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3,5-Dichloroaniline: Biotransformation and Mechanistic Aspects of Nephrotoxicity In Vitro

Christopher Robert Racine
racine.christopher@yahoo.com

3,5-DICHLOROANILINE: BIOTRANSFORMATION AND MECHANISTIC ASPECTS OF NEPHROTOXICITY IN VITRO

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Christopher Robert Racine
Approved by
Dr. Gary O. Rankin, Committee Chairperson
Dr. Monica Valentovic
Dr. Richard Niles
Dr. Travis Salisbury
Dr. Peter Harvison

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APPROVAL OF DISSERTATION

We, the faculty supervising the work of Christopher Robert Racine, affirm that the dissertation, *3,5-Dichloroaniline: Biotransformation and Mechanistic Aspects of Nephrotoxicity*, meets the high academic standards for original scholarship and creative work established by the Biomedical Science program and the Graduate College of Marshall University. This work also conforms to the editorial standards of our discipline and the Graduate College of Marshall University. With our signatures, we approve the manuscript for publication.

Dr. Gary Rankin	Committee Chairperson	11/3/16 Date
Dr. Monica Valentovic	Committee Member	11/3/16 Date
Dr. Richard Niles	Committee Member	11/2/16 Date
Dr. Travis Salisbury	Committee Member	11/2/16 Date
Dr. Peter Harvison	Committee Member	10/27/16 Date

DEDICATION

I dedicate this work to my loving wife, Holly, and to my daughter, Teegan. I am nothing without the love and support of you both, and this was never more evident than in the last few months. You gave me strength when I felt defeated and gave me hope when I felt despair. I will be forever grateful for the sacrifices you both made in order for me to complete this work.

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ABSTRACT

Chlorinated anilines have been used as important chemical intermediates in the production of a wide variety of pharmaceutical, industrial, and agricultural products. Exposure to chloroanilines can arise in both occupational and environmental settings and can lead to toxicity to multiple organs, including the kidney. Previous studies have established that of the mono- and dichlorinated anilines, 3,5-dichloroaniline (3,5-DCA) possesses the most nephrotoxic potential both in vitro and in vivo. However, little is known concerning the role of renal metabolism in nephrotoxicity. The studies presented in the following dissertation are the first to explore the toxicity, metabolism, and bioactivation of 3,5-DCA in isolated renal cortical cells (IRCC) obtained from male Fischer 344 rats, as well as to explore the nephrotoxic potential of putative 3,5-DCA metabolites. Results show that in IRCC, 3,5-DCA induced cytotoxicity in a concentration- and time-dependent manner. Further studies suggested that 3,5-DCA induced nephrotoxicity is due, at least in part, to bioactivation of 3,5-DCA to toxic metabolites via multiple enzyme systems. Additional studies suggested that CYP2C, FMO, and peroxidase metabolism of 3,5-DCA contributes to the bioactivation of 3,5-DCA to cytotoxic metabolites. In order to determine the ultimate toxic species, five putative metabolites were tested for their nephrotoxic potential. Of the five metabolites tested, only one, 3,5-DCAA was non-toxic. Further explorations were conducted to identify and quantify metabolites in IRCC after exposure to 3,5-DCA via HPLC analysis. The results of the HPLC studies show very little metabolism of 3,5-DCA in isolated renal cortical cells, which suggests that the ultimate toxicant is highly cytotoxic and most likely produced near its cellular target(s). However, the mechanism of 3,5-DCA induced cell death and cellular target remains to be determined.

CHAPTER I: AN IN-DEPTH REVIEW OF ANILINE AND ITS CHLORINATED DERIVATIVES

1.1 ANILINE AND CHLOROANILINE USE AND ROUTES OF EXPOSURE

Aniline and its mono- and dichlorinated derivatives have been used as chemical intermediates in the production of multiple agricultural, industrial, and pharmaceutical products. Aniline itself was first discovered in the early 1800s from indigo distillation and was produced following the reduction of nitrobenzene, although aniline was called crystallin or cyanol at the time. It wasn't until 1843 that Dr. August Hofmann showed that in fact crystallin and cyanol were actually the same compound, aniline (Hofmann, 1843). The earliest known use of aniline was in the production of synthetic dyes (Kahl et al., 2011). A student of Dr. Hofmann, William Perkins, accidentally produced the first aniline dye mauveine, also known as aniline purple (Perkin, 1879). The use of aniline dyes lead to one of the earliest studies demonstrating that aniline exposure can have toxic consequences (Muller, 1887). Currently, the major use of aniline is in the production of methylene dianiline, which is the precursor used in the production of urethane polymers (Kahl et al., 2011). The structure of methylene dianiline and aniline can be seen in figure 1.

Chlorinated anilines have also been shown to be important chemical intermediates in the manufacture of industrial compounds. For example, chlorhexidine, an antibacterial agent, uses 4-chloroaniline in its production (Rose and Swain, 1956). Chlorhexidine is widely used as topical disinfection to promote the healing of wounds in animals (Sanchez et al., 1988). Two products utilizing 4-chloroaniline as an intermediate are monolinuron and Pigment Red 184. Monolinuron is a broad leaf pesticide used to control weeds in potato, leek and French bean fields (Rossoff,

2002; Milne, 2005). Monolinuron's structure can be found in figure 1. Propanil, a post-emergent herbicide used to control weeds in rice and wheat fields, is synthesized from 3,4-dichloroaniline (Warren and Wyatt 2008). Vinclozolin, iprodione, and dimetaclone are all agricultural fungicides, which contain a 3,5-dichloroaniline moiety. The structure of each can be found in figure 1. These products highlight just a few uses of aniline and its chlorinated derivatives.

Generally, exposure to large amounts of pure chloroaniline only occurs in industrial settings, accidental poisonings, or suicide attempts. Exposure to high levels of pure chloroaniline leads to a variety of toxic conditions, which will be discussed in a later section. However, studies have suggested that chloroaniline exposure can also occur in smaller amounts via multiple environmental routes. Demers and Yates (1977) showed that chemical degradation of two common antimicrobial compounds, 3,4,4'-trichlorocarbanilide and 4,4'-dichloro-3-(trifluoromethyl) carbanilide, found in deodorant bars yielded 4-chloroaniline and 3,4-dichloroaniline. Chloroanilines have also been detected in soil following the microbial degradation of pesticides (Bartha and Pramer, 1967; Bartha, 1968; Kearney and Plimmer, 1972; You and Bartha, 1982). Khan, Marriage, and Saidak (1976) demonstrated that low levels of 3,4-dichloroaniline, a microbial breakdown product of Diuron, persisted in soil samples after treatment with Diuron. 3,5-Dichoroaniline is the major microbial breakdown product of two carboximide fungicides, iprodione and vinclozolin, in soil (Campos et al., 2015; Walker, 1987a, 1987b). Chlorinated anilines have also been found in groundwater (Wegman and de Korte, 1981) and industrial runoff (Lyons, Katz, and Bartha, 1984, 1985). Exposure to chlorinated anilines has been explored in great detail and results in hematotoxicity, splenotoxicity, and of most interest to the current work in our lab, nephrotoxicity.

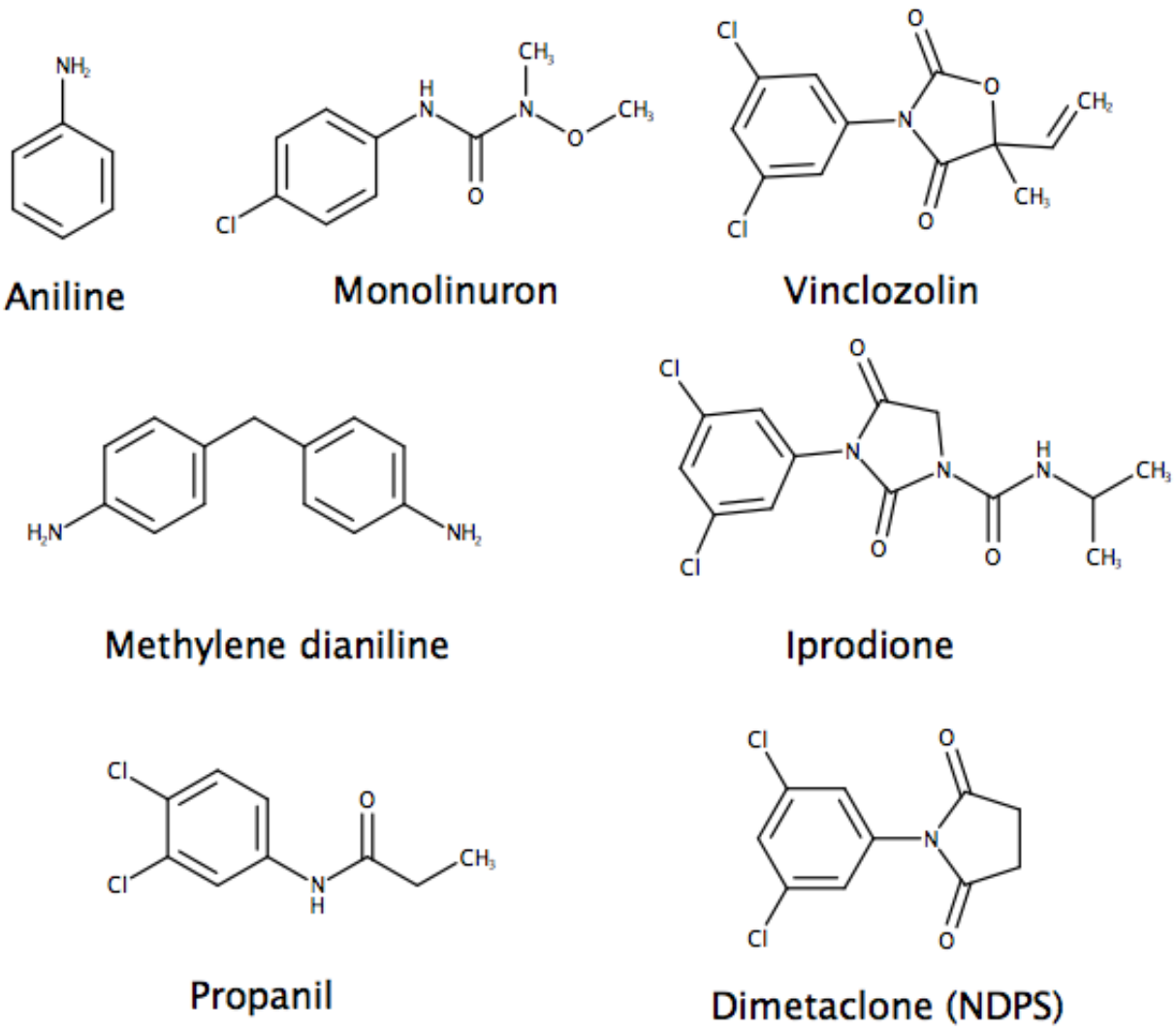

Figure 1. Chemical structure of aniline and related products

1.2 HEMATOTOXICITY OF ANILINE AND CHLOROANILINES

The major type of hematotoxicity seen following exposure to chlorinated anilines is methemoglobinemia. Methemoglobinemia occurs when the iron in the heme of hemoglobin is oxidized to the ferric (Fe^{3+}) form. This oxidation of the heme iron results in the formation of methemoglobin, and greatly reduces the ability of the blood to transport oxygen. The inability of methemoglobin to carry oxygen throughout the body can lead to varying degrees of cyanosis. Under normal physiological conditions, methemoglobin is present in the blood at concentrations less than 2%; however, levels up to 20% can be tolerated fairly well. Once the concentration of methemoglobin reaches greater than 70%, death can occur. Methemoglobinemia can be hereditary or acquired. Hereditary methemoglobinemia is the result of a deficiency in NADH cytochrome b5 reductase, the enzyme responsible for converting non-functioning methemoglobin to functioning hemoglobin. In contrast, acquired methemoglobinemia occurs following exposure to exogenous compounds. The treatment for acquired methemoglobinemia is the infusion of methylene blue, whose mechanism requires NAD or NADPH dependent methemoglobin reductase to reduce methemoglobin to hemoglobin (Nascimento, Pereira, de Mello, and Costa, 2008; Udeh, Bittikofer, and Sum-Ping, 2001). Careful attention must be paid to the concentration of methylene blue infused as a treatment for methemoglobinemia because at higher concentrations methylene blue can promote methemoglobin formation; resulting in a more severe methemoglobinemia and hemolytic anemia (Liao, Hung, and Yang, 2002).

Methemoglobinemia, as a consequence of aniline exposure, was first described in 1959 (Ramsey and Harvey, 1959). In this study, 17 newborn babies presented with methemoglobinemia after dermal exposure to diapers marked with aniline dye. These newborns presented with cyanosis and accelerated respiration. In some of the newborns, distention,

jaundice, enlargement of the spleen, hematuria, loose stool, and vomiting were also present. While exposure to aniline is the classic example of chemically induced methemoglobinemia, studies with p-chloroaniline in animals suggest that the p-chloroaniline is even more potent as a hematotoxicant than aniline (Nomura, 1975). Induction of severe methemoglobinemia has been well documented in case studies where workers were exposed to p-chloroaniline (Scotti and Tomasini, 1966; Faivre, Armand, Evreux, Duvermeuil, and Colin, 1971). For example, Pizon et al. (2009), examined a case where a 20-year old worker, whose job was to remove unknown waste from barrels and place the waste in an incinerator, was found cyanotic, lethargic, and in respiratory distress. By the time the worker was examined at the hospital, he was in a coma and had a methemoglobin level of 69%. Following three hours of methylene blue administration and mechanical ventilation, the patient recovered. Since the agent responsible for his condition was unknown, a comprehensive urine drug analysis was performed using GC/MS. Results of the urine analysis showed detectable levels of p-chloroaniline and p-chloroacetanilide, a known metabolite of p-chloroaniline, suggesting that the methemoglobin was caused to exposure to p-chloroaniline.

Valentovic et al. (1997) showed that acute exposure to 3,5-dichloroaniline was able to induce methemoglobinemia in male Fischer 344 rats. In this study, a single intraperitoneal (i.p) injection of 0.8 mmol/kg 3,5-dichloroaniline induced methemoglobinemia levels, which peaked at two hours post injection and returned to control levels after eight hours. Valentovic et al. (1997) also explored the ability of two putative metabolites of 3,5-dichloroaniline, 4-amino-2,6-dichlorophenol and 3,5-dichlorophenylhydroxylamine, to induce methemoglobinemia, since previous studies suggested that N-hydroxylated metabolites of aniline are responsible for the aniline-induced methemoglobinemia (Harrison and Jollow, 1987; Jenkins, Robinson, Gellatly,

and Slamond, 1972). In vitro studies using freshly isolated washed erythrocytes from male Fischer 344 rats showed that 3,5-dichloroaniline was able to induce methemoglobin formation after 60 minutes at concentrations greater than 4 mM. In contrast, 4-amino-2,6-dichlorophenol, a putative metabolite of 3,5-dichloroaniline, was able to generate methemoglobin in a concentration dependent manner after a 30 minute exposure at concentrations greater than 0.2 mM. Finally, 3,5-dichlorophenylhydroxylamine, a putative N-hydroxylation metabolite of 3,5-dichloroaniline, raised methemoglobin levels significantly after 30 minutes with concentrations greater than 2 μM (Valentovic et al., 1997). These studies were the first to introduce the idea that metabolites of aniline and its chlorinated derivatives can lead to increased toxicity and that the N-hydroxylation biotransformation pathway, which will be discussed later, may be critical in aniline and chloroaniline-induced methemoglobinemia.

Finally, severe methemoglobinemia following exposure to aniline can also result in hemolytic anemia (Bus and Popp 1987; Khan, Boor, Alcock, and Ansari, 1997a). Hemolytic anemia is when abnormal hemolysis of the red blood cells occurs, and this hemolysis is believed to play a critical role in the splenotoxicity.

1.3 ANILINE- AND CHLOROANILINE-INDUCED SPLENOTOXICITY

Splenotoxicity of aniline and some of its chlorinated derivatives have been documented in several animal species. Chhabra, Thompson, Elwell, and Gerken (1990), explored the toxicity of p-chloroaniline in rats (male and female Fischer 344 rats; seven weeks old) and mice (male and female B6C3F mice; nine weeks old) following exposure to p-chloroaniline (5, 10, 20, 40, or 80 mg/kg body weight for rats; 7.5, 15, 30, 60, or 120 mg/kg body weight for mice) in water, via oral gavage once daily, five days a week for 13 weeks. Results of this study showed a significant

increase in spleen weight, which was observed in a dose dependent manner in both rats and mice. Brain, lung, kidney, heart, thymus, and testis weights were unchanged in the p-chloroaniline treated groups. An increase in both incidence and severity of extramedullary hematopoiesis and pigmentation of the spleen was significantly higher than in the control group. This study supported previous studies in rats, which showed significant splenomegaly, as marked by congestion, pigmentation, and hematopoiesis, following treatment with aniline or p-chloroaniline (Gralla, Bus, Reno, Cushman, and Ulland, 1979; Bus, 1983). All of these studies suggested that the splenotoxicity observed following exposure to aniline or p-chloroaniline was a secondary consequence of hematotoxicity and increased deposition of damaged erythrocytes in the spleen. Since one of the major functions of the spleen is to remove aged and/or damaged erythrocytes, further studies by Khan, Kaphalia, Boor, and Ansari (1993) explored in more detail the relationship between hematoxicity and splenotoxicity. In this study, male Sprague-Dawley rats were given 600 ppm of aniline hydrochloride in drinking water for 30, 60, or 90 days. Similar to the previous studies, significant increases in spleen weight were observed after exposure to aniline. Red blood cell counts showed significant decreases at all three time points, and methemoglobin levels were significantly higher. Histological changes were only observed in the spleen and were marked by increased splenic sinusoids, fibroblasts, macrophages, and congestion of blood vessels in a time dependent manner. Spleen sections were stained for iron and showed significant accumulation of iron in a time dependent manner, with the greatest accumulation of iron seen after 90 days exposure. Additional studies also showed significant accumulation of iron in rats treated with aniline (Khan et al., 1993, 1997a; Khan, Kaphalia, Ansari, and Boor 1995a; Khan, Boor, Kaphalia, Alcock, and Ansari1995b; Khan, Wu, Kaphalia, Boor, and Ansari 1997b). Based on these studies, it was hypothesized that the accumulation of

damaged erythrocytes would lead to a deposition of iron in the spleen resulting in the generation of oxidative stress (Stadtman and Oliver, 1991 and Britton, 1996). The increase in oxidative stress would then contribute to aniline-induced splenotoxicity. Khan et al., (1997a, 1997b; Khan, Wu, Boor, and Ansari 1999) showed significant increases in oxidative stress markers, such as lipid peroxidation, following both acute (single i.p. injection) and subchronic exposure to aniline. These studies confirmed that lipid peroxidation and protein oxidation are critical to aniline-induced splenotoxicity.

A few studies have also shown that chronic exposure to aniline or p-chloroaniline resulted in a dose-dependent increase in splenic fibrosarcoma tumor formation in rats (Goodman, Ward, and Reichardt, 1984; Weinberger, Albert, and Montgomery, 1985). Khan et al. (1999) showed splenic development of fibrotic lesions and hyperplasia, a potential precursor to tumorgenesis, but no tumor formation was observed. Lipid peroxidation and oxidative damage has been shown to promote fibrogenesis by over expressing fibrogenic cytokines and increasing transcription and translation of collagen in animals and humans (Poli and Parola, 1996; Chojkier, Houglum, Solis-Herruzo, and Brenner, 1989; Geesin, Brown, Gordon, and Berg, 1993). However, the role of oxidative damage and lipid peroxidation in aniline- and chloroaniline-induced splenic tumorgenesis has yet to be determined in detail.

1.4 ANILINE- AND CHLOROANILINE-INDUCED HEPATOTOXICITY

There have been very few studies which have explored aniline- and chloroaniline-induced hepatotoxicity. One of the earliest reported cases of hepatotoxicity following exposure to aniline occurred in Madrid, Spain in 1981. From early May 1981 through late June 1981 several thousand patients presented to local hospitals with symptoms that presented as common

pneumonia. However, it was quickly determined that the symptoms were the result of contaminated rapeseed oil, which was denatured with aniline, and sold as olive oil. In a subset of patients, 170 children were admitted to the Hospital Clinico of the Complutense University Medical School after being exposed to the rapeseed oil. These patients were examined and it was determined that 55% of the children presented with hepatomegaly as a result of exposure to the aniline-contaminated oil (Casado de Frias, Andujar, Oliete, and Diaz, 1983). This was the first study that suggested aniline exposure could lead to hepatotoxicity.

Chhabra et al. (1990) showed increased Kupffer cell pigmentation and hematopoiesis in rat liver following exposure to p-chloroaniline. In the study, both male and female rats were exposed to various doses of p-chloroaniline (0, 5, 10, 20, 40, or 80 mg/kg) via oral gavage (once/day; five days/week; 13 weeks). The results showed increased Kupffer cell pigmentation at doses greater than or equal to 20 mg/kg in male rats and at doses greater than or equal to 10 mg/kg in female rats. Further studies were conducted by Valentovic et al. (1992) comparing the hepatotoxicity of aniline, 2-fluoroaniline (2-FA), 2-chloroaniline (2-CA), 2-bromoaniline (2-BrA) and 2-iodoaniline (2-IA). In these experiments, male Fischer 344 rats (four per group) were injected with a single i.p. injection of the hydrochloride salt of aniline, a 2-haloaniline (1.0 or 1.25 mmol/kg) or vehicle (0.9% saline, 2.5 ml/kg). Rats receiving an i.p. injection of vehicle were pair-fed with the groups receiving the aniline or 2-haloaniline in order to eliminate variability based on food intake. Alanine aminotransferase (ALT/GPT) activity, liver weight, and histological examinations were conducted 24-hours post treatment. Liver weight was unchanged in the 2-FA, 2-ClA and 2-BrA groups, as well as in the 1.25 mmol/kg aniline group, and 1.0 mmol/kg 2-IA groups, when compared to the corresponding pair fed control groups. Significant increases in liver weight were seen in the 1.0 mmol/kg aniline and 1.25 mmol/kg 2-IA treated

groups. ALT/GPT activity was significantly elevated in all 2-haloaniline treated groups, but not in the aniline treated groups. Similarly, histological examination demonstrated dose-dependent congestion, centrilobular degeneration, and reactive nuclei present after exposure to 2-haloanilines. No histological changes were seen following exposure to aniline. This data suggests that halo-substitution leads to increased hepatotoxicity, although further studies are required to determine the mechanism of hepatotoxicity.

1.5 ANILINE- AND CHLOROANILINE-INDUCED NEPHROTOXICITY

Although symptoms of renal damage following exposure to aniline and aniline derivatives had been documented as early as 1945 (Graubarth, Bloom, Coleman, and Solomon, 1945), it was not until the mid 1980s that studies were conducted to determine the nephrotoxic potential of aniline and chloroanilines. One of the earliest studies looked at the nephrotoxic potential of aniline, 2-chloroaniline, 3-chloroaniline, and 4-chloroaniline in male Fischer 344 rats after a single intraperitoneal (i.p.) injection and in a renal cortical slice model (Rankin et al., 1986a). Food intake, water intake, urine volume and content, blood urea nitrogen (BUN) concentration, histological examination of kidneys, and renal organic ion transporter function were assessed to determine nephrotoxic potential after 24 and/or 48 hours post injection. The results of the in vivo studies showed that of the compounds tested, 2-chloroaniline was nephrotoxic at 1.0 mmol/kg (single i.p. injection) as evident by increased BUN concentrations 48 hours post injection, decreased urine volume after 24 hours, and decreased organic ion transport. 3-Chloroaniline and 4-chloroaniline required 1.5 mmol/kg to elicit similar nephrotoxic effects. In contrast, aniline treatment induced very few renal effects, even at doses up to 1.5 mmol/kg. This study was the first to suggest that chlorine derivatives of aniline possessed enhanced nephrotoxic potential.

Around the same time, similar studies exploring the nephrotoxic potential of chlorine substitution on N-phenylsuccinimides were conducted. It was shown in those studies that increasing the number of chlorines on the aromatic ring led to an increased nephrotoxic potential. Of the chlorine substituted N-phenylsuccinimides tested, N-(3,5-dichlorophenyl) succinimide (NDPS) possessed the greatest nephrotoxic potential (Rankin et al., 1985; Yang, Lahoda, Brown, and Rankin, 1985a, 1985; Lo, Yang, Lahoda, and Rankin, 1985). Based on the toxicity and metabolism of NDPS (Ohkawa, Hisada, Fujiwara, and Miyamoto, 1974), it was hypothesized that 3,5-dichloroaniline, a known metabolite of NDPS, might be even more potent as a nephrotoxicant than the mono-chlorinated anilines previously tested. To test this hypothesis, Rankin, Yang, Teets, Lo, and Brown (1986) explored the nephrotoxic potential of 3,5-dichloroaniline in male Sprague-Dawley rats. The results showed that a single i.p. injection of 3,5-dichloroaniline (0.8 mmol/kg) was able to produce nephrotoxicity similar to that seen with the mono-chlorinated anilines previously explored. Specifically, decreased urine output was evident 24 hours post injection, while increased BUN concentrations and decreased basal and lactate-stimulated p-aminohippurate (PAH) accumulation by renal cortical slices was seen 48 hours post injection. Histological examination of exposed kidneys 48 hours post injection showed increased swelling in the proximal tubular cells. These effects were seen at a 3,5-dichloroaniline concentration of 0.8 mmol/kg, less than what was required for the mono-chlorinated derivatives already discussed. Another important difference in this study was the model used. Sprague-Dawley rats are less sensitive to nephrotoxicants, as compared to the Fischer 344 rat (Mazze, Cousins, and Kosek, 1973; Kosek, Mazze, and Cousins, 1974; McMurtry, Snodgrass, and Mitchell, 1978). Since 3,5-dichloroaniline was more nephrotoxic than the most toxic mono-chlorinated anilines in a less sensitive model it was concluded that, as with

the N-phenylsuccinimides, increasing the number of chlorines on the aromatic ring leads to increased nephrotoxicity.

Further studies were conducted to explore the nephrotoxic potential of the six dichlorinated aniline isomers, to determine the role of chlorine position in nephrotoxic potential. Also of interest in these studies was the ability of the dichlorinated anilines to induce nephrotoxicity directly to the kidney using a rat renal cortical slice model (Lo, Brown, and Rankin, 1990). To determine the in vivo nephrotoxic potential of each isomer, renal function of male Fischer 344 rats administered one of the dichloroaniline isomers (0.4, 0.8, or 1.0 mmol/kg; single i.p. injection) was monitored 24 and 48 hours post injection. In general, exposure to dichloroanilines resulted in increased proteinuria, hematuria, BUN concentration, and decreased PAH accumulation and urine volume, with the greatest changes seen after exposure to 3,5-dichloroaniline. Histological examination of renal tissue showed moderate to severe damage to the proximal tubular cells following exposure to most of the dichlorinated isomers, with 3,5-dichloroaniline (0.8 mmol/kg) producing the most severe effects, as determined by the number of cells involved and the extent of damage. Damage to the distal tubular cells and collecting ducts was negligible following exposure to most of the isomers and the glomeruli and loops of Henle were unaffected. This evidence further supported that the proximal tubular cells are the major target of chloroaniline-induced nephrotoxicity. The results of the in vivo study showed that 3,5-dichloroaniline was the most nephrotoxic dichloroaniline, followed by 2,5-dichloroaniline. 2,4-, 2,6-, and 3,4-Dichloroaniline were next and all possessed the same nephrotoxic potential, while 2,3-dichloroaniline proved to be the least potent nephrotoxicant of the dichlorinated aniline isomers.

To assess the in vitro nephrotoxic potential of the dichloroanilines, renal cortical slices from male Fischer 344 rats were exposed to various concentrations of each dichlorinated aniline isomer (0-10^{-3} M) for 120 minutes. Basal and lactate-stimulated PAH and tetraethylammonium (TEA) accumulation were used as a marker of in vitro renal function. Under normal conditions, PAH and TEA are accumulated in renal slices to intracellular levels approximately five-fold higher than the media concentration (Groves, Sheevers, and McGuiness, 1994). Results of Lo et al. (1990) showed that PAH (basal and lactate-stimulated) and TEA accumulation were significantly decreased at 10^{-3} M DCA concentrations of all six isomers in renal cortical slices. 3,5-Dichloroaniline resulted in the greatest decrease at this concentration and was also able to significantly decrease basal PAH accumulation at the 10^{-4} M concentration. As seen with the in vivo study, 3,5-dichloroaniline possessed the greatest nephrotoxic potential (Lo et al, 1990). These studies suggest that 3,5-dichloroaniline is the most nephrotoxic chlorinated aniline among the mono- and di-chlorinated anilines, and can be used as a prototypical compound to further understand chloroaniline-induced nephrotoxicity, since the nephrotoxic profile is similar among the chlorinated anilines.

1.6 XENOBIOTIC BIOTRANSFORMATION

One principle mechanism that organisms use to maintain homeostasis following exposure to exogenous molecules, also known as xenobiotics, is biotransformation. Biotransformation is facilitated by a number of xenobiotic metabolizing enzymes. These xenobiotic metabolizing enzymes have a wide range of both endogenous and exogenous substrates, and the reactions they catalyze can be separated into two main categories as first described by Richard Tecwyn Williams; phase I and phase II reactions (Williams 1959). The enzymes and the reactions they

catalyze were originally described as “detoxification” enzymes, because the reactions catalyzed resulted in increased compound hydrophilicity, which promotes the excretion and, ultimately, removal of the xenobiotic from the body. However, Williams suggested that in some instances “bioactivation” could occur following both phase I and phase II metabolism. Bioactivation occurs when xenobiotic metabolism results in a more physiologically active metabolite. The pharmaceutical industry has utilized bioactivation in drug development for a number of years, generally to increase bioavailability. Drugs, which require bioactivation, are known as prodrugs. One example of a prodrug is codeine, which is metabolized via both cytochrome P450 and UDP-glucuronosyltranferases. Cytochrome P450 2D6 is the major enzyme responsible for converting codeine to morphine (Dayer, Desmeules, Leemann, and Striberni, 1988). Another example of a commonly used prodrug is cyclophosphamide, which is converted to the active metabolite 4-hydroxycyclophophamide by liver CYPs. However, in some cases, bioactivation can result in increased toxicity as is seen with acetaminophen. Cytochrome P450 metabolism of acetaminophen to the reactive metabolite N-acetyl-p-benzoquinone imine (NAPQI) is responsible for acetaminophen-induced hepatotoxicity (Laine, Auriola, Pasanen, and Juvonen, 2009). Metabolizing enzymes are widely distributed throughout the body and levels of phase I and phase II enzymes can differ not only between tissues, but also within cellular compartments. Table 1 shows a list of phase I and phase II reactions, enzymes and cellular locations. While the liver has the highest concentration of phase I and phase II enzymes, these enzymes have been found in most other tissues, including the kidney.

Phase I reactions, also known as functionalization reactions, result in a slight increase in the hydrophilicity of a compound by exposing or introducing various functional groups (-OH, -SH, -NH_2, or -COOH). Sulfation, glucuronidation, acetylation, glutathione conjugation, amino

acid conjugation, and methylation are all examples of phase II reactions. Phase II reactions can result in substantial changes in compound hydrophilicity by conjugation of endogenous cofactors to functional groups either on the non-metabolized parent compound or groups introduced via phase I metabolism. In the following sections, we will explore in more detail a few important phase I and phase II enzyme-mediated reactions in aniline and chloroaniline metabolism.

Table 1. Summary of phase I and phase II reactions, enzymes, and cellular location.

REACTION	ENZYME	LOCATION
	Phase I	
Hydrolysis	Esterase	Microsomes, cytosol, lysosomes, blood
	Peptidase	Blood, lysosomes
	Epoxide hydrolase	Microsomes, cytosol
Reduction	Azo- and nitro-reduction	Microflora, microsomes, cytosol
	Carbonyl reduction	Cytosol, blood, microsomes
	Disulfide reduction	Cytosol
	Sulfoxide reduction	Cytosol
	Reductive dehalogenation	Microsomes
Oxidation	Alcohol dehydrogenase	Cytosol
	Aldehyde dehydrogenase	Mitochondria, cytosol
	Aldehyde oxidase	Cytosol
	Xanthine oxidase	Cytosol
	Monoamine oxidase	Mitochondria
	Diamine oxidase	Cytosol
	Prostagladin H synthase	Microsomes
	Flavin-monooxygenases	Microsomes
	Cytochrome P450	Microsomes
	Phase II	
	Glucuronide conjugation	Microsomes
	Sulfate conjugation	Cytosol
	Glutathione conjugation	Cytosol, microsomes
	Amino acid conjugation	Mitochondria, microsomes
	Acylation	Mitochondria, cytosol
	Methylation	Cytosol, microsomes, blood

Information from Parkinson (2001).

