CHARACTERIZATION OF THE PRECLINICAL CYANIDE ANTIDOTE SULFANEGEN

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Dedication

I dedicate this work to my parents.

Abstract

Cyanide is a metabolic poison that inhibits utilization of oxygen to form ATP. The consequences of acute cyanide exposure are severe: toxic doses result in loss of consciousness, cardio and respiratory failure, hypoxic brain injury, and dose-dependent death from within minutes to hours. In a mass casualty scenario such as an industrial accident or terrorist attack, currently available cyanide antidotes would leave many victims untreated in the short time available for successful medical countermeasure administration.

Therefore, there is a need for rapidly acting antidotes that can be quickly administered to large numbers of people who may be unconscious. Sulfanegen, a novel preclinical cyanide antidote, is being developed to meet this need. Sulfanegen is administered by intramuscular injection (IM), which has the advantages of requiring minimal training for first responders, as well as the potential for rapid antidotal administration to many affected people.

Due to ethical issues involved with testing cyanide antidotes in human subjects, sulfanegen cannot go through the normal clinical trial path to drug approval. However, the FDA has published guidelines for evidence needed to gain approval under the “Animal Rule”, which this thesis attempts to address. The “Animal Rule” allows for new drug approval if the following conditions are met: 1) Effective in more than one animal model with a response predictive for humans, 2) Pharmacokinetics understood well enough to determine effective dose, and 3) A well-understood mechanism of action and pathology of the disease.

We first elucidated the animal models predictive for human efficacy testing by MST species comparison of the blood of many common laboratory animals. Based on this preliminary screen, species closest to that of humans were chosen to characterize the MST activities in metabolically active tissues. Using those findings, we determined swine were not an appropriate model for predicting efficacy in humans, while murine and rabbit models were appropriate.

Next, we developed an isocratic UV-VIS HPLC method for characterizing the pharmacokinetics of sulfanegen in humans. Pre-column derivitization with NEM prevented the reformation of the 3-mercaptopyruvate (3MP) dimer and allowed for chromatographic detection. Moreover, this method is easily modified for applications to quality control, being able to separate sulfanegen from its precursor molecules and predicted decomposition products.

Finally, we examined the potential secondary mechanisms of sulfanegen administration. MST catalyzed detoxification of cyanide to thiocyanate is the primary mechanism of action, but secondary mechanisms involving 3MP alone or in conjunction with MST contribute to the overall efficacy of sulfanegen. 3MP was found to be a potent antioxidant, capable of quenching cyanide induced ROS. Additionally, H_2S generation by MST was confirmed after sulfanegen administration. Although it was confirmed that cyanohydrin formation was occurring, this probably does not contribute to reversing cyanide-induced cytochrome c oxidase inhibition.

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Abbreviations

Note: IUPAC conventions were followed for abbreviations of units

3MP: 3-Mercaptopyruvate

3MP-NEM: 3-Mercaptopyruvate derivitized with n-ethylmaleimide

3MP-MBB: 3-Mercaptopyruvate derivitized with monobromobimane

4-HC: 4-Hydroperoxycyclophosphamide

AA: Ascorbic Acid

ALS: Advanced Life Support

ATCA: 2-aminothiazoline-4-carboxylic acid

CBI: Cobinamide

CounterACT: Countermeasures Against Chemical Threats

CN: Cyanide

CNS: Central Nervous System

DMAP: 4-Dimethylaminophenol

DPPH: 2,2-diphenyl-1-picrylhydrazyl

DTMS: Dimethyl Trisulfide

DTT: Dithiothreitol

EDTA: Ethylenediaminetetraacetic acid

FDA: Food and Drug Administration

G6P: Glucose-6-Phosphate

GSH: Glutathione

HBEC: Human Bronchial Epithelial Cell

HCN: Hydrogen Cyanide

HPLC: High Pressure Liquid Chromatography

IACUC: Institutional Animal Care and Use Committee

IM: Intramuscular Administration

IO: Intraosseous Administration

IV: Intravenous Administration

MST: Mercaptopyruvate Sulfurtransferase

NAC: N-Acetyl-L-Cysteine

NBD-CL: Chloro-7-nitrobenzofurazan

NDRI: National Disease Research Interchange

NEM: N-Ethylmaleimide

NIH: National Institutes of Health

NO: Nitric Oxide

NZW: New Zealand White

NPDS: National Poison Data System

OB: Obliterative Bronchiolitis

PD: Pharmacodynamics

PHEMCE: Public Health Emergency Medical Countermeasures Enterprise

PK: Pharmacokinetics

PO: Oral Administration

ROS: Reactive Oxygen Species

RBC: Red Blood Cell

SCN: Thiocyanate

TRX: Thioredoxin

TXRX: Thioredoxin Reductase

WWI: World War I

WWII: World War II

CHAPTER I:

BACKGROUND

1. Introduction

Chemical threats such as cyanide pose an ever-increasing risk to human life due to their relative ease of accessibility and potential to cause mass casualties. The National Institutes of Health (NIH) Countermeasures Against Chemical Threats (CounterACT) Program classifies cyanide as a “higher priority threat”, highlighting the need for new antidotal developments. This chapter will cover cyanide’s history, symptoms, mechanisms of toxicity, as well as both current and future approaches to antagonism of toxicity. The goals of projects covered in the following chapters derive from unique characterization hurdles involved in approval of chemical countermeasures. The focus is on studies necessary for the clinical translation of sulfanegen, a next generation pre-clinical cyanide antidote. The studies include elucidation and validation of secondary mechanisms of action, justification of species chosen for efficacy studies, analytical method development suitable for pharmacokinetic studies, and examining the possibility of extension of this and similar compounds to other clinical uses.

1.1 History of Cyanide

Cyanide is an archetypal poison, whose use is documented throughout human history.[1] Historical poisonings usually occurred by means of plants containing cyanogenic glycosides such as almonds, cherry laurel, and cassava. Both the Egyptians and Romans used cyanogenic compounds in executions (peach pits and cherry laurel respectively).[1] The first written descriptions of cyanide poisoning dates back to Wepfer’s use of almond extract in 1679, and Maddern’s canine toxicity demonstrations in 1731 using the extracts of cherry

laurel.[2] However, the causative agent for the toxicity was not isolated until 1782 by Carl Windhelm Scheele.[3] Scheele heated Prussian blue with sulfuric acid, generating hydrogen cyanide (HCN), which he named prussic acid.[3]

1.2 Cyanide Exposure Scenarios

1.2.1 Industrial Accidents

Today, cyanide exposure can occur as a result of industrial accidents, smoke inhalation, naturally occurring sources, or deliberate release as an agent of war/terror. In the United States alone, about 2 billion pounds of cyanide are produced annually for broad uses in industry, including: the extraction of gold/silver in mining, metallurgy, pesticides, electroplating, the production of textiles, and in the aquatics industry for stunning fish prior to capture.[4–6] Therefore, an accidental exposure during either the use or transportation of cyanide is major cause for concern. These worries were realized in 1984 when gaseous methyl isocyanate and cyanide containing combustion products were released during a fire at a pesticide plant in the densely populated city of Bhopal, India resulting in 1,800-5,000 deaths and up to 200,000 injuries.[7]

1.2.2 Smoke Inhalation

Due to the use of nitriles in the production of textiles and other goods, smoke inhalation is another source of cyanide exposure. Incomplete combustion of nitrogen containing organics prepared from the above nitriles can result in release of hydrogen cyanide. Thus, cyanide poisoning is often overlooked in cases of smoke inhalation toxicity.[8–10] Combusting just 1 g of polyacrylonitrile, produces 1,500 ppm cyanide in a 15.6 L combustion chamber, and it has been

estimated just 2 kg of polyacrylonitrile is needed to produce toxic levels in the average sized living room.[11] During a fire, any delay in escape caused by decreased visibility or impedance of exits prolongs the duration of exposure to toxic smoke, increasing the chance of fatalities. Baud et al compared the blood cyanide and carbon monoxide levels of smoke inhalation victims and found large differences between fatal and nonfatal fires.[12] From 109 fire victims studied, 43 had died, and the cyanide concentration in the blood of the fatalities was 116.4 μM +/- 89.6, compared to 21.6 μM +/- 36.4 for those who had survived, and just 5.0 μM +/- 5.5 for control samples from the general population. Carbon monoxide levels between the two groups were 2.8 mM +/- 2.0 for fatal incidents and 0.7 mM +/- 0.7 for non-fatal incidents. While carbon monoxide levels also increased, some of the deceased had non-lethal levels of carbon monoxide, yet fatal levels of cyanide in the blood. It therefore follows that increased exposure to cyanide-containing fumes decreases the chance for survival in the case of fires, and that both carbon monoxide and cyanide exposure contribute to fire deaths.[12]

The importance of cyanide in smoke inhalation is highlighted by the Kiss nightclub fire disaster of 2013 in Brazil, which occurred during a crowded event. The incident ultimately left 242 dead and over 600 injured. The fire reportedly started when a member of the band lit a pyrotechnic device that ignited soundproofing material overhead releasing toxic cyanide-containing smoke. Officials reported that a lack of emergency exits contributed to the high fatality number.[13]

1.2.3 Naturally Occurring Cyanide

Cyanide has been detected throughout the universe in starbursts, and is released into the atmosphere via volcanoes.[14–16] However, most naturally occurring sources of cyanide leading to accidental exposures are confined to the plant kingdom. There are over 2000 plant species that contain cyanogenic glycosides, which can generate HCN when cells containing them are ruptured.[17,18] This is likely a defense mechanism in that herbivore consumption of these plants can cause illness or even death.[19] Such toxicity is well-documented in livestock, especially cattle.[20,21]

