prior to APAP injection (650 mg/kg) found that catalase, SOD (unspecified), and GPx were returned to control levels by NAC (Yousef et al., 2010). However, this is not a clinically relevant way to administer NAC to counteract APAP overdose.

The question remains whether NAC and SAMe are equivalent in preserving antioxidant enzyme function subsequent to APAP overdose. I do not believe that the two mechanisms of action are necessarily mutually exclusive since there is evidence by our laboratory that SAMe replenishes GSH levels similar to NAC (Terneus et al., 2008). Additionally, there is not a good comparison study looking at NAC and Mn-SOD, the mitochondrial variant of SOD. Yoshikawa and others found that knockdown of Mn-SOD greatly increases hepatotoxicity secondary to APAP overdose in rats (Yoshikawa et al., 2009). The major source of oxidative stress in APAP toxicity revolves around the mitochondria and, therefore, damage to Mn-SOD would be expected to worsen toxicity. By 4 hours, there is a clear depression of Mn-SOD activity in the APAP treatment group. SAMe partially corrects this decrease which could prove very advantageous to the cell. Whether this effect of SAMe on Mn-SOD is due to a unique action of SAMe, or a byproduct of GSH replenishment, remains to be determined. In summary, both SAMe and NAC seem to provide critical antioxidant enzyme protection following APAP overdose.

Mitochondrial Protection by SAMe

To my knowledge, our laboratory was the first to examine mitochondrial protein carbonyl formation associated with APAP toxicity. There are parallels in other mitochondrial toxicants, however. For instance, lipoic acid can reduce protein carbonyl formation on mitochondrial proteins brought about by methotrexate (Tabassum et al., 2010). Additionally, we found at both 2 and 4 hours, protection in the treatment S + A treatment groups (Figure 18).

Similarly, 3-NT formation on mitochondrial proteins has been investigated using a scavenger of peroxynitrite (Loguidice and Boelsterli, 2011). The authors found that scavenging with a peroxynitrite specific scavenger prevents mitochondrial 3-NT protein formation. This was obviously not the focus of their work, but a byproduct of the data collected. Our work shows for the first time that a non-peroxynitrite treatment for APAP overdose is able to prevent 3-NT mitochondrial protein formation. Further confirmation of the reduced oxidative stress is noted by our findings that oxidized glutathione is maintained at normal levels when SAMe is given after APAP (Figure 29). Additionally, we discovered that SAMe prevents leakage of proteins known to increase in concentration in the cytoplasm following APAP overdose.

The literature lays out a clear case for cytochrome c leakage from the mitochondria during APAP toxicity. There is also evidence that treatment of APAP overdose can prevent cytochrome c release from the mitochondria (Latchoumycandane et al., 2006). NAC has been examined preventing the mitochondrial swelling with APAP toxicity, but not cytochrome c release. Additionally, SAMe protects against ethanol induced mitochondrial cytochrome c release in the liver, but had not previously been examined with APAP toxicity (Cabrales-Romero Mdel et al., 2006).

A 2002 study found that cytochrome c release is governed by two factors. First, mitochondria become electrodestabilized (Ott et al., 2002). The authors argue that lipid peroxidation could be a cause of this destabilization. Second, the cytosolic protein Bax must translocate to the mitochondria to allow release of the newly freed cytochrome c. A recent study by Kumari and Kakkar demonstrates that preventing Bax translocation to the mitochondria decreases cytochrome c release due to APAP toxicity (Kumari and Kakkar, 2012). The literature on SAMe and Bax mainly focuses on the paradoxical ability of SAMe to induce apoptosis in cancer cell lines (Zhao et al., 2002). In the PC12 cell lines, SAMe increases Bax levels thereby facilitating apoptosis. However, the role of SAMe in cancer treatment is beyond the scope of the current work. What we can infer is that SAMe decreases mitochondrial dysfunction and, theoretically, Bax translocation to the mitochondria, thereby creating the observed decrease in cytochrome c leakage into the cytosol. Future studies should investigate Bax translocation in our APAP toxicity model to further elucidate the role of SAMe in Bax translocation.

Finally, we assessed mitochondrial swelling to determine whether SAMe reduced this sequelae of APAP toxicity. At 2 and 4 hours SAMe did not prevent mitochondrial swelling when given after APAP (Figure 20). Thus, SAMe does not prevent all the mitochondrial effects of APAP toxicity. As discussed earlier, there is depletion of GSH within 15 minutes of APAP administration and we did not give SAMe for a full hour after APAP. Therefore, significant mitochondrial damage may already have occurred. We also used a harsh swelling assay that might have ensured swelling even in mitochondria partially protected by SAMe.