1.7 CYTOCHROME P450 (CYP)

Cytochrome P450s (CYPs) are a super family of heme-containing proteins and represent the largest group of phase I reaction enzymes, both in number of substrates and number of isoforms (Guengerich, 1987; Waterman and Johnson 1991). Cytochrome P450s were first identified and characterized as a single enzyme found in the endoplasmic reticulum of rabbit liver able to oxidize xenobiotic compounds in 1955 by Axelrod (1955) and Brodie et al. (1955). Additional studies showed that this enzyme had a maximum absorbance at 450 nm due to its ability to bind CO (Garfinkel 1958; Klingenberg 1958). Further studies demonstrated that this enzyme was a hemoprotein and was named cytochrome P450 (CYP) after its absorbance (Omura and Sato 1964a, 1964b). Since those earlier studies, there are now more than 6000 known isoforms of CYP, in all species, (Macherey and Dansette, 2008), which are named and grouped based on amino acid sequencing similarities (Gonzalez, 1989; Nelson et al., 1993). Cytochrome P450 enzymes with amino acid sequence similarity of 40 percent or less are separated into multiple gene families, represented by the number immediately following CYP (i.e., CYP1, CYP2, etc). The CYPs are then further separated in subfamilies with conserved amino acid sequence between 40-55 percent and are designated by a letter (i.e, CYP1A, CYP1B, etc.). Finally, if 55 percent or more of the amino acid sequence is similar, then the CYP is classified as members of the same subfamily (i.e. CYP1A1, CYP1A2, etc.). Since the nomenclature is based on amino acid structure rather than function or evolutionary relationship, CYPs are named in a amino acid sequence sequential manner. For example, CYP2A6 is the only functional CYP2A in human liver and CYP2A1 is the member of the CYP2A subfamily found in mouse. CYP1A1, CYP1A2, CYP1B1, and CYP2E1 are the only exception to the nomenclature as the function and regulation is highly conserved across all mammalian species (Parkinson, 2001).

The expression and activity of CYPs are affected by both genetic and environmental factors. Genetic polymorphisms have been shown in a number of CYPs. These polymorphisms can lead to significant changes in the CYP metabolism of xenobiotics. CYP2D6 is one such CYP. The CYP2D6 family is important in the metabolism of a number of drugs, including codeine, tamoxifen, and antidepressants. Polymorphisms within the CYP2D6 allele lead to up to four groups of patients that can be grouped based on their ability to metabolize CYP2D6 substrates. The three groups are poor metabolizers, normal metabolizers, and ultrarapid metabolizers. Of the Caucasian population, 6-10% fall within the poor metabolizer group, due to the presence of a CYP2D6 allele with decreased function, while ~2% of the Asian population are poor metabolizers (Bradford, 2002). In contrast, Middle Eastern and North African populations tend to have more ultrarapid metabolizers (McLellan, Oscarson, Seidefard, Evans, and Ingelman-Sundberg, 1997) within the population.

Environmental factors, which can affect CYP expression and activity, are social habits (smoking, alcohol consumption), foods (cruciferous vegetables), medications (isoniazid, barbiturates), and disease states (pregnancy, diabetes). The discovery that medicines and other environmental factors can alter CYP activity has allowed researchers to search for compounds that can inhibit CYP metabolism altogether. These CYP inhibitors have become useful in determining the metabolism of xenobiotics. CYP inhibitors can have isoform selective or non-selective activity, and these differences can also be species specific. For example, ketoconazole has been shown to be a potent CYP3A4 inhibitor in humans, but has much less isozyme selective activity in rats (Eagling, Tijia, and Back, 1998). The species specific differences in CYP inhibition is most likely due to differences in the CYP catalytic and/ or binding sites between species. For this reason caution must be taken when extrapolating results between species

(Spatzenegger, Born, and Halpert, 2003). Table 2 shows the species which is most similar to humans in terms of CYP activity.

Table 2. Species with CYP activity most comparable to humans.

Human P450	Species Most Similar
CYP1A1	Rat, Rabbit
CYP1A2	Rat, Mouse
CYP2A6	Monkey, Mouse, Hamster, Rabbit
CYP2B6	Mouse
CYP2C19	Monkey
CYP2D6	Dog
CYP2E1	No large species differences
CYP3A4	Mouse, Male Rat
CYP4A11	No large species differences

Data adapted from Spatzenegger et al (2003).

CYPs are found in the greatest concentration in the endoplasmic reticulum of liver tissues; however, they have also been found in other tissues such as lung, skin, blood, and kidneys. In humans, CYP1A1, CYP1A2, and CYP4A1 protein isoforms have been found in the kidney (Ioannides and Parke, 1990; Hardwick, Song, Hubeman, and Gonzalez, 1987). Gonzalez (1989) found CYP2 and CYP4 family genes are expressed in the human kidney. Finally, CYP3A4 is expressed in approximately 80 percent of human kidneys. In contrast, Cummings, Zangar, and Novak (1999) demonstrated that freshly isolated renal proximal and distal tubular cells from male Fischer 344 rats expressed CYP2E1, CYP2C11, CYP2B1/2, and CYP4A3/4. CYP4A2 is also present in rat kidneys (Kimura, Hanioka, Matsunga, and Gonzalez, 1989a; Kimura, Hardwick, Kozak, and Gonzalez, 1989b).

CYPs are responsible for 75-80% of all phase I metabolism (Nelson, 2004; Guengerich, 2008; Zanger, Turpeinen, Klein, and Schwab, 2008). CYP enzymes catalyze monooxygenase reactions, where one atom of molecular oxygen is added to the substrate, and a second atom of oxygen is reduced to water with reducing equivalents from NADPH. In the endoplasmic reticulum, the electron from NADPH is enzymatically transferred to the CYP enzyme via NADPH-cytochrome P450 reductase. The general reactions for CYP can be expressed as Substrate (RH) + O_2 + NADPH + H^+ ➔ Product (ROH) + H_2O + $NADP^+$. Figure 2 shows the cyclic catalytic nature of CYP reactions (Dawson, 1988; Schlichting, 2000). While CYPs catalyze a number of oxidation reactions, two are important reactions in aniline and chloroaniline metabolism: aromatic ring hydroxylation and N-hydroxylation. The role of these reactions in aniline and its chlorinated derivative biotransformation will be explored in more detail in a later section.

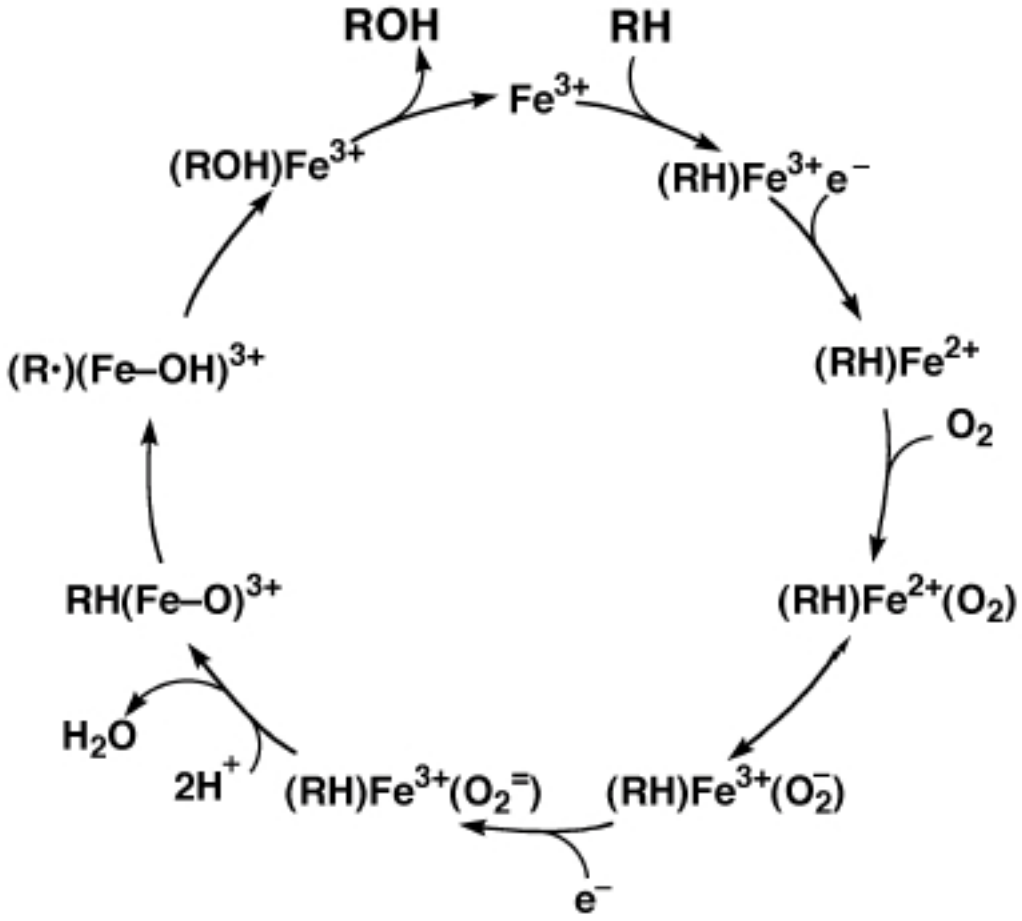

Figure 2. Cytochrome P450 Catalytic Cycle. The substrate is RH and the oxidized substrate is ROH.

1.8 FAD-CONTAINING MONOOXYGENASES (FMOs)

Another key phase I xenobiotic metabolism enzyme system is the flavin adenine dinucleotide (FAD)-containing monooxygenase (FMO) enzymes. First discovered in the early 1970s in pig liver by Dr. Daniel Ziegler, FMOs were described as a single flavoprotein enzyme that was able to oxidize a number of amines to nitro compounds (Ziegler, 1980). There are now five known mammalian FMO isozymes, designated as FMO1, FMO2, FMO3, FMO4, and FMO5 (Lawton et al., 1994; Cashman, 1995, 1999; Van Berkel, Namerbeek, and Fraaije, 2006). FMOs are located in the endoplasmic reticulum of almost all tissues including the liver, lungs, and kidney. Similar to the cytochrome P450s discussed earlier, species and tissue specific differences in expression have been explored in some detail. A summary of these studies can be found in table 3. For example, FMO1 has been shown to be expressed at high levels in the human kidney, but very low in human liver. In contrast, FMO1 expression is high in both rat kidney and liver tissue (Cashman, 1995; Dolphin et al., 1991; Parkinson, 2001). Along with FMO1, FMO2 and FMO4 are expressed at high levels in rat kidneys (Cashman, 1995). While differences occur in expression, the amino acid sequence for the five mammalian isozymes are 50-58% identical, with each FMO having two highly conserved regions near the active site, one that binds the FAD moiety and the second that binds NADPH (Phillips et al., 1995; Kubo, Itoh, Itoh, and Kamataki, 1997; Lawton and Philpot, 1993).

Ziegler discovered that the FAD moiety bound to the enzyme in the presence of oxygen forms a 4α-hydroxyperoxyflavin intermediate (Ziegler 1980). This 4α-hydroxyperoxyflavin is uncharacteristically stable for a peroxyflavin, which allows it to oxygenate substrates (Entsch and van Berkel, 1995). Figure 3 shows the catalytic cycle of FMOs, starting with the reduction of FAD to $FADH_2$ by NADPH, which in the present of O_2 generates the 4α-hydroxyperoxyflavin.

4α-Hydroxyperoxyflavin is then converted to 4α-hydroxyflavin as the flavin peroxide is transferred to the xenobiotic substrate. Finally, 4α-hydroxyflavin is dehydrated and $NADP^+$ is released, resetting the FAD moiety of the enzyme, priming it to react again (van Berkel et al, 2006). Most FMO substrates are soft nucleophile heteroatoms (nitrogen or sulfur containing compounds) and undergo N-oxidation or sulfoxidation; although hydroxylation, epoxidation, selenide oxidation, and phosphate ester oxidation can be catalyzed by FMOs (van Berkel et al., 2006; Jokanovic, 2001; Rooseboom, Commandeur, Floor, Rettie, and Vermeulen, 2001; Lohr, Willsky, and Acara, 1998). A few substrates and reactions catalyzed by FMO can be seen in Figure 4. Benadryl, imipramine, nicotine, morphine, and chlorpromazine are all examples of drugs that have been shown to form N-oxide metabolites (Lohr et al., 1998). The N-oxide metabolite of meperidine was the major metabolite in perfused isolated rat kidney (Acara, Gessner, Greizerstein, and Trudnowski, 1981). While the majority of FMO substrates are tertiary amines, there has been evidence in support of FMO oxidation of primary amines (Tynes , Sabourin, Hodgson, and Philpot, 1986).

Table 3. Putative tissue levels of FMO forms present in animals and humans. NP, apparently not present. A question mark indicates that no data are available or the presence of FMO form is in doubt. Adapted from Cashman, 1995.

	FMO1	FMO2	FMO3	FMO4	FMO5
Liver					
Mouse	Low	NP	High	?	Low
Rat	High	?	Low	?	Low
Rabbit	High	NP	Low	?	Low
Human	Very Low	Low	High	Very Low	Low
Kidney					
Mouse	High	?	High	?	Low
Rat	High	?	High	High	Low
Rabbit	Low	Low	Very Low	High	Low
Human	High	Low	?	?	?
Lung					
Mouse	?	High	Very Low	NP	Low
Rat	?	?	?	NP	Low
Rabbit	?	Very High	?	NP	NP
Human	?	Low	?	NP	?

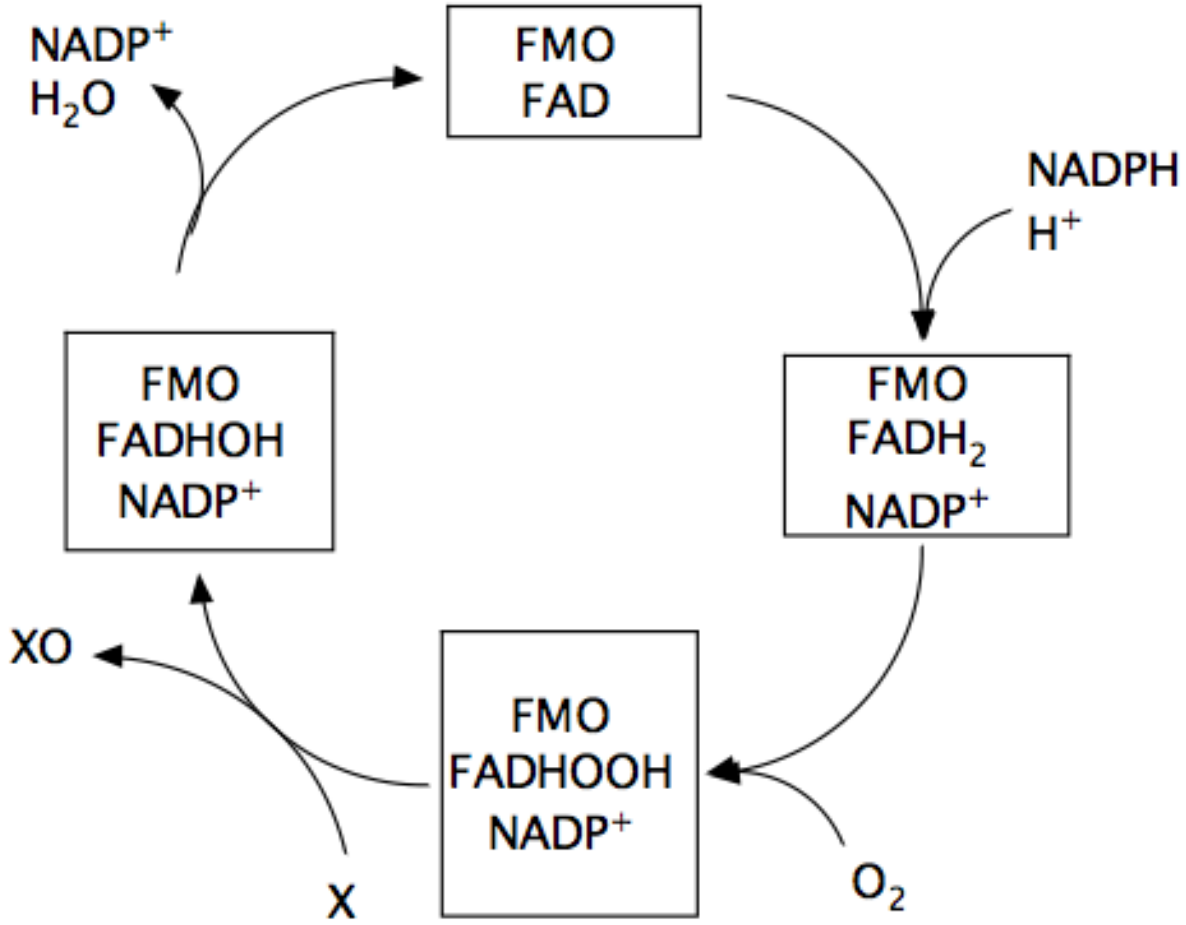

Figure 3. FMO Catalytic Cycle. X is the xenobiotic substrate; XO is the oxygenated product; FADHOOH is the 4α-hydroperoxyflavin; FADHOH is the 4α-hydroxyflavin

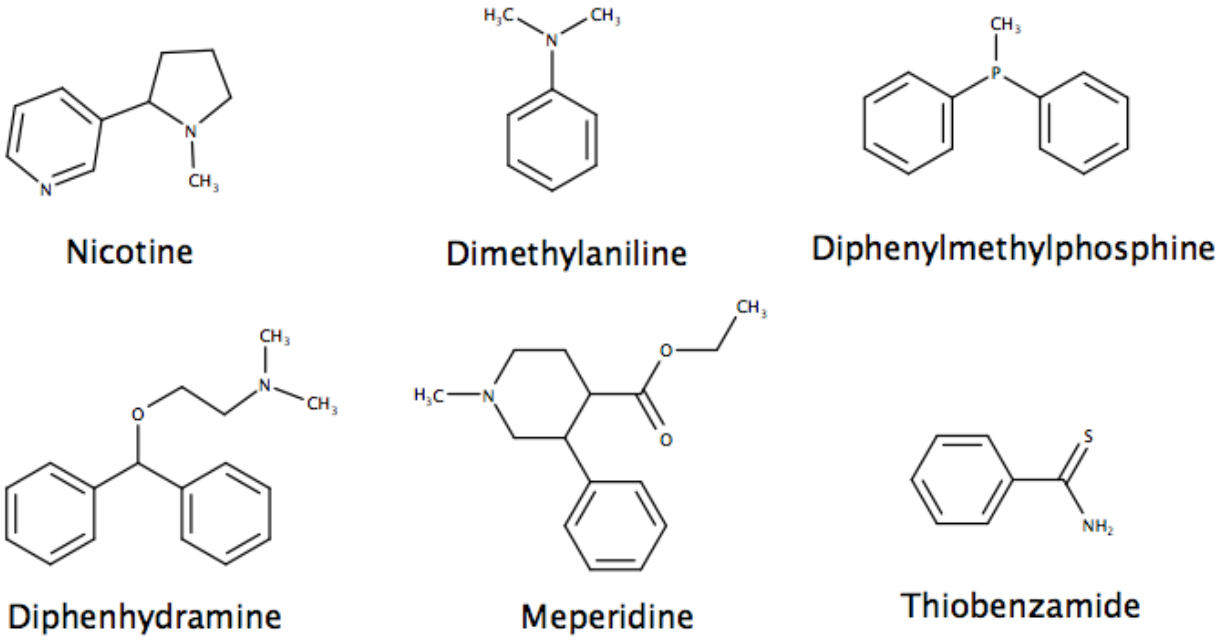

Figure 4. Examples of FMO substrates.

1.9 PEROXIDASE-DEPENDENT COOXIDATION

Peroxidase cooxidation has been shown to play a role in xenobiotic metabolism, especially in extrahepatic tissues that have low levels of CYPs. Unlike the other oxidative biotransformation pathways that require the reduction of cofactors such as NADPH and NADH, during peroxidase cooxidation the reduction of hydrogen peroxide and other lipid peroxides is coupled to the oxidation of xenobiotics and other substrates (Eling, Thompson, Foureman, Curtis, and Hughes, 1990). There are a number of peroxidases, which can catalyze xenobiotic biotransformation. One of the most commonly studied peroxidases involved in xenobiotic biotransformation is prostaglandin H synthase (PHS). There are two main pathways for PHS-dependent cooxidation. The first involves the direct transfer of peroxide oxygen to the substrate, while the second utilizes the xenobiotcs as electron donors, which can be oxidized by free radicals during the reduction of hydrogen peroxide. One example of xenobiotic metabolism by PHS is the two-electron oxidation of acetaminophen to N-acetyl-benzoquinoneimine. It has been suggested that a single electron intermediate, N-acetyl-benzosemiquinonemine is formed via PHS cooxidation. Further studies have suggested that N-acetyl-benzosemiquinonemine contributes to the nephrotoxicity of acetaminophen. PHS is expressed in kidney medulla, brain, lung, urinary bladder epithelium, and GI tract (Parkinson, 2001). While peroxidase-dependent cooxidation has not been shown as a major pathway in aniline and chloroaniline metabolism, it is a potential pathway whose role needs to be explored in more detail.

1.10 GLUCURONIDE AND SULFATE CONJUGATION REACTIONS

Glucuronidation and sulfation are two major phase II reactions that primarily detoxify xenobiotics. These reactions require endogenous cofactors to react with functional groups of the xenobiotics to increase polarity of the parent compound or metabolite, which will increase the

rate of excretion. As mentioned before, phase II reactions can occur prior to or following phase I metabolism. It is also important to note the phase II reactions generally occur at a greater rate than phase I reactions (Parkinson, 2001).

Glucuronidation is catalyzed by UDP-glucuronosyltranferases (UGTs). Initially, UGTs were separated into four groups; however, it is now apparent that there are only two gene families, UGT1 and UGT2 (Parkinson, 2001), found in rats. The members of UGT2 can be further separated into two sub-families, UGT2A and UGT2B. The members of the UGT2 arise from different genes. In contrast, all members of UGT1 arise from a single gene, with multiple UGTs encoded. UGTs have been found in the endoplasmic reticulum of the multiple tissues, including the liver and kidney. In glucuronidation, uridine diphosphate-glucuronic acid (UDP-glucuronic acid) is added to electron rich heteroatoms, such as oxygen, nitrogen, or sulfur. Over 350 UGT substrates have been discovered (Tukey and Strassburg, 2000). Figure 5 shows a sampling of substrates. For example, aniline and its chlorinated derivatives have been shown to undergo glucuronidation as will be discussed in more detail in the next section (Smith and Williams, 1949; Bohme and Grunow, 1969; Parke, 1960). Since glucuronidation requires UDP-glucuronic acid, UDP-glucuronic acid availability can limit the rate of glucuronidation. Depletion of UDP-glucuronic acid can lead to increased toxicity, if glucuronidation is the major detoxifying pathway (Whitcomb and Block, 1994).

Sulfation on the other hand is catalyzed by the cytosolic enzyme sulfotransferase. There are a number of sulfotranferase enzymes that have been characterized in mammals. While there is not a universally agreed upon nomenclature, the sulfotranferase enzymes can be separated into five distinct gene families, SULT1A-SULT1E (Nagata and Yamazoe, 2000). This nomenclature uses amino acid sequence similarities to group members into one of the five gene families.

Sulfotransferase has been found in a number of tissues, including the kidney (Mulder and Jakoby, 1990). The function of sulfotransferase is to transfer sulfonate from 3'-phosphoadenosine-5'-phosphosulfate (PAPS) to the substrate (Figure 5). Some examples of xenobiotics that undergo sulfation are acetaminophen and phenol. While phenols and aliphatic alcohols represent the largest group of substrates that are sulfated, a number of aromatic amines have been shown to be sulfated. Aniline and its chlorinated derivatives have been shown to undergo both O- and N-sulfation during metabolism (Hong and Rankin, 1998; Parkinson, 2001; Ehlhardt and Howbert, 1991; Bohme and Grunow, 1969). The metabolism of aniline will be explored in more detail in the following section.

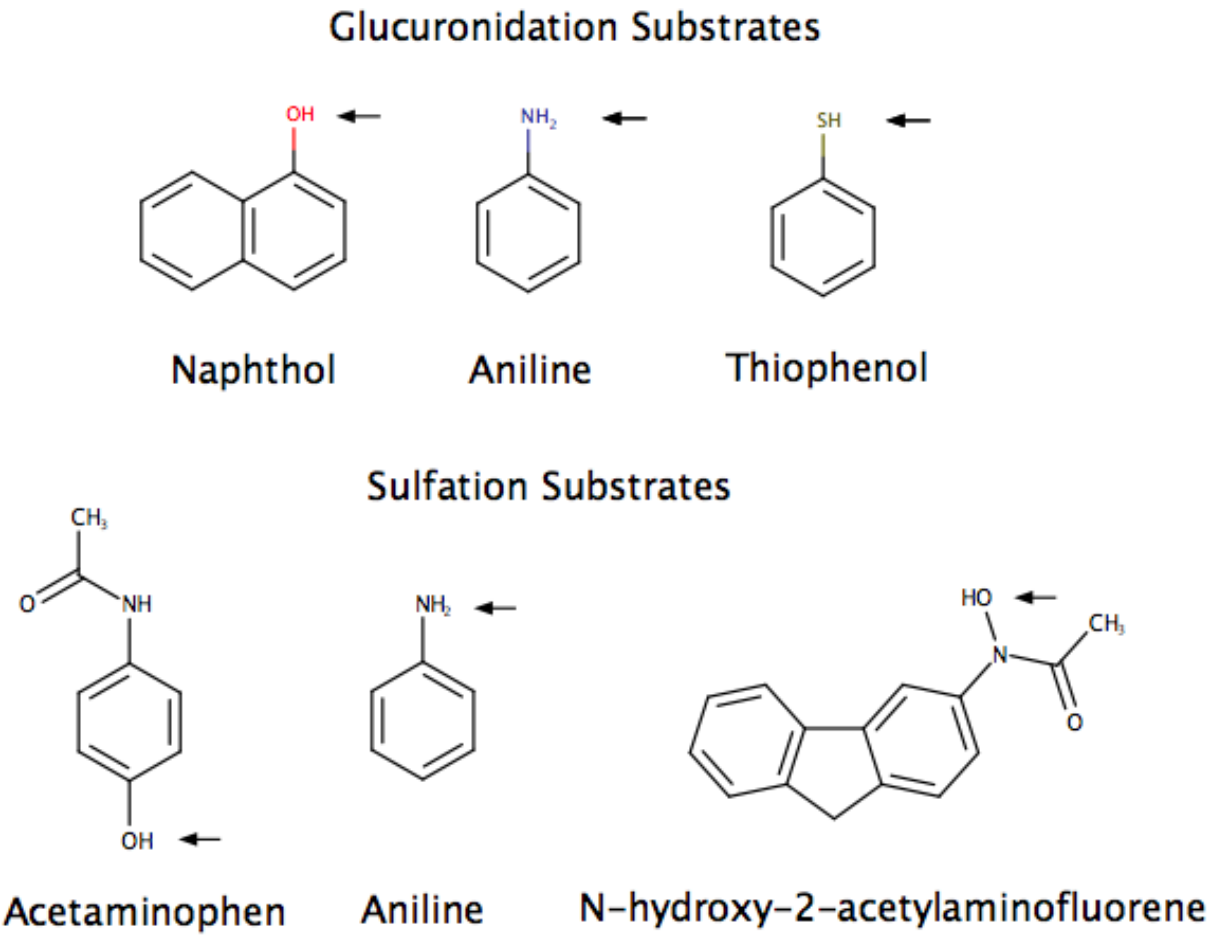

Figure 5. Examples of substrates, which undergo glucuronidation and/or sulfation.

Arrow indicates site of glucuronidation or sulfation.

1.11 ANILINE AND CHLOROANILINE METABOLISM

The biotransformation of aniline has been explored in great detail in both mammalian and non-mammalian species. One of the first known accounts of human exposure and resulting metabolism of aniline was a human poisoning case with 25 g of aniline. This study showed that the patient's urine contained sulfate conjugated p-aminophenol, as well as unchanged aniline (Muller 1887). Additional studies where dogs (Schmiedeberg, 1878) and rats (Elson, Goulden, and Warren, 1946) were fed aniline found similar results to Muller's results in that the urine contained p-aminophenol after acid hydrolysis. In 1949, Smith and Williams investigated the fate of aniline in rabbits. In this study, rabbits were given 200 mg/kg aniline via an oral dose. The results of the study demonstrated that while conjugated p-aminophenol metabolites represented 40-45% of the aniline fed, the major metabolite was the N-glucuronide of aniline itself (Figure 6). With the availability of [^{14}C]-aniline in the early 60s, it became possible to account for 75-100% of a single dose of aniline in rabbits and other animals. The results showed that in rabbits 51% of the dose was eliminated as p-aminophenol, 9% was o-aminophenol, 0.1 % was m-aminophenol, 3.5% was aniline-N-glucuronide, 5.4% was phenylsulphamic acid, 0.2% was acetanilide, and finally, 8.2% was eliminated as unchanged aniline (Parke, 1960).

Kao, Faulkner, and Bridges, (1978) provided the next major breakthrough in the metabolism of aniline. Their study found that the major urinary metabolite of aniline in male Wistar albino rats was N-acetyl-p-aminophenol sulfate (~56%). Minor metabolites included O-conjugates of o- and p-aminophenol (~20%), acetanilide (~3%) and N-acetyl-p-aminophenol (~10%), as determined by thin layer chromatography. In contrast to the previous studies already discussed, neither N-glucuronides and sulfates of aniline, nor free aniline were detected in the urine. This difference can be explained by the use of enzymatic hydrolysis of sulfate and

glucuronide conjugates, as opposed to chemical hydrolysis used in previous studies. It is possible that the strong acids used in the previous studies could lead to deacetylation, as well as hydrolysis of both the sulfate and glucuronide conjugates (Kao et al., 1978).

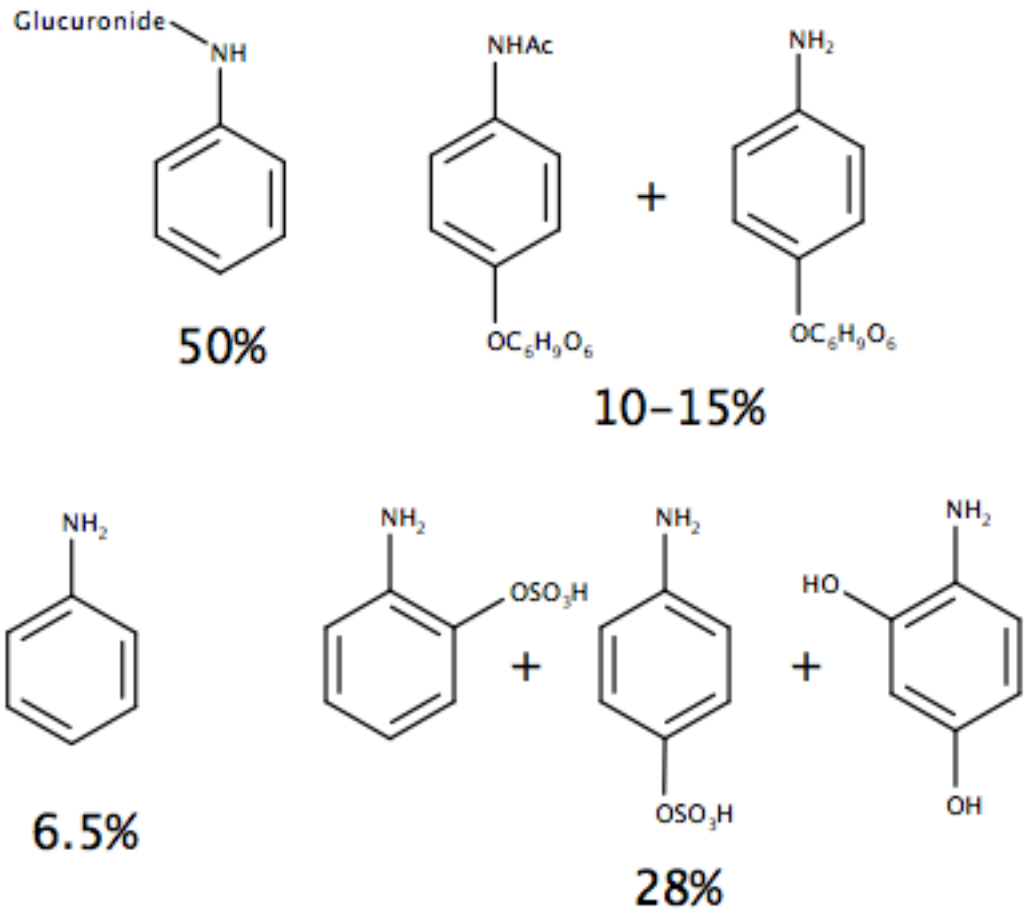

Figure 6. Metabolites of aniline. Information is from Smith and Williams (1949). Percentages displayed are the percent of dose recovered in urine after rabbits were given 200 mg/kg of aniline via an oral dose.