1.2.4 Agent of War

Deliberate use of weaponized cyanide was not employed on the battlefield in modern times until World War I (WWI). The development of trench warfare at the first battle of the Aisne in 1914 gave the tactical advantage to defenders, and the frontlines ground to a halt with each side digging in.[22] The war of maneuver that contemporaries of the day believed would end quickly had transformed into a stalemate. To overcome this stalemate, both sides began innovating outside of the norms of conventional warfare. The Hague convention of 1899 banned the use of projectiles to spread chemical weapons.[23,24] However, German forces were the first to break this convention, with the use of chlorine gas in the second battle of the Ypres in April 1915. Chlorine gas (498 tons) was discharged from 20,730 cylinders.[22,25] German leadership argued this did not violate the Hague conventions, as they simply opened canisters of gas and let the wind blow the gas to the enemy lines, rather than deliver it via munitions.[26] It is important to note many countries were considering chemical warfare before that point, with

US patents being filed for incendiary devices containing hydrogen cyanide and the UK considering the use of sulfur dioxide as early as 1914.[27,28] After that incident, gas attacks became more commonly used by both sides, with some calling WWI the “chemists war”.[29,30] Unlike other conventional weapons, both sides developed and deployed effective countermeasures in the form of charcoal containing gas masks. French forces started experimenting with HCN containing artillery shells in 1916, partly due to HCN’s ability to pass through these gas masks. Eventually, 4,000 tons were manufactured for war.[31] However, HCN’s high volatility limits its usefulness in trench warfare as it disperses too rapidly to be effective in open-air. As a result, both the French and Austrians experimented with cyanogen chloride/bromide respectively, which had a cumulative effect and was more effective than HCN alone.[22,25]

Following WWI, the Geneva protocol was ratified in 1925, prohibiting the use of biological and chemical weapons in warfare.[32] However, these laws would be tested again merely a decade later. During World War II (WWII), chemical weapons played a smaller role on the battlefield, partly due to the deterrent of both sides having large stockpiles of chemical weapons as well as the necessary protective equipment. Between 1922 and 1945, the United States produced 500 tons of HCN and 11,400 tons of cyanogen chloride.[22,33] Meanwhile, Japan produced 255 tons of HCN from 1939 to 1945 and German production after 1944 was 20 tons per month.[22,34] However, Zyklon B, which was a commercially available pesticide containing HCN, was used by the Nazis in gas chambers to kill millions of civilians as well as enemy combatants in death camps. HCN was

detected on the walls of the gas chambers nearly 50 years later, demonstrating its extensive use.[35]

Following the great wars, cyanide use on battlefields declined, with the next reports of its use occurring in the 1980s when it was used during the Iran-Iraq wars.[36,37] However, declining use of cyanide in war has not been the end of cyanide's deliberate release to impact human health.

1.2.5 Use of Cyanide in Suicide, and Murders

As cyanide availability increased due to its broad adaptation for applications in industry, incidents of cyanide related suicides, murder, and terrorism became more common. Possibly the best known example of cyanide's role in suicide is the Jonestown mass suicide of 1978.[38] Jim Jones was the leader of a cult known as "The Peoples Temple", which relocated to Guyana in 1974 after the tide of public perception began to turn against them. However, their troubles did not stop there, pressure from relatives back in the US eventually lead US representative Leo Ryan to visit Jonestown. Jones' increasing paranoia about the potential for negative stories leading to persecution as a result of this visit lead him to order the assassination of Ryan. Knowing the backlash this action would cause, he convinced over 900 people to consume a Flavor-Aid drink laced with cyanide, among other intoxicants.[38]

Deliberate use of cyanide in murders has been known throughout history, and is still relevant today. Recent examples of cyanide being employed in murder are the Chicago Tylenol murders of 1980s, which killed 7 people and resulted from cyanide laced Tylenol packages being placed on store shelves and

sold to consumers.[39] This incident lead to consumer protection reform and the resulting tamper-resistant packaging laws. The perpetrator behind these poisonings was never caught. More recent examples of deliberate cyanide poisonings in the news are the deaths of potential Whitey Bulger witness Stephen Rakes in 2013, million dollar Chicago lottery winner Urooj Khan in 2012, and University of Pittsburgh neuroscientist Autumn Klein in 2013.[40–42]

1.2.6 Agent of Terror

Cyanide as an agent of terror is an increasing threat. Compared to its relative ineffectiveness on the battlefield due to its volatility, cyanide's efficacy as a weapon is greatly increased in enclosed spaces. Additionally, the large quantities of cyanide used in industry provide relatively easy access compared to other chemical or biological threats of interest. This threat materialized in 2002, when a commercial truck carrying nearly 8 tons of sodium cyanide in Mexico was stolen.[43] To this day, 6 of 96 barrels remain unaccounted for after the robbery.[44] In another example, US counterterrorism forces had credible threats of Al-Qaeda's intent in 2013 to release cyanide into the New York subway system. The attack was reportedly called off by Al-Qaeda leadership for unknown reasons.[45] These plans were reminiscent of the Tokyo subway terror attacks of 1995, where members of the Aum Shinrikyo cult used sarin, resulting in 12 deaths and over 5,000 injuries.[46] Other examples include the 2002 indictment of Joseph Konopk ("Dr. Chaos") for storing cyanide in the Chicago subway system, and the 2008 arrest Jeffrey Detrixhe for attempting to sell 100 pounds of sodium cyanide to a member of the Aryan Brotherhood during an FBI sting operation.[47,48] Detrixhe

reportedly bragged the amount was enough to "kill a city".[48] Thus, the use of cyanide in terrorist attacks clearly remains a genuine threat.

1.3 Incidence Rates

Cyanide poisoning occurrences in the United States are tracked by the American Association of Poison Control Center's National Poison Data System (NPDS). Data included in the annual reports from 2010-2015 are given in **Table 1**. The number of case mentions, single exposures, intentionality of the exposure, hospital visits, and deaths are reported therein.[49–54]

Year	Cases	Single Exposures	Accidental Exposures	Intentional Exposures	Hospital Visits	Deaths
2015	205	143	107	14	86	3
2014	245	187	126	19	134	9
2013	358	294	244	18	103	8
2012	202	148	103	22	85	7
2011	246	177	133	14	97	3
2010	225	145	103	18	89	8
Total	1481	1094	921	105	594	38

Table 1. Cyanide exposures in the United States from 20010 to 2015 as reported by the NPDS. [49–54]

Reports of unintentional exposures to cyanide outnumber those of intentional exposures; this contrasts with the perception of cyanide being primarily used in high profile suicides that are well known in American culture. Many of these unintentional exposures result from small-scale accidents during the use of cyanide in industry or cyanide-containing smoke inhalation during fires. Overall, the relatively few cases of cyanide exposure each year are no excuse for a lack of preparedness. For the most part, the current antidotes (covered in section 1.8) perform adequately in a setting where few are exposed

provided a rapid medical response (shortcomings of current antidotes are covered in section 1.9). However, current readiness is inadequate for mass casualty exposure, where stockpiles of available antidote, properly trained medical personnel, and limitations of the antidotes themselves play a large factor between life and death for those exposed.

1.4 Preparedness

The risk of cyanide toxicity in smoke inhalation, and industrial/laboratory use means emergency response personnel need rapidly acting medical countermeasures. RTI International published a rare look into the national cyanide disaster readiness in 2006 that detailed advanced life support (ALS) preparedness and perceived threat levels in the US post-September 11, 2001.

Cyanide's toxicity is dose dependent and can cause death within minutes to hours (mechanisms of toxicity covered in section 1.5 below), making availability an antidotes paramount to saving lives. However, the results from this survey showed that in 76% of standard emergency vehicles no cyanide antidote was available. Additionally, cyanide antidotes were ranked as low importance compared to other supplies on the vehicle by 28% of ALS providers. The average number of antidote kits available in hospitals was 25.7 kits per 100,000 people. Approximately a third (32.1%) of providers surveyed did not have access to a larger antidote cache as part of a disaster readiness plan, and 31.7 % did not know if they had such a cache available. More than two-thirds (68.8%) of high cyanide interest areas reported access to large caches of antidotes available in 1 hour or less.[55] However, these larger caches are unlikely to be of much use in

response to deliberate release of cyanide in a confined area, as the therapeutic window necessitates rapid antidote administration.