Polyamine Metabolism Following APAP Overdose, A New Perspective on SAMe Treatment of Toxicity

Previous attempts at preventing toxicity by increasing polyamine levels focused largely on injecting Put to boost levels of both Spd and Spm (Daikuhara et al., 1979). SAMe represents a promising therapeutic alternative to Put due to its well characterized human pharmacokinetics. Additionally, SAMDC and ODC have been examined during liver regeneration. Activity of both SAMDC and ODC is increased following a partial hepatectomy in rats (Pegg et al., 1982). However, the distinction between a partial hepatectomy and active fulminant liver necrosis must be noted. Given the role of components of the polyamine pathway at not only protecting the liver, but also aiding in liver regeneration, it is surprising that no one had examined SAMe as a potential therapeutic intervention aimed at increasing polyamine levels.

We attempted to examine levels of ODC and SAMDC via Western blot (data not shown). However, the results were unsuccessful and future studies will need to examine not only their expression, but also their function. In contrast, we were able to produce an intriguing time course study on Spd and Spm levels. Unfortunately, low levels of Put, coupled with background noise in the HPLC histogram, prevented us from determining the levels of Put. Since SAMe would not enter at the level of Put, but rather, dcSAMe would enter polyamine synthesis at Spd and Spm, our inability to measure Put does not detract from our findings Spd is increased in both the APAP and S + A groups in the 4 hour treated mice. The fact that the S + A group had significantly increased Spd compared with the APAP minus SAMe group also indicates that exogenous SAMe cycles through the polyamine synthesis pathway following APAP overdose. Since Spd is required for growth and differentiation of cells this increase in Spd could be linked to subsequent liver regeneration.

The effect of APAP and SAMe on polyamines needs further investigation. Long-term measurements of liver regeneration could potentially distinguish between the effectiveness of SAMe vs. NAC as a therapeutic intervention for APAP toxicity. If SAMe enhances liver regeneration, then its therapeutic potential could expand to a myriad of liver toxicants. Any potential therapy that could induce regeneration of the liver and reduce the need for liver transplants would be very beneficial.

Conformation of a Mitochondrial 4-HNE Adducted Protein CPS-1

My thesis work focused heavily on mitochondrial function protection by SAMe when used as an antidote to APAP toxicity. Previous work on the subject by Dr. Marcus Terneus demonstrated SAMe protection against liver homogenate 4-HNE protein adduction (Terneus et al., 2008). This work has been part of an ongoing collaboration with Dr. Serrine Lau at the University of Arizona in an attempt to examine specific proteins adducted with 4-HNE following APAP toxicity. In 2005, Dr. Lau's laboratory determined protein targets of 4-HNE (data not shown). Adduction by the lipid peroxidation byproduct 4-HNE could increase APAP toxicity and represents a new and heretofore unexamined pathway of APAP toxicity. Therefore, we sought to examine one of the earlier targets identified by Dr. Lau to confirm adduction, CPS-1. Additionally, we examined global mitochondrial 4-HNE adduction and the ability of SAMe to prevent the adduction.

4-HNE adducts the mitochondrial protein SIRT3 and inhibit its activity (Jacobs and Marnett, 2010). Therefore, modification by 4-HNE adduction could play a critical role in toxicity. We were able to successfully confirm with Western blotting the earlier work of Dr. Lau's laboratory that CPS-1 was adducted by 4-HNE. While perhaps not directly implicated in cellular death following APAP toxicity, CPS-1 dysfunction could increase ammonia concentrations in the blood of recovering patients. Additionally, our findings support prior work that APAP toxicity

significantly reduces CPS-1 activity (Gupta et al., 1997). There is evidence in rats that this increase in ammonia disrupts the blood brain barrier in rats (Scorticati et al., 2004). It is likely that the buildup of ammonia is predominantly a result of the observed necrosis of the liver disrupting the urea cycle, with CPS-1 modification playing a minor role. In addition to adducting proteins, 4-HNE is detoxified in part by GSH further depleting stores in the hepatocytes and increasing toxicity (Fritz and Petersen, 2011). Furthermore, SAMe clearly reduces mitochondrial 4-HNE adduction of CPS-1 and other proteins. These effects would prove advantageous to the survival of the cell by helping maintain proper energy gradients within the hepatocytes. Additionally, SAMe prevents both 4-HNE adduction and oxidative damage following APAP overdose. This is a novel finding involving SAMe effects on APAP toxicity and warrants further study.