During this same time period, experiments exploring the metabolism of halogenated anilines were being conducted. The major halogenated aniline explored was the chlorinated derivative of aniline, p-chloroaniline, sometimes referred to as 4-chloroaniline. In Fischer 344 rats, the major urinary metabolite found was 2-amino-5-chlorophenylsulfate (Ehlhardt and Howbert, 1991), following a single dose of 4-chloroaniline given intragastrically by syringe. N-Acetyl-2-amino-5-chlorophenylsulfate was a minor metabolite and accounted for ~7% of the dose. Once again, no glucuronides were found in the urine. Since hydroxylation of the para-position is effectively blocked with the addition of chlorine, the o-hydroxylation and subsequently conjugated product would be expected to be the major metabolite. Similarly to aniline metabolism, the ortho-hydroxylated metabolite is excreted as free aniline sulfate rather than its N-acetylated derivative. In similar studies with 3-chloroaniline, aromatic ring hydroxylation to 4-amino-2-chlorophenol and 2-amino-4-chlorophenol was the major biotransformation pathway (Bohme and Grunow 1969). Both of these metabolites were then N-acetylated and/or conjugated to form a sulfate or glucuronide metabolites. These studies have demonstrated that phenyl ring hydroxylation, N-acetylation, glucuronidation, and sulfonation are all important metabolic pathways for aniline and chloroaniline metabolism, in vivo.

Another proposed metabolic pathway that has been explored in aniline metabolism is N-hydroxylation. The N-hydroxylation metabolite of aniline, phenylhydroxylamine, has only been shown in blood (Harrison and Jollow 1986, 1987). Harrison and Jollow (1986) measured the formation of 2-aminophenol, 4-aminonophenol, 4-hydroxyacetanilide, and phenylhydroxylamine in the blood of male Sprague-Dawley rats following a single i.p. injection of aniline (1.5 mmol/kg). Phenylhydroxylamine was measured as phenylhydroxyalamine + nitrosobenzene as previously described (Harrison and Jollow, 1983). The results showed the major unconjugated

metabolite found in blood for the first five hours post injection was the phenylhydroxylamine + nitrosobenzene, as measured by HPLC. The peak levels of phenylhydroxylamine occurred just ten minutes post injection. In contrast, both the 4-aminophenol and 4-hydroxyacetanilide concentrations did not peak until 6-8 hours post injection. N-Hydroxylation of aniline and chloroaniline has been shown to play a role in aniline-induced methemoglobinemia, as previously discussed (Valentovic et al, 1997; Harrison and Jollow, 1987; Jenkins et al., 1972). While N-hydroxylation metabolites have not been found in urine following exposure to either aniline or chloroanilines, N-hydroxylation is still a potential biotransformation pathway for these compounds that needs to be explored in greater detail.

1.12 CHLOROANILINE METABOLITE INDUCED NEPHROTOXICITY

The nephrotoxic potential of select chloroaniline metabolites have been previously described both in vivo and in vitro (Rankin et al., 1994, 1996; Rankin, Racine, Sweeney, Kraynie, Anestis, and Barnett 2008a; Valentovic, Ball, Sun, and Rankin, 2002; Hong et al., 1997). Most of these studies explored the nephrotoxicity of putative metabolites, which arise from phenyl ring hydroxylation to aminophenolic compounds because 4-aminophenol, the para-hydroxylation product of aniline, was shown to cause diuresis, elevated kidney weight, increased BUN levels, proteinuria, glucosuria, proximal tubular necrosis, and decreased organic ion accumulation in Fischer 344 and Sprague-Dawley rats (Newton, Kuo, Gemborys, Mudge, and Hook, 1982; Davis et al., 1983; Gartland, Bonner, Trimbell, and Nicholson, 1989). One of the few studies to explore the nephrotoxic potential of putative chlorinated anilines was conducted by Rankin et al. (1994). In that study, male Fischer 344 rats were given a single i.p. injection of 4-amino-2,6-dichlorophenol (0.25, 0.38, or 0.50 mmol/kg). Results of the study show that a

single i.p. injection of 0.38 mmol/kg 4-amino-2,6-dichlorophenol was able to produce nephrotoxicity similar to that seen with 3,5-dichloroaniline. The nephrotoxicity was characterized by significant increases in hematuria, proteinuria, glucosuria, BUN concentration, and kidney weight, as well as significantly decreasing organic ion accumulation in vivo. In vitro studies showed significant increases in lactate dehydrogenase (LDH) release, a marker of cytotoxicity, from renal cortical slices following the exposure to concentrations greater than 1 x 10^{-5} M for 90 minutes (Rankin et al., 1994). Comparisons with 3,5-dichloroaniline suggest that the putative metabolite, 4-amino-2,6-dichlorophenol, is a more potent nephrotoxicant than 3,5-dichloroaniline (Rankin et al., 1994). However, further studies are required to determine the role of 4-amino-2,6-dichlorophenol and other putative 3,5-dichloroaniline metabolites in 3,5-dichloroaniline-induced nephrotoxicity.

1.13 SCOPE OF CURRENT WORK

It was hypothesized that 3,5-DCA would be nephrotoxic in IRCC from male Fischer 344 rats, and that renal metabolism of 3,5-DCA in IRCC contributed to nephrotoxicity, at least in part. The current studies were designed to explore the metabolism of 3,5-dichloroaniline (3,5-DCA), the nephrotoxic potential of 3,5-DCA and its putative metabolites, the role of metabolism in 3,5-DCA induced nephrotoxicity, and the role of oxidative stress as a mechanism of cell death, using a Fischer 344 rat isolated renal cortical cell (IRCC) model. 3,5-DCA was chosen for further exploration because it proved to possess the greatest nephrotoxic potential among the mono- and dichlorinated anilines, both in vivo and in vitro using rat renal slices, as discussed above (Lo et al., 1990; Valentovic, Ball, Anestis, and Rankin 1995a; Valentovic et al.,1996). While most of the previous in vitro work was performed in a renal slice model, the current study

was performed in IRCC. The IRCC model was chosen because it is enriched for proximal tubular cells, the major target of 3,5-DCA induced nephrotoxicity. Fischer 344 rats were chosen as the animal species because they have been shown to respond to nephrotoxicants more like humans do than other rat strains (Mazze et al., 1973, Kosek et al., 1974; McMurtry et al., 1978).

CHAPTER II: THE ROLE OF BIOTRANSFORMATION AND OXIDATIVE STRESS IN 3,5-DICHLOROANILINE (3,5-DCA) INDUCED NEPHROTOXICITY IN ISOLATED RENAL CORTICAL CELLS FROM MALE FISCHER 344 RATS

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Christopher R. Racine[1], Travis Ferguson[1], Debbie Preston[2], Dakota Ward[1], John Ball[1], Dianne Anestis[1], Monica Valentovic[1], Gary O. Rankin[1]

[1]Department of Pharmacology, Physiology, and Toxicology, Joan C. Edwards School of Medicine, Marshall University, Huntington, WV 25755, USA

[2]Department of Pediatrics, Joan C. Edwards School of Medicine, Marshall University, Huntington, WV 25755, USA

Correspondence should be addressed to Gary O. Rankin; rankin@marshall.edu

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ABSTRACT

Among the mono- and dichloroanilines, 3,5-Dichloroaniline (3,5-DCA) is the most potent nephrotoxicant in vivo and in vitro. However, the role of renal biotransformation in 3,5-DCA induced nephrotoxicity is unknown. The current study was designed to determine the in vitro nephrotoxic potential of 3,5-DCA in isolated renal cortical cells (IRCC) obtained from male Fischer 344 rats, and the role of renal bioactivation and oxidative stress in 3,5-DCA nephrotoxicity. IRCC (~4 million cells/ml) from male rats were exposed to 3,5-DCA (0-1.0 mM) for up to 120 min. In IRCC, 3,5-DCA was cytotoxic at 1.0 mM by 60 min as evidenced by the increased release of lactate dehydrogenase (LDH), but 120 min was required for 3,5-DCA 0.5 mM to increase LDH release. In subsequent studies, IRCC were exposed to a pretreatment (antioxidant or enzyme inhibitor) prior to exposure to 3,5-DCA (1.0 mM) for 90 min. Cytotoxicity induced by 3,5-DCA was attenuated by pretreatment with inhibitors of flavin-containing monooxygenase (FMO; methimazole, N-octylamine), cytochrome P450 (CYP; piperonyl butoxide, metyrapone), or peroxidase (indomethacin, mercaptosuccinate) enzymes. Use of more selective CYP inhibitors suggested that the CYP 2C family contributed to 3,5-DCA bioactivation. Antioxidants (glutathione, N-acetyl-L-cysteine, α-tocopherol, ascorbate, pyruvate) also attenuated 3,5-DCA nephrotoxicity, but oxidized glutathione levels and the oxidized/reduced glutathione ratios were not increased. These results indicate that 3,5-DCA may be activated via several renal enzyme systems to toxic metabolites, and that free radicals, but not oxidative stress, contribute to 3,5-DCA induced nephrotoxicity in vitro.

Abbreviations

3,5-DCA, 3,5-dichloroaniline; IRCC, isolated renal cortical cells; LDH, lactate dehydrogenase; ALT, alanine aminotransferase; GPT, glutamic-pyruvic transaminase; GSH, glutathione; GSSG, oxidized glutathione; DMSO, dimethyl sulfoxide; DNP, 2,4-dinitrophenylhydrazone; FMO, flavin-containing monooxygenase; CYP, cytochrome P450; PiBx, piperonyl butoxide; DEDTCA, diethyldithiocarbamate; ASC, ascorbate; NAC, N-acetyl-L-cysteine; 3,5-DCAA, 3,5-dichloroacetanilide; 3,5-DCPHA, 3,5-dichlorophenylhydroxylamine; 3,5-DCNB, 3,5-dichloronitrobenzene

2.1 INTRODUCTION

Toxicity arising from exposure to anilines, important chemical intermediates used in the production of agricultural, industrial and pharmaceutical products (Lindh, Littorin, Amilon, and Jönsson, 2007; Unger, 1996), has been well established. Exposure to aniline and its chlorinated derivatives has been associated with hematotoxicity (methemoglobinemia, hemolytic anemia; Chhabra et al., 1990; Guilhermino, Sores, Carvalho, and Lopes, 1998; Pauluhn, 2004; Valentovic et al., 1997), splenotoxicity (splenomegaly, elevated erythropoietic activity, hyperpigmentation, fibrosis; Chhabra et al., 1990; Khan, Wu, Boor, and Ansari, 1999; Ma, Wang, Abdel-Rahman, Boor, and Kahn, 2008, 2013), hepatotoxicity (hepatomegaly, elevated serum ALT/GPT levels, centralobular necrosis; Valentovic et al., 1992, 1995a; Valentovic, Lo, Brown, and Rankin, 1995b), and nephrotoxicity (Hong, Anestis, Henderson, and Rankin, 2000; Lo et al., 1990; Racine et al., 2014; Valentovic et al., 1995a). Chloroaniline induced nephrotoxicity in vivo is characterized by oliguria, decreased kidney weight, proteinuria, hematuria, elevated blood urea nitrogen (BUN) concentration, and decreased organic ion transport in the proximal tubule cells (Rankin et al., 1986; Lo et al., 1990; Valentovic et al., 1995a). Morphological changes occur in both the proximal and distal tubules and collecting ducts, with the greatest abnormalities seen in the proximal tubular cells. These morphological changes include blebbing and vacuolization of proximal tubular cells, occluded lumina with sloughed microvilli and enlarged lumina in the distal tubule (Lo et al., 1990). In vitro exposure of rat renal cortical slices to chloroanilines leads to a significant increase in cytotoxicity as seen by a decrease in organic ion accumulation and an increase in lactate dehydrogenase (LDH) release (Valentovic et al., 1992, 1995a, 1995b). Among the mono- and dichloroanilines, 3,5-dichloroaniline (3,5-DCA) was the most potent nephrotoxicant both in vivo and in vitro (Lo et al., 1990; Valentovic et al., 1995a, 1996).

Biotransformation via N-oxidation, N-acetylation, and phenyl ring oxidation are all known pathways of chloroaniline metabolism (Ehlhardt and Howbert, 1991; Hong and Rankin, 1998; Racine et al., 2014). Based on these studies a proposed biotransformation pathway of 3,5-DCA can be seen in figure 7. Two of these pathways (N-oxidation and phenyl ring oxidation) have the potential to lead to the formation of toxic metabolites from aniline compounds. For example, N-oxidation of aniline leads to toxic metabolites responsible for hematoxicity (Harrison and Jollow, 1987, 1986) and splenotoxicity (Khan, Wu, and Ansari, 2000; Ma et al., 2013, 2008), while formation of the 4-aminophenol metabolite can contribute to nephrotoxicity (Harmon, Terneus, Kiningham, and Valentovic, 2005; Rankin et al., 1996; Tarloff, Goldstein, Morgan, and Hook, 1989). A small number of studies have shown that bioactivation can also contribute to the nephrotoxicity associated with some chloroanilines (Racine et al., 2014; Valentovic et al., 1995b). Putative metabolites of chloroanilines arising from N-oxidation or phenyl ring oxidation are also toxic to the kidney (Hong et al., 1997, 1996; Rankin et al., 2008a). Nonetheless, the enzyme systems responsible for the bioactivation of chloroanilines studied to date and the ultimate nephrotoxic metabolites formed from these compounds are not clearly defined.

Oxidative stress may contribute to the mechanism of cell death with aniline compounds. Harmon et al. (2005) found that oxidative stress played a role in 4-aminophenol-induced nephrotoxicity in vitro, while Hong et al. (1997) showed that in vivo 4-amino-2,6-dichlorophenol (ADCP), a putative metabolite of 3,5-DCA, increased the oxidized to reduced glutathione ratio in kidney, suggesting that ADCP induced renal oxidative stress. In addition, ADCP nephrotoxicity was prevented by pretreatment with antioxidants. However, it is unclear if oxidative stress plays a role in 3,5-DCA nephrotoxicity.

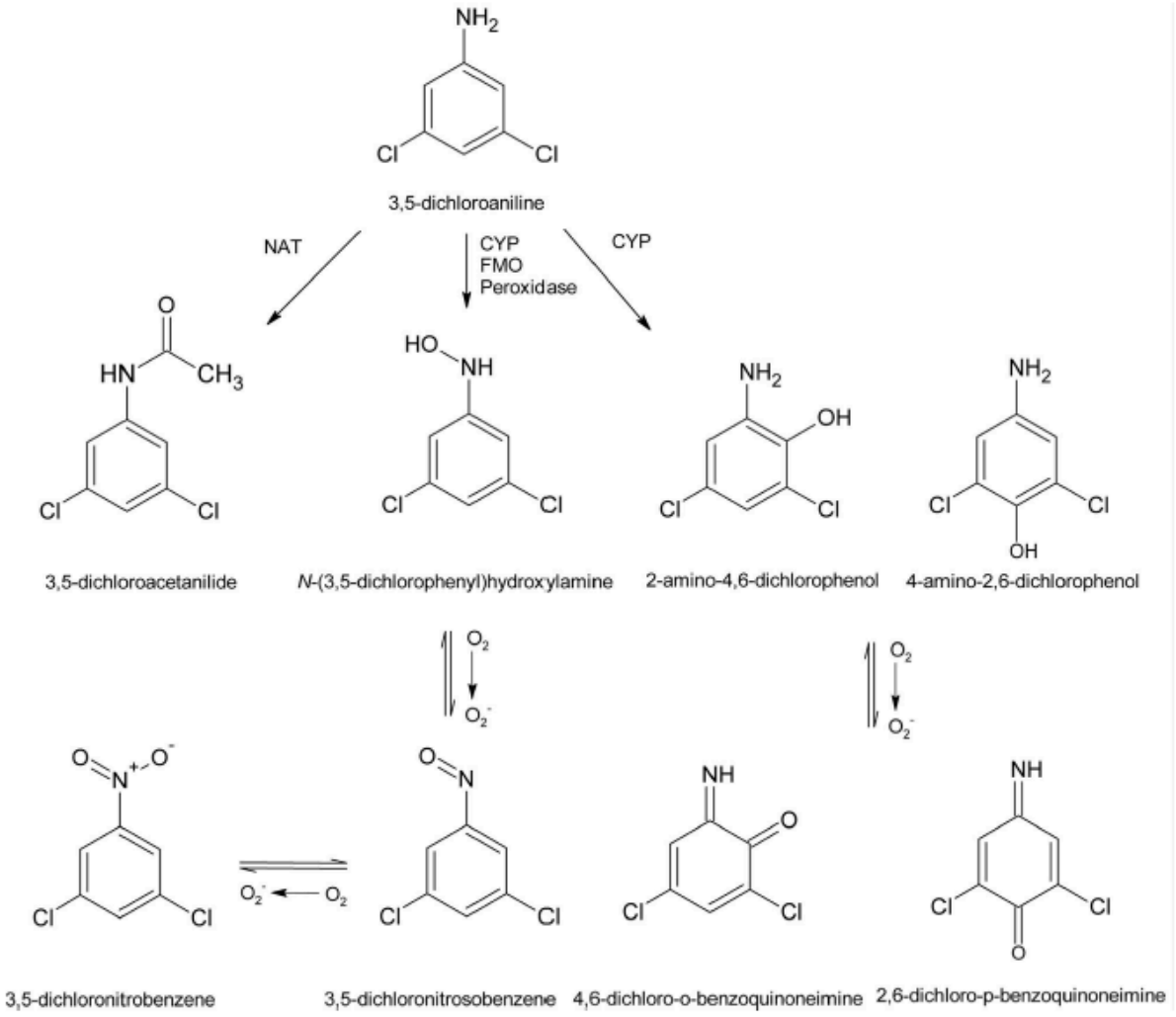

Figure 7. Proposed Renal Biotransformation Pathway of 3,5-DCA. Abbreviations:

CYP=Cytochrome P450, FMO=Flavin-containing Monooxygenase, NAT= N-acetyltransferase.

The purpose of this study was to explore, in more detail, the in vitro nephrotoxicity induced by 3,5-DCA. The in vitro cytotoxicity of 3,5-DCA was determined in isolated rat renal cortical cells (IRCC) from male Fischer 344 rats, and the role of renal metabolizing enzyme systems, including CYP isozymes, in the bioactivation of 3,5-DCA to nephrotoxic metabolites was also examined. Lastly, the role of free radicals and oxidative stress in 3,5-DCA-induced nephrotoxicity was explored. The IRCC model was selected for use because this model contains the target cells for 3,5-DCA and maintains metabolic and transport capabilities seen in vivo for several hours, including CYP activity (Cummings et al., 1999; Lash, 1998).

2.2 MATERIALS AND METHODS

2.2.1 Experimental Animals

Male Fischer 344 rats (200-250 g) from Hilltop Lab Animals, Inc. (Scottdale, PA) were used for these experiments. All animals were kept in a controlled environment with a regulated light cycle (12 h on/12 h off), temperature (21-23^{o}C), and humidity (40-55%) with food (Purina Rat Chow) and water available *ad libitum*. Rats were allowed at least one week to acclimate to the animal facilities prior to use in any experiments. Animal use for these experiments was approved by the Marshall University Institutional Animal Care and Use Committee, and animal use was conducted in accordance with the Guide for the Care and Use of Laboratory Animals as adopted by the National Institutes of Health.

2.2.2 Chemicals

All chemicals used were the highest purity available and were purchased from Sigma Aldrich (St. Louis, MO) or Fisher Scientific (Pittsburgh, PA).

2.2.3 Isolated Renal Cortical Cells (IRCC) preparation and treatment

Untreated male rats were anesthetized with pentobarbital (75 mg/kg, ip) and isolated renal cortical cells (IRCC) were obtained using the collagenase perfusion method of Jones, Sundby, Ormstad, and Orrenius (1979). Cell viability was initially determined by trypan blue (2% w/v) exclusion and lactate dehydrogenase (LDH) release. IRCC were counted and resuspended in Krebs-Henseleit buffer, pH 7.37, containing 25 mM Hepes and 2% (w/v) bovine serum albumin at a concentration of ~4.2 million cells/ml. IRCC (3 ml) were added to 25 ml polycarbonate Erlenmeyer flasks for a five min pre-incubation period in a Dubnoff shaking water

bath incubator (37˚C, 60 cycles/min) under an atmosphere of 95% oxygen and 5% carbon dioxide. Cells were exposed to various concentrations of 3,5-dichloroaniline (3,5-DCA; 0.5 or 1.0 mM) or vehicle (DMSO) for 60, 90 or 120 min. After the incubation period, flasks were removed, placed on ice, and samples (0.5 ml) taken for lactate dehydrogenase (LDH) release assays. Briefly, samples were centrifuged (3000xg, 3 min), the supernatant was decanted and saved, and the pellet was disrupted with 1 ml of 10% Triton X-100 solution to release cellular LDH. LDH activity was then determined in each fraction as previously described using a kinetic assay based on the amount of NADH produced from NAD (Rankin et al., 2008b). LDH released into the media was expressed as the percent of total (media + pellet).

In separate experiments, IRCC were pretreated with either an antioxidant or an enzyme system inhibitor (Table 4) before exposure to 1.0 mM 3,5-DCA for 90 min, to determine the role of biotransformation of 3,5-DCA on cytotoxicity. Males were chosen for these experiments because our previous studies were performed in male rats (Lo, Valentovic, Brown, and Rankin 1994; Valentovic et al., 1995b). All pretreatment times and concentrations were based on previously published studies (Baliga, Zhang, Baliga, Ueda, and Shah, 1998; Harleton et al., 2004.; Harmon et al., 2005; Harmon, Kiningham, and Valentovic, 2006; Katsuda et al., 2010; Lau and Monks, 1987; Lock, Cross, and Schnellmann, 1993; O'Brien and Siraki, 2005; Rodriguez and Acosta, 1997; Suzuki and Sudo, 1990; Valentovic et al., 1999).

Table 4. List of antioxidants and enzyme inhibitors with primary targeted enzyme systems.

Pretreatment (PreTx) Compound	PreTx Conc.	PreTx Time (min)	Enzyme	Reference
N-Acetyl-L-cysteine	2.0mM	30	Antioxidant	Katsuda et al. 2010
α-Tocopherol	1.0mM	5	Antioxidant	Suzuki et al. 1990
Glutathione	1.0mM	30	Antioxidant	Lau et al. 1987
Ascorbate	2.0mM	5	Antioxidant	Lock et al.1993
Pyruvate	1.0mM	15	Antioxidant	Harmon et al. 2006, 2005
Methimazole	1.0mM	30	FMO	Rodriquez et al. 1997
N-Octylamine	2.0mM	5	FMO	Rodriquez et al.1997
Indomethacin	1.0mM	15	Cyclooxygenase	Lau et al. 1987
Mercaptosuccinate	0.1mM	15	Peroxidase	Racine et al. 2014
PiBx	1.0mM	15	NS CYP	Baliga et al.1998
Metyrapone	1.0mM	5	NS CYP	Lock et al. 1993
Oleandomycin triacetate	0.5mM	30	CYP3A1/2	Racine et al. 2014
Thio-tepa	0.1 mM	5	CYP2B1/2	Harleton et al. 2004
Isoniazid	1.0mM	5	CYP2E1	Racine et al. 2014
DEDTCA	0.1mM	30	CYP2C > CYP2E1	Eagling et al.1998
Omeprazole	0.01mM	30	CYP2C	Racine et al. 2014
Sulfaphenazole	0.1mM	30	CYP2C	Eagling et al. 1998, Kobayashi, Urashima, Shimada, and Chiba 2003

2.2.4 Glutathione determination

Following treatment with 3,5-DCA (0.5 or 1.0 mM) or vehicle (DMSO) for 60 or 90 min, IRCC (2.0 ml; 4.2 million cells/ml) were homogenized in 0.5% sulfosalicylic acid (250 μl). Using a glutathione and NADPH coupled reaction; total glutathione (GSH) was determined as previously described (Anderson, 1985; Griffith, 1980; Valentovic, Terneus, Harmon, and Carpenter, 2004). To measure glutathione disulfide (oxidized glutathione; GSSG) 2-vinylpyridine derivation was used as described by Griffith (1980). The ratio of oxidized/reduced GSH was then determined and all results were expressed as percent of control for each set of experiments.

2.2.5 Protein carbonyl measurement

Protein carbonyl levels were measured after exposure to vehicle (DMSO) or 3,5-DCA (0.5, 1.0 mM) for 60 or 90 min as previously described by Terneus, Kiningham, Carpenter, Sullivan, and Valentovic, (2007). Briefly, a sample from treated IRCC (0.5 ml) was homogenized in 5x volume Krebs buffer, and total protein was determined using a Coomassie blue spectrophotometric method (Bradford, 1976). Protein OxyBlot kits (Millipore) were utilized to determine protein carbonyl levels following the manufacturer's recommended protocol. The assay utilizes a 2,4-dinitrophenylhydrazine (DNP) specific antibody to detect DNP moiety on proteins. DNP moieties arise from the reaction of carbonyl side chains formed under oxidative stress conditions with 2,4-dinitrophenylhydrazine (Terneus et al., 2007).

2.2.6 Statistics

Data for LDH release is presented as mean ± S.E.M. with an $N \geq 4$ separate isolation experiments. Data for oxidized/reduced GSH and Oxyblot experiments are presented as mean percent of control ± S.E.M. with an $N \geq 4$ experiments. Data was analyzed by a one-way analysis of variance followed by a Student-Newman-Keuls Test using GraphPad Prism 6.0. Statistical significance was determined at $P < 0.05$.

2.3 Results

2.3.1 Time and concentration cytotoxicity studies

To determine the nephrotoxic potential of 3,5-DCA in IRCC, concentration response curves were performed at 60, 90 or 120 min. Cytotoxicity, as determined by increased LDH release, was seen at 90 and 120 min at a 3,5-DCA concentration of 1.0 mM. Cytotoxicity was also seen with 0.5 mM 3,5-DCA, but only at the 120 min exposure time (Fig. 8). Based on these observations, the 3,5-DCA concentration selected for further study was 1.0 mM with an exposure time of 90 min.

2.3.2 Effects of flavin-containing monooxygenase (FMO), cyclooxygenase, or peroxidase inhibition

To determine the role of FMOs in 3,5-DCA bioactivation, IRCC were pretreated with the FMO inhibitors methimazole or N-octylamine. The cyclooxygenase activity of prostaglandin H synthase was inhibited with indomethacin, while general peroxidase activity was inhibited with mercaptosuccinate. All pretreatments significantly attenuated 3,5-DCA induced cytotoxicity (Fig. 9).

2.3.3 Effects of nonselective cytochrome P450 (CYP) inhibition

The role of CYPs in 3,5-DCA induced cytotoxicity was first examined by using the nonselective CYP inhibitors piperonyl butoxide [PiBx], and metyrapone. Results showed PiBx and metyrapone both significantly attenuated 3,5-DCA 1.0 mM induced cytotoxicity (Fig. 10).

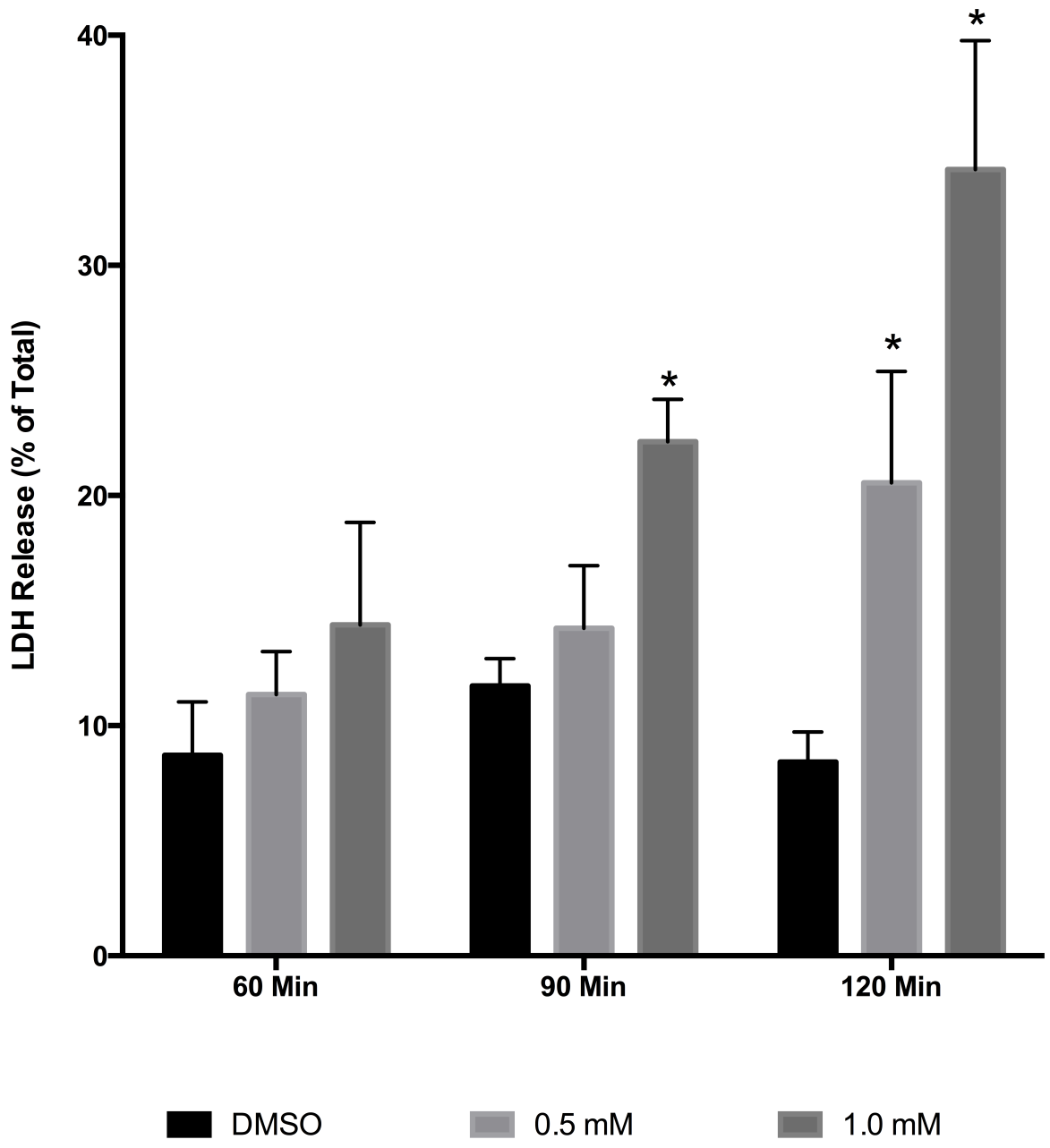

Figure 8. 3,5-DCA induced cytotoxicity in isolated renal cortical cells obtained from male Fischer 344 rats following exposure for 60, 90 and 120 min. Each bar represents the mean ± S.E.M. for N=4-5 separate isolation experiments. *Significantly different from DMSO control, $P<0.05$.