Additionally, access to antidotes is only part of the preparedness problem. Another crucial area for disaster readiness is availability of trained medical personnel. Only 16% of ALS personnel in the RTI International study had experience administering a cyanide antidote; exposing another pain point in the chain of successful countermeasure deployment.[55]

1.5 Mechanisms of Toxicity

Cyanide is classified as a metabolic poison. Its primary mechanism of toxicity is attributed to cytochrome c oxidase inhibition.[56] Cytochrome c oxidase is the terminal electron acceptor in the electron transport chain. The electron transport chain utilizes NADH and $FADH_2$, the end products of the citric acid cycle, to pump protons across the inner mitochondrial membrane, forming a proton gradient. It accomplishes this using four complexes (I-IV), coenzyme Q, and cytochrome C. Electrons donated from NADH and $FADH_2$ move through the complexes by means of redox chemistry, releasing the energy needed at each step to move protons from inside the inner membrane to the intermembrane space. Ubiquinone (coenzyme Q) receives electrons from complex I & II (donated by NADH and $FADH_2$ respectively) and passes them onto Complex III. Electron transport terminates at complex IV, also known as cytochrome C oxidase, where oxygen is the ultimate electron recipient, forming water. The proton gradient is then employed by ATP synthase which pumps protons from the intermembrane space across the inner membrane to produce ATP **(Figure**

1). Cyanide inhibits cytochrome C oxidase by binding to the reduced form of cytochrome a_3 preventing oxidative phosphorylation.[57] Cells can temporarily adapt to this inhibition by switching ATP production to the less productive glycolysis pathway, but buildup of the resulting lactic acid leads to metabolic acidosis and symptoms of toxicity (covered in section 1.6).[56]

In addition to loss of ATP production, cyanide also leads to production of reactive oxygen species (ROS) and results in oxidative stress and lipid peroxidation.[58,59] This ROS generation is dependent on NMDA receptor activation and Ca^{2+} influx.[59] Cyanide also inhibits other heme-containing enzymes, some of which contribute to the antioxidant defense system such as superoxide dismutase and glutathione reductase.[58]

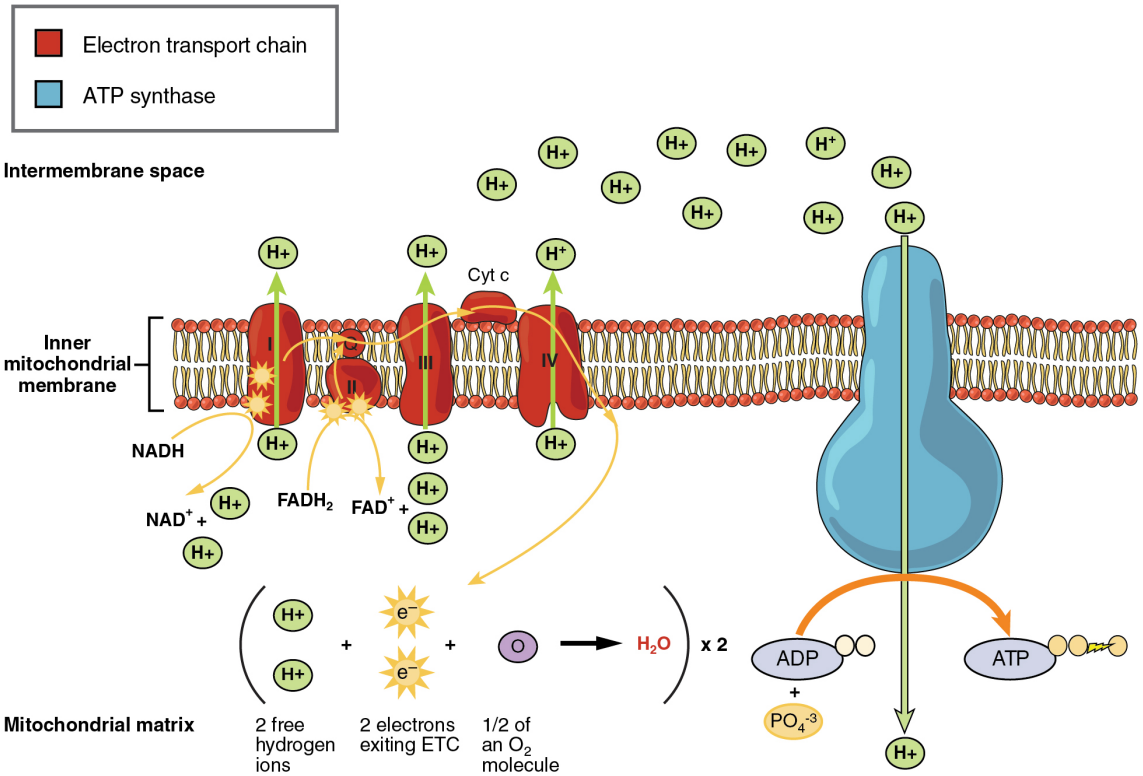


Figure 1. The electron transport chain and ATP synthase. Electrons travel from complex I through complex IV, pumping hydrogen ions across the inner mitochondrial membrane, which ATP synthase uses to drive ATP. [Image modified from[60]. No permission required for educational use (CC BY-NC-SA 4.0)]

1.6 Symptoms of Cyanide Exposure

1.6.1 Acute Symptoms

Symptoms of cyanide poisoning are dose dependent, and ultimately stem from biological mechanisms of toxicity in preventing oxygen utilization (Lethal doses of cyanide in humans are 1.52 mg/kg orally or 500 ppm over 10 minutes via inhalation).[5] Due to inhibition of aerobic respiration, the body attempts to increase oxygen levels. As a result, the symptoms are very similar to tissue hypoxia, with the noted exception of oxygenated venous blood in cyanide-poisoned persons. Metabolically active tissues such as the central nervous

system (CNS) and heart are the most sensitive due to their high oxygen demand. Cyanide poisoning presents clinically in hyperventilation, tachycardia, nausea, dizziness and metabolic acidosis. In severe cases apnea or hypopnea, cardiopulmonary failure, loss of consciousness, and death follow.[5] The above symptoms likely result from the cardiac and neuronal tissues' aforementioned greater sensitivity to cyanide.[5] An almond scented breath has also been described, however this is of limited diagnostic utility; the odor is faint and only a fraction of the population can detect cyanide via smell, with estimates of 20-30% of the population failing to detect it.[61] Death is dose dependent and occurs within minutes to hours. Thus, a rapid response to cyanide exposure is paramount to saving lives.

1.6.2 Chronic Symptoms

Patients who survive high dose cyanide intoxication (usually resulting from failed suicide attempts) are relatively rare, but those who do survive often develop a Parkinsons-like set of symptoms. Published case studies following survivors are rare, but do exist.[36,62–68] These symptoms do not respond to standard Parkinson's therapies such as L-dopamine supplementation, illuminating a key difference in pathology, even in the presence of similar symptoms. [36,62–68]

In contrast to sub-lethal acute poisonings, chronic sub-lethal ingestion of cyanide, usually in the form of cyanogenic glycosides, can lead to a condition known as konzo.[36,69] Konzo is a poorly understood and troubling condition resulting in loss of visual acuity and paraparesis, or partial paralysis of the lower

limbs. Symptoms set in within 1 day for 90% of cases and the disease typically results in chronic disabling paraparesis.[70–73] Currently, prevention is the only known treatment for this debilitating disease.[74] The cassava root is a carbohydrate staple in the diets of many central African nations and the Congos. Cassava contains the cyanogenic compound linamarin. During times of drought, people of the Congo depend heavily on this crop for sustenance, and its consumption when improperly prepared is linked with konzo.[75] Proper preparation of the cassava plant by boiling breaks down the linamarin and prevents ingestion of cyanogenic compounds. There is also emerging interest in cyanide antidotes taken concurrently with cassava-containing meals, as administration of antidote with potential sources cyanide ingestion may prevent the onset of konzo.

1.7 Endogenous Detoxification Pathways

The persistence of cyanide in the environment has applied evolutionary pressure on aerobic organisms for mechanisms of defense against it. The key pathways for mammalian defense are the enzymes rhodanese and mercaptopyruvate sulfurtransferase. These enzymes each detoxify cyanide by conversion to thiocyanate (SCN). Thiocyanate is much less toxic than cyanide and is eliminated principally by renal excretion. The clinically relevant differences between these cyanide detoxification enzymes are their substrates, reaction rates, and compartmentalization of the enzyme within the body.

Rhodanese (EC 2.8.1.1) was the first cyanide detoxification enzyme discovered (1933), and was purified in 1953. Rhodanese catalyzes the transfer of

sulfur from thiosulfate to cyanide, via an enzyme bound persulfide intermediate forming sulfite and thiocyanate. Rhodanese is compartmentalized mainly in the mitochondrial fractions of liver and kidneys, with little activity found elsewhere.

Mercaptopyruvate Sulfurtransferase (MST) (EC 2.8.1.2) was first described in 1953, and purified in 1955.[76–78] The function of MST in cysteine catabolism can also detoxify cyanide by catalyzing the transferring a sulfur to cyanide, forming thiocyanate. The intermediate in this reaction, a persulfide at cysteine^{248} (cys^{248}), is similar to that of rhodanese.[79] The results of the MST reaction, in the presence of cyanide, are the formation of pyruvate and thiocyanate. However, MST and rhodanese diverge with respect to their compartmentalization. Unlike rhodanese, MST is found in both the mitochondrial and cytosolic fractions of many tissues including the blood, brain, liver, heart, and kidneys. As a result, MST may have a greater potential than rhodanese for detoxifying cyanide.

Cyanide may also react non-enzymatically with other components of the cellular makeup. Cyanomethemoglobin can form upon addition of cyanide to methemoglobin in red blood cells, which decreases free cyanide concentrations at the expense of oxygen carrying capacity of the blood. Cyanide can also react with cysteine to form 2-aminothiazoline-4-carboxylic acid (ATCA), and endogenous keto-acids and sugars to form cyanohydrins.