In Conclusion

My thesis research over the past six years has led to a better explanation of the mechanism by which SAMe is able to prevent APAP toxicity (Figure 30). While a mechanistic understanding is not essential for use as a therapeutic intervention, one wonders if APAP would have been able to obtain FDA approval today if it was unknown how it worked. Additionally, work in Italy in the past decade has found use for SAMe in a medical prevention of liver toxicity. Researchers found patients being treated for cancer were able to extend treatment windows with reduced hepatotoxicity when SAMe was used as adjuvant therapy (Santini et al., 2003). I firmly believe that SAMe has great potential not only for APAP toxicity treatment, but a host of other liver toxicity disorders. Best of all, it is a natural product that our bodies produce large quantities of every day. I am excited to see what the future holds for SAMe as a therapeutic intervention.

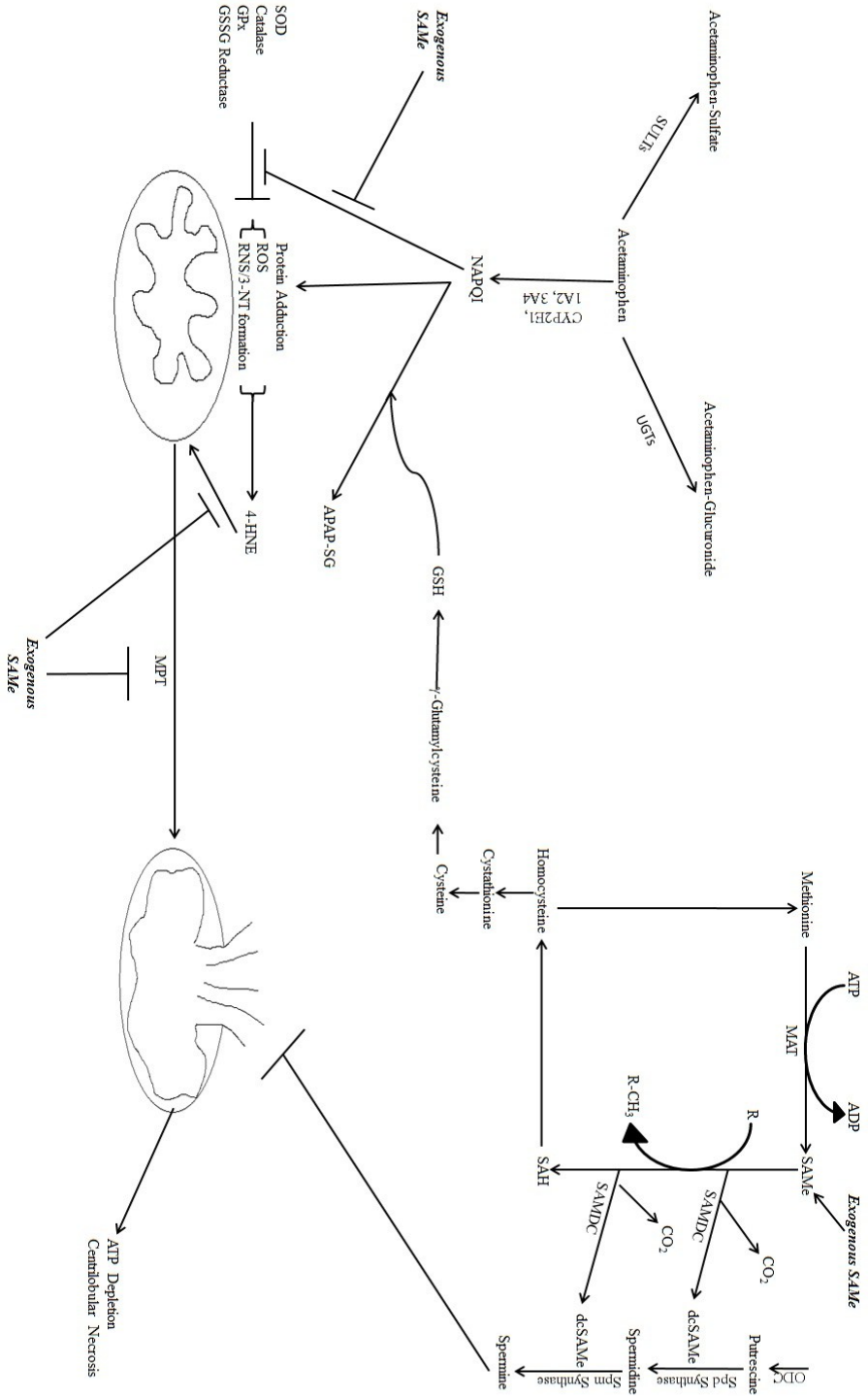

Figure 30. Summary of SAMe protection against APAP toxicity. I propose that exogenously administered SAMe protects against APAP toxicity by preventing antioxidant enzyme dysfunction. Protection of the antioxidant enzymes prevents formation of ROS and RNS and subsequent adduction by 4-HNE and formation of 3-NT on mitochondrial proteins. Exogenous SAMe is also able to maintain SAMe levels inside the cell which enables SAMe to participate in both GSH synthesis and Spd and Spm synthesis. GSH, Spd, and Spm are all protective to the

cell. Spm may possess direct mitochondrial protective ability itself. My findings offer greater understanding on how SAMe is able to so effectively prevent APAP toxicity when given following APAP overdose.