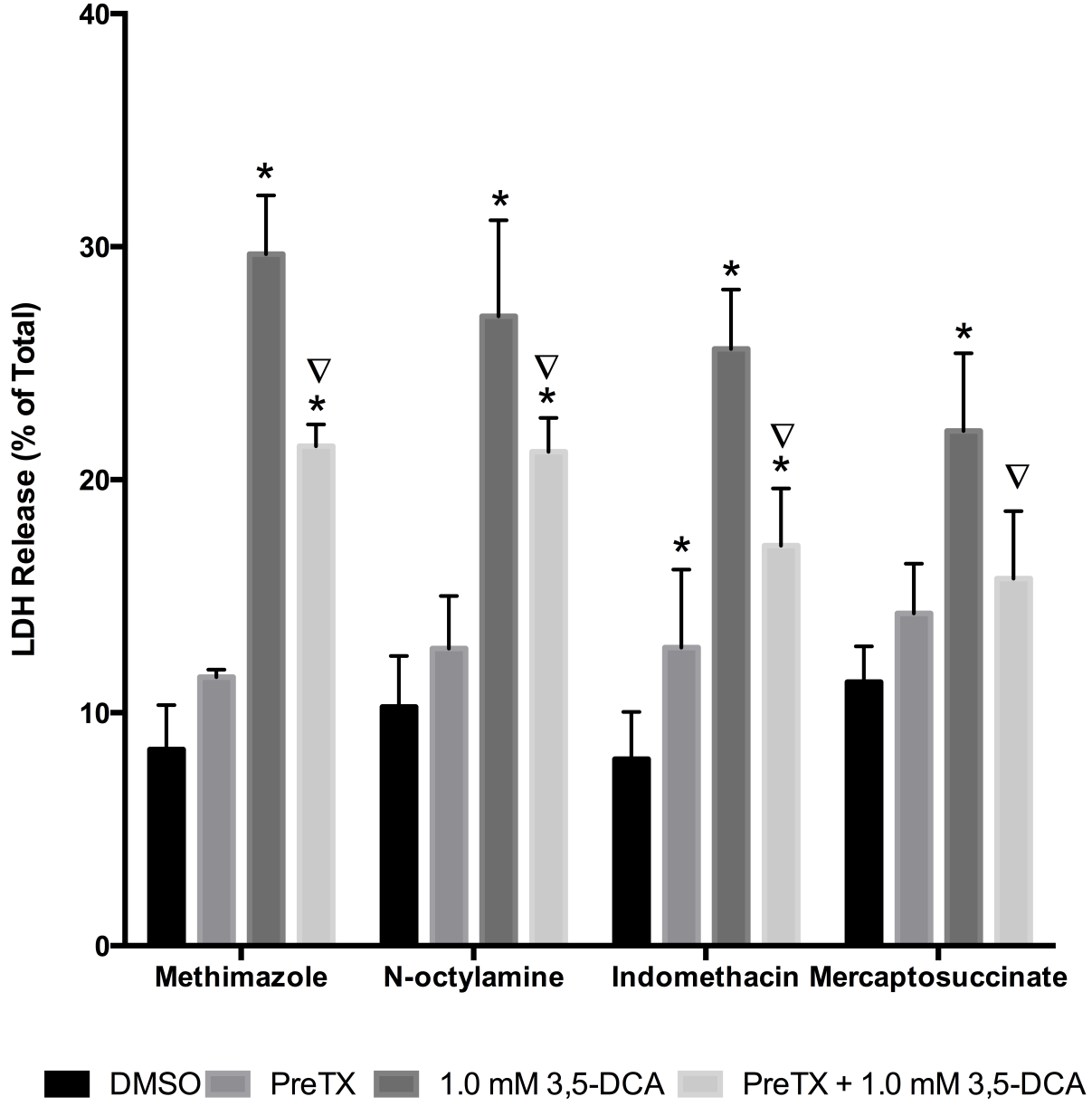

Figure 9. Effects of FMO, cyclooxygenase, or peroxidase inhibitor pretreatment (PreTX) on 3,5-DCA cytotoxicity in isolated renal cortical cells obtained from male Fischer 344 rats after 90 min. Each bar represents the mean ± S.E.M. for N=4-5 separate isolation experiments. *Significantly different from DMSO control, $P<0.05$. ∇Significantly different from the 1.0 mM 3,5-DCA value, $P<0.05$.

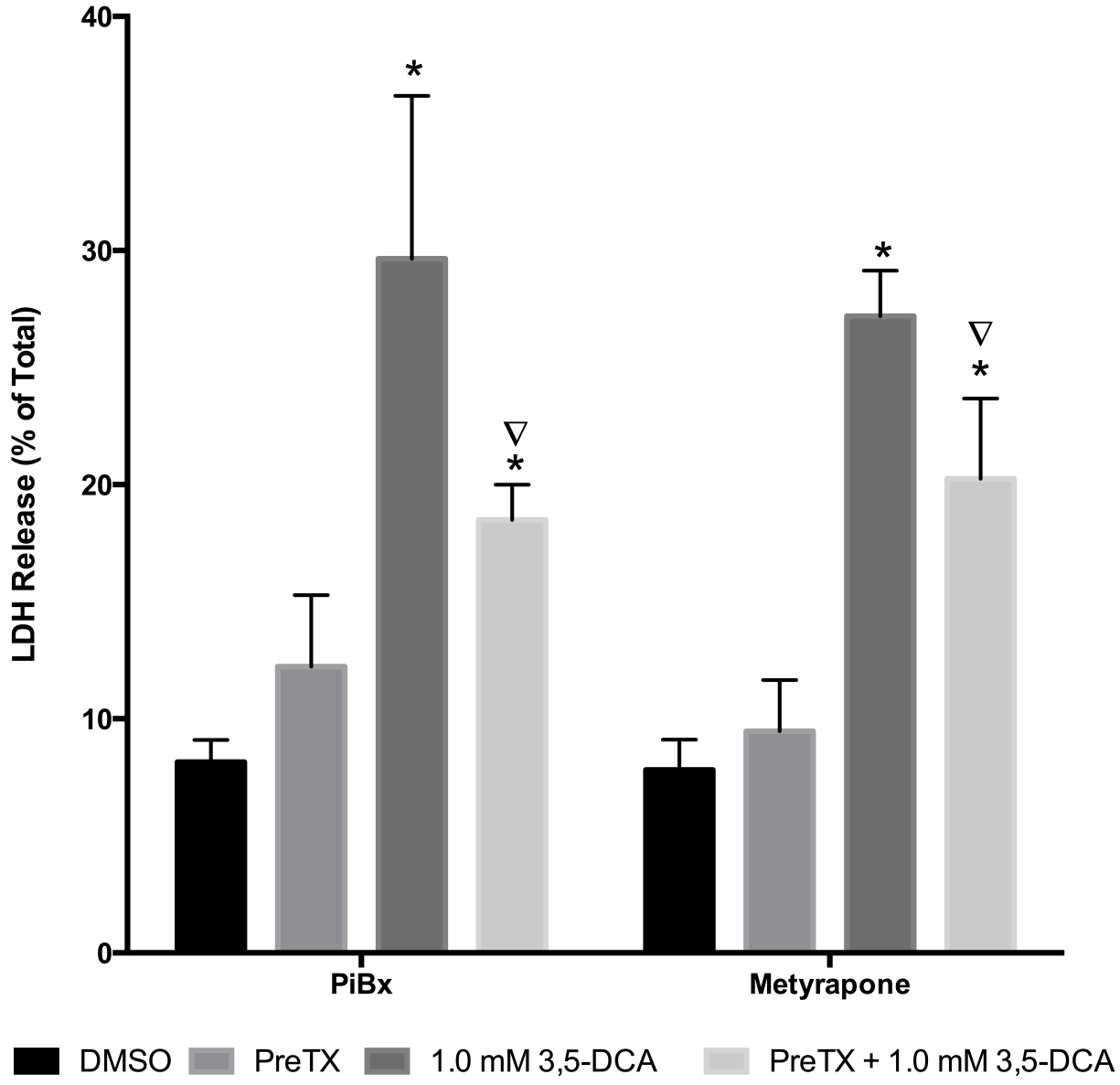

Figure 10. Effects of nonselective cytochrome P450 inhibitor pretreatment (PreTX) on 3,5-DCA cytotoxicity in isolated renal cortical cells obtained from male Fischer 344 rats after 90 min. Each bar represents the mean ± S.E.M. for N=4-5 separate isolation experiments. *Significantly different from DMSO control, P<0.05. ∇Significantly different from the 1.0 mM 3,5-DCA value, P<0.05. PiBx, piperonyl butoxide.

2.3.4 Effects of isozyme selective CYP inhibition

Based on the results from studies using nonselective CYP inhibitors, further studies were designed to explore the role of select renal CYP isozymes on 3,5-DCA induced cytotoxicity. To determine the role of CYP3A, CYP2E1, and CYP2B1/2 isozymes in 3,5-DCA bioactivation, oleandomycin triacetate, isoniazid, and thio-tepa were used to inhibit CYP3A, CYP2E1, and CYP2B1/2 isozymes, respectively. Cytotoxicity induced by 3,5-DCA was not significantly attenuated by any of these pretreatments (data not shown). To determine the role of the CYP2C family in 3,5-DCA bioactivation, the effects of three CYP2C family inhibitors (omeprazole, diethyldithiocarbamate (DEDTCA), and sulfaphenazole) on 3,5-DCA cytotoxicity were examined. Results show that inhibition of the CYP2C family significantly reduced cytotoxicity (Fig. 11).

2.3.5 Effects of antioxidants on 3,5-DCA cytotoxicity

The effect of pretreating IRCC with antioxidants on 3,5-DCA cytotoxicity was also examined to explore whether oxidative stress or free radicals played a significant role in 3,5-DCA (1.0 mM) induced cytotoxicity. Results showed all five antioxidants (N-acetyl-L-cysteine, α-tocopherol, glutathione, ascorbate and pyruvate) significantly attenuated 3,5-DCA cytotoxicity (Fig. 12).

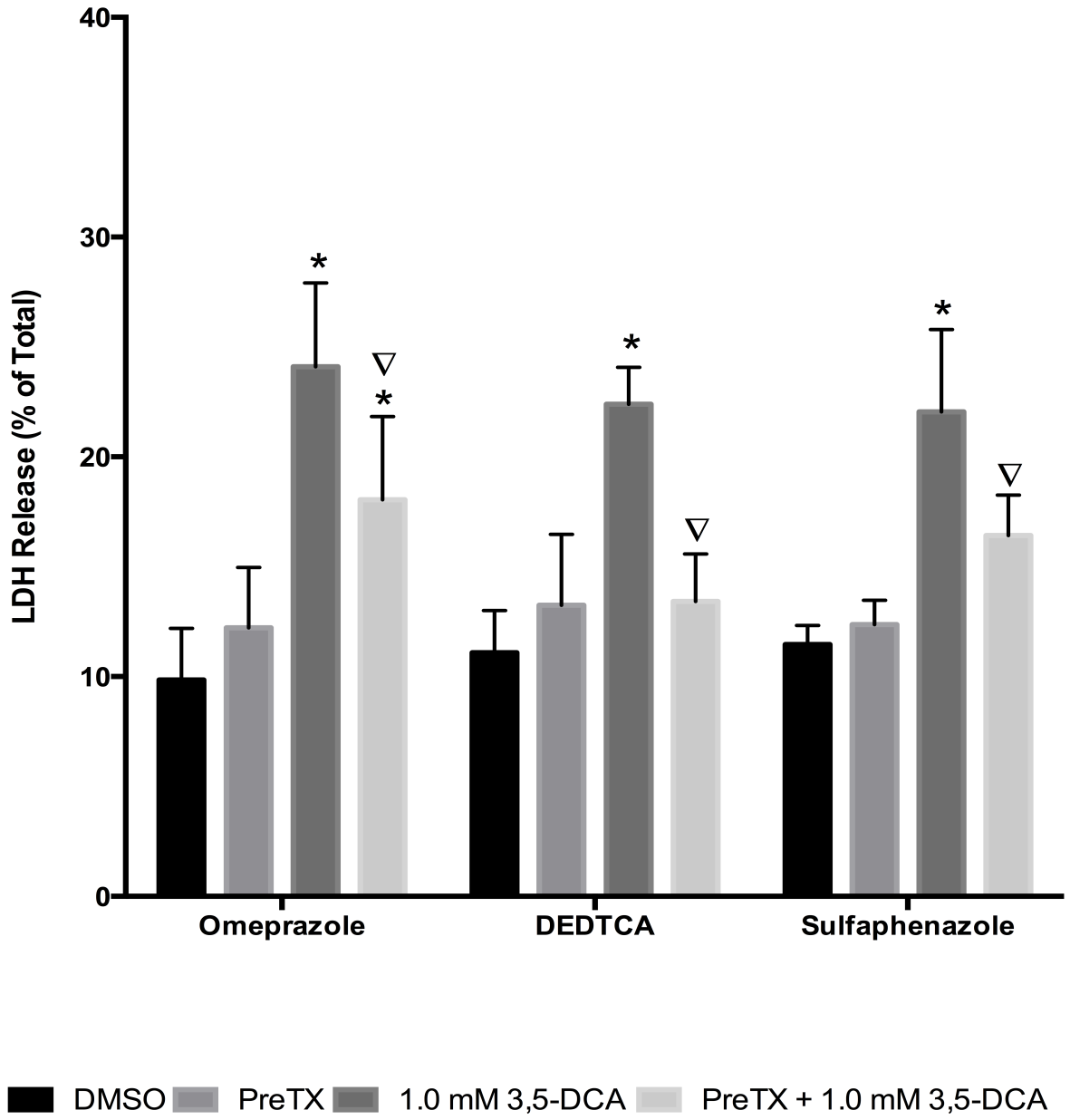

Figure 11. Effects of CYP2C isozyme selective inhibitor pretreatment (PreTX) on 3,5-DCA cytotoxicity in isolated renal cortical cells obtained from male Fischer 344 rats after 90 min. Each bar represents the mean ± S.E.M. for N=4-5 separate isolation experiments. *Significantly different from DMSO control, $P<0.05$. ∇Significantly different from the 1.0 mM 3,5-DCA value, $P<0.05$.

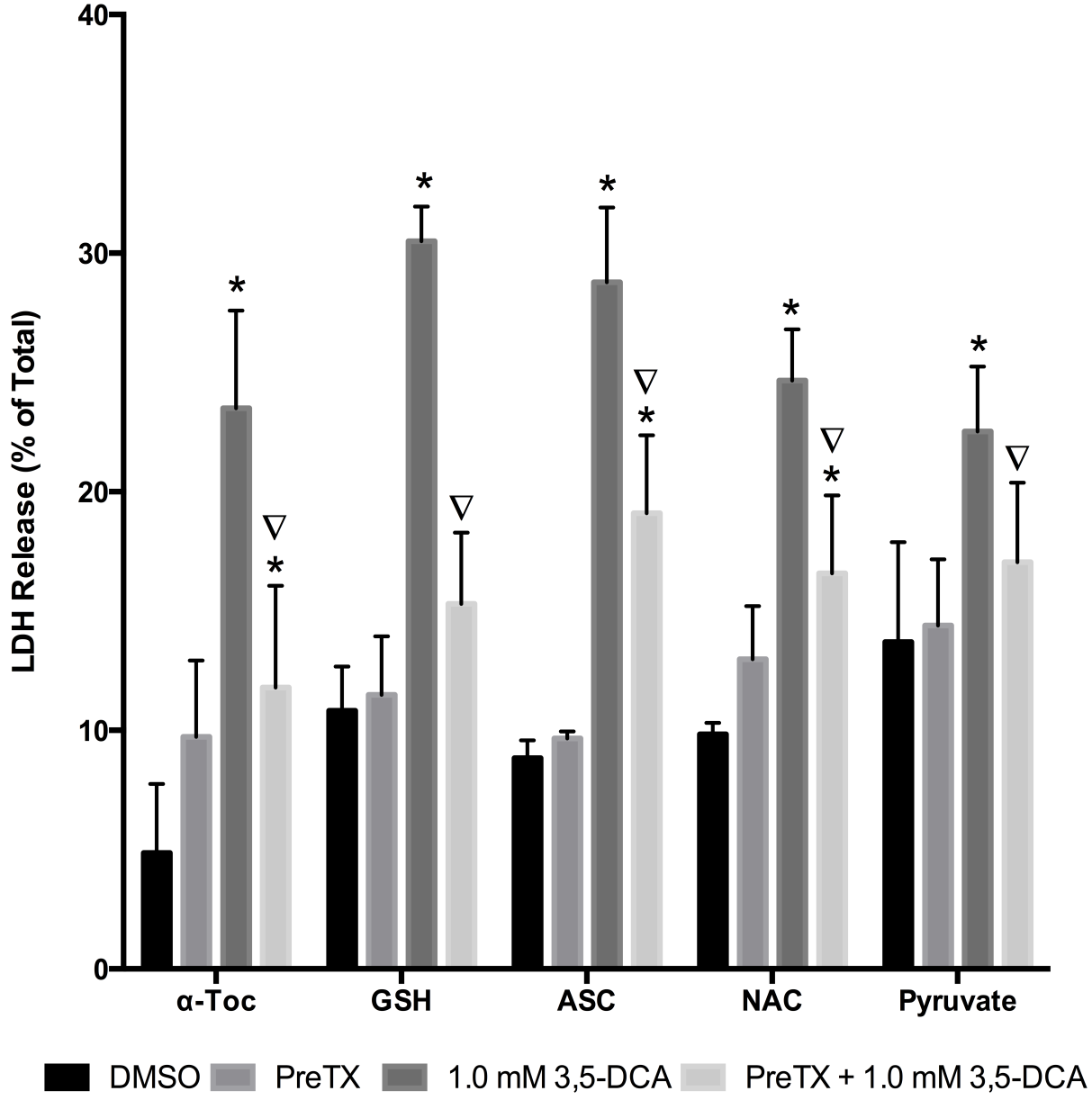

Figure 12. Effects of antioxidant pretreatment (PreTX) on 3,5-DCA cytotoxicity in isolated renal cortical cells obtained from male Fischer 344 rats after 90 min. Each bar represents the mean ± S.E.M. for N=4-5 separate isolation experiments. *Significantly different from DMSO control, $P<0.05$. ∇Significantly different from the 1.0 mM 3,5-DCA value, $P<0.05$.

2.3.6 Determination of oxidative stress following exposure to 3,5-DCA

Since antioxidant pretreatment significantly attenuated cytotoxicity, studies were designed to determine whether oxidative stress following exposure to 3,5-DCA played a role in cytotoxicity. The presence of oxidative stress was determined by examining the ratio of oxidized/reduced glutathione (GSH) after exposure to 3,5-DCA (0.5 and 1.0 mM) at 60 and 90 min. These results were then correlated with the appearance of cytotoxicity. Results showed no significant changes in the ratio of oxidized/reduced GSH at any 3,5-DCA concentration or time point (Fig. 13), even though some cell death was occurring with the 1.0 mM concentration at the 90 min time point (Fig. 8). However, total GSH and reduced GSH were significantly reduced following exposure to 3,5-DCA at both concentrations (0.5 mM and 1.0 mM) and times (60 min, 90 min). Oxidized GSH was unchanged at 60 min and was significantly reduced at 90 min with both concentrations of 3,5-DCA (0.5 mM and 1.0 mM). (Fig. 13)

Protein carbonyl levels were examined as a second marker of oxidative damage to determine if oxidative stress was a causative factor in cell death. Protein carbonyl levels were only significantly increased following exposure to 1.0 mM 3,5-DCA after 90 min (Fig. 14), a time when cell death was evident (Fig. 8). Thus, oxidative damage appears to be secondary to the causative mechanism of cell death.

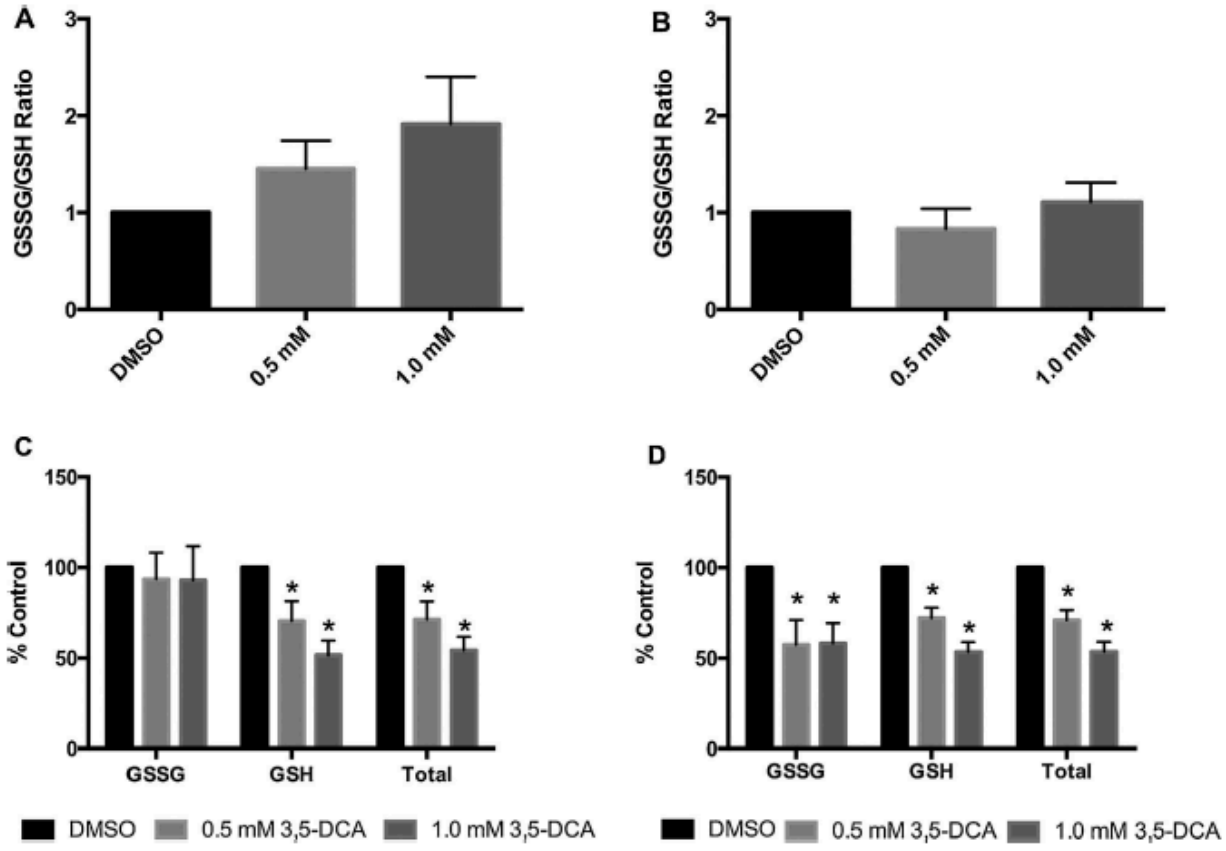

Figure 13. The oxidized/reduced glutathione (GSSG/GSH) ratio following exposure to 3,5-DCA for 60 (A) or 90 (B) min. Total, oxidized, and reduced GSH following exposure to 3,5-DCA for 60 (C) and 90 (D) min. Each bar represents the percent control ± S.E.M. for N=4-6 separate isolation experiments. *Significantly different from DMSO control, $P<0.05$.

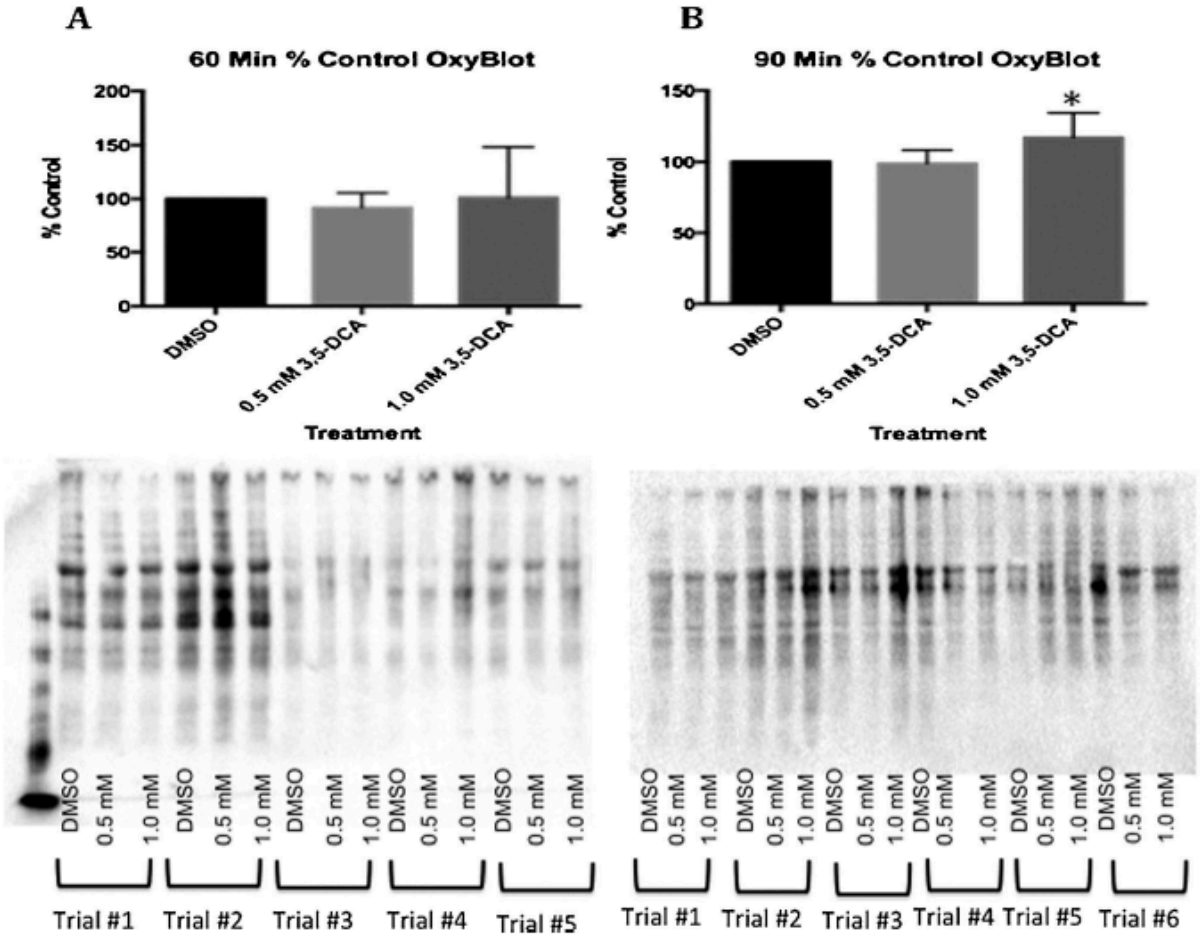

Figure 14. Oxyblot data following exposure to 3,5-DCA for 60 (A) and 90 (B) min. Each bar represents the percent control ± S.E.M. for N=4-6 separate isolation experiments. *Significantly different from DMSO control, $P<0.05$

2.4 DISCUSSION

Human exposure to 3,5-DCA can occur in a variety of settings, including during the manufacture of 3,5-DCA-based pesticides (e.g. iprodione, vinclozolin), dyes, etc. and through environmental exposure (e.g. application of pesticides, accumulation in waste water). Human exposure data to 3,5-DCA is extremely limited and blood levels of 3,5-DCA following pesticide or 3,5-DCA exposure are not reported for humans. Exposure to vinclozolin during manufacture has been monitored by measuring 3,5-DCA levels in the urine, which can be as high as ~1.4 mg/g creatinine (Zober et al., 1995). However, these levels have not resulted in harmful renal effects. Thus, nephrotoxicity in humans would most likely occur only through accidental poisoning, as is seen for aniline (Gosselin, Smith, and Hodge, 1984). A single nephrotoxic dose of 3,5-DCA in Fischer 344 rats (0.8 mmol/kg, ip) would result in an estimated blood level of 1.25 mM (Lo et al., 1990), a concentration 2.5 times higher than the minimal nephrotoxic concentration used in this study. However, whether the human kidney would be exposed to these minimal in vitro nephrotoxic concentrations of 3,5-DCA is unclear at this time.

This study was the first to show the importance of renal biotransformation in 3,5-DCA induced cytotoxicity. Inhibition of FMO, CYP and peroxidase activity all attenuated cytotoxicity suggesting that a toxic metabolite is formed following exposure to 3,5-DCA and that multiple enzyme systems are capable of bioactivating 3,5-DCA and/or its metabolites (Fig. 7). Antioxidants also offered protection from 3,5-DCA induced cytotoxicity, suggesting that free radicals are involved in the mechanisms of 3,5-DCA bioactivation and/or cytotoxicity. If the parent compound alone was responsible for cytotoxicity, inhibition of biotransformation systems would lead to either no changes in cytotoxicity or an increase in toxicity if one of the pathways

inhibited was a major detoxifying mechanism. Thus, renal bioactivation of 3,5-DCA contributes to 3,5-DCA induced cytotoxicity in an IRCC model.

N-Oxidation, *N*-acetylation, and phenyl ring oxidation are three primary routes of chloroaniline biotransformation (Ehlhardt and Howbert, 1991; Hong and Rankin, 1998). Based on these observations and the biotransformation of other chloroanilines in rats, a proposed biotransformation pathway for 3,5-DCA is shown in Fig. 7. Of these potential biotransformation pathways, it is known that chloroacetanilides, which arise from *N*-acetylation catalyzed via *N*-acetyltransferase enzymes, possess much reduced nephrotoxic potential compared to the parent chloroanilines (Rankin et al., 1993, 1995). Therefore, it is unlikely that *N*-acetylation of 3,5-DCA to 3,5-dichloroacetanilide (3,5-DCAA) would contribute to the renal cytotoxicity of 3,5-DCA. N-Acetyltransferase inhibitors were not evaluated for their ability to reduce 3,5-DCA nephrotoxicity for this reason. However, studies with 3,5-DCAA are required to confirm its renal effects and role in 3,5-DCA nephrotoxicity.

Another purposed metabolic pathway that could contribute to 3,5-DCA induced nephrotoxicity is *N*-oxidation. *N*-Oxidation of aromatic amines is known to be catalyzed by multiple enzymes including CYPs, FMOs, prostaglandin H synthase, and peroxidases (Bakkenist, Plat, and Wever, 1981; Corbett, Chipko, and Batchelor, 1980; Golly and Hlavica, 1985; McMillan, Leakey, Arlotto, McMillan, and Hinson, 1990; Ochiai, Sakurai, Nomura, Itoh, and Tanaka, 2006; Sun et al., 2007; Yanni et al., 2010). N-Oxidation of 3,5-DCA would lead to 3,5-dichlorophenylhydroxylamine (3,5-DCPHA) (Fig. 8), whose nephrotoxic potential is yet to be determined. However, it would not be surprising if 3,5-DCPHA contributed to 3,5-DCA induced nephrotoxicity because 3,4-dichlorophenylhydroxylamine is a known nephrotoxicant in vitro using a rat renal cortical slice model (Valentovic, Ball, Stoll, and Rankin, 2001). The results

of the current study indicate that multiple enzymatic systems that catalyze N-oxidation can contribute to 3,5-DCA bioactivation, and that N-oxidation is a potential pathway leading to one or more ultimate, cytotoxic 3,5-DCA metabolites.

Further oxidation of 3,5-DCPHA would result in 3,5-dichloronitrosobenzene, which can be oxidized to 3,5-dichloronitrobenzene (3,5-DCNB). 3,5-DCNB has been shown to reduce renal gluconeogenesis at 1.0 mM and increase LDH release at 2.0 mM or higher concentrations after a 90 min exposure in renal cortical slices from Fischer 344 rats (Hong, Anestis, Ball, Valentovic, and Rankin, 2002). It is unlikely that 3,5-DCNB is the ultimate toxic species following 3,5-DCA 1.0 mM, since a sufficient concentration of 3,5-DCNB to cause LDH release could not be reached with the 3,5-DCA concentration used in the current study. However, *N*-hydroxyl and nitroso compounds have been shown to alter cellular function by covalently binding to cellular nucleophiles (e.g. GSH, thiols of proteins, etc.) or redox cycling to produce reactive oxygen species, leading to increased cell death (Eyer and Ascherl, 1987; Kiese and Taeger, 1976; Stiborová, Frei, Schmeiser, Wiessler, and Anzenbacher, 1992; Umbreit, 2007; Valentovic et al., 1997). Therefore, any 3,5-dichloronitrosobenzene formed from 3,5-DCPHA could be contributing to 3,5-DCA cytotoxicity.

Aromatic ring oxidation is another potential biotransformation pathway that could contribute to 3,5-DCA bioactivation. CYPs can catalyze aromatic ring oxidation, and since CYP inhibition reduces 3,5-DCA cytotoxicity (Fig. 10), production of phenolic metabolites of 3,5-DCA could be an important contributing factor in 3,5-DCA nephrotoxicity. Oxidation of the aromatic ring can lead to two possible metabolites, 2-amino-4,6-dichlorophenol and 4-amino-2,6-dichlorophenol (Fig. 7). While not much is known about 2-amino-4,6-dichlorophenol nephrotoxicity, 4-amino-2,6-dichlorophenol has been shown to be a potent nephrotoxicant both

in vivo and *in vitro* (Hong et al., 1997; Rankin et al., 1994, 2008a; Valentovic et al., 1997). Interestingly, addition of a chloro group to the 4-position of 3,5-DCA to form 3,4,5-trichloroaniline produces a 3,5-DCA derivative without the ability to form significant amounts of 4-amino-2,6-dichlorophenol. However, 3,5-DCA and 3,4,5-trichloroaniline have equal nephrotoxic potential at 90 min, and 3,4,5-trichloroaniline is more potent as a nephrotoxicant than 3,5-DCA at 120min in IRCC (Racine et al., 2014). Thus, although 4-amino-2,6-dichlorophenol is a nephrotoxicant, it does not appear to be the ultimate nephrotoxic metabolite arising from 3,5-DCA in vitro. Studies with 2-amino-4,6-dichlorophenol are ongoing to determine its nephrotoxic potential. Thus, the role of aminophenol metabolites in 3,5-DCA cytotoxicity remains to be fully determined, but oxidation at the 4-position of 3,5-DCA does not appear to be a critical bioactivation pathway.