1.8 Current Approved Antidotes

Currently there are two FDA-approved cyanide antidotes available in the United States, nithiodote™ and hydroxocobalamin. Nithiodote™ consists of

intravenous infusions of sodium nitrate and sodium thiosulfate. The efficacies of the nitrates were originally thought to stem from nitrate's ability to oxidize hemoglobin into methemoglobin, which bound cyanide. However, methemoglobin formation is too slow and inefficient to be effective. Peterson and Pearce demonstrated the primary mechanism of nitrate mediated antidotal efficacy was reduction of nitrite to of nitric oxide (NO), which competes with cyanide for the cytochrome C oxidase binding site.[80] The other primary component of the cyanide antidote kit, sodium thiosulfate, is a substrate for the enzyme rhodanese. Rhodanese catalyzes the transfer of sulfur from thiosulfate to cyanide, forming thiocyanate (see above). When combined, these antidotes work together to scavenge, compete for the binding site, and detoxify free cyanide. The potential of this thiosulfate/nitrate combination was summarized in 1944 by Chen et al:

> "The two substances, intravenously injected…are capable of detoxifying approximately twenty lethal doses of sodium cyanide in dogs, and are effective even after respiration has stopped. As long as the heart is still beating, the chances of recovery by this method of treatment are very good."[81]

Hydroxocobalamin, also known as vitamin B_{12a} and marketed as cyanokit™, is administered by intravenous infusion similarly to some components of the cyanide antidote kit. However, it differs from the cyanide antidote kit in its mechanism of action. Hydroxocobalamin contains a cobalt atom at its center, and similar to iron-containing enzymes, this cobalt directly binds and sequesters cyanide, decreasing free cyanide in the body **(Figure 2)**. The Food and Drug

Administration (FDA) approved hydroxocobalamin in December 2006 under the FDA's "Animal Rule", which will be discussed later.

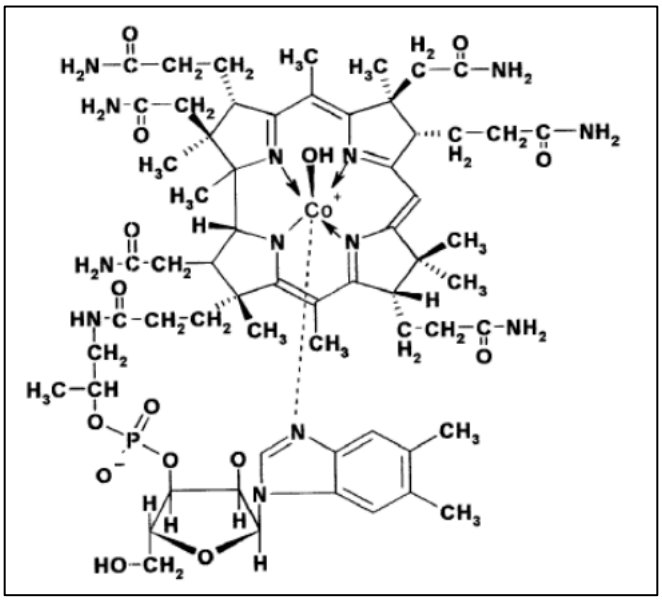


Figure 2. Structure of hydroxocobalamin. The cobalt ion in hydroxocobalamin binds and sequesters cyanide. Image from Cyanokit™ package insert.[82] Permission not required for educational use.

There are other cyanide antidotes used outside of the United States. 4-Dimethylaminophenol (DMAP) is an antidote used in Germany and Austria whose antidotal mechanism of action is attributed to production of methemoglobin from hemoglobin.[37] Dicobalt EDTA is another chelating agent used in Europe. However, the utilities of both of these antidotes are limited by their side effects. Cobalt complexes (with noted exceptions being the corrins like hydroxocobalamin) are quite toxic, and DMAP administration can lead to hemolysis and multi organ failure in the absence of cyanide.[37] For these reasons, the remainder of this document will focus on approved therapeutics in the US.

1.9 Issues with Current Antidotes

Both of the current FDA approved cyanide antidotes are not without their limitations. Nitrates (a component of nithiodote™) are dangerous to use in cases of smoke inhalation, as the resulting methemoglobin will decrease the oxygen carrying capacity of the blood. This reduced carrying capacity is further complicated by both: 1) exposure to carbon monoxide also present in many cases of smoke inhalation and 2) nitrate-mediated hypotension.[83] For this reason, nitrates are contraindicated in cases of smoke inhalation, which is a major source of cyanide exposure. In instances where nithiodote™ is indicated, side effects require administration with thiosulfate to lower the required nitrite dose to limit this toxicity.[83]

Thiosulfate (a component of nithiodote™) has fewer side effects than nitrates, but is slower acting and requires an IV infusion. This slower onset is a function of its mechanism as a substrate for the enzyme rhodanase, which is concentrated mainly in the mitochondria of livers and kidneys. Sole administration of thiosulfate may leave critical, susceptible areas of cyanide toxicity such as the CNS or heart unprotected. However, emerging research has demonstrated sodium thiosulfate and nitrite administration (both IM) is more efficacious than previously thought.[84]

Hydroxocobalamin (cyanokit™) is a cyanide scavenger and is safe for use in smoke inhalation scenarios. Hydroxocobalamin's low aqueous solubility requires a large volume intravenous infusion slowly administered (200 mL over 15 minutes) in order to deliver a therapeutic dose. Additionally, its dark red

coloration persists in the body fluids for days, turning skin, blood, urine, sweat and tears a deep red. As a result, administration of hydroxocobalamin may interfere with diagnostic tests such as pulse oximetry **(Figure 3)**.[85]

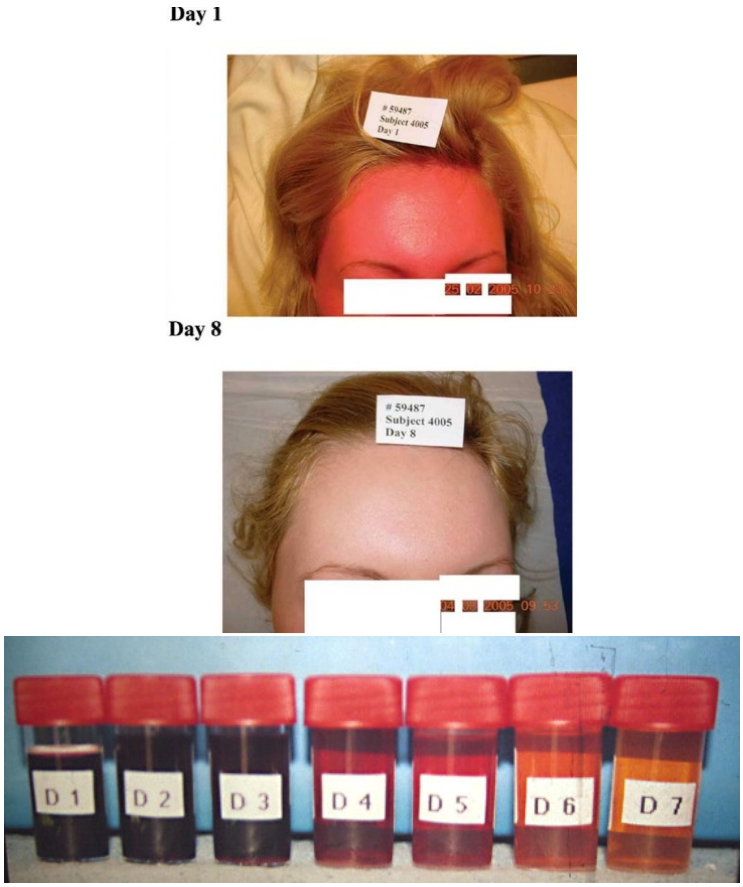


Figure 3. Side effects of hydroxocobalamin administration. Red coloration of hydroxocobalamin persists in skin (above) and urine (below) for days. Images from Uhl and Borron.[86,87] Permission not required for educational use.

1.10 Properties of an Ideal Cyanide Antidote

The ideal antidote for cyanide poisoning needs to address the requirements for both the specific toxicant and likely exposure scenarios. For cyanide, antidotes need to be fast acting. Due to the rapid decline of individuals who are exposed to cyanide, the ideal antidote must start working rapidly. For

this reason, antidotes that take advantage of the rhodanese system are not ideal, because of the need for these compounds to distribute to the liver and kidney mitochondria, where most rhodanese is compartmentalized.

The antidote also must be safe. Currently there are no field-deployable methods for rapid identification of cyanide and medical countermeasures must be administered quickly; victims may not survive transportation to a hospital. Therefore, the antidote needs to be safe enough to administer even in instances where exposure to cyanide is only suspected.

Due to cyanide specific exposure scenarios such as smoke inhalation or mass casualty settings during an accidental or terrorist related release, the ideal antidote needs to account for these scenarios and retain efficacy and safety. For smoke inhalation scenarios, the oxygen carrying capacity of the blood should not be compromised. For this reason, antidotes which detoxify cyanide by forming methemoglobin are not ideal. Additionally, in mass casualty scenarios rapid and facile administration becomes of utmost importance. Currently approved cyanide antidotes all require some component of the antidote to be given intravenously (IV), which requires both trained medical personnel onsite as well as time to get the IV line started. Due to this rate-limiting step, in a mass casualty scenario there would be people who would succumb to the poison before medical countermeasure therapy could be administered. For this reason, the ideal cyanide antidote would not be given IV. Additionally, due to loss of consciousness and apnea associated with cyanide exposure, both oral and

inhalational routes of administration would not be optimal. Either intramuscular (IM) or intraosseous (IO) administration are preferred for cyanide antidotes.