Literature Cited

Acharya, M., and Lau-Cam, C.A. (2010). Comparison of the protective actions of N-acetylcysteine, hypotaurine and taurine against acetaminophen-induced hepatotoxicity in the rat. J Biomed Sci *17 Suppl 1*, S35.

Andringa, K.K., King, A.L., Eccleston, H.B., Mantena, S.K., Landar, A., Jhala, N.C., Dickinson, D.A., Squadrito, G.L., and Bailey, S.M. (2010). Analysis of the liver mitochondrial proteome in response to ethanol and S-adenosylmethionine treatments: novel molecular targets of disease and hepatoprotection. Am J Physiol Gastrointest Liver Physiol *298*, G732-745.

Cabrales-Romero Mdel, P., Marquez-Rosado, L., Fattel-Fazenda, S., Trejo-Solis, C., Arce-Popoca, E., Aleman-Lazarini, L., and Villa-Trevino, S. (2006). S-adenosyl-methionine decreases ethanol-induced apoptosis in primary hepatocyte cultures by a c-Jun N-terminal kinase activity-independent mechanism. World J Gastroenterol *12*, 1895-1904.

Daikuhara, Y., Tamada, F., Takigawa, M., Takeda, Y., and Mori, Y. (1979). Changes in polyamine metabolism of rat liver after administration of D-galactosamine. Favorable effects of putrescine administration on galactosamine-induced hepatic injury. Gastroenterology *77*, 123-132.

Dybing, E., Holme, J.A., Gordon, W.P., Soderlund, E.J., Dahlin, D.C., and Nelson, S.D. (1984). Genotoxicity studies with paracetamol. Mutat Res *138*, 21-32.

Finkelstein, J.D. (1990). Methionine metabolism in mammals. J Nutr Biochem *1*, 228-237.

Fritz, K.S., and Petersen, D.R. (2011). Exploring the biology of lipid peroxidation-derived protein carbonylation. Chem Res Toxicol *24*, 1411-1419.

Gad, S., ed. (2007). Animal Models in Toxicology, 2nd edn (Boca Raton, FL, CRC Press).

Gupta, S., Rogers, L.K., Taylor, S.K., and Smith, C.V. (1997). Inhibition of carbamyl phosphate synthetase-I and glutamine synthetase by hepatotoxic doses of acetaminophen in mice. Toxicol Appl Pharmacol *146*, 317-327.

Jacobs, A.T., and Marnett, L.J. (2010). Systems analysis of protein modification and cellular responses induced by electrophile stress. Acc Chem Res *43*, 673-683.

Knight, T.R., Kurtz, A., Bajt, M.L., Hinson, J.A., and Jaeschke, H. (2001). Vascular and hepatocellular peroxynitrite formation during acetaminophen toxicity: role of mitochondrial oxidant stress. Toxicol Sci *62*, 212-220.

Kumari, A., and Kakkar, P. (2012). Lupeol prevents acetaminophen-induced in vivo hepatotoxicity by altering the Bax/Bcl-2 and oxidative stress-mediated mitochondrial signaling cascade. Life Sci *90*, 561-570.

Latchoumycandane, C., Seah, Q.M., Tan, R.C., Sattabongkot, J., Beerheide, W., and Boelsterli, U.A. (2006). Leflunomide or A77 1726 protect from acetaminophen-induced cell injury through inhibition of JNK-mediated mitochondrial permeability transition in immortalized human hepatocytes. Toxicol Appl Pharmacol *217*, 125-133.

Loguidice, A., and Boelsterli, U.A. (2011). Acetaminophen overdose-induced liver injury in mice is mediated by peroxynitrite independently of the cyclophilin D-regulated permeability transition. Hepatology *54*, 969-978.

Ott, M., Robertson, J.D., Gogvadze, V., Zhivotovsky, B., and Orrenius, S. (2002). Cytochrome c release from mitochondria proceeds by a two-step process. Proc Natl Acad Sci U S A *99*, 1259-1263.

Oz, H.S., McClain, C.J., Nagasawa, H.T., Ray, M.B., de Villiers, W.J., and Chen, T.S. (2004). Diverse antioxidants protect against acetaminophen hepatotoxicity. J Biochem Mol Toxicol *18*, 361-368.

Pegg, A.E., Poso, H., Shuttleworth, K., and Bennett, R.A. (1982). Effect of inhibition of polyamine synthesis on the content of decarboxylated S-adenosylmethionine. Biochem J *202*, 519-526.