Since the general CYP inhibitors (piperonyl butoxide and metyrapone) were able to significantly attenuate cytotoxicity, further studies were conducted looking at the role of selective CYP isozymes which are found in the kidney. Cummings et al. (1999) found CYP2E1, CYP2C11, CYP2B1/2, and CYP4A2/3 in freshly isolated rat proximal and distal tubular cells. CYP2E1 expression was higher in distal tubular cells than proximal tubular cells, while CYP2C11 was higher in proximal tubular cells than distal tubular cells. CYP3A1/2 was not detected in the proximal tubular cells but was found in total kidney homogenate, which may indicate why oleandomycin, a CYP3A inhibitor, was not effective in attenuating 3,5-DCA cytotoxicity. The inability of thio-tepa (CYP2B inhibitor) and isoniazid (CYP2E inhibitor) to attenuate 3,5-DCA cytotoxicity, suggests that these CYPs are not critical for 3,5-DCA bioactivation. Of the selective CYP inhibitors we used, only sulfaphenazole, omeprazole, and diethyldithiocarbamate (DEDTCA) were able to attenuate 3,5-DCA cytotoxicity. These three

inhibitors all show a preference to inhibit the 2C family of rat isozymes (Eagling et al., 1998; Kobayashi, Urashima, Shimada, and Chiba, 2003), suggesting that the 2C family may play a role in the bioactivation of 3,5-DCA. The CYP2C family in rats facilitates *N*-hydroxylation, as well as aromatic ring oxidation (Cribb, Spielberg, and Griffin, 1995), which supports one or both of these pathways as contributing to 3,5-DCA bioactivation.

Both *N*-hydroxylation and aromatic ring oxidation can lead to an increase in free radicals: either as metabolites undergoing redox cycling or directly from oxidation during metabolism (Harmon et al., 2006; Michail, Baghdasarian, Narwaley, Aljuhani, and Siraki 2013), and N-hydroxyl, N-nitroso and aminophenol metabolites can induce cell death via oxidative stress mechanisms (Harmon et al., 2005; Lock et al., 1993; Umbreit, 2007; Valentovic et al., 1997). Antioxidant pretreatment proved to be highly effective in attenuating 3,5-DCA cytotoxicity, with all antioxidants offering protection, suggesting that free radicals may play a role in cytotoxicity. Oxidative stress was measured by looking at the ratio of GSSG/GSH and increases in protein carbonyl levels. If oxidative stress played a significant role in the mechanism of cellular death, an increase in the GSSG/GSH ratio should occur prior to cytotoxicity, as seen with compounds such as *para*-aminophenol (Harmon et al., 2005). However, in the case of 3,5-DCA, there was no significant increase in the GSSG/GSH ratio, and the significant increase in protein carbonyl levels only occurred after there was an increase in cytotoxicity. These data suggest that oxidative stress is not responsible for cell death in 3,5-DCA induced nephrotoxicity in vitro, and that the antioxidants may be offering protection, at least in part, by scavenging one or more radical metabolites produced during the metabolism of the amino group or an aminophenol metabolite (e.g. 2-amino-4,6-dichlorophenol) (Fowler, Moore, Foster, and Lock, 1991; Fowler, Foster, and Lock, 1993).

Total glutathione levels decreased following 3,5-DCA exposure, even though the GSSG/GSH ratio didn't significantly change (Fig. 13). In the absence of oxidative stress, it is likely that the reduction in total glutathione levels was due to the formation of a reactive 3,5-DCA metabolite(s) that was(were) detoxified by reaction with reduced glutathione. Both addition of GSH and N-acetyl-L-cysteine, which protect cells by being converted to GSH (Lauterburg, Corcoran, and Mitchell, 1983), to IRCC attenuated 3,5-DCA cytotoxicity. Thus, at least part of the mechanism of protection from GSH could come from reacting with a reactive 3,5-DCA metabolite. It is also possible that the reduction of the GSSG levels seen at 90 min was an effort to restore GSH concentrations to a level offering cellular protection. However, further studies are needed to more clearly determine the role(s) of glutathione in attenuating 3,5-DCA cytotoxicity.

2.5 Conclusions

The results of this study determined that, as in rat renal cortical slices, 3,5-DCA is toxic to IRCC in a time- and concentration-dependent manner. Results also suggest that 3,5-DCA is potentially bioactivated in the kidney via several biotransformation pathways and that the CYP2C family is the major contributing renal CYP family for bioactivation. In addition, oxidative stress does not appear to play a significant role in the causative mechanism of 3,5-DCA cytotoxicity. While these studies provide insight into the role of biotransformation enzymes in bioactivating 3,5-DCA to nephrotoxic metabolites, studies are underway to determine the ultimate toxicant(s) in 3,5-DCA induced nephrotoxicity.

2.6 Acknowledgements

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2.7 Conflict of Interest

The authors have no conflict of interest to declare.

CHAPTER III: 3,4,5-TRICHLOROANILINE NEPHROTOXICITY IN VITRO: POTENTIAL ROLE OF FREE RADICALS AND RENAL BIOTRANSFORMATION

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Christopher Racine[1], Dakota Ward[1], Dianne K. Anestis[1], Debbie Preston[2], Gary O. Rankin[1].

[1]Department of Pharmacology, Physiology, and Toxicology, Joan C. Edwards School of Medicine, Marshall University, Huntington, WV 25755, USA

[2]Department of Pediatrics, Joan C. Edwards School of Medicine, Marshall University, Huntington, WV 25755, USA

Correspondence should be addressed to Gary O. Rankin; rankin@marshall.edu

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ABSTRACT

Chloroanilines are widely used in the manufacture of drugs, pesticides and industrial intermediates. Among the trichloroanilines, 3,4,5-trichloroaniline (TCA) is the most potent nephrotoxicant in vivo. The purpose of this study was to examine the nephrotoxic potential of TCA in vitro and to determine if renal biotransformation and/or free radicals contributed to TCA cytotoxicity using isolated renal cortical cells (IRCC) from male Fischer 344 rats as the animal model. IRCC (~4 million cells/mL; 3 mL) were incubated with TCA (0, 0.1, 0.25, 0.5 or 1.0 mM) for 60-120 min. In some experiments, IRCC were pretreated with an antioxidant or a cytochrome P450 (CYP), flavin monooxygenase (FMO), cyclooxygenase or peroxidase inhibitor prior to incubation with dimethyl sulfoxide (control) or TCA (0.5 mM) for 120 min. At 60 min, TCA did not induce cytotoxicity, but induced cytotoxicity as early as 90 min with 0.5 mM or higher TCA and at 120 min with 0.1 mM or higher TCA, as evidenced by increased lactate dehydrogenase (LDH) release. Pretreatment with the CYP inhibitor piperonyl butoxide, the cyclooxygenase inhibitor indomethacin or the peroxidase inhibitor mercaptosuccinate attenuated TCA cytotoxicity, while pretreatment with FMO inhibitors or the CYP inhibitor metyrapone had no effect on TCA nephrotoxicity. Pretreatment with an antioxidant (α-tocopherol, glutathione, ascorbate or N-acetyl-L-cysteine) also reduced or completely blocked TCA cytotoxicity. These results indicate that TCA is directly nephrotoxic to IRCC in a time and concentration dependent manner. Bioactivation of TCA to toxic metabolites by CYP, cyclooxygenase and/or peroxidase contributes to the mechanism of TCA nephrotoxicity. Lastly, free radicals play a role in TCA cytotoxicity, although the exact nature of the origin of these radicals remains to be determined.

3.1 INTRODUCTION

Chloroanilines are commonly used as chemical intermediates to manufacture dyes, agricultural chemicals, drugs and industrial compounds. Exposure to chloroanilines can occur in occupational settings, through the release or formation during the metabolism of compounds in mammals (Aizawa, 1989; Ehlhardt, 1991; Rickert and Held, 1990) or by the degradation of pesticides in the environment (Aizawa, 1989; Lee et al., 2008; Santos et al., 1998; Mercadier, Vega, and Bastide, 1998). In addition, the detection of chloroanilines in human urine or blood can be used as a biomarker for exposure to chloroaniline-based pesticides (Lindh et al, 2007; Kutting et al., 2009; Turci, Barisano, Baldducci, Colosio, and Minoia, 2006; Vitelli et al., 2007). The toxicity associated with exposure to mono- and dichloroanilines includes hematotoxicity (e.g. methemoglobinemia or anemia) (Chhabra et al., 1990; Guilhermino et al., 1998; Valentovic et al. 1997, splenotoxicity (Chhabra et al, 1990; Ward, Reznik, and Garner, 1980), hepatotoxicity (Valentovic et al., 1992, 1995a, 1995b) and nephrotoxicity (Valentovic et al., 1995b; Hong et al., 2000, Lo et al., 1990). Because of their adverse health effects and release into the environment in agricultural areas following the breakdown of pesticides, chloroanilines are considered priority pollutants in environmental risk assessments (Boehncke, Kielhorn, Konnecker, Pohlenz-Michel, and Mangelsdorfer, 2003; Vangnai et al., 2012).

Trichloroanilines have similar uses as the mono- and dichloroanilines, including use in drug development (Craciunescu, Furlani, Scarcia, Ghirvu, and Doadrio, 1985; Imai, Takahashi, Watanabe, Nakazawa, and Yamanaka, 1991; Limban, Marutescu, and Chifiriuc, 2011), dye manufacturing (Peters and Yang, 1996) and production of agricultural agents (Aggarwal, Kumar, Dureja, and Rawat, 2009). While the potential adverse health effects of mono- and dichloroanilines have been studied in some detail, little information is available about the

toxicity induced by trichloroanilines, including the nephrotoxic potential of trichloroanilines or their mechanisms of inducing nephrotoxicity. Lo, Brown, and Rankin (1991) examined the in vivo and in vitro effects of four trichloroanilines (2,3,4-, 2,4,5-, 2,4,6- and 3,4,5-trichloroaniline) on the renal function of male Fischer 344 rats. They noted that of the four trichloroanilines tested, 3,4,5-trichloroaniline (TCA) had the greatest nephrotoxic potential in vivo as evidenced by oliguria, increased kidney weight, elevated blood urea nitrogen concentration and altered renal organic ion accumulation. In vitro, TCA was also the most potent nephrotoxicant of the four trichloroanilines tested, decreasing tetraethylammonium accumulation by renal cortical slices at 1.0 μM concentration (Lo et al., 1991).

Although it is known that metabolites of mono- and dichloroanilines are toxic to the kidney in vivo and in vitro (Hong et al., 1996; Hong et al., 1997; Rankin, Hong, Anestis, Ball, and Valentovic, 2008a; Valentovic et al., 2001), no studies have examined the role of biotransformation in trichloroaniline nephrotoxicity. It is also unknown if the kidney bioactivates parent chloroanilines to nephrotoxic metabolites, or if the parent chloroaniline is toxic to the kidney without bioactivation. The purpose of this study was to begin to examine the role of biotransformation of a trichloroaniline in the nephrotoxicity it produces in vitro as well as determine if free radicals contributed to the cytotoxicity. TCA was selected for study because it is the most potent trichloroaniline nephrotoxicant in vivo and in vitro. The Fischer 344 rat was selected as the animal model because our previous studies with chloroaniline-induced nephrotoxicity have been conducted in this animal model. The inhibitor pretreatments, concentrations and times selected for study were based on previous reports (Lock et al., 1993; Baliga et al., 1998; Rodriguez and Acosta, 1997; Valentovic et al., 1999; O'Brien and Siraki, 2005; Lau and Monks, 1987; Katsuda et al., 2010 Suzuki and Sudo, 1990).

3.2 RESULTS

3.2.1 Time and concentration cytotoxicity studies

To obtain information concerning the nephrotoxic potential of TCA in isolated renal cortical cells (IRCC), a concentration response study was performed at 60, 90 and 120 min. IRCC exposed to TCA at concentrations up to 1.0 mM for 60 min did not exhibit any cytotoxicity (data not shown). A 90 min exposure to TCA at a concentration of 0.5 mM or higher induced cytotoxicity (increased LDH release), while at 120 min of exposure, cytotoxicity was evident at a concentration of 0.1 mM TCA or higher (Figure 15). Based on these findings, a concentration of 0.5 mM and an exposure time of 120 min were selected for use in the antioxidant and inhibitor pretreatment studies.

3.2.2 Effects of antioxidants on TCA cytotoxicity

The effects of pretreating IRCC with an antioxidant on TCA cytotoxicity was examined next (Figure 16). All four antioxidants (α-tocopherol, ascorbate, glutathione and N-acetyl-L-cysteine) provided at least some degree of attenuation of TCA cytotoxicity. Glutathione and ascorbate were most effective and α-tocopherol was the least effective at attenuating TCA cytotoxicity.

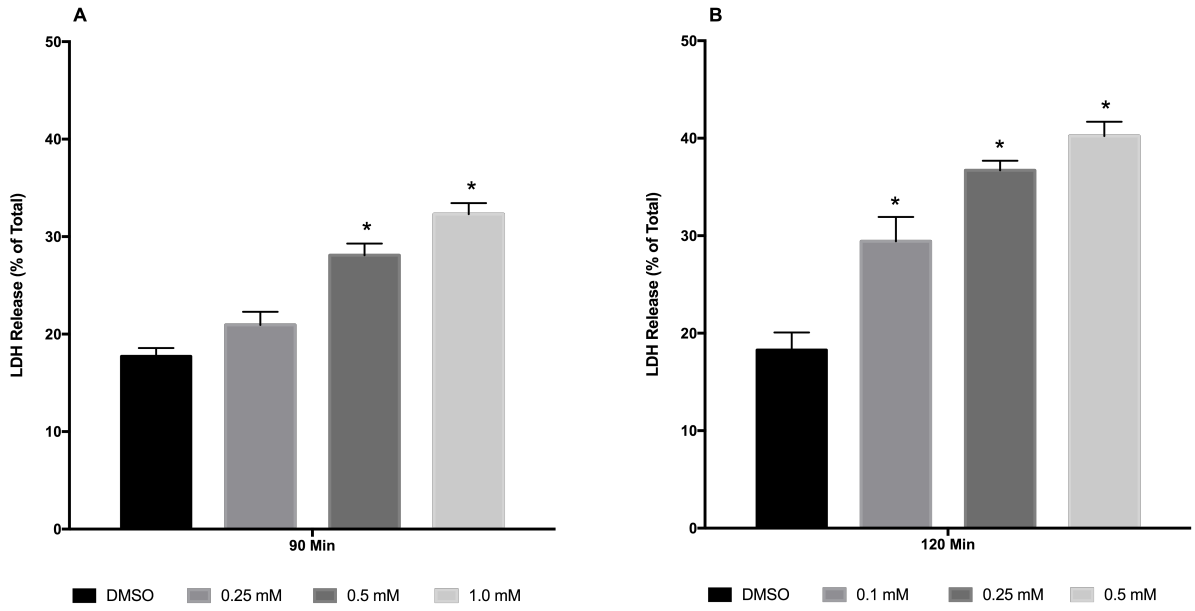

Figure 15. Cytotoxic effects of TCA at 90 min (Panel A) and 120 min (Panel B) in IRCC. An asterisk indicates significantly different from the DMSO control group value, $P<0.05$.

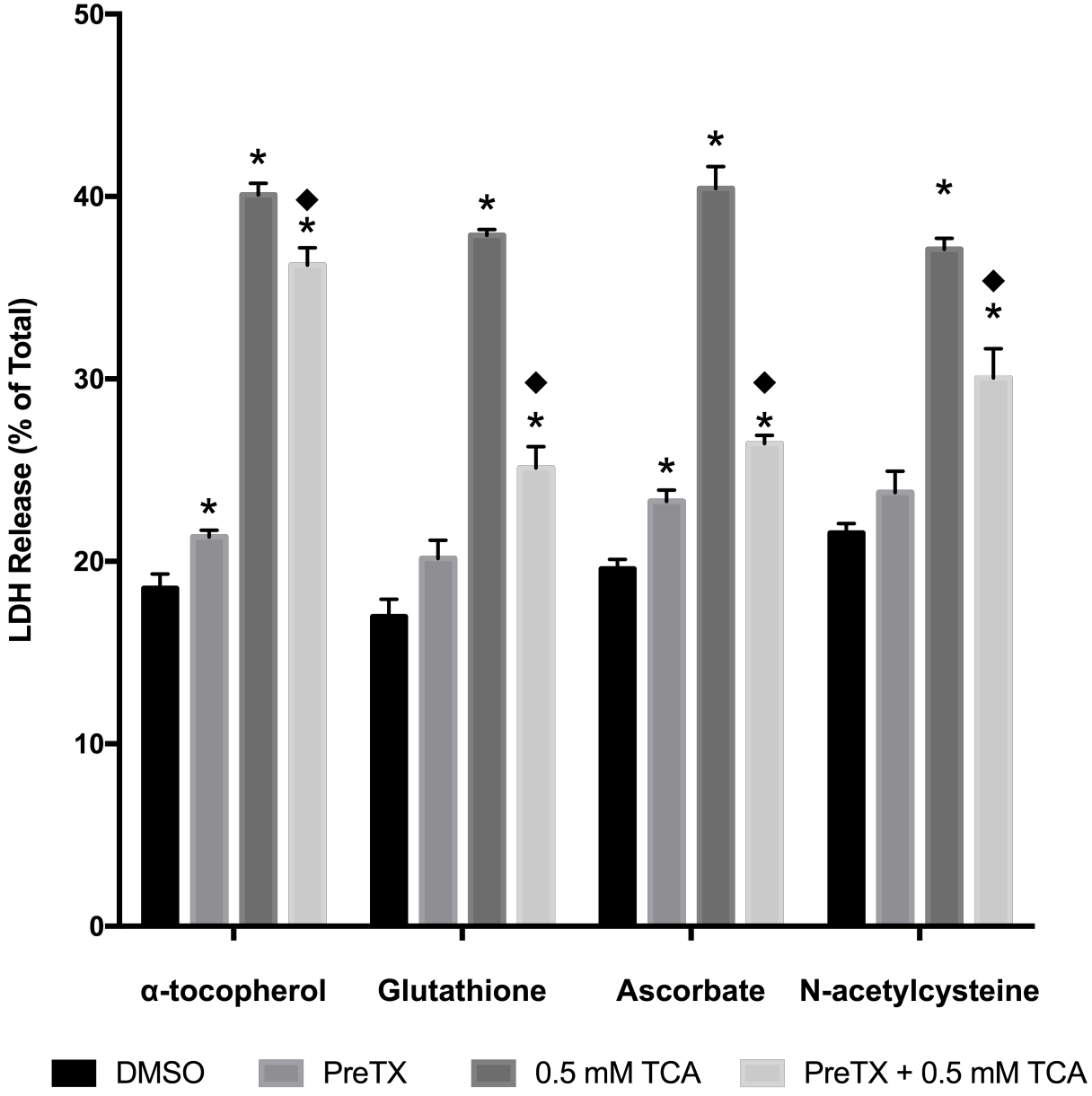

Figure 16. Effect of antioxidant pretreatment on TCA cytotoxicity at 120 min. An asterisk indicates significantly different from the DMSO control group value, $P<0.05$. A diamond indicates significantly different from the 0.5 mM TCA alone value, $P<0.05$

3.2.3 Effects of cytochrome P450 (CYP) and flavin monooxygenase (FMO) inhibition

The effects of inhibiting CYP and FMO activity on TCA cytotoxicity were examined using nonselective CYP (piperonyl butoxide [PiBX] and metyrapone) and FMO (methimazole and N-octylamine) inhibitors. Inhibition of CYPs with PiBX, but not metyrapone, attenuated TCA cytotoxicity (Figure 17). Inhibition of FMOs with either methimazole or N-octylamine had no effect on TCA induced cell death (Figure 17).

3.2.4 Effects of cyclooxygenase and peroxidase inhibition

The effect of inhibiting the cyclooxygenase activity of prostaglandin H synthase on TCA cytotoxicity was determined using indomethacin pretreatment, while mercaptosuccinate was used as a general peroxidase inhibitor. Both indomethacin pretreatment and mercaptosuccinate pretreatment reduced TCA cytotoxicity (Figure 18).

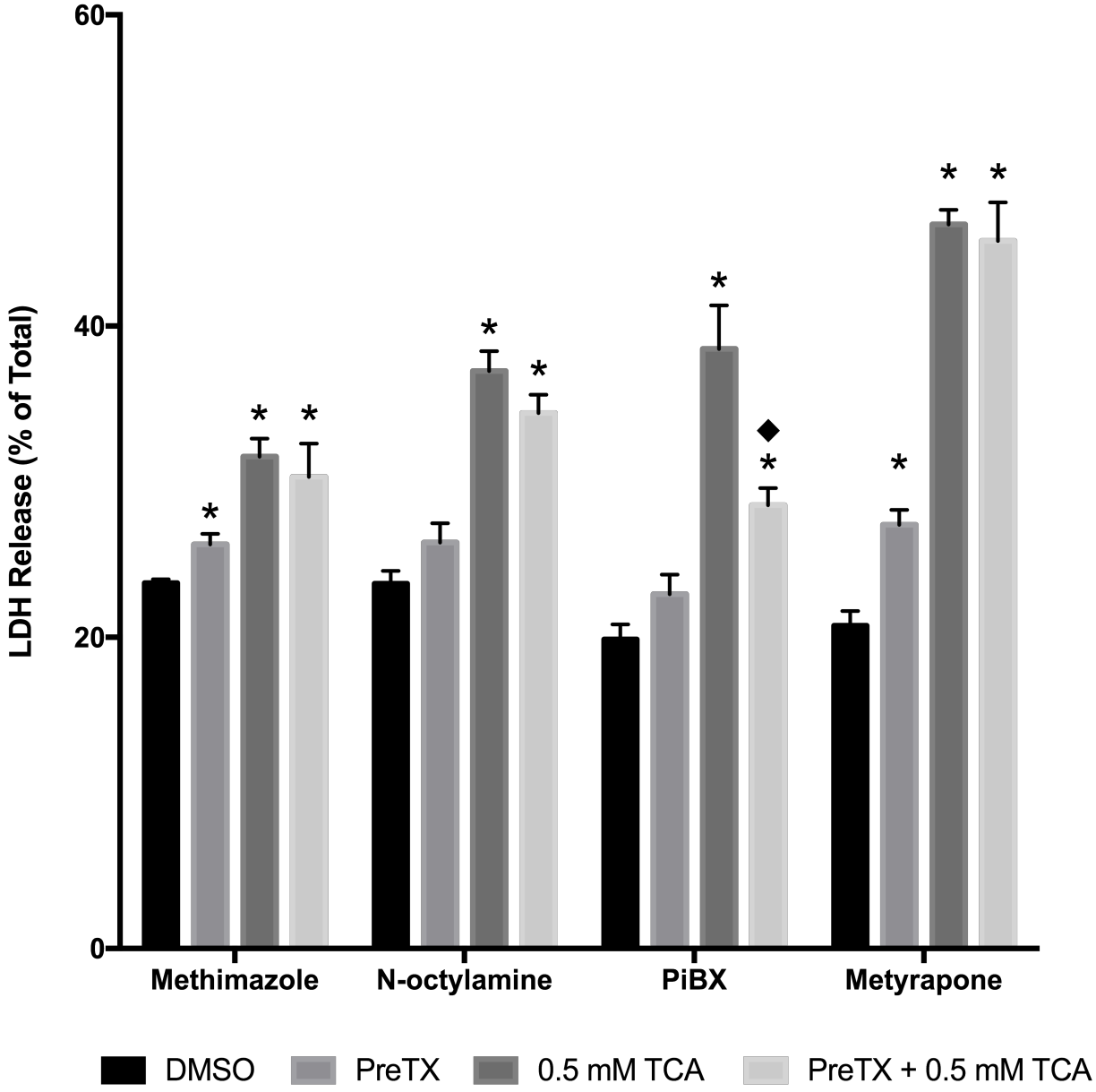

Figure 17. Effect of FMO or CYP inhibition on TCA cytotoxicity at 120 min. An asterisk indicates significantly different from the DMSO control group value, $P<0.05$. A diamond indicates significantly different from the 0.5 mM TCA alone value, $P<0.05$.

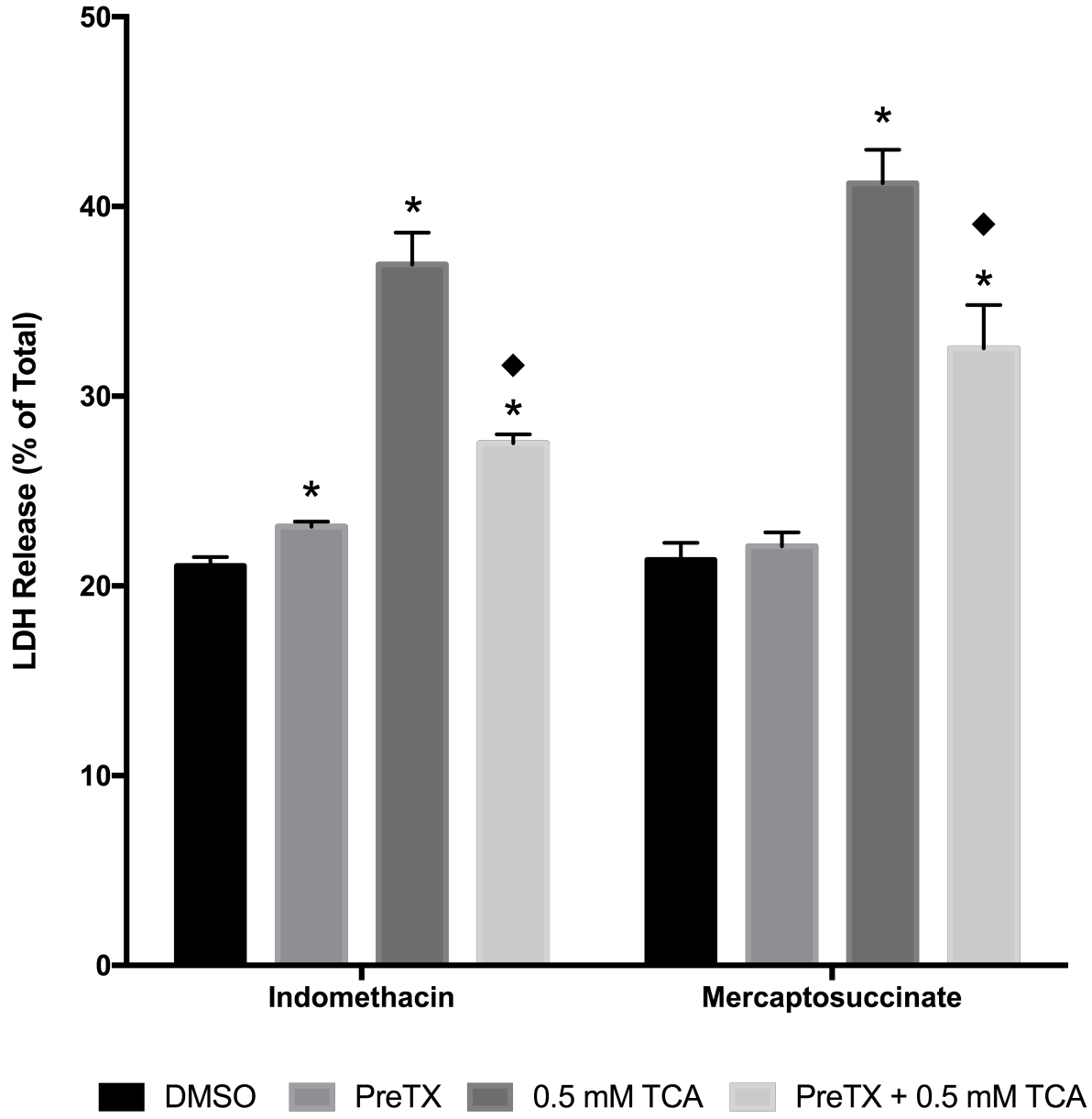

Figure 18. Effect of cyclooxygenase or peroxidase inhibition on TCA cytotoxicity at 120 min. An asterisk indicates significantly different from the DMSO control group value, $P<0.05$. A diamond indicates significantly different from the 0.5 mM TCA alone value, $P<0.05$.

3.3 DISCUSSION

This study is the first report to demonstrate the direct cytotoxic effects of TCA on the kidney. In a previous study, the in vitro effects of TCA on organic ion transport by renal cortical slices from male Fischer 344 rats suggested that TCA could affect renal function, decreasing organic cation accumulation at concentrations as low as 1.0 μM, and affecting both organic anion and cation accumulation at 1.0 mM (Lo et al., 1991). However, TCA is a weakly basic compound. Thus, the possibility existed that the effects seen at μM concentrations of TCA in the work by Lo et al. (1991) were more related to interactions at the organic cation transporter level than cytotoxicity, and that cytotoxicity was not observed until TCA concentrations reached mM levels. Results from the present study clearly demonstrate that TCA can induce cytotoxicity at μM concentrations, as evidenced by increased LDH release at concentrations of TCA as low as 100 μM at 120 min, and that TCA induces cytotoxicity in a time and concentration dependent manner.

The ability of the various inhibitors used in this study to attenuate TCA cytotoxicity suggests that metabolites of TCA contribute to TCA nephrotoxicity in vitro. The biotransformation of TCA has only been reported in fish (De Wolf, Seinen, and Hermens, 1993). However, based on studies of the metabolism of other chloroanilines in rats (Hong et al., 1998; Ehlhardt and Howbert, 1991; McMillan, Leakey, Arolotto, McMIllan, and Hinson, 1990; McMillan, Jensen, and Jollow, 1998), a potential biotransformation pathway for TCA can be proposed which includes acetylation, N-oxidation and aromatic ring oxidation (Figure 19).

N-Acetylation is catalyzed by cytosolic N-acetyltransferase enzymes, and it is unlikely that any of the pretreatments used in this study would alter this biotransformation reaction. In addition, acetylation of chloroanilines produces chloroacetanilides, which have greatly reduced

nephrotoxic potential in vivo (Rankin et al., 1993). Thus, it is unlikely that acetylation would be a mechanism for bioactivation of 3,4,5-trichloroaniline.

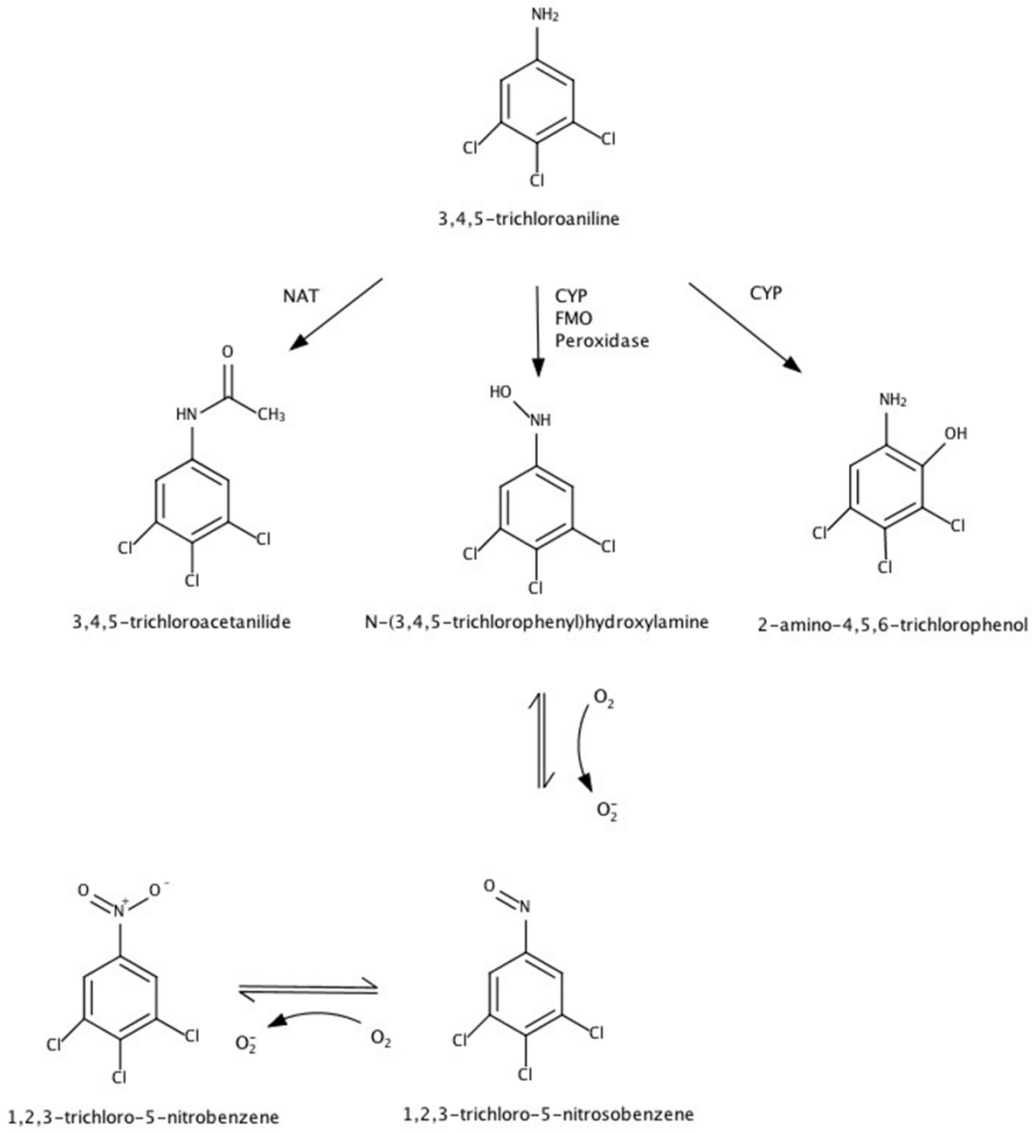

Figure 19. Potential metabolic pathways for TCA. NAT = N-acetyltransferase, CYP = cytochrome P450, FMO = flavin-containing monooxygenase

Oxidation of the aromatic ring to produce 2-amino-4,5,6-trichlorophenol would be a potential bioactivation mechanism for TCA, as many aminophenols are known nephrotoxicants (Hong et al., 1996, 1997; Lock et al., 1993; Rankin et al., 1994, 2008a). Production of an aminophenol metabolite of TCA would be catalyzed by CYPs, as McMillan et al. (1990, 1998) found that aromatic ring oxidation of 3,4-dichloroaniline was catalyzed by CYPs. Whether 2-amino-4,5,6-trichlorophenol is produced in the kidney from TCA and contributes to TCA nephrotoxicity remains to be determined. However, a structurally-related metabolite, 2-amino-4,5-dichlorophenol, is directly toxic to renal cortical slices from male Fischer 344 rats (Valentovic et al., 2002). Attenuation of TCA cytotoxicity by the CYP inhibitor PiBX suggests that aromatic ring oxidation may be a potential route of TCA bioactivation. Nonetheless, the inability of another general CYP inhibitor, metyrapone, to reduce TCA cytotoxicity indicates that further study is needed to clarify which CYPs may contribute to TCA bioactivation to toxic metabolites and the role of 2-amino-4,5,6-trichlorophenol in TCA nephrotoxicity. The use of additional, more specific CYP inhibitors will help define the role and nature of the CYPs contributing to the production of toxic metabolites in TCA metabolism.