1.11 Next Generation Cyanide Antidotes

To address the issues with currently approved cyanide antidote countermeasures outlined above, next generation antidotes are in development with mass casualty scenarios in mind. Bhattacharya et al in India are developing α-ketoglutarate.[58,88] The proposed mechanism of action for α-ketoglutarate is cyanohydrin formation at the α-keto acid moiety. Cyanohydrin formation is a reversible reaction, but any cyanohydrin present decreases the overall free cyanide available to inhibit cytochrome c oxidase. In a human dose fixation study, adverse events of α-ketoglutarate administration were vomiting and tachycardia, but did not halt development as an antidote.[89]

Cobinamide (Cbi) is an antidote under development by Boss and colleagues at the University of California, San Diego. Cobinamide is a biosynthetic precursor to the approved hydroxycobalamin and binds cyanide with greater affinity. It is also more soluble than hydroxocobalamin, as a result more amenable to other routes of administration in addition to IV.[90] In both cell and animal models, Cbi has demonstrated efficacy at binding cyanide released by nitroprusside administration.[91] It has also demonstrated efficacy in both non-lethal and lethal cyanide doses in animal models by oral (PO) and intramuscular (IM) routes of administration.[92–95] Cobinamide has not yet entered phase I clinical trials, but remains a promising new therapy.

Rockwood, Petrikovics *et* al are developing an IM injectable formulation of dimethyl trisulfide (DMTS). DTMS is a sulfur donor, and is capable of converting cyanide into thiocyanate either in the presence or absence of rhodanase.[96] This reduced reliance on an enzyme catalyst enables detoxification of cyanide in rhodanase deficient tissues. Moreover, the increased lipid solubility of DMTS when compared to thiosulfate allows DMTS to distribute more efficiently to the mitochondria, where rhodanase is compartmentalized.[96] As with cobinamide, DMTS is in pre-clinical development.

Sulfanegen is an antidote undergoing evaluation by Nagasawa, Patterson, *et al* at the University of Minnesota and whose development, characterization, and clinical translation is the focus of this dissertation. Sulfanegen is a prodrug of 3-mercaptopyruvate, the substrate for mercaptopyruvate sulfurtransferase. As discussed previously, this enzyme's catalyzed reaction generates thiocyanate and pyruvate **(Figure 4)**. Sulfanegen has demonstrated efficacy in murine, rabbit, and swine animal models of cyanide poisoning at both toxic sub-lethal and lethal cyanide doses.[97–101] It has also shown efficacy when combined with other antidotes.[102] The development of sulfanegen was recently summarized in the literature.[99] As with cobinamide and DMTS, clinical trials for sulfanegen have not yet commenced.

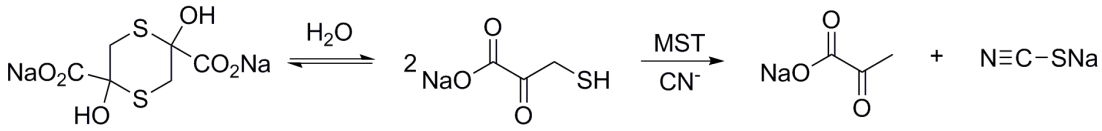


Figure 4. The mechanism of Action of sulfanegen. Sulfanegen disassociates into two molecules of 3MP, which is the substrate for MST. MST catalyzes the conversion of cyanide to thiocyanate.

1.12 Hurdles to Clinical Translation

Key data in the approval of any new drug is obtained via phase III human clinical efficacy studies. However, the ethical issues in evaluating cyanide efficacy in human populations present an additional challenge to the normal drug development process. The United States FDA recognizes this hurdle to clinical translation, and has published "Approval of New Drugs when Human Efficacy Studies are not Ethical or Feasible" (CFR 314 Subpart 1), more commonly referred to as the "animal rule".[103] These guidelines allow reliance on animal-only efficacy data for determining drug approval in cases when the following criteria are met:

1) There is a reasonably well-understood pathophysiological mechanism of the toxicity of the substance and its prevention or substantial reduction by the product;

2) The effect is demonstrated in more than one animal species expected to react with a response predictive for humans, unless the effect is demonstrated in a single animal species that represents a sufficiently well-characterized animal model for predicting the response in humans;

3) The animal study endpoint is clearly related to the desired benefit in humans, generally the enhancement of survival or prevention of major morbidity; and

4) The data or information on the kinetics and pharmacodynamics of the product or other relevant data or information, in animals and humans, allows selection of an effective dose in humans.[103]

Criterion #1, the pathophysiological mechanism of cyanide toxicity, is well documented to be competitive inhibition of cytochrome C oxidase and resulting inhibition of oxidative phosphorylation. In addition, the previous animal model work completed by our lab shows clear benefit to sulfanegen administration in cases of cyanide exposure as well as endpoints clearly related to the desired outcome in humans (criterion #3). However, the two remaining criteria in the "animal rule" remain hurdles to clinical translation. Even though sulfanegen has shown efficacy in multiple animal models of cyanide poisoning, those models must have justification for why the results seen in those studies are expected to be predictive for humans. Additionally, the pharmacokinetics (PK) and pharmacodynamics (PD) must be understood well enough to determine an effective dose in humans. Addressing these remaining hurdles to clinical translation of sulfanegen is a major focus of this thesis.

CHAPTER II:

IN-VIVO MERCAPTOPYRUVATE SULFURTRANSFERASE SPECIES COMPARISON IN HUMANS AND COMMON LABORATORY ANIMALS

1. Introduction

Upon exposure to cyanide, substrates for the endogenous detoxification enzymes MST or rhodanase are rapidly depleted. The resulting inability to detoxify cyanide results in inhibition of cytochrome C oxidase, leads to buildup of lactic acid and resulting metabolic acidosis.[56] Time to death is dose dependent, occurring in minutes to hours if medical countermeasures in the form of either additional substrate (such as thiosulfate) or chelators (hydroxocobalamin) are not administered. Discussion of cyanide's mechanism of toxicity and antidotal approaches are covered in Chapter 1.

Sulfanegen is a prodrug of 3-mercaptopyruvate (3MP), the endogenous substrate for mercaptopyruvate sulfurtransferase (MST), and represents a novel mechanism of action for cyanide antidotes **(Figure 5)**.[101] However, ethical issues of testing the efficacy of chemical threat countermeasures in human clinical trials render phase 2 and 3 efficacy trials impossible. Therefore, additional care must be taken to justify the animal models for efficacy testing in order to determine a response predictive for humans, as laid out in the "animal rule" discussed in chapter 1. For MST based therapies, this would involve characterizing not only the MST activity, but also the localization of the enzyme activity for each species and comparison with human MST activity localization.

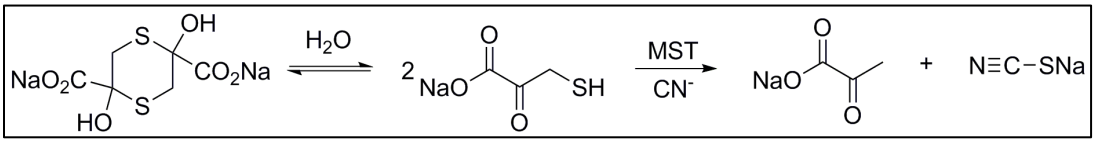


Figure 5. Reaction scheme of sulfanegen (shown here as the sodium salt, sulfanegen sodium) and route of cyanide detoxification by MST.

We report here a species comparison study of MST activity to identify and select appropriate animal models to address point 2 (above). First, MST activity comparison in erythrocytes from commonly used laboratory species was carried out by my colleagues Dr. Daune Crankshaw and Ms. Jacquie Briggs. Based on these data, species with activities closest to that of human blood were more thoroughly examined in clinically relevant tissues. For this, liver and kidney samples were examined because MST is more abundantly expressed in these organs compared to others.[5] These data should help selection and justification of animal models for sulfanegen use in humans.

2. Methods

All procedures involving collection of blood and tissue from animals were reviewed and approved by the University of Minnesota Institutional Animal Care and Use Committee (IACUC). The use of de-identified human organ samples from deceased individuals was ruled exempt by the University of Minnesota Investigational Review Board.

2.1 Chemicals

Tris-HCL, sodium cyanide, iron (III) nitrate nonahydrate, sucrose, ethylenediaminetetraacetic acid (EDTA), mammalian protease inhibitor cocktail, dithiothreitol (DTT), nitric acid, potassium chloride, and formaldehyde solution (38%) were purchased from Sigma Aldrich Co. (St. Louis, MO, USA). Sodium mercaptopyruvate was synthesized by Dr. Alexandre Monteil as described previously.[101]

2.2 Blood

Fresh whole blood samples from healthy animals were collected in K_2 EDTA coated BD Vacutainer tubes, transported on ice, and centrifuged at 1,500 x g for 10 minutes. The pellet containing red blood cells (RBC's) was suspended in isotonic saline for manual hemocytometer counting and subsequently ruptured by freeze/thaw cycling prior to assay. All species had n=6 for both sexes, except for male Wistar rat (n=5), male human (n=8), male and female NZW rabbit (n=3), and male and female Yorkshire pig (n=4 and 5 respectively).