Prudova, A., Bauman, Z., Braun, A., Vitvitsky, V., Lu, S.C., and Banerjee, R. (2006). S-adenosylmethionine stabilizes cystathionine beta-synthase and modulates redox capacity. Proc Natl Acad Sci U S A *103*, 6489-6494.

Ramirez, T., Garcia-Montalvo, V., Wise, C., Cea-Olivares, R., Poirier, L.A., and Herrera, L.A. (2003). S-adenosyl-L-methionine is able to reverse micronucleus formation induced by sodium arsenite and other cytoskeleton disrupting agents in cultured human cells. Mutat Res *528*, 61-74.

Reid, A.B., Kurten, R.C., McCullough, S.S., Brock, R.W., and Hinson, J.A. (2005). Mechanisms of acetaminophen-induced hepatotoxicity: role of oxidative stress and mitochondrial permeability transition in freshly isolated mouse hepatocytes. J Pharmacol Exp Ther *312*, 509-516.

Santini, D., Vincenzi, B., Massacesi, C., Picardi, A., Gentilucci, U.V., Esposito, V., Liuzzi, G., La Cesa, A., Rocci, L., Marcucci, F.*, et al.* (2003). S-adenosylmethionine (AdoMet) supplementation for treatment of chemotherapy-induced liver injury. Anticancer Res *23*, 5173-5179.

Scorticati, C., Prestifilippo, J.P., Eizayaga, F.X., Castro, J.L., Romay, S., Fernandez, M.A., Lemberg, A., and Perazzo, J.C. (2004). Hyperammonemia, brain edema and blood-brain barrier alterations in prehepatic portal hypertensive rats and paracetamol intoxication. World J Gastroenterol *10*, 1321-1324.

Shirota, F.N., DeMaster, E.G., Shoeman, D.W., and Nagasawa, H.T. (2002). Acetaminophen-induced suppression of hepatic AdoMet synthetase activity is attenuated by prodrugs of L-cysteine. Toxicol Lett *132*, 1-8.

Song, Z., Zhou, Z., Song, M., Uriarte, S., Chen, T., Deaciuc, I., and McClain, C.J. (2007). Alcohol-induced S-adenosylhomocysteine accumulation in the liver sensitizes to TNF hepatotoxicity: possible involvement of mitochondrial S-adenosylmethionine transport. Biochem Pharmacol *74*, 521-531.

Stramentinoli, G. (1987). Pharmacologic aspects of S-adenosylmethionine. Pharmacokinetics and pharmacodynamics. Am J Med *83*, 35-42.

Sykora, P., Kharbanda, K.K., Crumm, S.E., and Cahill, A. (2009). S-adenosyl-L-methionine co-administration prevents the ethanol-elicited dissociation of hepatic mitochondrial ribosomes in male rats. Alcohol Clin Exp Res *33*, 1-9.

Tabassum, H., Parvez, S., Pasha, S.T., Banerjee, B.D., and Raisuddin, S. (2010). Protective effect of lipoic acid against methotrexate-induced oxidative stress in liver mitochondria. Food Chem Toxicol *48*, 1973-1979.

Terneus, M.V., Brown, J.M., Carpenter, A.B., and Valentovic, M.A. (2008). Comparison of S-adenosyl-L-methionine (SAMe) and N-acetylcysteine (NAC) protective effects on hepatic damage when administered after acetaminophen overdose. Toxicology *244*, 25-34.

Varela-Moreiras, G., Ragel, C., and Ruiz-Roso, B. (1994). Effects of prolonged aspirin or acetaminophen administration to rats on liver folate content and distribution. Relation to DNA methylation and S-adenosylmethionine. Int J Vitam Nutr Res *64*, 41-46.

Yoshikawa, Y., Morita, M., Hosomi, H., Tsuneyama, K., Fukami, T., Nakajima, M., and Yokoi, T. (2009). Knockdown of superoxide dismutase 2 enhances acetaminophen-induced hepatotoxicity in rat. Toxicology *264*, 89-95.

Yousef, M.I., Omar, S.A., El-Guendi, M.I., and Abdelmegid, L.A. (2010). Potential protective effects of quercetin and curcumin on paracetamol-induced histological changes, oxidative stress, impaired liver and kidney functions and haematotoxicity in rat. Food Chem Toxicol *48*, 3246-3261.

Zappia, V., Galletti, P., Porcelli, M., Ruggiero, G., and Andreana, A. (1978). Uptake of adenosylmethionine and related sulfur compounds by isolated rat liver. FEBS Lett *90*, 331-335.

Zhao, W.Q., Williams, Z., Shepherd, K.R., Reuben, J.S., Lee, E.S., Darling-Reed, S., Lamango, N., Soliman, K.F., and Charlton, C.G. (2002). S-adenosyl-methionine-induced apoptosis in PC12 cells. J Neurosci Res *69*, 519-529.