N-Oxidation can be catalyzed by several enzyme systems, including CYPs, FMOs, prostaglandin H synthase and peroxidases (McMillan et al., 1998; Ochiai et al., 2006; Yanni et al., 2010; Golly and Hlavica, 1985; Corbett et al., 1980, Bakkenist et al., 1981). The N-oxidation pathway has the potential to lead to metabolites that could damage cells via multiple pathways (Figure 19). Metabolites, such as the N-hydroxyl and nitroso metabolites can redox cycle to produce reactive oxygen species and other free radicals which can damage membranes and lead to oxidative stress (Valentovic et al., 1997; Umbreit, 2007). In addition, N-hydroxyl and nitroso metabolites have the ability to be further activated to form covalent bonds with cellular

nucleophiles to alter cellular function and led to cell death (Kiese and Taegar, 1976; Eyer and Ascherl et al., 1987; Stiborová, Frei, Schmeiser, Wiessler, and Anzenbacher, 1992). The ability of PiBX, indomethacin and mercaptosuccinate, but not methimazole or N-octylamine, to attenuate TCA cytotoxicity suggests that multiple enzyme systems may be involved in bioactivating TCA to toxic metabolites via the N-oxidation pathway, but renal FMOs do not appear to contribute to this bioactivation mechanism. That N-oxidation would contribute to TCA cytotoxicity is not completely surprising, as 3,4-dichlorophenylhydroxylamine, the N-oxidation metabolite of 3,4-dichloroaniline, is a known nephrotoxicant in vitro to rat renal cortical slices (Valentovic et al., 2001).

The ability of the four antioxidants to attenuate TCA cytotoxicity suggests that free radicals contribute to the mechanism of renal injury induced by TCA. These free radicals may be produced during the oxidation of an aromatic amine or its metabolites (Loew and Goldblum, 1985), or as a consequence of redox cycling of aminophenol or phenylhydroxylamine/nitrosobenzene metabolites to produce reactive oxygen species and oxidative stress (Umbreit, 2007; Harmon et al., 2006). Further work is required to determine if either or both of these potential pathways explains the mechanism of protection by antioxidants on TCA cytotoxicity. Preliminary work with another chloroaniline, 3,5-dichloroaniline, suggests that oxidative stress may not contribute to the in vitro nephrotoxicity induced by 3,5-dichloroaniline (Racine et al., 2016), but studies with TCA have not been conducted to determine how free radicals contribute to TCA nephrotoxicity in vitro and are necessary to define the exact role of free radicals in TCA nephrotoxicity.

It is interesting to note that α-tocopherol, a lipophilic compound, was the weakest of the four antioxidants in attenuating TCA nephrotoxicity. It would be expected that α-tocopherol would

enter IRCC via a passive diffusion mechanism, while renal transporters can promote the accumulation of ascorbate, glutathione and N-acetyl-L-cysteine (Lee et al., 2006; Lash et al., 2011; Koh, Simmons-Willis, Pritchard, Grassel, and Ballatori, 2002). Glutathione can also be accumulated in proximal tubular cells via processing at the luminal membrane and re-synthesis from the accumulated amino acids. Thus, higher intracellular concentrations of ascorbate, glutathione and N-acetyl-L-cysteine may be achieved in IRCC as compared to α-tocopherol. In addition, while all of the antioxidants have the ability to scavenge and detoxify a variety of free radicals and reactive oxygen and nitrogen species to varying degrees (Machlin and Bendich, 1987), α-tocopherol associates with membranes and appears to have a primary role in preventing lipid peroxidation which can lead to cell death. Thus, if TCA does not induce oxidative stress and lipid peroxidation as its primary mechanism for inducing nephrotoxicity, α-tocopherol would be expected to be less effective than the other antioxidants used in this study. However, additional studies are needed to clarify the role of oxidative stress and lipid peroxidation in TCA nephrotoxicity.

3.4 EXPERIMENTAL SECTION

3.4.1 Experimental animals

Male Fischer 344 rats (220-280g) from Hilltop Lab Animals (Scottdale PA) were used for all experiments. All animals were kept under controlled environments consisting of regulated light cycle (on 12 hours, off 12 hours), temperature (21-23^{o}C), and humidity (40-55%) and were housed in standard plastic cages (two rats per cage). Animals were allowed to acclimate for at least one week before being used in experiments. Purina Rat Chow and water were available *ad libitum.* The Marshall University Institutional Care and Use Committee approved all animal use. Studies were performed at an AAALAC (Association for the Assessment and Accreditation of Laboratory Animal Care International) accredited facility and all animal care was in accordance with the American Association of Laboratory Animal Sciences (AALAS) Policy on the Humane Care and Use of Laboratory Animals (http://www.aalas.org).

3.4.2 Chemicals

All chemicals used were of the highest purity available and were purchased from Sigma Aldrich (St. Louis, MO).

3.4.3 Isolated Renal Cortical Cell (IRCC) preparation and treatment

Naïve rats were anesthetized with pentobarbital (75 mg/kg, i.p.) and isolated renal cortical cells (IRCC) were obtained using the collagenase perfusion method of Jones et al. (1979). Cell viability was initially determined by trypan blue (2% w/v) exclusion and lactate dehydrogenase (LDH) release. IRCC were counted and re-suspended in Krebs-Henseleit buffer, pH 7.4, containing 25 mM Hepes and 2% (w/v) bovine serum albumin at a concentration of ~4.0

million cells/mL. IRCC (3 mL) were added to a 25 mL polycarbonate Erlenmeyer flask for a five min pre-incubation period in a shaking water bath incubator (37˚C, 60 cycles/min) under a 95% oxygen/5% carbon dioxide atmosphere. IRCC were then exposed to various concentrations of TCA (0, 0.1, 0.25, 0.5, or 1.0 mM) for 60, 90 or 120 minutes. After the incubation period, flasks were removed and placed on ice. Samples (0.5 mL) were taken for lactate dehydrogenase (LDH) release assays. Briefly, samples were centrifuged (3000xg, 3 min), the supernatant was decanted and saved, and the pellet was disrupted with 1 mL of 10% Triton X-100 solution to release cellular LDH activity. LDH activity was then determined in each fraction (supernatant and pellet) as previously described using a kinetic assay based on the amount of NADH produced from NAD (Rankin et al., 2008b). LDH released was expressed as % of total (supernatant plus pellet).

In separate experiments, IRCC were pretreated with either an antioxidant or an enzyme system inhibitor before exposure to 0.5 mM TCA for an additional 120 minutes as described above. The concentrations and pretreatment times for all of the pretreatments are shown in Table 5.

Table 5. List of pretreatments and mechanisms/targeted enzyme systems.

Pretreatment	Concentration (mM)	Pretreatment Time (Min)	Mechanism of Action
N-Acetyl-L-cysteine	2.0	30	Antioxidant
α-Tocopherol	1.0	5	Antioxidant
Glutathione	1.0	30	Antioxidant
Ascorbate	2.0	5	Antioxidant
Methimazole	1.0	30	FMO Inhibitor
N-Octylamine	2.0	5	FMO Inhibitor
Indomethacin	1.0	15	Cyclooxygenase Inhibitor
Piperonyl Butoxide	1.0	15	Non-specific CYP Inhibitor
Metyrapone	1.0	5	Non-specific CYP Inhibitor
Mercaptosuccinate	0.1	15	Peroxidase Inhibitor

3.4.4 Statistics

Data are presented as mean ± S.E.M. with an $N \geq 4$. Data were analyzed by one-way analysis of variance followed by a Student-Newman-Keuls Test. Statistical significance was determined at $P < 0.05$, $\alpha=0.5$.

3.5 CONCLUSIONS

The results of this study demonstrate that TCA is toxic to the kidney in a time and concentration dependent manner. Toxicity was evident as early as 90 min with 0.5 mM TCA and with 0.1 mM TCA at 120 min. Since several of the enzyme inhibitor pretreatments attenuated 3,4,5-cytotoxicity, metabolites appear to contribute to the renal toxicity induced by TCA. Bioactivation of TCA to nephrotoxic metabolites via renal CYPs, cyclooxygenase and/or peroxidases, but not FMOs, are potential mechanisms of TCA nephrotoxicity. Free radicals also play a role in TCA nephrotoxicity, as evidenced by the inhibition of TCA cytotoxicity by antioxidants. However, whether the free radicals are intermediates in TCA biotransformation or reactive oxygen species produced during TCA metabolism remains to be determined.

3.6 ACKNOWLEDGEMENTS

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3.7 AUTHORS CONTRIBUTIONS

Gary O. Rankin designed the research; Christopher Racine, Dakota Ward, Dianne K. Anestis, Travis Ferguson and Debra Preston performed the experiments; Christopher Racine, Debra Preston and Dianne Anestis contributed to the analysis of the data; Gary O. Rankin and Christopher Racine wrote the paper.

3.8 CONFLICTS OF INTEREST

None of the authors have a conflict of interest with this work.

CHAPTER IV: METABOLISM OF 3,5-DICHLOROANILINE AND THE COMPARATIVE NEPHROTOXIC POTENTIAL OF PUTATIVE METABOLITES IN ISOLATED RENAL CORTICAL CELLS FROM MALE FISCHER 344 RATS

Christopher R. Racine[1], Connor Tyree[1], Dakota Ward[1], Dianne Anestis[1], Monica A. Valentovic[1] and Gary O. Rankin[1]

[1]Department of Pharmacology, Physiology, and Toxicology, Joan C. Edwards School of Medicine, Marshall University, Huntington, West Virginia 25755

ABSTRACT

Previous studies have suggested that 3,5-dichloroaniline (3,5-DCA; 1.0 mM; 90 min exposure), an intermediate used in the production of agricultural and industrial products, may undergo renal bioactivation resulting in toxic metabolite(s) in isolated renal cortical cells (IRCC) obtained from male Fischer 344 rats. Studies using renal cortical slices have also shown that 4-amino-2,6-dichlorophenol (4A26DCP), a putative metabolite of 3,5-DCA, is a potent nephrotoxicant, however, the metabolism of 3,5-DCA and the nephrotoxic potential of putative metabolites has not yet been explored in IRCC. The current study was designed to explore the nephrotoxic potential of five putative 3,5-DCA metabolites (3,5-dichloroacetanilide, 3,5-DCAA; 3,5-dichlorophenylhydroxylamine, 3,5-DCPHA; 4-amino-2,6-dichlorophenol, 4A26DCP; 2-amino-4,6-dichlorophenol, 2A46DCP; 3,5-dichloronitrobenzene, 3,5-DCNB) and to determine the metabolism of 3,5-DCA by IRCC. IRCC were exposed to various concentrations of 3,5-DCAA (0.5,1.0, or 1.5 mM), 3,5-DCNB (0.5, 1.0, or 1.5 mM), 3,5-DCPHA (0.25, 0.5, or 1.0 mM), 2A46DCP (0.5, 1.0, or 1.5 mM), or 4A26DCP (0.1, 0.25, or 0.5 mM). Of these metabolites, only 3,5-DCAA proved noncytotoxic at the concentrations tested. In contrast, the other four metabolites proved to be nephrotoxic at concentrations and/or exposure times equal to or lower than 3,5-DCA (1.0 mM; 90 min). To determine the metabolic profile of 3,5-DCA in IRCC, IRCC were exposed to 0.5 mM or 1.0 mM 3,5-DCA for 45 or 90 minutes. HPLC analysis demonstrated minimal metabolism occurs in IRCC following exposure to 3,5-DCA. In fact, only two minor metabolites (3,5-DCNB and 3,5-DCAA) and 3,5-DCA were detected. Since 3,5-DCNB arises from N-oxidation of 3,5-DCA, and previous studies showed that DEDTCA, a CYP2C selective inhibitor, can significantly attenuate 3,5-DCA induced toxicity, additional studies were conducted to determine the metabolic profile of 3,5-DCA in IRCC following pretreatment with

DEDTCA. Results showed a decrease in the production of 3,5-DCNB following DEDTCA pretreatment. This result suggests that N-oxidation may play a role in 3,5-DCA induced nephrotoxicity, while further studies are required to determine the ultimate toxicant(s) species, as well as the mechanism of cell death.

ABBREVIATIONS:

3,5-DCA, 3,5-dichloroaniline; 3,5-DCAA, 3,5-dichloroacetanilide;l 2A46DCP, 2-amino-4,6-dichlorophenol; 3,5-DCPHA, 3,5-dichlorophenylhydroxylamine; IRCC, isolated renal cortical cells; LDH, lactate dehydrogenase; DMSO, dimethyl sulfoxide; FMO, flavin-containing monooxygenase; CYP, cytochrome P450; DEDTCA, diethyldithiocarbamate; 3,5-DCNB, 3,5-dichloronitrobenzene, 4-amino-2,6-dichlorophenol; 4A26DCP

5.1 INTRODUCTION

Chlorinated aniline-induced hematotoxicity (Chhabra et al., 1990; Guilhermino et al., 1998; Pauluhn, 2004; Valentovic et al., 1997), splenotoxicity (Chhabra et al., 1990; Khan et al., 1999; Ma et al., 2008, 2013), hepatotoxicity (Valentovic et al., 1995a, 1995b, 1992), and nephrotoxicity (Hong et al., 2000; Lo et al., 1990; Racine et al., 2014; Valentovic et al., 1995a) has been well established. These studies have shown that of the chlorinated anilines, 3,5-dichloroaniline (3,5-DCA) is the most potent as a nephrotoxicant. In vivo exposure to 3,5-dichloroaniline, in rats, results in oliguria, elevated blood urea nitrogen (BUN) concentration, decreased organic ion transport in proximal tubule cells, decreased kidney weight, increased proteinuria, and hematuria (Rankin et al., 1986; Lo et al., 1990; Valentovic et al., 1995a). The greatest morphological changes occur in the proximal tubular cells, but the distal tubular and collecting ducts were also affected to a lesser degree (Lo et al., 1990). Further studies using isolated renal cortical cells (IRCC) from male Fischer 344 rats demonstrated that 1.0 mM 3,5-DCA exposure for 90 min leads to increased cytotoxicity as evidenced by increased lactate dehydrogenase (LDH) release, a marker of cytotoxicity. These studies also demonstrated that 3,5-DCA nephrotoxicity can be attenuated by inhibiting multiple enzyme systems, suggesting that biotransformation, especially N-oxidation, plays a role in 3,5-DCA-induced nephrotoxicity in vitro (Racine et al., 2016). However, the ultimate toxic metabolite(s) is not currently known.

Based on studies with aniline and its mono-chlorinated derivatives, N-oxidation, N-acetylation, and phenyl ring oxidation are all potential biotransformation pathways of 3,5-DCA (Figure 20; Ehlhardt and Howbert, 1991; Hong and Rankin, 1998; Racine et al., 2014; 2016). In the case of 3,5-DCA, it has been demonstrated that one putative metabolite, resulting from phenyl ring oxidation of 3,5-DCA at the para-position, 4-amino-2,6-dichlorophenol (4A26DCP)

is a potent nephrotoxicant in Fischer 344 rats both in vivo and in vitro using a renal slice model (Hong et al., 1996; Rankin et al., 2008a). However, there are few studies that have explored the metabolism of 3,5-DCA in mammals, and none of these were conducted in the isolated renal cortical cell (IRCC) model. Marbouh et al. (2002) explored the urinary metabolites of 3,4- and 3,5-DCA following both percutaneous and oral administration. In this study, male Sprague-Dawley rats were exposed to 12 mg of 3,5-DCA in methanol applied topically or via oral gavage. Urine was collected for 24 hours post exposure. HPLC analysis of the urine found 0.04% of the dose was 3,5-dichloroacetanilide (3,5-DCAA), less than 0.01% was 2-amino-4,6-dichlorophenol (2A46DCP), and 0.57% of the dose was recovered as 3,5-DCA. Following acid hydrolysis to determine conjugated metabolites, 4.79% of the dose was 3,5-DCA and 0.02% of the dose was 2A46DCP, suggesting that conjugated metabolites of 3,5-DCA and 2A46DCP were formed after exposure to 3,5-DCA. 3,5-DCAA was not detected following acid hydrolysis. Deacetylation following acid hydrolysis has been shown in previous studies (Kao et al., 1978).

The current study was designed to determine the metabolism of 3,5-DCA in IRCC from male Fischer 344 rats as well as to explore the nephrotoxic potential of five putative 3,5-DCA metabolites. Based on previous studies with aniline and mono-chlorinated derivatives, it was hypothesized that only those putative metabolites arising from N-oxidation (3,5-dichlorophenylhydroxylamine, 3,5-DCPHA; 3,5-dichloronitrosobenzene, 3,5-DCNSB; 3,5-dichloronitrobezene, 3,5-DCNB) and/or phenyl ring oxidation (4-amino-2,6-dichlorophenol, 4A26DCP; 2-amino-4,6-dichlorophenol, 2A46DCP) would be cytotoxic while the acetylated product, 3,5-DCAA, would show a reduced nephrotoxic potential when compared to 3,5-DCA.

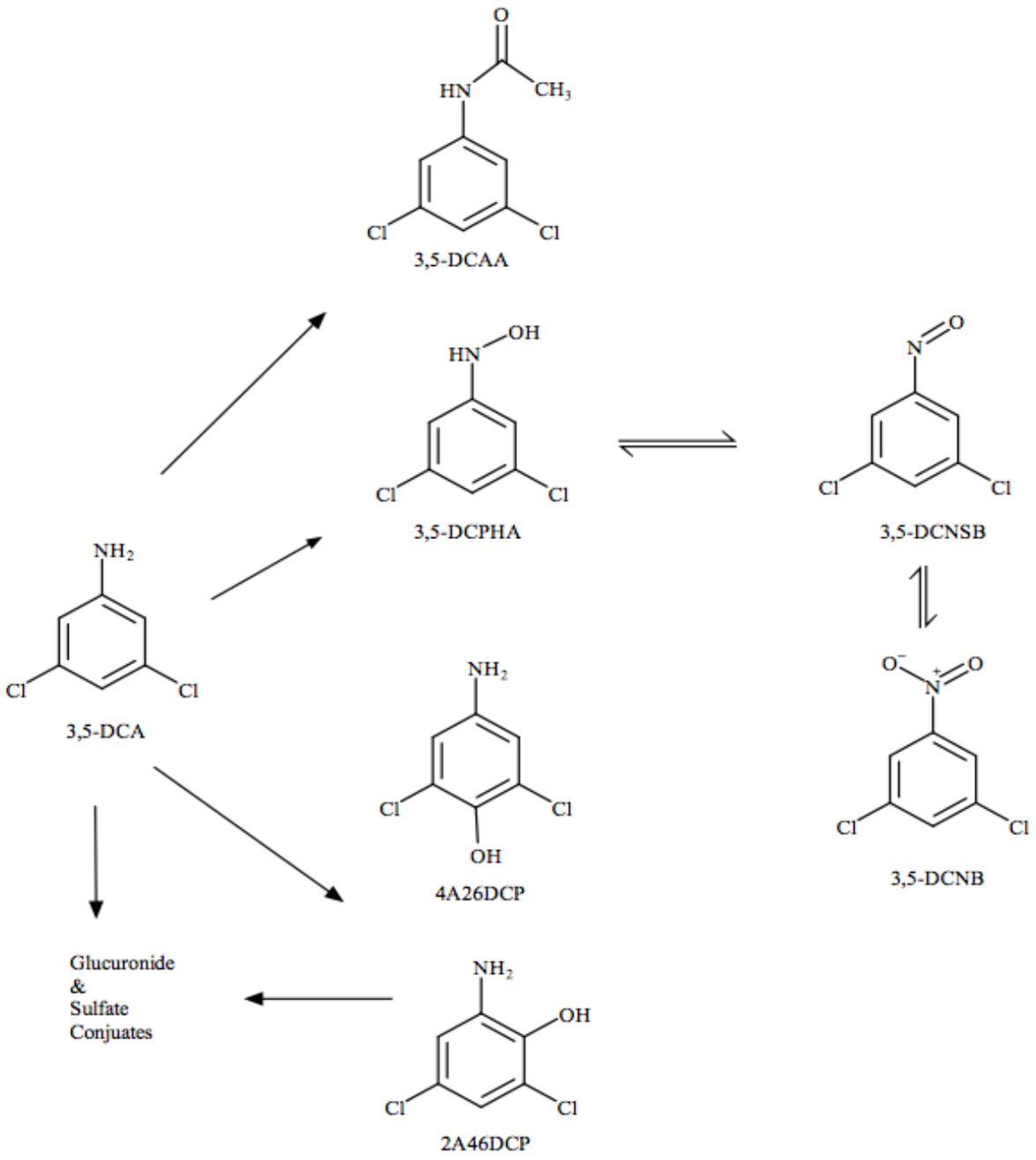

Figure 20. Proposed renal biotransformation pathway of 3,5-DCA. 3,5-DCA ,3,5-dichloroaniline, 3,5-DCAA, 3,5-dichloracetanilide; 3,5-DCPHA, 3,5-dichlorophenylhydroxylamine; 3,5-DCNSB, 3,5-dichloronitrosobenzene; 3,5-DCNB, 3,5-dichloronitrobenzene; 4A26DCP, 4-amino-2,6-dichlorophenol; 2A46DCP, 2-amino-4,6-dichlorophenol.

5.2 MATERIALS AND METHODS

5.2.1 Animals

Male Fischer 344 rats (200-250g) were obtained from Hilltop Lab Animals, Inc. (Scottsdale, PA). Animals were housed 2 rats/cage with food and water available *ad libitum*, while temperature (21-23 C), humidity (40-55%), and light (12 h on/12 h off) are controlled. Prior to use, all animals were allowed one week to acclimate. All animals use was approved by the Marshall University Institutional Animal Care and Use Committee, and animal use experiments were conducted in accordance with the Guide for the Care and Use of Laboratory Animals, adopted by the National Institute of Health.

5.2.2 Chemicals

All chemicals were purchased at the highest purity available from either Fischer Scientific (Pittsburgh, PA) or Sigma Aldrich (St. Louis, MO), except for 3,5-DCPHA, 2A46DCP, 3,5-DCAA, which were synthesized in our laboratory. 3,5-DCPHA was synthesized using previously described methods (Rondestvedt and Johnson, 1977). Briefly, 3,5-DCNB was reduced with hydrazine-palladium on carbon, prior to recrystallization using benzene-petroleum ether. 2A46DCP was synthesized and purified using a modified method previously described by Christiansen (1923) and Newell, Argus, and Ray. (1960). 3,5-DCAA was synthesized and purified using a modified method described by Searle and Cupery (1954). Purity was determined using melting point, thin-layer chromatography, infrared spectrophotometry, and nuclear magnetic resonance spectroscopy techniques.

5.2.3 Preparation and treatment of Isolated Renal Cortical Cells (IRCC)

Renal cortical cells were obtained from untreated male Fischer 344 rats anesthetized with pentobarbital (75 mg/kg, ip) via the Jones et al. (1979) collagenase perfusion method. Initial cell viability was determined by lactate dehydrogenase (LDH) release and trypan blue (2% w/v) exclusion. Prior to incubation, IRCC were counted and resuspended in Krebs-Henseleit (pH 7.37; 25 mM Hepes; 2% w/v bovine serum albumin) buffer at a concentration of ~4.2 million cells/ml. IRCC (3 ml) were pre-incubated in 25 ml polycarbonate Erlenymyer flasks for five min at 37°C under 95:5 oxygen/carbon dioxide. Following pre-incubation, cells were exposed to various concentrations of 3,5-dichloronitrobenzene (3,5-DCNB; 0.5, 1.0, or 1.5 mM), 3,5-dichloroacetanilide (3,5-DCAA; 0.5, 1.0, or 1.5 mM), 3,5-dichlorophenylhydroxylamine (3,5-DCPHA; 0.25, 0.5, or 1.0 mM), 2-amino-4,6-dichlorophenol (2A46DCP; 0.5, 1.0, or 1.5 mM), 4-amino-2,6-dichlorophenol (4A26DCP; 0.1, 0.25, or 0.5 mM) or vehicle (30 μL DMSO) for 60 or 90 min. At the conclusion of the allotted incubation period, samples (0.5 ml) were taken for LDH release assay, as previously described (Rankin et al., 2008a, Racine et al., 2014).

5.2.4 HPLC determination of 3,5-DCA metabolism

To determine the metabolism of 3,5-DCA, IRCC were exposed to 3,5-DCA (0.5 mM or 1.0 mM) for 90 min. Following incubation, a 1.0 mL aliquot was taken for HPLC analysis. HPLC samples were centrifuged, 0.9 mL of supernatant was collected and labeled as media. The cell pellet was then rinsed with 1.0 mL Krebs Heinselt (KH) buffer without BSA and 0.9 mL of the rinse was collected, and the pellet was saved for analysis. Methanol (MeOH; 0.9 mL) was added to the rinse and media fractions, while 1.0 mL of MeOH was added to the cell lysate. Following the addition of MeOH to precipitate protein, all samples were sonicated for 30 sec

prior to being centrifuged @3000xg for 10 min @4°C, supernatant was filtered through 0.45 micron syringe filters, and stored at -20°C until analysis.

Separate experiments were conducted to determine whether conjugated metabolites of 3,5-DCA are formed in IRCC. In these experiments, IRCC were incubated with either DMSO (30 µL) or 3,5-DCA (1.0 mM) for 45 or 90 minutes. Following the incubation, three aliquots (0.9 mL each) were taken for each treated group. The first was prepared as discussed above. The other two underwent the same processing, until the addition of MeOH. Instead of adding MeOH each fraction (media, cells, and rinse) was treated with either β-glucuronidase (6500 units/mL; final concentration) or arylsulfatase (200 units/mL: final concentration) containing saccharolactone (20mM: final concentration) in 0.1 M acetate buffer (pH 5.0) for 18 h at 37°C. Saccharolactone inhibited any β-glucuronidase activity which may be present in the arylsulfatase preparation (Kao et al., 1978). The β-glucuronidase and arylsulfatase incubations were terminated by the addition of cold MeOH (2.4 mL). Once the MeOH was added, all samples were sonicated for 30 seconds prior to being centrifuged @3000xg for 10 min @4°C, supernatant was filtered through 0.45 micron syringe filters, and stored in -20°C, until analyzed using HPLC. 3,5-DCA stability was determined by incubating 3,5-DCA in KH buffer (as previously described) without BSA only for 18 h at 37°C before a sample was taken as described above and compared to a non-incubated 3,5-DCA sample.

Finally, since previous studies suggested that the diethyldithiocarbamic acid (DEDTCA), a CYP2C selective inhibitor, was able to attenuate 3,5-DCA induced cytotoxicity to control levels (Racine et al., 2016), experiments were conducted to determine the effect of DEDTCA on 3,5-DCA metabolism. In this experiment cells were pretreated with DEDTCA (0.1 mM; 30 min) before being exposed to 3,5-DCA (1.0 mM; 90 min), as previously described (Racine et al.,

2016). Following incubation, samples were collected and prepared for HPLC analysis as described above.

5.2.5 HPLC Parameters

All HPLC samples (75 µL) were analyzed on a Waters Alliance e2695 HPLC system, utilizing a Waters 2489 variable UV/Vis detector at 254 nM. Chromatograms were collected and integrated using Empower 3 software from Waters. A Waters X Select HSS T3 C18 column (3.5µm; 4.6 x 150 mm) fitted with a Waters XSelect HSS T3 VanGuard Cartridge (3.5 µm; 3.9 x 5 mm) was used for separation of compounds. The mobile phase consisted of 50:50 MeOH:H_2O with a flow rate of 0.75 mL/min. Standard curves, limits of detection, and extraction coefficients were determined for 3,5-DCA and putative metabolites. Extraction coefficients were determined by comparing the integration of a 1.0 mM 3,5-DCA or metabolite sample that had undergone sample processing (i.e., the addition of MeOH and centrifugation) to that of a 1.0 mM 3,5-DCA or metabolite sample unprocessed.

5.2.6 Statistics

Data is presented as mean ± S.E.M. with an $N \geq 4$ separate isolation experiments and was analyzed by one-way analysis of variance followed by Student Newman-Keuls Test using GraphPad Prism 7.0. Significance was determined at $p < 0.05$.

5.3 RESULTS

5.3.1 Nephrotoxic potential of putative 3,5-DCA metabolites

To determine the nephrotoxic potential of the putative metabolites, concentration and time course studies were conducted for each of the metabolites listed above. Exposure to 3,5-dichloroacetanilide (3,5-DCAA) did not significantly increase LDH release (Figure 21A), a marker of cytotoxicity, at any time or concentration tested. In contrast, exposure to 2-amino-4,6-dichlorophenol (2A46DCP) resulted in significant increases in LDH release at all concentrations after 90 min, but no significant increases at 60 min. (Figure 21B). 3,5-Dichloronitrobenzene (3,5-DCNB; Figure 22) and 3,5-dichlorophenylhydroxylamine (3,5-DCPHA; Figure 23), 4-amino-2,6-dichlorophenol (4A26DCP; Figure 24) exposure resulted in a time and concentration dependent increase in LDH release. Significant LDH release was seen at all concentrations (0.5, 1.0, and 1.5 mM) after 90 minutes for 3,5-DCNB and at 1.0 mM or greater after 60 min. 3,5-DCPHA induced a significant increase in LDH release at 0.5 mM or greater after 60 min and at all concentrations (0.25, 0.5, and 1.0 mM) examined at 90 min. 4A26DCP significantly increased LDH release at a concentration of 0.5 mM after 60 min and at concentrations greater than or equal to 0.25 mM after 90 min.