2.3 Tissues

Liver and kidneys from healthy Swiss-Webster mice (n=8), C57BL/6 mice (n=8), New Zealand White (NZW) rabbits (n=8), and Yorkshire pigs (n=3) were excised and snap-frozen at -80 °C until ready for homogenization. Human samples from smokers (n=6), nonsmokers (n=7), and those who had previously smoked but successfully quit (n=5) were obtained from the National Disease Research Interchange (NDRI). The homogenization buffer consisted of 200 mM sucrose, 1 mM EDTA, 2 mM DTT, 1.15% potassium chloride, Protease Inhibitor Cocktail, and 20 mM Tris-HCl pH 7.4. Samples were homogenized using a Fisher Scientific PowerGen 125 homogenizer, and centrifuged at 650 x g for 10 minutes at 4 °C to remove cellular debris. The supernatant was collected and centrifuged at 17,000 x g for 10 minutes at 4 °C. The resulting supernatant was collected as the cytosolic fraction, and the pellet was suspended in homogenization buffer as the mitochondrial fraction. Samples were frozen at -80 °C until ready to assay.

2.4 MST Enzyme Assay

2.4.1 Blood

MST activity in erythrocytes was measured according to the method of Valentine et al.[104] Enzyme units were defined as μmoles of pyruvate generated per minute per 10^{10} RBC's at 37 °C under assay conditions.

2.4.2 Tissues

Tissue cytosolic or mitochondrial fractions were diluted to a 1 mg / mL working solution in protease inhibitor as described below. The enzyme activity was then determined by the method of Taniguchi and Kimura.[105] Specific activity was defined as μmoles thiocyanate formed per mg protein per minute.

2.5 Protein Assay

Protein concentrations were determined by Bradford assay using bovine serum albumin as a standard.[106]

2.6 Statistical Analysis

The data are expressed as the mean ± Standard Deviation (SD). Significance differences among the groups were determined by either unpaired t-test with Welch's correction or one-way ANOVA and followed by Dunnet's test where appropriate. The software GraphPad Prism (Version 6.0, San Diego, CA, USA) was used for statistical analysis and the statistical significance was set at $p < 0.05$.

3. Results

MST activity in blood was detectable in all species tested except for swine. In this species the blood MST activity was below the limit of detection (**Figure 6**). There were no significant differences between sexes within a species except for

NZW rabbits (p=0.0050) when analyzed using an unpaired t-test with Welch's correction. Human activity (113.3 +/- 7.5) was flanked by Wistar rat (430.0 +/- 43.7) and Swiss-Webster mouse (140.1 +/- 16.8) being greater than human, and male NZW rabbit (72.3 +/- 6.0), macaque (40.8 +/- 3.6), and C57BL/6 mice (38.6 +/- 18.1) being less than human. The data for all species were statistically significant from human when analyzed using a one-way ANOVA with Dunnett's test.

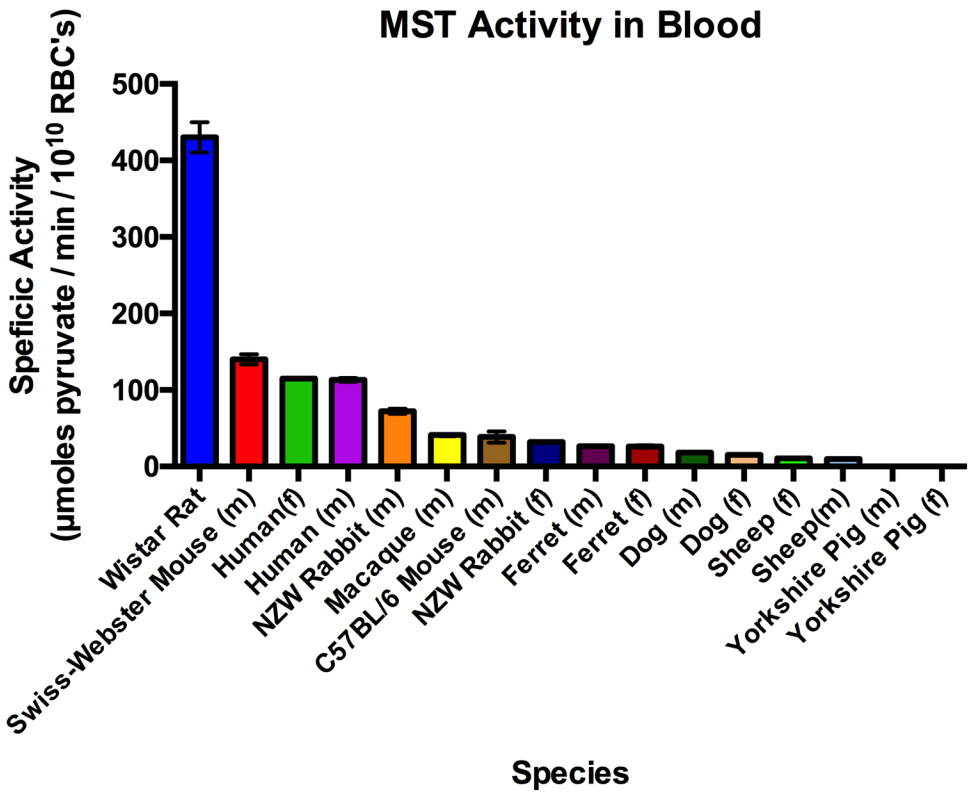


Figure 6. Species Comparison of MST activity in RBC. Enzyme units were defined as µmoles of pyruvate generated per minute per 10^{10} RBC's at 37 °C under assay conditions. Data were collected by Briggs and Crankshaw, and are plotted as mean +/- SEM.

Using the results from the RBC assay, male NZW rabbit, Swiss-Webster mouse, and C57BL/6 mouse were chosen to further quantify MST activities in the liver and kidneys as they were the species with the closest activity to humans among those tested. Choosing two different mouse breeds also allows for investigation into how activity might vary between different breeds of the same species, and could illustrate the importance of breed selection within species. The Yorkshire pig was also chosen because although the RBC MST activity in that model was below the limit of detection for our method, sulfanegen was demonstrated effective in rescue of Yorkshire pigs from a lethal dose of cyanide. The results from the liver specific MST comparison are summarized in **Figure 7**. NZW rabbit had the highest activity in cytosolic samples (0.84 +/- 0.15) and were similar to human (0.69 +/- 0.12) and C57BL/6 mouse (0.48 +/- 0.12). In the mitochondrial liver samples, Swiss-Webster mouse had the highest activity (0.59 +/- 0.06), and both NZW rabbit (0.33 +/- 0.09) and C57B/6 mouse (0.36 +/- 0.06) were similar to human (0.26 +/- 0.03). MST results from the kidney samples are summarized in **Figure 8**. Swiss-Webster mouse had the highest cytosolic activity (0.49 +/- 0.08) followed by NZW rabbit (0.40 +/- 0.10), humans (0.17 +/- 0.04), and C57BL/6 mouse (0.14 +/- 0.08). NZW rabbit had the closest kidney mitochondrial activity to humans (0.18 +/- 0.04 and 0.14 +/- 0.04 respectively). Interestingly, Yorkshire pig had very low MST activity once again in all samples tested. A one-way ANOVA with Dunnett's test to compare samples to human activity showed no statistical difference from human in the liver mitochondrial fraction from NZW rabbits, and in the cytosolic samples from the Swiss-Webster

mouse. In the kidney, NZW rabbits were once again statistically non-significant for the mitochondrial, as was the C57BL/6 mouse for the cytosolic fraction.

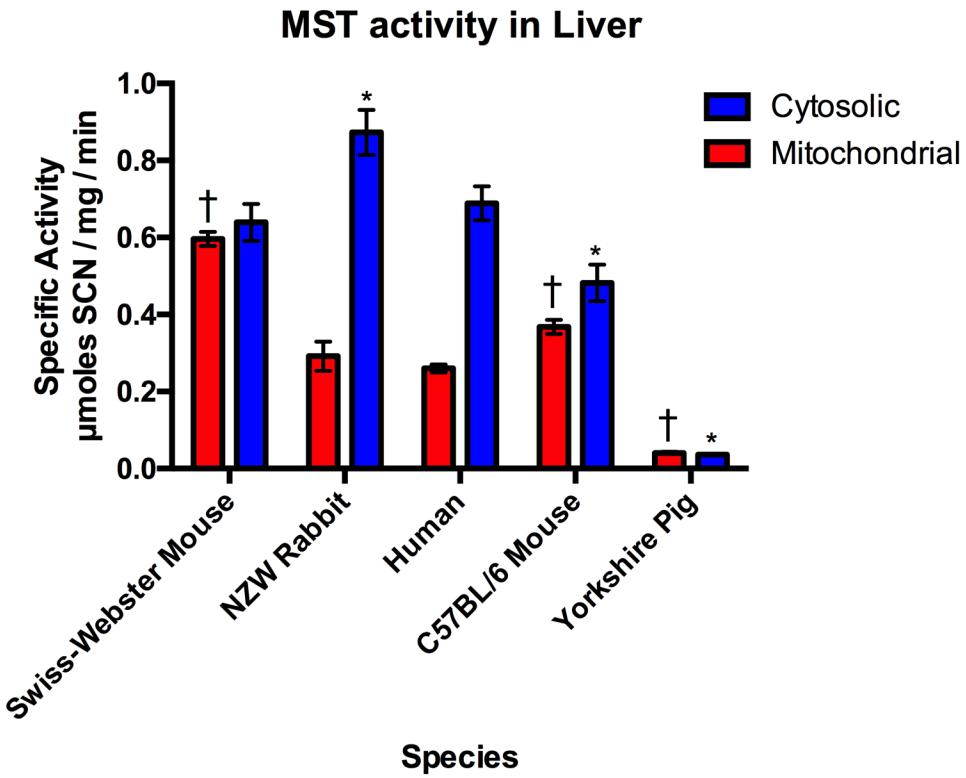


Figure 7. Mercaptopyruvate Sulfurtransferase liver production by subcellular fraction and species. Data are plotted as mean +/- SEM. Specific Activity was defined as µMoles SCN produced per mg protein per minute under assay conditions. * Signifies statistical significant differences from human cytosolic samples, while † signifies statistically significant differences from human mitochondrial samples.