Appendix A: Institutional Review Board

MARSHALL UNIVERSITY
www.marshall.edu

Office of Research Integrity

July 17, 2012

James M. Brown
Department of Pharmacology
Joan C Edwards School of Medicine
Marshall University

Dear Mr. Brown:

This letter is in response to the submitted dissertation abstract titled "A Mechanistic Study of S-Adenosyl-L-Methionine Protection Against Acetaminophen Hepatotoxicity." After assessing the abstract it has been deemed not to be human subject research and therefore exempt from oversight of the Marshall University Institutional Review Board (IRB). The Institutional Animal Care and Use Committee (IACUC) has reviewed and approved the study under protocols #372 and #475. The applicable human and animal federal regulations have set forth the criteria utilized in making this determination. If there are any changes to the abstract you provided then you would need to resubmit that information to the Office of Research Integrity for review and determination.

I appreciate your willingness to submit the abstract for determination. Please feel free to contact the Office of Research Integrity if you have any questions regarding future protocols that may require IRB review.

Sincerely,

Bruce F. Day, CIP
Director
Office of Research Integrity

Appendix B: Curriculum Vitae

J. Michael Brown
612 11th Avenue
Apartment 2B
Huntington, WV 25701
(304) 617-7775
jamesbrown@ucwv.edu

August 20, 2012

Education & Training

August 2010 - Present	Doctor of Pharmacy (Student), University of Charleston School of Pharmacy, Charleston, WV
July 2006 – August 2012	Doctor of Philosophy in Biomedical Sciences, Marshall University School of Medicine, Huntington, WV
August 2002 - May 2006	Bachelor of Arts in Chemistry, West Virginia Wesleyan College, Buckhannon, WV

Professional Licensure & Certifications

August 2010 – Present	Registered Pharmacy Intern in the State of West Virginia, License # IN0006447

Professional Experience

Internship

June 2012-August 2012

Veterans Affairs Hospital, Huntington, WV
Veterans Affairs Learning Opportunities Residency Program (VALOR)
Director of Pharmacy: Christine Waugh, Pharm.D.

- Daily activities include: Rotations through all of the different pharmacy departments within the Huntington VA hospital, medication discharge counseling, and various leadership development projects.

Pharmacy Practice Rotations

January 2012-April 2012

Community Pharmacy
Griffith and Feil Pharmacy, Kenova, WV
Preceptor: Rickey Griffith, R.Ph. and Heidi Griffith-Romero, Pharm.D.

- My daily activities include: fielding phone calls from patients and taking prescriptions from providers over the phone, counseling patients under the supervision of the PIC, and aiding in the flow of prescriptions through the pharmacy.

August 2011-November 2011

Institutional Pharmacy
Holzer Medical Center, Gallipolis, OH
Preceptor: Jill Strauch, R.Ph.

- Activities included: mixing parenteral drugs, antibiotic and anticoagulation clinics, and medication audits for The Joint Commission among others.

Research

July 2006 - Present

"Attenuation of acetaminophen toxicity in a recovery scenario using S-adenosyl-L-methionine (SAMe)"

- Collaborators: Monica Valentovic, Ph.D. (PI); John Ball (Technician)
- Research conducted at Marshall University School of Medicine as part of the requirement for the degree of Doctor of Philosophy
- Funding: I was awarded a grant through the NASA West Virginia Space Grant Consortium Graduate Research Fellowship Program to support the work which was used to supplement a grant from Dr. Valentovic through the West Virginia IDeA Network of Biomedical Research Excellence (NIH Grant 5P20RR016477)

Teaching

January 2008-August 2010

Instructor. The Princeton Review MCAT Preparation, Huntington, WV

- Biology and Organic Chemistry

August 2009-December 2009

Tutor. Marshall University Forensic Science Program, Huntington, WV

- Tutor in the Biochemistry course required for the program

October 2007

Seminar Lecture. Marshall University Biomedical Sciences program Huntington, WV

- Fifty minute lecture required for completion of a Seminar course given to the faculty and students of the Biomedical Sciences program

Peer-Reviewed Publications

June 2012

Brown, J.M., Ball, J.G., Wright, M.S., Van Meter, S., and Valentovic, M.A. Novel protective mechanisms for S-adenosyl-l-methionine against acetaminophen hepatotoxicity: Improvement of key antioxidant enzymatic function. Toxicol Lett. 2012 Aug 3; 212(3): 320-328.