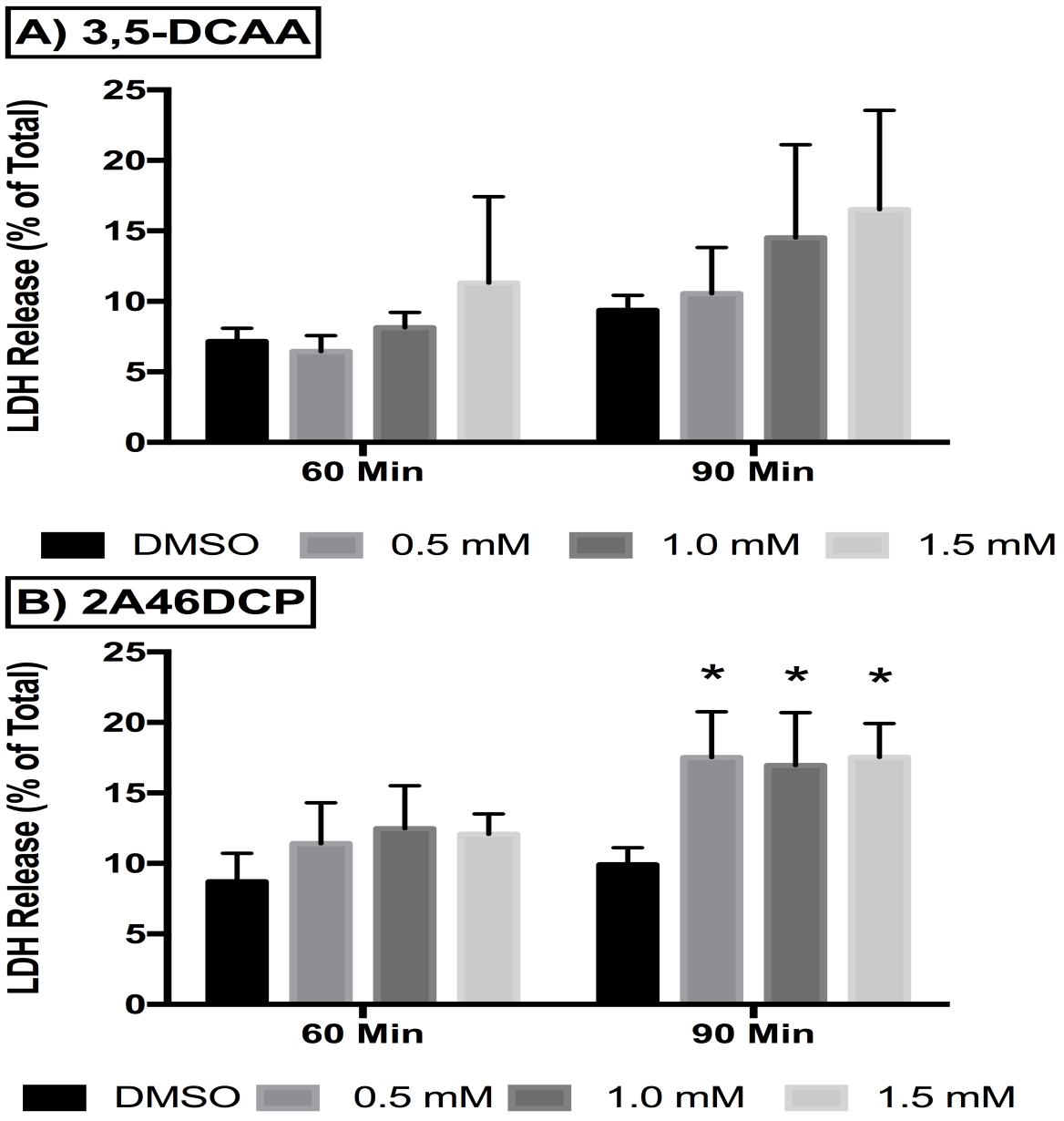

Figure 21. 3,5-Dichloroacetanilide and 2-amino-4,6-dichlorophenol induced cytoxicity in IRCC from male Fischer 344 rats. **A)** 3,5-DCAA induced LDH release in isolated renal cortical cells obtained from male Fischer 344 rats following exposure for 60 or 90 min. **B)** 2A46DCP induced cytotoxicity in isolated renal cortical cells obtained from male Fischer 344 rats following exposure for 90 min. Each bar represents the mean ± S.E.M. for N=4-5 separate isolation experiments. *Significantly different from DMSO control, $P<0.05$.

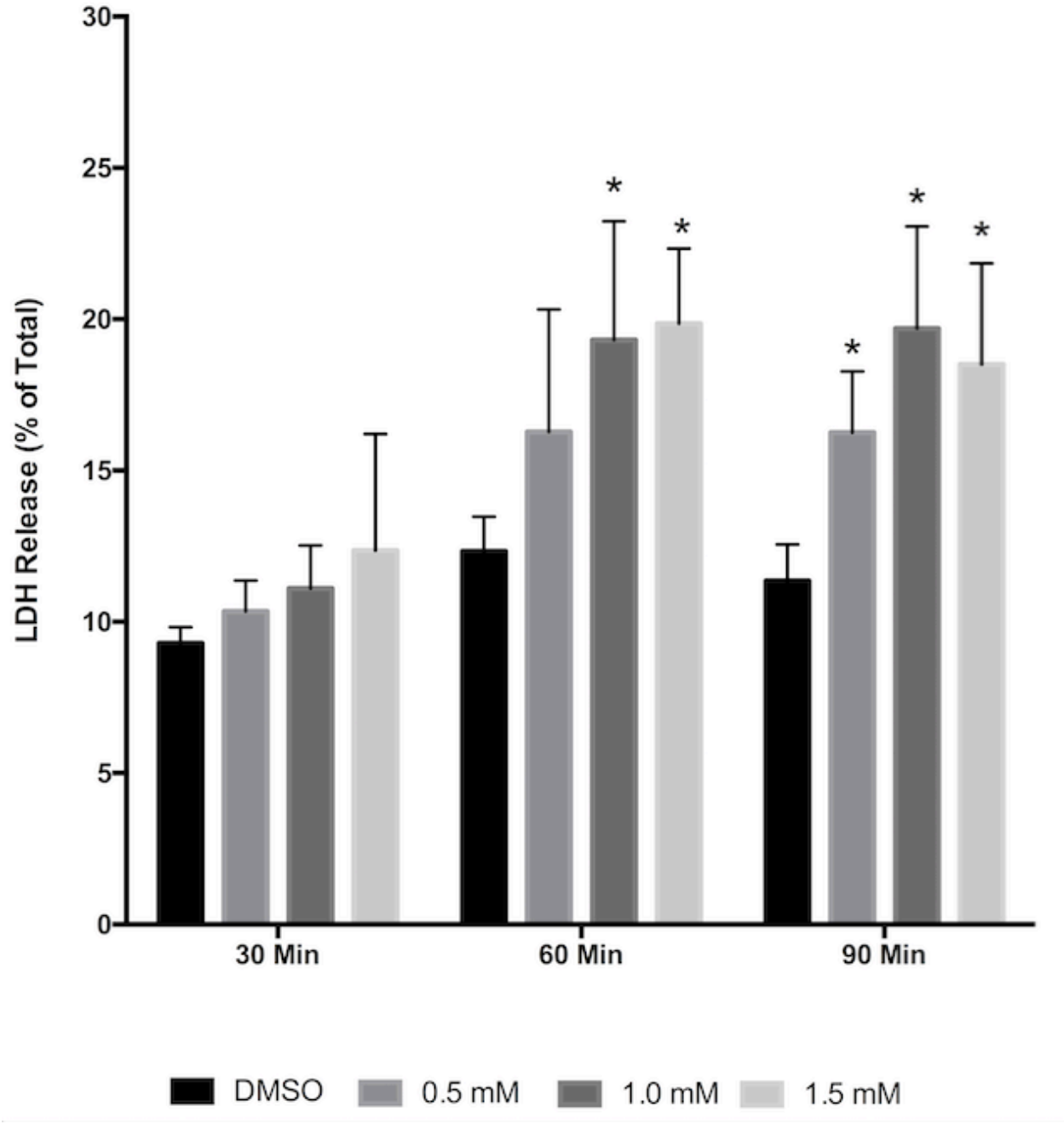

Figure 22. 3,5-Dichloronitrobenzene induced cytotoxicity in IRCC from male Fischer 344 rats. Cells were exposed for 30, 60, or 90 min. Each bar represents the mean ± S.E.M. for N=4-5 separate isolation experiments. *Significantly different from DMSO control, $P<0.05$.

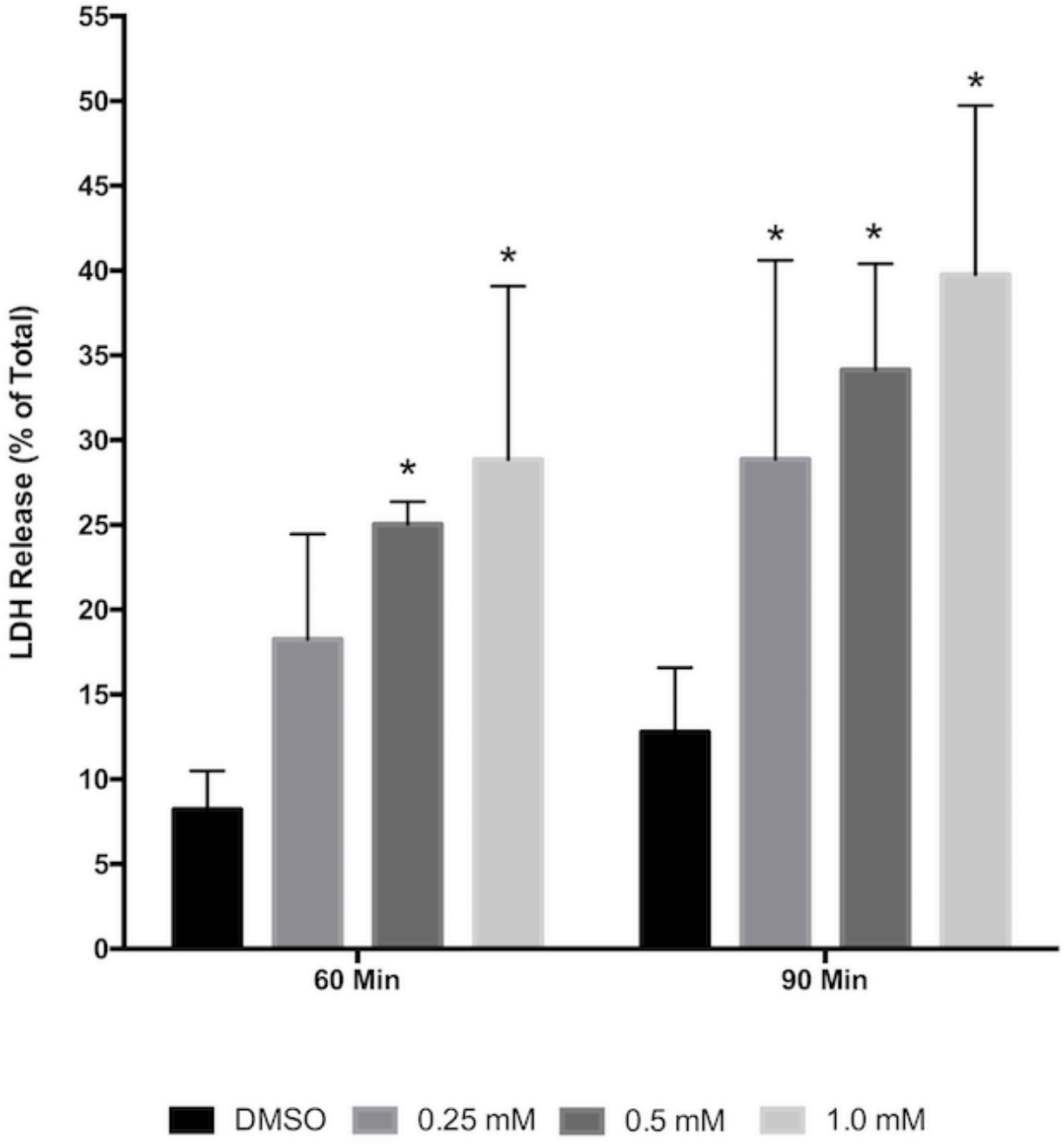

Figure 23. 3,5-Dichlorophenylhydroxylamine induced cytotoxicity in IRCC from male Fischer 344 rats. Cells were exposed for 60 or 90 min. Each bar represents the mean ± S.E.M. for N=4-5 separate isolation experiments. *Significantly different from DMSO control, $P<0.05$.

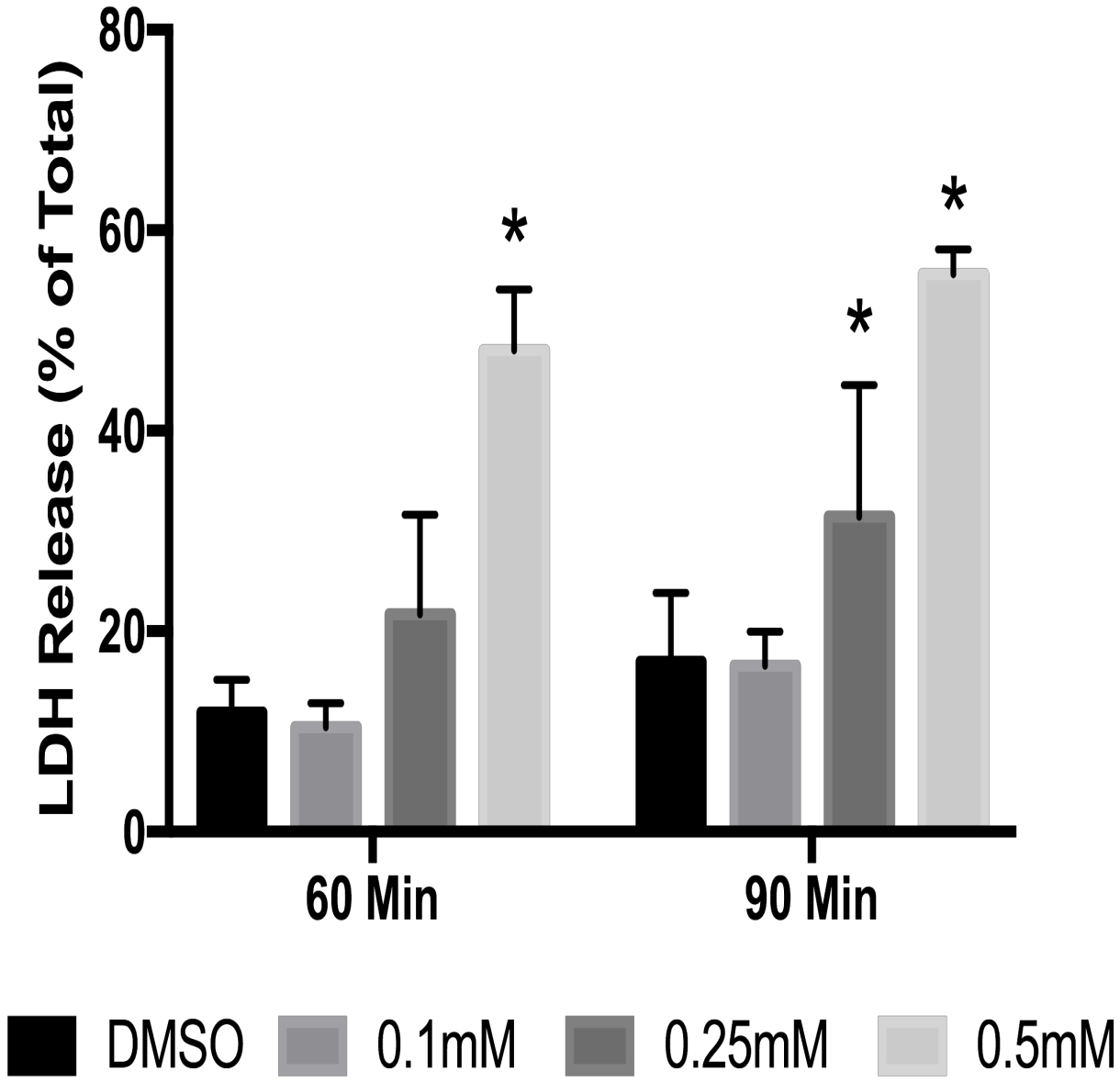

Figure 24. 4-Amino-2,6-dichlorophenol induced cytotoxicity in IRCC from male Fischer 344 rats. Cells were exposed for 60 or 90 min. Each bar represents the mean ± S.E.M. for N=4-5 separate isolation experiments. *Significantly different from DMSO control, P<0.05.

5.3.2 Identification of 3,5-DCA metabolites in IRCC

Retention times, limits of detection, and extraction coefficients were determined for all putative metabolites and 3,5-DCA (Figure 25; Table 6), except for 3,5-DCPHA. 3,5-DCPHA is very unstable and was undetectable after following the processing utilized for all HPLC experiments.

5.3.3 Metabolism of 3,5-DCA in IRCC

Three separate sets of experiments were conducted in order to determine the metabolism of 3,5-DCA. This first set of experiments determined the metabolic profile of two different concentrations of 3,5-DCA (0.5 mM or 1.0 mM) after a 90 minute exposure. Results of these experiments show that 93.973 ± 5.516% (0.5 mM) and 88.830 ± 5.136% (1.0 mM) of the 3,5-DCA dose was found in the media, while 7.300 ± 3.319% (0.5 mM) and 13.530 ± 5.136% (1.0 mM) was found in the cells. As shown in table 7, there were only two minor metabolites detected in both the media and cell fractions. 3,5-Dichloroacetanilide (3,5-DCAA; 1.863 ± 0.417%) was detected in the media of all three samples, but was only detected in one of the tissue samples. 3,5-Dichloronitrobenzene (3,5-DCNB; 2.803 ± 2.803%) was detected in the media and cells, but only in one of three samples. The remaining 3,5-DCA was found as un-metabolized 3,5-DCA. After exposure to 1.0 mM 3,5-DCA for 90 minutes, 3,5-DCNB was detected in both the media (1.193 ± 0.566%) and cells (0.340 ± 0.192%) in three samples. 3,5-DCAA was detected in the media of all four samples, but was undetected in the cells. Once again the remaining 3,5-DCA was found as the unchanged parent compound. A representative chromatography of 3,5-DCA metabolism can be seen in figure 26.

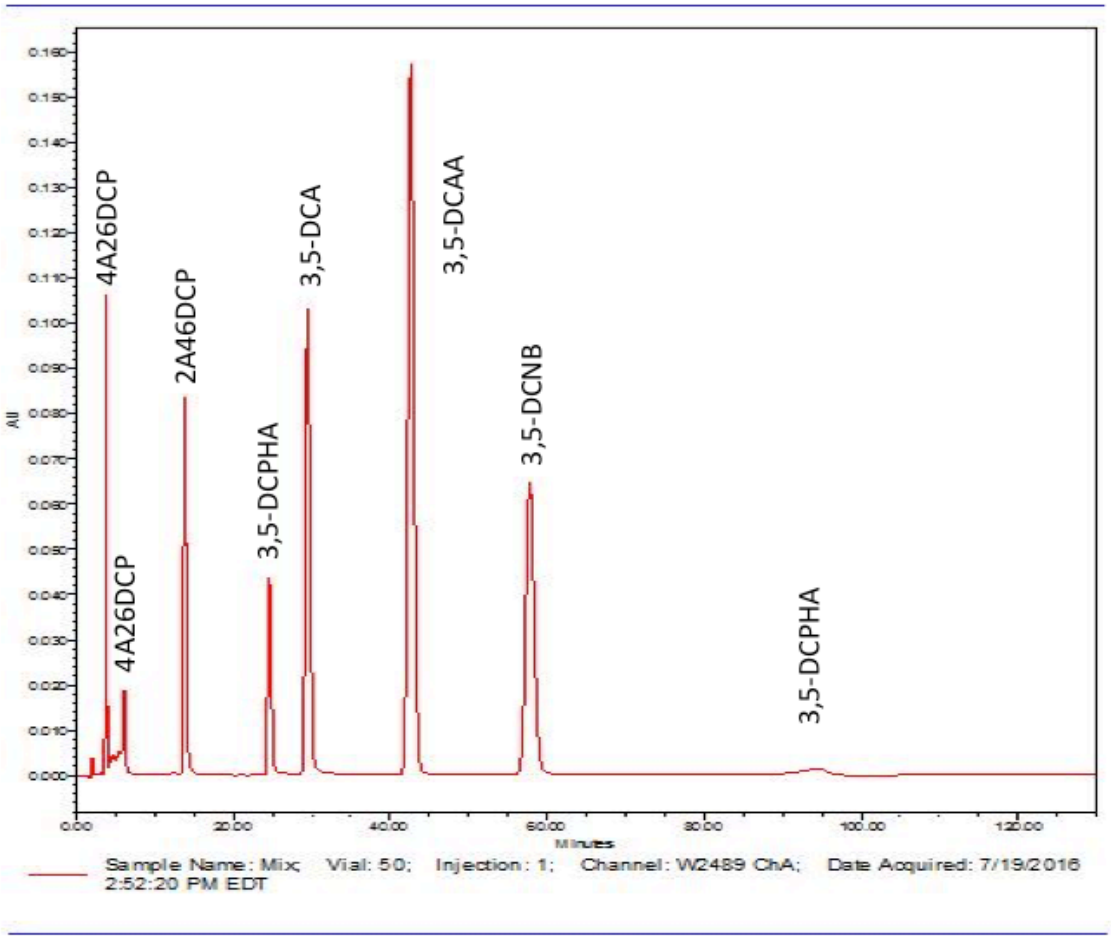

Figure 25. Chromatogram of 3,5-DCA and putative metabolites. Displayed is a representative chromatogram of a mixture of authentic 3,5-DCA and putative metabolite standards.

Table 6. Retention times, limits of detection, and extraction efficiency for authenticated standards of 3,5-dichloroaniline and its putative metabolites.

Compound	Retention Time (min)	Limit of Detection (ug)	Extraction Efficiency (%)
4-amino-2,6-dichlorophenol	3.8 6.1	0.0178 0.0178	75.8 100
2-amino-4,6-dichlorophenol	13.8	0.0178	97.8
3,5-dichlorophenylhydroxylamine	24.6	17.8	0.0
3,5-dichloroaniline	29.4	0.00198	89.5
3,5-dichloroacetanilide	42.6	0.0024	96.1
3,5-dichloronitrobenzene	57.7	0.00192	79.7

Table 7. Metabolism of 3,5-dichloroaniline (3,5-DCA; 0.5 mM or 1.0 mM; 90 min) in IRCC.

	Percentage of dose in media (%)	
Compound	0.5 mM 3,5-DCA (N=3)	1.0 mM 3,5-DCA (N=4)
4-amino-2,6-dichlorophenol	ND	ND
2-amino-4,6-dichlorophenol	ND	ND
3,5-dichlorophenylhydroxylamine	ND	ND
3,5-dichloroaniline	89.307 ± 6.322	87.133 ± 4.562
3,5-dichloroacetanilide	1.863 ± 0.417	0.505 ± 0.072
3,5-dichloronitrobenzene	2.803^	1.193 ± 0.566*
Total in media	93.973 ± 5.516	88.830 ± 5.136
	Percentage of dose in cells (%)	
Compound	0.5 mM 3,5-DCA	1.0 mM 3,5-DCA
4-amino-2,6-dichlorophenol	ND	ND
2-amino-4,6-dichlorophenol	ND	ND
3,5-dichlorophenylhydroxylamine	ND	ND
3,5-dichloroaniline	6.823 ± 3.332	13.190 ± 2.122
3,5-dichloroacetanilide	0.067^	ND
3,5-dichloronitrobenzene	0.410^	0.340 ± 0.192*
Total in tissue	7.300 ± 3.319	13.530 ± 5.136
Combined total	101.273 ± 8.834	102.360 ± 6.473

Data is presented as mean ± S.E.M., N as listed in table; ^found in one sample,*found in three samples. ND means not detected.

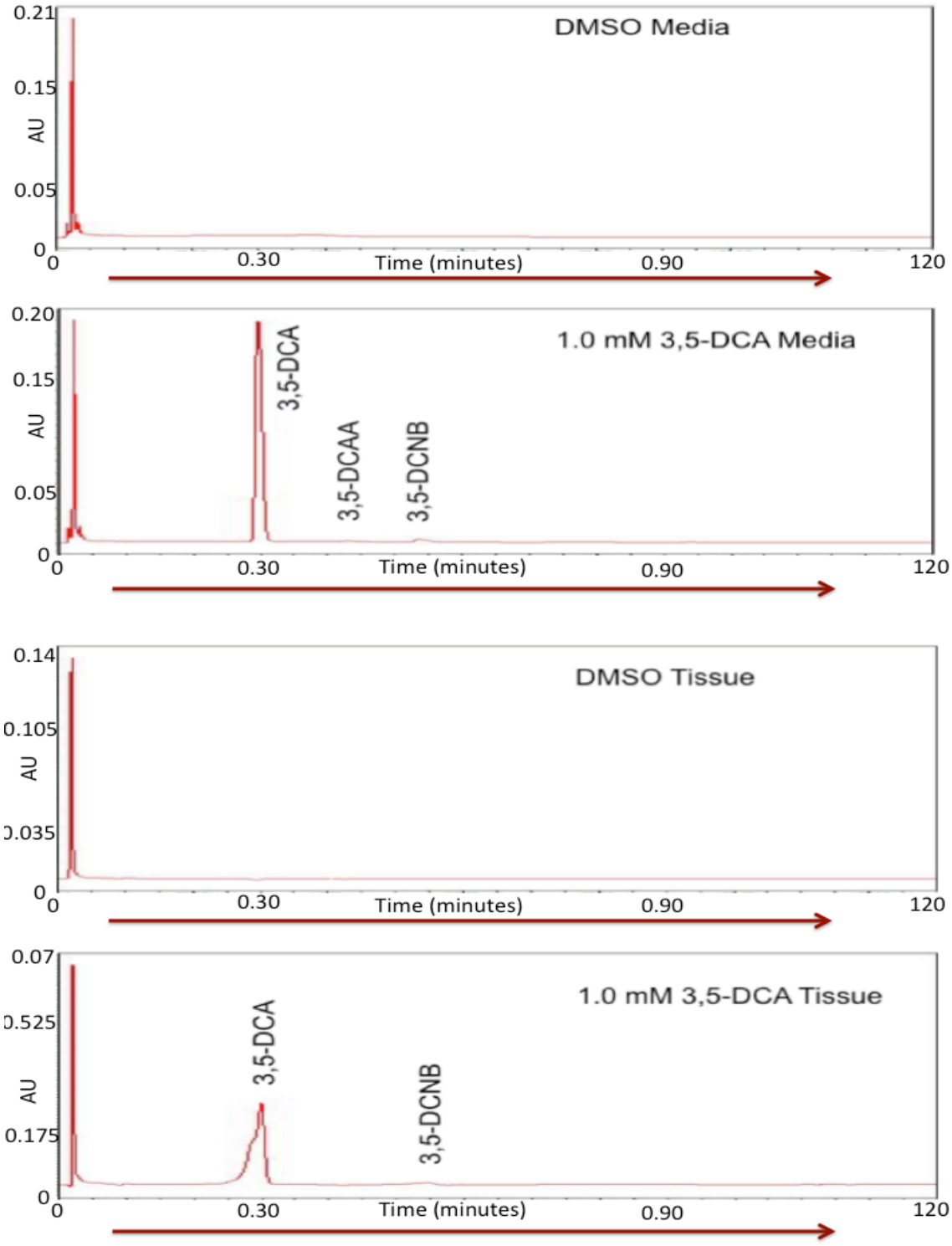

Figure 26. HPLC chromatograms of 3,5-DCA metabolism in IRCC. IRCC were exposed to either DMSO or 3,5-DCA for 90 minutes. Treatment is listed in each panel.

Further experiments were conducted to determine the presence of glucuronide and/or sulfated conjugates following exposure to 1.0 mM 3,5-DCA at two time points [Table 8 (45 min exposure) or Table 9 (90 min exposure)]. No significant changes in the metabolic profile were seen after treatment with either β-glucuronidase or arylsulfatase. A change was seen in the total recovery following the 18 hr incubation required for the glucuronidase and arylsulfatase experiments. Because of this change further studies into the stability of 3,5-DCA after incubation for 18 h at 37°C, showed that ~20% of 3,5-DCA is lost during the 18 hr incubation. The stability of putative metabolites was not determined.

Since previous studies demonstrated that pretreatment with DEDTCA was able to significantly attenuate 3,5-DCA-induced cytotoxicity in vitro, a final set of experiments was conducted to explore the effect of DEDTCA pretreatment on 3,5-DCA metabolism. Results of this study can be found in table 10. As seen with the other experiments conducted in the current study, the majority of 3,5-DCA was found to be unchanged in both the DEDTCA + 3,5-DCA treated and 3,5-DCA only treated groups. 3,5-DCNB was detected in two of the four samples and accounted for 0.305 ± 0.185% of the 3,5-DCA dose in media in the 3,5-DCA only group. In contrast, 3,5-DCNB was only detected in one of the four samples, and it was only 0.088% of the 3,5-DCA dose (in media), suggesting that the N-oxidation pathway was being inhibited by DEDTCA pretreatment.

Table 8. Metabolism of 3,5-DCA (1.0 mM; 45 min) in IRCC.

	Percentage of dose in media (%)		
Compound	Untreated	Arylsulfatase treated	β-glucuronidase treated
4-amino-2,6-dichlorophenol	ND	ND	ND
2-amino-4,6-dichlorophenol	ND	ND	ND
3,5-dichlorophenylhydroxylamine	ND	ND	ND
3,5-dichloroaniline	74.515 ± 3.015	51.050 ± 2.330*	58.925 ± 2.715*
3,5-dichloroacetanilide	0.770 ± 0.130	1.290 ± 0.050	ND
3,5-dichloronitrobenzene	ND	ND	ND
Total in media	75.285 ± 3.145	52.340 ± 2.280	58.925 ± 2.715
	Percentage of dose in cells (%)		
Compound	Untreated	Arylsulfatase treated	β-glucuronidase treated
4-amino-2,6-dichlorophenol	ND	ND	ND
2-amino-4,6-dichlorophenol	ND	ND	ND
3,5-dichlorophenylhydroxylamine	ND	ND	ND
3,5-dichloroaniline	12.875 ± 2.805	5.315 ± 2.545*	9.400 ± 2.580*
3,5-dichloroacetanilide	ND	ND	ND
3,5-dichloronitrobenzene	ND	ND	ND
Total in tissue	12.875 ± 2.805	5.315 ± 2.545	9.400 ± 2.580
Combined total	88.170 ± 5.940	68.325 + 5.295	57.095 ± 4.385

Data is presented as mean ± S.E.M. N=2. *Uncorrected for ~20% 3,5-DCA loss during 18 hr incubation. ND means not detected

Table 9. Metabolism of 3,5-DCA (1.0 mM; 90 min) in IRCC.

	Percentage of dose in media (%)		
Compound	Untreated	Arylsulfatase treated	β-glucuronidase treated
4-amino-2,6-dichlorophenol	ND	ND	ND
2-amino-4,6-dichlorophenol	ND	ND	ND
3,5-dichlorophenylhydroxylamine	ND	ND	ND
3,5-dichloroaniline	75.425 ± 0.895	55.205 ± 3.405*	58.525 ± 4.174*
3,5-dichloroacetanilide	0.7095 ± 0.155	0.895 ± 0.005	ND
3,5-dichloronitrobenzene	ND	ND	ND
Total in media	76.220 ± 1.050	56.100 ± 3.410	58.525 ± 4.174
	Percentage of dose in cells (%)		
Compound	Untreated	Arylsulfatase treated	β-glucuronidase treated
4-amino-2,6-dichlorophenol	ND	ND	ND
2-amino-4,6-dichlorophenol	ND	ND	ND
3,5-dichlorophenylhydroxylamine	ND	ND	ND
3,5-dichloroaniline	11.610 ± 2.820	6.055 ± 2.315*	9.380 ± 1.700*
3,5-dichloroacetanilide	ND	ND	ND
3,5-dichloronitrobenzene	ND	ND	ND
Total in tissue	11.610 ± 2.820	6.055 ± 2.315	9.380 ± 1.700
Combined total	87.835 ± 3.665	62.155 ± 1.085	67.910 ± 2.470

Data is presented as mean ± S.E.M. N=2. *Uncorrected for ~20% 3,5-DCA loss during 18 hr incubation. ND means not detected

Table 10. Effect of DEDTCA (0.1 mM; 30 min) pretreatment on 3,5-DCA (1.0 mM; 90 min) metabolism in IRCC.

	Percentage of dose in media (%)	
Compound	Untreated	DEDTCA pre-treated
4-amino-2,6-dichlorophenol	ND	ND
2-amino-4,6-dichlorophenol	ND	ND
3,5-dichlorophenylhydroxylamine	ND	ND
3,5-dichloroaniline	89.428 ± 5.656	91.143 ± 3.041
3,5-dichloroacetanilide	0.498 ± 0.032	0.300 ± 0.052
3,5-dichloronitrobenzene	0.305 ± 0.185^{+}	0.088^
Total in media	90.230 ± 5.652	91.530 ± 3.056
	Percentage of dose in cells (%)	
Compound	Untreated	DEDTCA pre-treated
4-amino-2,6-dichlorophenol	ND	ND
2-amino-4,6-dichlorophenol	ND	ND
3,5-dichlorophenylhydroxylamine	ND	ND
3,5-dichloroaniline	16.010 ± 2.019	14.670 ± 1.782
3,5-dichloroacetanilide	0.018^	ND
3,5-dichloronitrobenzene	0.125^	0.060^
Total in tissue	16.135 ± 2.013	14.730 ± 1.819
Combined total	106.365 ± 6.762	106.260 ± 3.956

Data is presented as mean ± S.E.M. N=4, except ^found in one sample, $^{+}$found in two samples.