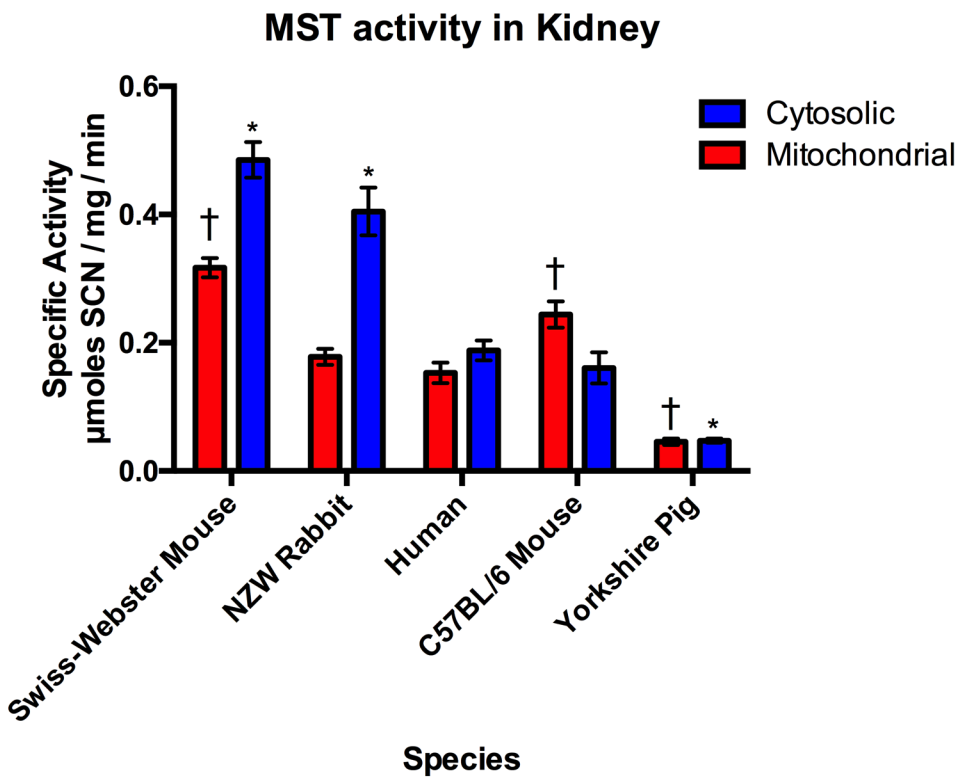


Figure 8. Mercaptopyruvate Sulfurtransferase kidney production by subcellular fraction and species. Data are plotted as mean +/- SEM. Specific Activity was defined as µMoles SCN produced per mg protein per minute under assay conditions. * signifies statistically significant differences from human cytosolic samples, while † signifies statistically significant differences from human mitochondrial samples.

Environmental factors may also influence MST activities. Cigarette smoke contains cyanide, and as previously reported, cyanide levels in blood peak immediately following smoking a cigarette (0.484 +/- 0.100 µmol/L compared to 0.125 +/- 0.035 µmol/L basal levels).[107] It is unknown what effects, if any, this chronic ingestion may have on the endogenous cyanide detoxification pathways. To examine the effects of chronic, low-level cyanide exposure on MST activity, human samples from smokers, non-smokers, and those who had previously smoked but quit were analyzed separately. In the liver, there was no change

seen in cytosolic MST activities between the three groups. However, in the mitochondria, the p value between the smokers and nonsmokers was statistically significant (p=0.0003) using a one-way ANOVA with Dunnett's test. This is summarized in **Figure 9**. No significant differences were seen in the kidney samples between the three groups.

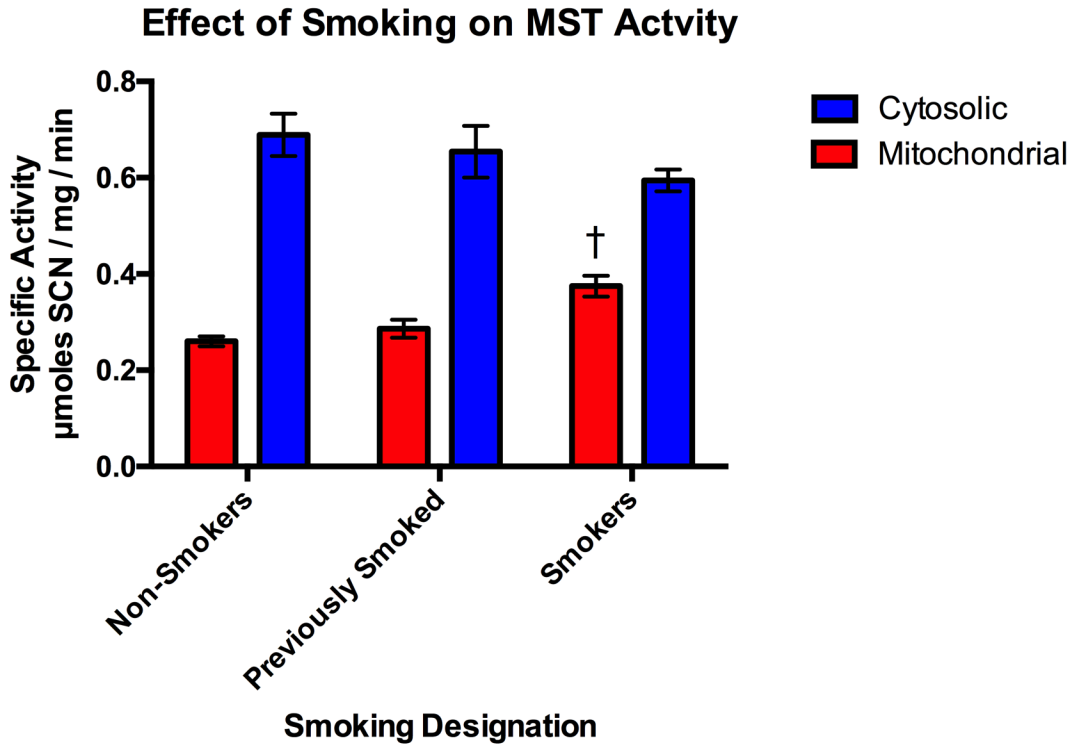


Figure 9. Effect of smoking on liver MST activity. Data are plotted as mean +/- SEM. Specific activity is defined as µMoles SCN produced per mg protein per minute under assay conditions. † signifies statistically significant differences from non-smoking mitochondrial samples.

4. Discussion

Ideal animal models should closely reflect human system intended to model. For cyanide, this involves mirroring both the signs and symptoms (lactic acidosis, apnea, cardiac arrest and death) seen in human exposure. Wide diversity exists in the models used for cyanide research. There are differences in

cyanide administration mode, cyanide dose, and endpoint (i.e. lethality, recovery, neurological condition). These models are summarized in **Table 2.**

Group	Species/Breed	Administration	Dose	Reference
Canine	Beagle	IV	Lethal	[108]
	Beagle	IV	Lethal	[109]
	Beagle	IV	Non-Lethal	[109]
Feline	Domestic Cat	IV	Non-Lethal	[110]
Murine	C57BL/6	IP	Lethal	[102]
	C57BL/6	Inhalation	Lethal	[102]
	NSA	IP	Non-Lethal	[111]
	Swiss Webster	IP	Non-Lethal	[112]
	Swiss Webster	IP	Non-Lethal	[113]
Rabbit	NZW	IV	Non-Lethal	[92]
	NZW	IV	Lethal	[114]
	NZW	Oral	Lethal	[93]
Swine	Yorkshire	IV	Lethal	[115]
	Yorkshire	IV	Lethal	[116]
Aquatic	Zebrafish	Environmental	Lethal	[117]

Table 2. Common animal models of cyanide intoxication.

The above species are reasonable models for cyanide exposure, since the symptoms following such exposure closely resemble those seen in humans, including lactic acidosis, apnea, and when appropriate death, with neurological deficit among survivors. However, animal models of antidotal treatment must take into account not only that the animal represents a reasonable model of human exposure to cyanide, but should also take into account the antidotal mechanism of the drug.

In our judgment, in addition to being an appropriate model for cyanide intoxication, a reasonable animal model for evaluation of sulfanegen should also mimic the human metabolic pathways of the drug. Therefore the model's expression of MST should be similar to the human expression, both in degree

and distribution of MST activities. An animal model that fits the above should then reasonably allow prediction of human cyanide antidotal efficacy and toxicology. However, the Yorkshire pig (swine) model appears to be an exception, despite our reproducible observation that sulfanegen treatment fully protected Yorkshire pigs from lethal doses of cyanide swine have very low MST activity.[116] Possible explanations for this observation could be that a low level of MST activity is sufficient, or that secondary mechanisms could offset the decreased thiocyanate production.