August 2010

Brown JM, Ball JG, Hogsett A, Williams T, Valentovic M. Temporal study of acetaminophen (APAP) and S-adenosyl-L-methionine (SAMe) effects on subcellular hepatic SAMe levels and methionine adenosyltransferase (MAT) expression and activity. Toxicol Appl Pharmacol. 2010 Aug 15; 247(1):1-9.

February 2008

Terneus MV, **Brown JM**, Carpenter AB, Valentovic MA. Comparison of S-adenosyl-L-methionine (SAMe) and N-acetylcysteine (NAC) protective effects on hepatic damage when administered after acetaminophen overdose. Toxicology. 2008 Feb 3; 244(1):25-34.

Presentations

Oral

January 2011	"S-Adenosyl-L-methionine Attenuation of Oxidative Stress Induced by Acetaminophen Toxicity." Presented to the American Association of Pharmaceutical Scientists of the University of Charleston School of Pharmacy as part of the Brown Bag research lunch series.
October 2008	"S-Adenosyl-L-methionine (SAMe) attenuation of acetaminophen (APAP) toxicity: New insights." Presented to West Virginia Wesleyan College faculty and students, Buckhannon, WV.
November 2007	"Effect of S-adenosyl-L-methionine (SAMe) treatment given after acetaminophen (APAP) overdose on hepatic transmethylation substrates." Presented to Fairmont State University and West Virginia Wesleyan College faculty and students, Fairmont, WV and Buckhannon, WV.
March 2006	"S-Adenosyl-L-methionine alterations of transmethylation reactions in cases of acetaminophen overdose in C57Bl/6 mice." Presented to West Virginia Wesleyan College faculty and students, Buckhannon, WV.

Poster

May 2011	"S-Adenosyl-L-methionine Attenuation of Lipid Peroxidation Markers Following Acetaminophen Toxicity" by **Brown, JM**, Ball, JG, and Valentovic, MA. Presented at the Keystone Symposia: Lipid Biology and Lipotoxicity held at Ireland's National Events & Conference Centre, Co. Kerry, Killarney, Ireland.
March 2011	"Acetaminophen (APAP) and S-Adenosyl-L-methionine (SAMe) Effects on Oxidative Stress Enzymes: Attenuation by SAMe" by **Brown, JM**, Ball, JG, and Valentovic, MA. Presented at the Society of Toxicology Annual Meeting, Washington, DC.
March 2010	"Acetaminophen alterations of antioxidant enzyme function negated by S-adenosyl-L-methionine" by **Brown, JM**, Ball, JG, and Valentovic, MA. Presented at Marshall University School of Medicine Research Day, Huntington, WV
March 2010	"Acetaminophen alterations of subcellular S-adenosyl-L-methionine levels and hepatic metabolism" by **Brown, JM**, Ball, JG, and Valentovic, MA. Presented at the Society of Toxicology Annual Meeting, Salt Lake City, UT
March 2009	"Temporal study of acetaminophen (APAP) and S-adenosyl-L-methionine (SAMe) on subcellular hepatic SAMe levels and methionine adenosyltransferase (MAT) expression" by **Brown, JM**, Ball, JG, Hogsett, AA, Williams, T, and Valentovic, MA. Presented at the Society of Toxicology Annual Meeting, Baltimore, MD
March 2009	"Potential role of oxidative stress in resveratrol protection of cisplatin in vitro renal toxicity" by Valentovic, MA, Ball, JG, and **Brown, JM.** Presented at the Society of Toxicology Annual Meeting, Baltimore, MD
March 2009	"S-adenosyl-L-methionine (SAMe) reversal of acetaminophen (APAP) effects on hepatic glutathione peroxidase and hepatic SAMe levels" by Williams, T, **Brown, JM**, Ball, JG, Hogsett, AA, and Valentovic, MA. Presented at the Society of Toxicology Annual Meeting, Baltimore, MD

March 2008	"Effect of SAMe treatment given after acetaminophen (APAP) overdose on hepatic transmethylation substrates" by **Brown, JM** and Valentovic, MA. Presented at the Society of Toxicology Annual Meeting, Seattle, WA
March 2007	"Hepatic transmethylation alterations induced by acetaminophen overdose: SAMe:SAH ratio" by **Brown, JM**, Ball, JG, and Valentovic, MA. Presented at Marshall University School of Medicine Research Day, Huntington, WV

Honors & Awards

April 2012	University of Charleston School of Pharmacy Academic Excellence Award
April 2012	University of Charleston School of Pharmacy Service Award
April 2011	University of Charleston School of Pharmacy Academic Excellence Award
April 2011	University of Charleston School of Pharmacy Service Award
August 2010-Present	University of Charleston School of Pharmacy Fellow
August 2010	Best Overall Performance as a Graduate Student • Award granted for a combination of outstanding research and service to the Marshall University Biomedical Science Program
March 2010	Finalist for the Carl Smith Mechanisms Specialty Section Award in the Society of Toxicology • Award granted for the best research by an active doctoral student
March 2010	Society of Toxicology Travel Award Recipient
March 2009	Finalist for the Carl Smith Mechanisms Specialty Section Award in the Society of Toxicology • Award granted for the best research by an active doctoral student