5.4 DISCUSSION

3,5-Dichloroaniline (3,5-DCA) has been shown to be the most potent nephrotoxicant among the dichlorinated anilines both in vivo and in vitro (Lo et al., 1990; Valentovic et al., 1995a). Further studies in vitro have suggested that 3,5-DCA induced cytotoxicity is at least in part, due to bioactivation of 3,5-DCA to a toxic metabolite(s) (Racine et al., 2016). Previous work with aniline and other chloroanilines have shown there to be three major routes of biotransformation, N-oxidation, phenyl ring oxidation, and N-acetylation (Ehlhardt and Howbert, 1991; Hong and Rankin, 1998). Glucuronide and sulfate conjugates have also been shown to be major metabolites in aniline and chloroaniline metabolism, in vivo (Hong and Rankin, 1998; Smith and Williams, 1949; Kao et al., 1978). Based on these studies, the proposed metabolism of 3,5-DCA can be seen in Figure 20.

Results of the current study show that the metabolite arising from N-acetylation, 3,5-dichloroacetaniline (3,5-DCAA), was not toxic at the concentrations and time points examined. This result is in agreement with earlier studies with 3,4-dichloroaniline (3,4-DCA) that show N-acetylation of 3,4-DCA results in a metabolite with reduced nephrotoxic potential as compared to the parent compound (Rankin et al., 1993, 1995). The results of the HPLC analysis suggests that 3,5-DCAA is a minor metabolite detectable following exposure to 3,5-DCA in IRCC and would not be expected to contribute significantly to 3,5-DCA cytotoxicity in this model.

In contrast, putative metabolites resulting from phenyl ring oxidation are both nephrotoxicants. 2-Amino-4,6-dichlorophenol (2A46DCP), which is formed via ortho-hydroxylation of 3,5-DCA, proved to be nephrotoxic in our IRCC model. However, 2A46DCP was not formed at detectable levels in the current study. The putative metabolite of 3,5-DCA which arises from oxidation of 3,5-DCA at the para-position, 4-amino-2,6-dichloropenol

(4A26DCP), had been shown to be a potent nephrotoxicant in previous studies using a renal cortical cell model (Rankin et al., 2008a). The current study showed that 4A26DCP is a potent nephrotoxicant in IRCC, as well. However, further studies with 3,4,5-trichloroaniline have shown that the addition of chlorine at the para-position, which would inhibit para-oxidation, leads to increased nephrotoxicity (Racine et al., 2014), suggesting that 4A26DCP, while a toxic compound, most likely does not play a significant role in 3,5-DCA induced nephrotoxicity. As seen with 2A46DCP, 4A26DCP was not detected by HPLC analysis. These results suggest that while phenyl-ring oxidation would ppotentially lead to potent nephrotoxicants, it is not a major metabolic pathway of 3,5-DCA in IRCC.

N-Oxidation has also been shown to be an important pathway of chloroaniline metabolism (Hong and Rankin, 1998; Ehlhardt and Howbert, 1991). A previous study has suggested that N-oxidation may play a significant role in 3,5-DCA induced nephrotoxicity in IRCC (Racine et al., 2016). There are three major metabolites that can arise via N-oxidation of 3,5-DCA, as shown in figure 20; 3,5-dichlorophenylhydroxylamine (3,5-DCPHA), 3,5-dichloronitrosobenzene (3,5-DCNSB), and 3,5-dichloronitrobenzene (3,5-DCNB). The nephrotoxic potential of two of these metabolites, 3,5-DCNB and 3,5-DCPHA, was determined in the current study. The third, 3,5-dichloronitrosobenzene, is highly unstable and for that reason no experiments were conducted using it. Both 3,5-DCNB (Figure 22) and 3,5-DCPHA (Figure 23) proved to be more potent nephrotoxicants than 3,5-DCA (Racine et al., 2016), suggesting that both may contribute to 3,5-DCA induced nephrotoxicity in IRCC if formed. HPLC analysis was unable to detect 3,5-DCPHA. However, 3,5-DCNB is a minor metabolite of 3,5-DCA in IRCC. The presence of 3,5-DCNB suggests that 3,5-DCA undergoes N-oxidation in IRCC. Additional studies showed that pretreatment of IRCC with DEDTCA, a selective CYP2C

inhibitor, led to a decrease in the production of 3,5-DCNB, indicating that the N-oxidation pathway for the 3,5-DCA was inhibited by DEDTCA. Earlier studies with DEDTCA pretreatment led to a significant attenuation of 3,5-DCA-induced nephrotoxicity in IRCC (Racine et al., 2016). Combining these observations supports a role for N-oxidation in 3,5-DCA-induced toxicity, in vitro.

5.5 CONCLUSION

This study was designed to explore the nephrotoxic potential of putative metabolites of 3,5-dichloroaniline (3,5-DCA), since previous studies with 3,5-DCA suggested that metabolites contributed to 3,5-DCA induced cytotoxicity, at least in part. Since toxicity with 3,5-DCA required a concentration of 1.0 mM for 90 minutes, the results of the current study suggest that 3,5-DCNB, 3,5-DCPHA, 2A46DCP, and 4A26DCP are nephrotoxicants in vitro and may contribute to 3,5-DCA induced nephrotoxicity. However previous work has suggested that 4A26DCP most likely does not contribute (Racine et al., 2014). Furthermore, HPLC analysis showed that there is minimal detectable metabolism of 3,5-DCA in IRCC. In fact, only two minor metabolites were detected, 3,5-DCAA and 3,5-DCNB. 4A26DCP, 2A46DCP, and 3,5-DCPHA were not formed in detectable levels in this study. Also absent in this study was the detection of glucuronidated or sulfonated conjugates. These data, along with previous studies that showed metabolites contribute to 3,5-DCA-induced nephrotoxicity, suggest that the ultimate toxic metabolite(s) is/are extremely potent and is/are most likely formed close to its molecular target. Future studies are required to determine the molecular target(s) and mechanism of cellular death for 3,5-DCA.

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5.7 CONFLICT OF INTEREST

The authors have no conflict of interest to declare.

CHAPTER V: CONCLUSIONS AND FUTURE DIRECTIONS

Chloroanilines are important intermediates in the production of a variety of agricultural and industrial compounds (Kahl et al., 2011). Exposure to chloroanilines has been associated with hematotoxicity (Nomura, 1975; Pizon et al., 2009; Valentovic et al., 1997), splenotoxicity (Gralla et al., 1979; Bus, 1983; Kahn et al., 1993), and nephrotoxicity (Rankin et al., 1986; Lo et al., 1990). 3,5-DCA was shown to be the most potent nephrotoxicant of the mono- and dichlorinated anilines both in vivo and in vitro, using renal cortical cells (Rankin et al., 1986; Lo et al., 1990; Valentovic et al., 1995a). In vivo, 3,5-DCA induced nephrotoxicity is characterized by proximal tubular cell necrosis, increased BUN concentration, kidney weight, proteinuria, and hematuria (Rankin et al., 1986; Lo et al., 1990). In vitro studies have shown that LDH release is significantly increased and organic ion accumulation is significantly decreased following exposure to 3,5-DCA in renal cortical slices from rats (Valentovic et al., 1995a; Lo et al., 1990). Studies have also shown one putative metabolite of 3,5-DCA, 4A26DCP, possesses increased nephrotoxic potential both in vivo and in vitro, when compared to the parent compound 3,5-DCA (Rankin et al., 1994). 4A26DCP is formed via phenyl ring hydroxylation, which has been shown to be a major metabolic pathway in aniline metabolism (Kao et al., 1978; Parke, 1960). However, the role of metabolism in 3,5-DCA-induced nephrotoxicity had yet to be explored in any great detail. The work presented in this thesis was designed to fill this void in knowledge.

Initial studies were designed to explore the nephrotoxic potential of 3,5-DCA in isolated renal cortical cells (IRCC). IRCC was chosen as the model for multiple reasons. Firstly, IRCC are enriched for proximal tubular cells, the target cell type in 3,5-DCA-induced nephrotoxicity in vivo (Lash, 1998). Secondly, IRCC have been shown to maintain their metabolic enzyme activity (Cummings et al., 1999). As summarized in Chapter 2, 3,5-DCA was capable of significantly

increasing lactate dehydrogenase (LDH) release, a marker of cytotoxicity, in a concentration and time dependent manner. Cytotoxicity was evident at concentrations as low as 0.5 mM after 120 minutes exposure, and as early as 90 minute at 1.0 mM. Since previous studies had suggested that putative metabolites of 3,5-DCA possessed increased nephrotoxicity, further studies were conducted to determine the role of renal metabolism in 3,5-DCA induced cytotoxicity. For these studies, IRCC were pretreated with inhibitors of biotransforming enzymes prior to exposure to 1.0 mM 3,5-DCA for 90 minutes. Inhibition of the CYP2C, FMO, and peroxidase activity was able to significantly attenuate toxicity, suggesting that renal metabolism contributes to cytotoxicity, especially metabolic pathways which facilitate N-oxidation.

Since renal metabolism of 3,5-DCA was indicated to play a role in cytoxicity in IRCC, further studies were conducted to determine the ultimate toxicant(s) following exposure to 3,5-DCA. The first set of experiments can be found in chapter 4. In these experiments the role of para-hydroxylation of 3,5-DCA was determined. The nephrotoxic potential of TCA was determined in IRCC from male Fischer 344 rats. TCA was used because the addition of a chlorine on the para-position effectively inhibits para-hydroxylation. In these studies TCA was shown to significantly increase LDH release at concentrations as low as 0.1 mM after 120 minutes and 0.5 mM after 90 minutes. When compared with 3,5-DCA cytotoxicity, TCA was shown to be a more potent nephrotoxicant. These data indicate that blocking the para-position to hydroxylation produces a compound with greater nephrotoxic potential than 3,5-DCA. Thus, production of 4A26DCP would not be the major bioactivation pathway for 3,5-DCA in IRCC, and that while 4A26DCP is a potent nephrotoxicant in renal cortical slices, it is unlikely that it plays a significant role in 3,5-DCA-induced in vitro nephrotoxicity. However, more studies were

required to determine the nephrotoxic potential of additional putative metabolites of 3,5-DCA and to determine 3,5-DCA metabolism in IRCC.

The nephrotoxic potential of five putative metabolites of 3,5-DCA in IRCC can be found in chapter 4. The metabolites tested were 3,5-DCAA, 3,5-DCPHA, 3,5-DCNB, 4A26DCP, and 2A46DCP. These metabolites arise from N-acetylation, N-oxidation, and phenyl ring oxidation, all known pathways of aniline and chloroaniline metabolism (Kao et al., 1978; Parke, 1960; Ehlhardt and Howbert, 1991; Harrison and Jollow, 1986; 1987). Results of this study demonstrated that 3,5-DCPHA, 3,5-DCNB, 4A26DCP, and 2A46DCP were all nephrotoxicants at either concentrations or exposure times less than that of the parent compound 3,5-DCA, as measured by LDH release. These data suggest that any or all four of these metabolites could contribute to 3,5-DCA cytotoxicity; however, as previously discussed it is highly unlikely that 4A26DCP would contribute. In contrast, 3,5-DCAA was not cytotoxic at any of the concentrations or time points tested. This result matches previous studies with 3,4-dichloroacetanilide that showed the acetylated metabolite possessed a much lower nephrotoxic potential than the parent compound 3,4-dichloroaniline (Rankin et al., 1993; 1995).

While it is widely accepted that biotransformation enzymes are found at higher concentrations in the liver, the kidney has been shown to possess many of the same enzymes (Cashman, 1995; Cummings et al., 1999; Kimura et al., 1989a, 1989b; Dolphin et al., 1991). One study suggested that the renal biotransformation enzyme activity is between 15-40% of hepatic biotransformation enzyme activity (Litterst, Mimnaugh, Reagan, and Gram, 1975). For the current study, it was hypothesized that renal metabolism of 3,5-DCA played a role in 3,5-DCA induced nephrotoxicity, despite the lower levels of biotransformation enzymes found in renal tissue, as compared to the liver, since any resulting toxic metabolite(s) is produced in the target

tissue. The results of the current study showed minimal renal metabolism of 3,5-DCA in IRCC. In fact, only two metabolites were found, 3,5-DCAA and 3,5-DCNB. Furthermore, the formation of 3,5-DCNB and the ability of DEDTCA to not only limit 3,5-DCNB's formation but also to attenuate 3,5-DCA cytotoxicity strongly support N-oxidation as a contributing pathway in 3,5-DCA induced nephrotoxicity in IRCC.

Preliminary studies into the mechanism of cell death in 3,5-DCA induced cytotoxicity have also been conducted. It was initially hypothesized that free radical induced oxidative stress may be the mechanism of cell death in 3,5-DCA induced nephrotoxicity, since earlier studies demonstrated multiple antioxidants (glutathione, ascorbate, N-acetyl-L-cysteine, pyruvate, and α-tocopherol) were able to significantly attenuate cytotoxicity in IRCC (Racine et al., 2016). However, additional experiments showed that oxidative stress was only present following the exposure to toxic levels of 3,5-DCA, as previously discussed in Chapter 2, suggesting that oxidative stress was a consequence of toxicity rather than the mechanism of cell death. Since minimal metabolism and oxidative stress were detected in IRCC following exposure to 3,5-DCA, yet inhibitors of biotransforming enzymes and antioxidants significantly attenuate cytotoxicity, it is further hypothesized that the ultimate toxicant(s) species of 3,5-DCA is most likely a highly reactive radical and/or alkylating intermediate arising via N-oxidation, formed close to its molecular target(s).

One potential molecular target for 3,5-DCA is the mitochondria. Previous studies with 4-chloroaniline (4-CA) and 2-chloroaniline (2-CA) found the presence of both 4-CA and 2-CA in mitochondrial fractions of renal cortical slices from male Fischer 344 rats given a single i.p. injection of [^{14}C]-4CA or [^{14}C]-2CA (0.5 or 1.0 mM) three hours post injection (Dial, Anestis, Kennedy, and Rankin, 1998). Studies with submitochondrial particles (SMP) from beef heart

mitochondria showed increased toxicity with increased chlorine substitution of aniline (Argese et al., 2001). SMP are stable preparations of inverted inner mitochondrial membrane, which maintain mitochondrial respiration functions. The SMP assay is based on the process of reverse electron transfer that can be negatively affected by inhibitors and/or uncouplers of electron transport. One downfall of this assay is that any compound that affects membrane integrity can also negatively affect the assay (Argese et al., 2001). While there have been some studies exploring mitochondrial dysfunction with a few select chloroanilines, the role of mitochondrial dysfunction in 3,5-DCA and 3,5-DCA metabolite-induced nephrotoxicity has yet to be determined. To that end, the molecular target of 3,5-DCA could be explored by exposing IRCC to 14C isotopically labeled 3,5-DCA. Following exposure, the subcellular location of 3,5-DCA can be determined using methods described in Dial et. al (1998). It is hypothesized that a fraction of radioactivity will be found in the mitochondria following exposure to 3,5-DCA.

To further explore mitochondrial involvement in 3,5-DCA-induced cytotoxicity, mitochondrial dysfunction can be determined following exposure to 3,5-DCA. One way to determine mitochondrial dysfunction would be by measuring mitochondrial respiration of IRCC, using a Seahorse XF cell mito stress test, with some modifications. In these experiments, IRCC would be exposed to various concentrations of 3,5-DCA (0.0, 0.5, 1.0 mM) for either 60 or 90 minutes. Following exposure IRCC can be seeded and loaded on to the Seahorse XF cell mito stress test 96 well plate at a density of 3.0×10^5 cells/well in triplicate and then analyzed following the manufacturer's protocol. One downfall of the Seahorse XF cell mito stress test, which could limit its ability to determine the effect of 3,5-DCA on mitochondria function, is the viability of the IRCC. In the case that IRCC viability limits the use of Seahorse XF cell mito stress test, further studies could be performed using HK-2 cells, which have been shown to be

another model for renal toxicity. While cellular respiration is the best measure of mitochondrial dysfunction, another way to measure mitochondrial dysfunction is to determine the expression, concentration, and activity of electron transport complexes, using western blot analysis (Brand and Nicholls, 2011). In these experiments, IRCC would be exposed to 3,5-DCA (0.0, 0.5, 1.0 mM; 60 or 90 min). Following exposure, an aliquot of cells can be taken, prepared, and analyzed using commercially available western blot kits. The two main electron transport complexes, which are measured to explore mitochondrial dysfunction, are complex I and complex IV (Brand and Nicholls, 2011). If mitochondria are the molecular target of 3,5-DCA in IRCC, as hypothesized, not only would significant increases in LDH release be observed but significant decreases in the mitochondrial respiration, as well as the expression, concentration, and activity of complex IV and I should also be observed. Mitochondrial function of IRCC following exposure to 3,5-DCA metabolites should also be determined using the same methods as described for 3,5-DCA.

The purpose of the current studies was to explore the nephrotoxic potential of 3,5-DCA in IRCC, determine the role of metabolism in 3,5-DCA-induced nephrotoxicity, and explore oxidative stress as a potential mechanism of cell death. In the current studies, 3,5-DCA proved to be nephrotoxic in IRCC from male Fischer 344 rats. Further studies suggested that metabolism of 3,5-DCA contributes to nephrotoxicity, at least in part. These studies suggested that N-oxidation of 3,5-DCA is most likely the major contributing metabolic pathway, as determined by the ability of inhibitors of N-oxidation to significantly reduce both the formation of 3,5-DCNB and the release of LDH following exposure to 3,5-DCA. These data suggest that the ultimate toxicant is most likely a highly toxic and highly reactive radical intermediate formed via N-oxidation. However, further studies are required to determine the ultimate toxicant. The results of

the current study also suggested that oxidative stress is not the mechanism of cell death in 3,5-DCA-induced toxicity but rather a result of toxicity. Therefore, further studies are required to determine the mechanism of cell death, as well as any molecular targets of 3,5-DCA-induced cytotoxicity.

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APPENDIX A: INSTITUTIONAL REVIEW BOARD APPROVAL

MARSHALL UNIVERSITY
www.marshall.edu

Office of Research Integrity

August 9, 2016

Chris Racine
914 Francis Ct
Huntington, WV 25701

Dear Chris:

This letter is in response to the submitted dissertation abstract entitled *"3,5-Dichloroaniline: Biotransformation and Mechanistic Aspects of Nephrotoxicity."* 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 #447 and #531. 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 a 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, ThD, CIP
Director
Office of Research Integrity

APPENDIX B: LIST OF ABBREVIATIONS

2A46DCP…2-amino-4,6-dichlorophenol

2-BrA…2-bromoaniline

2-CA…2-chloroaniline

2-FA…2-fluoroaniline

2-IA…2-iodoaniline

3,5-DCA…3,5-dichloroaniline

3,5-DCAA…3,5-dichloroaceataniline

3,5-DCNB…3,5-dichloronitrobenzene

3,5-DCNSB…3,5-dichloronitrosobenzene

3,5-DCPHA…3,5-dichlorophenylhydroxylamine

4A26DCP…4-amino-2,6-dichlorophenol

4-CA…4-chloroaniline

α-Toc… α-tocopherol

AAALAC…Association for the Assessment and Accreditation of Laboratory Animal Care International

AALAS…American Association of Laboratory Animal Sciences

ADCP…4-amino-2,6-dichlorophenol

ALT/GPT…alanine aminotransferase

ASC…ascorbate

BUN…blood urea nitrogen

CYP…cytochrome P450

DCA…dichloroaniline

DEDTCA…diethyldithiocarbamate

DMSO…dimethyl sulfoxide

DNP…2,4-dinitrophenylhydrazone

GPT…glutamic-pyruvic transaminase

GSH…gluthathione

GSSG…oxidized glutathione

FAD/$FADH_2$…flavin adenine dinucleotide

FMO…FAD-containing monooxygenase, Flavin-containing monooxygenase

HPLC…high pressure liquid chromatography

i.p….intraperitoneal

IRCC…isolated renal cortical cells

KH…Krebs Heinselt

LDH…lactate dehydrogenase

MeOH…methanol

NAC…N-acetyl-L-cysteine

NAD/NADH…nicotinamide adenine dinucleotide

NAPQI…N-acetyl-p-benzoquinone

NAT…N-acetyltranferase

NDPS…N-(3,5-dichlorophenyl)-succinimide

PAH…p-aminohippurate

PAPS…3'-phosphoadenosine-5'-phosphosulfate

PHS…prostaglandin H synthase

PiBX…piperonyl butoxide

SMP…submitochondrial particles

TCA…3,4,5-trichloroaniline

TEA…tetraethylammonium

UDP…uridine disphosphate

UGT…UDP-glucuronosyl tranferase

CURRICULUM VITAE

CHRISTOPHER R. RACINE, Ph.D.

Office
Marshall University
Joan C. Edwards School of Medicine
Dept. of Pharmacology, Physiology
& Toxicology
One John Marshall Dr.
Huntington, WV 25755

Home
914 Francis Ct.
Huntington, WV 25701
racine.christopher@yahoo.com
(703)-508-9852

EDUCATION

Marshall University
Huntington, WV 25755
Ph.D. (2016). Biomedical Sciences. 3,5-Dichloroaniline: Biotransformation and Mechanistic Aspects of Nephrotoxicity

West Virginia State University
Institute, WV 25112
M.S. (2010). Biotechnology. In Vitro Screening of Hibiscus sabdariffa Extract for Anti-tumor Properties and Effects of Vascular Smooth Muscle Cell Migration and Proliferation

Davis and Elkins College
Elkins, WV 26241
B.S. (2008). Biology & Chemistry. Mechanistic Aspects of Propanil Nephrotoxicity.

TEACHING EXPERIENCE

Teaching Assistant- Department of Biology, West Virginia State University (2008-2009)

Teaching Assistant/Tutor- Departments of Chemistry & Department of Biology and Environmental Science, Davis and Elkins College (2005-2008)

EXPERIMENTAL TECHNIQUES

Perform surgical isolation of renal cortical cells from rats via a collagenase perfusion method.

Explore cytotoxicity using spectrometry techniques, including lactate dehydrogenase release assays, cell titer glo assays, and MTT viability assays

Counted cells using trypan blue exclusion hemocytometry methods

Determined oxidative stress generation via western blot analysis

Grow and maintain human cell lines using sterile cell culture techniques

Synthesized and explored the chemical characteristics of aminophenol and phenylhydroxylamine compounds using thin layer chromatography, melting point, etc.

Performing experiments to quantify and determine metabolites of 3,5-dichloroaniline in isolated renal cortical cells using Waters e2695 HPLC system with a Waters 2489 UV/Vis Dectector and Empower 3 Software

PUBLICATIONS

Racine C.R., Ferguson T., Preston D., Ward D., Ball J., Anestis D., Valentovic M., and Rankin G.O. 2016. The Role of biotransformation enzymes and oxidative stress in 3,5-dichloroaniline (3,5-DCA) induced nephrotoxicity in isolated renal cortical cells from male Fischer 344 rats. Toxicology. 341-343; 47-55. Epub: Jan 22, 2016.

Racine C, Ward D., Anestis D.K., Ferguson T., Preston D., Rankin G.O. 2014. 3,4,5-Trichloroaniline nephrotoxicity in vitro: Potential role of free radicals and renal biotransformation, Int. J. Mol. Sci. 15, 20900-20912.

Rankin G.O., Sweeney A., **Racine C.,** Ferguson T., Preston D., Anestis D.K. 2014. 4-Amino-2-chlorophenol: Comparative in vitro nephrotoxicity and mechanisms of bioactivation. Chemico-Biological Interactions. 222, 126-132. Epub; Oct 5, 2014.

Rankin G.O., **Racine C.,** Sweeney A., Kraynie A., Anestis D.K., Barnett J.B. 2008. In vitro nephrotoxicity induced by propanil. Environment. Toxicol. Aug; 23(4), 435-442. Epub: Jan 23, 2008

ABSTRACTS AND PRESENTATIONS: NATIONAL AND INTERNATIONAL

Rankin G.O., **Racine C.,** Ward D., Tyree C., Pope D., Sharp J., Anestis D.K. Nephrotoxic Potential of 3,5-Dichloroaniline Putative Metabolites in Isolated Renal Cortical Cells from Fischer 344 Rats. Presented by G.O. Rankin at the 2016 Experimental Biology Meeting in San Diego, CA. April 2-6, 2016.

Racine C.R., Ward D., Tyree C., Pope D., Sharp J., Anestis D., Rankin G.O. In vitro renal cytotoxicity induced by putative metabolites of 3,5-dichloroaniline. Presented at the 55th Society of Toxicology Annual Meeting in New Orleans, LA. March 13-17, 2016. Toxicologist 150 (1), 495, 2016. Program # 3109

Rankin G.O., **Racine C.,** Anestis D., Valentovic M. Role of biotransformation and free radicals in 3,4-dichloroaniline nephrotoxicity in vitro. Presented by G.O. Rankin at the 13th European ISSX Meeting, June 22-25, 2015. Glasgow Scotland.

Racine C., Anestis D., Ball J.B., Valentovic M., Rankin G.O. Oxidative stress induced following exposure to 3,5-dichloroaniline (3,5-DCA) in vitro: role in nephrotoxicity. Presented at the 2015 Experimental Biology Meeting in Boston, MA. March 27-April 1. FASEB J. 29: 938.7.

Tate J., **Racine C.R.,** Vermudez S., Tyree C., Ward D., Anestis D., Rankin G.O. Role of renal bioactivation enzyme systems on 3,5-dichloronitrobenene (3,5-DCNB) induced nephrotoxicity in vitro. Presented by J. Tate at the 54th Society of Toxicology Annual Meeting in San Diego, CA. March 22-26, 2015. Toxicologist 144 (1), 489, 2015. Program #2278

Racine C., Anestis D., Ball J.B., Valentovic M., Rankin G.O. Exploring the role of oxidative stress in 3,5-dichloroaniline (3,5-DCA) induced nephrotoxicity in vitro. Presented at the 54th Society of Toxicology Annual Meeting in San Diego, CA. March 22-26, 2015. Toxicologist 144 (1), 490, 2015. Program #2283

Rankin G.O., Anestis D., **Racine C.** Role of renal biotransformation in 3,4,5-trichloroaniline nephrotoxicity in vitro. Presented at the 2014 Experimental Biology Meeting in San Diego, CA. April 2014. FASEB J 28: 1063.1

Racine C., Anestis D., Rankin G.O. Exploration of the role of renal biotransformation in 3,4,5,-trichloroaniline nephrotoxicity in vitro. Presented at the 53rd Society of Toxicology Meeting in Phoenix, AZ. March 2014. Toxicologist 138(1),543, 2014. Program number 2064.

Racine C., Fergson T., Preston D., Anestis D., Rankin G.O. 2013. Attenuation of 1,2,3-trichloro-4-nitrobenzene nephrotoxicity by antioxidants and inhibitors of biotransformation. Presented at the 2013 Experimental Biology Meeting in Boston, MA. April 2013. FASEB J27: 889.9.

Rankin G.O., **Racine C.,** Ferguson T., Preston D., Anestis D. 2013. Effect of cytochrome P450 isozyme inhibitors on 3,5-dichloroaniline nephrotoxicity in vitro. FASEB J27: 889.2.

Racine C.R., Ferguson T., Baski S., Preston D., Anestis D., Rankin G.O. 2013. Role of renal cytochrome P450 isozymes in the bioactivation of 3,5-dichloroaniline in vitro. Presented at the 52nd Society of Toxicology Annual Meeting in San Antonio, TX. March 2013. Toxicologist 132(1), 466, 2013. Program #2181.

Rankin G.O., Ferguson T., Baksi S., Anestis D., **Racine C.** 2012. 4-Amino-2-chlorophenol nephrotoxicity in vitro: alteration of cytotoxicity by antioxidants. FASEB J26: 1051.10

Racine C., Ferguston T., Baksi S., Anestis D., Rankin G.O. 2012. Mechanistic aspects of 4-amino-2-chlorophenol nephrotoxicity in vitro. Presented at the 51st Society of Toxicology Annual Meeting in San Francisco, CA. March 2012. Toxicologist 126(1), 261, 2012. Program #1212.

Racine C.R., Seidler M.E., Moore D.L., Hankins G.R. 2010. Screening of hibiscus sabdariffa extracts for anti-tumor properties. Joint meeting of the American Society of Pharmacognosy and the Phytochemical Society of North America.

Racine C., Sweeney A., Kraynie A., Baksi S., Anestis D., Rankin G.O. 2008. Mechanistic aspects of propanil nephrotoxicity in vitro. Presented at the 47th Society of Toxicology Annual Meeting in Seattle, WA. March 2008. Toxicologist 102(1). Abstract #445.

ABSTRACTS AND PRESENTATIONS: REGIONAL

Racine C., Tate J., Vermudez S., Tyree C., Ward D., Anestis D., Rankin G.O. 2014. Role of renal bioactivation enzyme on 3,5-dichloronitrobenzene (3,5-DCNB) induced nephrotoxicity in vitro. Presented at the Ohio Valley Regional Chapter of the Society of Toxicology Annual Meeting. September 26, 2014. Dayton, OH.

Vermudez S.A., Tate J., Anestis D.K., **Racine C.R.,** Tyree C., Ward D., Rankin G.O. Trichloroitrobenzene nephrtotoxicity in isolated renal cortical cells (IRCC) from Fischer 344 Rats. Presented at the Annual WV-INBRE Summer Research Symposium, July 28 2014, Morgantown, WV.

Racine C., Ferguson T., Preston D., Anestis D., Rankin G.O. 2013. Role of renal biotransformation in 1,2,3-trichloro-4-nitrobenzene induced nephrotoxicity in vitro. Presented at the Ohio Valley Regional Chapter of the Society of Toxicology Annual Meeting. September 23, 2013. Louisville, KY.

Racine C., Ferguson T, Preston D., Anestis D., Rankin G.O. 2013. Effect of cytochrome P450 isozyme inhibitors on 3,5-dichloroaniline nephrotoxicity in vitro. Presented at the 25th Annual Joan C. Edwards School of Medicine Research Day, March, 19, 2013, Huntington, WV.
Kim J.H., Anestis D.K., **Racine C.R.,** Baksi S.R., Ferguson T.L., Rankin G.O. 2011. Mechanistic aspects of 3,4-dichloroaniline nephrotoxicity in vitro. Annual WV-INBRE Summer Research Symposium, July 30, 2012, Huntington, WV.

Racine C., Seidler M.E., Moore D.L., Hankins G.R. 2010. In vitro screening of Hibiscus sabdariffa extract for anti-tumor properties. Presented at the WV Academy of Science Annual Meeting 2010, Morgantown WV.

Racine C., Sweeney A., Kraynie A., Baksi S., Anestis D.K., Rankin G.O. 2007. Mechanistic aspects of propanil nephrotoxicity. Presented at the Annual WV-INBRE Summer Research Symposium, August 2, 2007, Huntington, WV.

PROFESSIONAL/HONORARY MEMBERSHIP

The American Society of Pharmacology and Experimental Therapeutics
2013-Present

Society of Toxicology 2012-present
Mechanism Specialty Section 2012-present
Ohio Valley Region Chapter 2013-present

Marshall University Graduate Student Organization (Biomedical Sciences Program)
Member 2010-Present
Treasurer 2013-2014

West Virginia State University Graduate Student Association
Member 2008-2010
Faculty Assembly Student Representative - 2009

Phi Beta Lambda-Business Fraternity-Davis and Elkins College Chapter
Member 2007-2008

Chi Beta Phi-National Science Honorary- Davis and Elkins College Chapter
Member 2005-2008
President 2007-2008

Davis and Elkins College Student Assembly
Member 2006-2008

AWARDS/HONORS

Best Research Performance This Academic Year 2014-2015, Awarded by Marshall University Biomedical Sciences Program – August 2015

Best Abstract Award Finalist, awarded by the Division of Toxicology of the American Society of Pharmacology and Experimental Therapeutics (ASPET) – May 2015

Graduate Student Travel Award to attend 2015 Experimental Biology in Boston MA, awarded by The American Society for Pharmacology and Experimental Therapeutics (ASPET) - May 2015

Ohio Valley Regional Chapter and Charles River Laboratories Best PhD Student Poster Presentation Award, Ohio Valley Regional Chapter of the Society of Toxicology Annual Meeting 2013

Distinguished Graduate of the Honors Program: Davis and Elkins College 2008

Academic Achievement Award, Department of Chemistry: Davis and Elkins College 2008

Undergraduate Award for Achievement in Organic Chemistry, ACS Division of Polymer Chemistry: POLYED 2007

Academic Achievement Award, Department of Biology & Environmental Science: Davis and Elkins College 2007

CRC Press Achievement Award in Chemistry, CRC Press 2006

INVITED SPEAKER

How did I get here? My Journey As A Student. Davis and Elkins College Senior Seminar. Presented March 3, 2016.

3,5-Dichloroaniline: Biotransformation and mechanistic aspects of nephrotoxicity. Davis and Elkins College Senior Seminar. Presented March 21, 2013.