Professional Activities

May 2012-Present	Phi Lambda Sigma at University of Charleston School of Pharmacy • Member of the Gamma Psi Chapter
February 2012-Present	ACPE Student Representative Class of 2014, University of Charleston School of Pharmacy
November 2011-Presesnt	Pharmacy Practice Department Chair Committee, University of Charleston School of Pharmacy
August 2011-Present	Professional Standards & Conduct Committee, University of Charleston School of Pharmacy
April 2011-Present	Education Committee Member West Virginia Society of Health System Pharmacists
March 2011-August 2011	Scholarship Committee Member, First United Methodist Church
March 2011	RxIMPACT Pharmacy Advocacy Project. Washington, D.C.
September 2010-August 2011	Student Affairs Committee Representative for the University of Charleston School of Pharmacy Class of 2014

September 2010-Present	American Society of Health System Pharmacy-Student Society of Health System Pharmacy at University of Charleston School of Pharmacy • **President**-April 2012-Present • **Treasurer**-April 2011-April 2012
September 2010-September 2011	Student National Pharmaceutical Association at University of Charleston School of Pharmacy • Member of the Programming Committee
September 2010-Present	American Association of Pharmaceutical Scientists
December 2006-Present	Society of Toxicology Student Member through Marshall University School of Medicine
July 2006-Present	Marshall University School of Medicine Graduate Student Organization • **President**- May 2009-April 2010 • **Vice President**- April 2008-May 2009 • **Secretary/Treasurer**- April 2007-April 2008

Community Service

June 2012	Volunteer. Charleston Area Medical Center HealthFest Charleston, WV.
April 2012	Volunteer. Health Fair at the West Virginia Society of Health System Pharmacists Annual Meeting in Charleston, WV.
April 2012	Volunteer and Planning Committee. Charleston Against Cervical Cancer.
March 2012	Volunteer. Prescription Drug Abuse Community Meeting at the Charleston Boys and Girls Club with the School of Pharmacy Fellows (3 hours)
March 2012	Volunteer. Health Fair for Operation Diabetes in conjunction with APhA (2 hours)
March 2012	Volunteer and Planning Committee. Zumbathon for Charleston Against Cervical Cancer (2.5 hours)
October 2011	Volunteer. Trunk or Treat Halloween at the Salvation Army in St. Albans, WV (1.5 hours)
October 2011	Volunteer. Susan G. Komen Foundation Walk in Huntington, WV (1 hour)
September 2011	Volunteer. Zumbathon for Ovarian Cancer with the Student Society of Health System Pharmacists (3 hours)
March 2011	Volunteer and Planning Committee. "Charleston Against Cervical Cancer" held in Charleston, WV with the Student Society of Health System Pharmacists (10 hours)
March 2011	Volunteer. National Kidney Foundation KEEP Screening held in Charleston, WV with the Student National Pharmaceutical Association (3 hours)
December 2010	Volunteer. Folding clothes to go to local shelters in Charleston, WV with the Student National Pharmaceutical Association (1 hour)
December 2010	Volunteer. Bead for Life bead sale to benefit impoverished regions of Uganda, Africa and in support of World AIDS day with the Student National Pharmaceutical Association (3 hours)

November 2010	Volunteer. Health Fair for Blood Pressure Screening held in Charleston, WV at Wal-Mart with the Student National Pharmaceutical Association (6 hours)
October 2010	Volunteer. Health Fair for Blood Glucose Screening held in Point Pleasant, WV at Fruth Pharmacy with the Student National Pharmaceutical Association (4 hours)
September 2010	Volunteer. Fundraising sale for the Student Society of Health System Pharmacy to benefit ovarian cancer research at the University of Charleston School of Pharmacy (1 hour)

Other Activities

Meetings Attended

April 2012	West Virginia Society of Health Systems Pharmacists, Charleston, WV.
October 2011	West Virginia Pharmacists Association Meeting, Charleston, WV
April 2011	West Virginia Society of Health Systems Pharmacists, Morgantown, WV
March 2011	Society of Toxicology Annual Meeting, Washington, D.C.
January 2011	Issues and Eggs Breakfast Sponsored by the West Virginia Chamber of Commerce, Charleston, WV
September 2010	West Virginia Pharmacists Association Meeting at Stonewall Jackson Resort, WV
March 2010	Society of Toxicology Annual Meeting, Salt Lake City, UT
March 2009	Society of Toxicology Annual Meeting, Baltimore, MD