5. Conclusions

A careful consideration of both overall MST activity and intracellular compartmentalization of this activity is imperative in the selection of appropriate animal models for consideration under the animal rule. However, because no animal model tested was a perfect match to humans, demonstrated antidotal efficacy in animals with MST activity flanking both above and below that of humans lends credibility to predicted efficacy in humans. We report the comparability of NZW rabbits and C57BL/6 mice to humans, since MST activities in these species were most similar to humans in physiologically relevant tissues. The Swiss-Webster mouse comparability to human blood activity should also warrant consideration when choosing an animal model, as there may be an advantage to using both murine breeds as models. Although sulfanegen rescued Yorkshire pigs from a lethal dose of cyanide, the low levels of MST activities we observed in blood as well as in the liver and kidneys exclude it from further consideration. Nevertheless, this effect suggests efficacy could be expected even

in MST deficient humans. In addition, the metabolic fate of thiocyanate, the product of sulfanegen's action on cyanide, should also be seriously considered. Thiocyanate is excreted in urine, thus animal models should eliminate thiocyanate via renal excretion with half-lives similar to humans. Although we only addressed MST location and activity here, these data should be consolidated with the thiocyanate elimination data to more accurately identify the justified species for MST efficacy. Comparative thiocyanate kinetics will be addressed in due course.

This account was published in Toxicology Letters:

Moeller BM, Crankshaw DL, Briggs J, Nagasawa H, Patterson SE. In-Vitro Mercaptopyruvate Sulfurtransferase Species Comparison in Humans and Common Laboratory Animals. Toxicol Lett. 2017. doi:10.1016/j.toxlet.2017.04.005.[118]

The author of this dissertation's contribution to the above paper was the liver and kidney follow-up studies to blood MST results. This involved the procurement, homogenization, differential centrifugation, data collection and analysis for tissue specific species comparison, as well as intellectual input on selection of appropriate species for the liver and kidney studies.

CHAPTER III:

HPLC METHOD DEVELOPMENT FOR

PHARMACOKINETICS OF SULFANEGEN

1. Introduction

Upon absorption, a drug must be distributed to the target in order to achieve the therapeutic effect; absorption into the blood must then be followed by exit of the drug from the cardiovascular system (except for cardiovascular drugs e.g. antihypertensives), entry to targeted system and this process involves crossing multiple biological or cellular membranes. This complex interaction between the body and drug is mediated by the physiochemical properties of the drug: size, charge, and hydrophobic properties determine efficiency in crossing biological membranes and distribution to the target.[119] Additionally, the desired effect can only be achieved as long as an effective concentration is maintained at the target. For most cases, concentration of the drug is decreased by metabolization into an inactive form and/or excretion in urine.[119] The study of the rate of drug absorption, distribution, metabolism, and excretion is pharmacokinetics (PK). Characterizing PK is an integral part of the drug development process,[120] should help explain sulfanegen's rapid reversal of cyanide toxicity, and will be required to satisfy the animal rule.

The second condition of the "Animal Rule" for drug approval is that the pharmacokinetics are understood well enough to determine an effective dose.[103] The Logue and Patterson labs recently published pharmacokinetic data for sulfanegen in cyanide naïve rabbits **(Figure 10)**.[122] Plasma concentrations of 3MP were 1775 μM at C_{max}, with $T_{1/2}$ of nearly 2 hours. Furthermore, efficient catalytic turnover requires a sulfur acceptor such as cyanide. In the absence of such an acceptor, enzyme bound persulfide accumulates – rendering the

enzyme unable to catalyze the desulfuration of 3MP. This inhibits further metabolism of 3MP, establishing the need for characterizing PK both with and without concurrent cyanide administration.

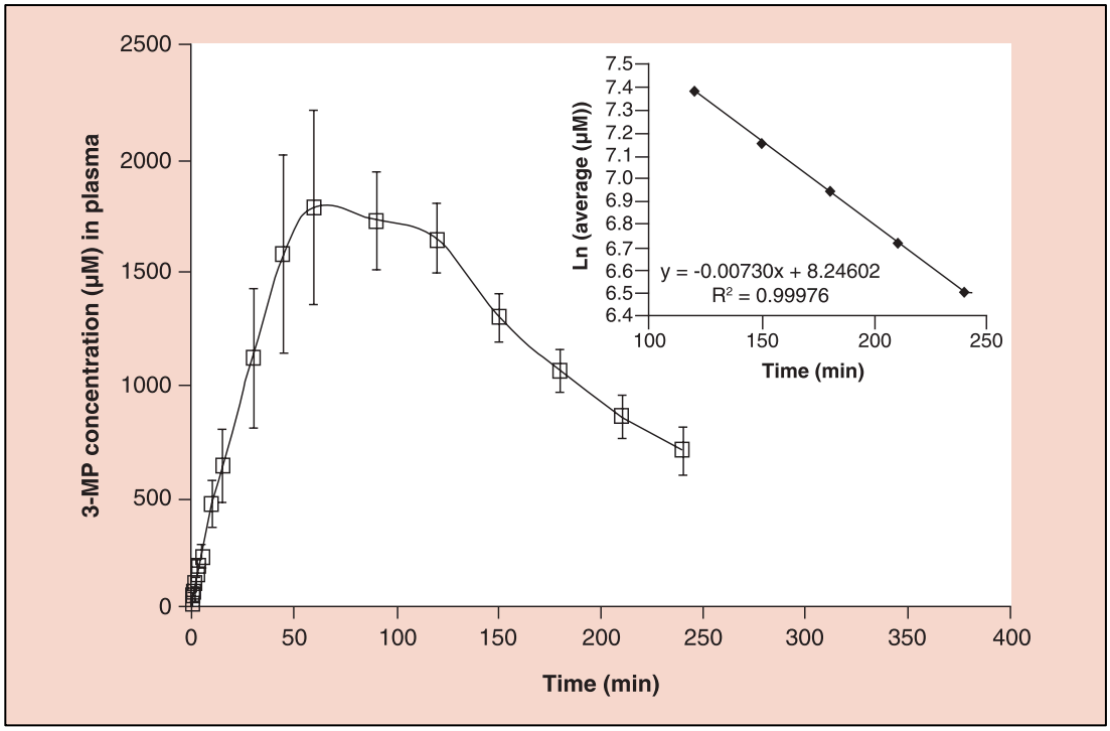


Figure 10. Pharmacokinetics of Sulfanegen in rabbits. Plasma concentrations of 3MP in the absence of cyanide were 1775 µM at C_{max}, with $T_{1/2}$ of nearly 2 hours. Image from reference [122]. Permission not needed for educational use.

Finally, an additional need for clinical translation of sulfanegen is a validated stability-indicating method that can separate sulfanegen from precursor molecules used in synthesis (bromopyruvic acid), as well as decomposition products such as β-hydroxypyruvic acid). This is necessary for quality assurance of the drug as well as determination of shelf-life and acceptable storage conditions. Here, we describe a simple UV-VIS HPLC method for sulfanegen detection. The method is suitable for sulfanegen purity and stability

determination, and is amenable to biological specimens such as human plasma with minimal modification.

2. Methods

2.1 Reagents

All solvents were HPLC grade unless stated otherwise. Sulfanegen Sodium was synthesized in our lab by Dr. A Monteil and Dr M Baran as described previously.[97] N-ethylmaleimide (NEM), monobromobimane (MBB), β-hydroxypyruvic acid, bromopyruvic acid, and Sulfuric Acid were purchased from Sigma-Aldrich (Saint Louis, MO, USA), Acetone and acetonitrile (ACN) was purchased from Fischer Scientific (Waltham, MA, USA). Maleic acid was purchased from Sigma-Aldrich (Saint Louis, MO, USA) and used as an internal standard. A 2.5 mM standard solution of Sulfanegen sodium (resulting in 5 mM 3-MP) was freshly prepared and serially diluted in Millipore filtered water or plasma. Commercial reagents were used without further purification.

2.2 Biological fluids

Pooled plasma from n=12 humans was purchased from Memorial Blood Centers (St. Paul, MN) and stored at -80 °C until ready to use.

2.3 Preparation of Samples

2.3.1 Biological Samples

Sample preparation followed the method of Stutelberg *et al*, with the following modifications: NEM derivitization rather than MBB, maleic acid as an internal standard, and reconstitution in deionized water containing NEM.[114] Blank or 3MP spiked human plasma (100 µL) and 50 µM maleic acid (internal standard) were added to a 1.5 µL microcentrifuge tube. Plasma proteins were precipitated

by addition of 300 µL acetone followed by centrifugation at 16,500 x g for 30 minutes at 4 °C. The supernatant was dried under N_2 and reconstituted in 100 µL NEM followed by incubation at room temperature for 15 minutes. NEM derivitizes thiols, which prevents reformation of the reversible 3-MP dimer **(Figure 11)**. Non-derivitized 3MP produces chromatograms unfit for quantification because the equilibrium between the sulfanagen dimer and 3MP monomer results in unacceptably broad peaks. Prepared samples were stored at 4 °C until ready for HPLC analysis.

2.4 Biological HPLC Analysis of 3MP-NEM

An Agilent 1200 series HPLC coupled to an Agilent G1315B diode array detector (DAD) was used for UV/Vis HPLC analysis. Separation was performed on a Phenomenex ROA Organic Acid H+ (8%) column (300 x 7.8 mm, 8 µM) with an injection volume of 10 µL. The mobile phase consisted of isocratic 0.0075 M H_2SO_4 with 15% acetonitrile at a flow rate of 0.5 mL / min. Column temperature was set to 80 °C, with a total run time of 30 minutes. All analytes were detected by absorption at 212 nm.

2.5 Purity and Stability HPLC Analysis of 3MP-MBB

An Agilent 1200 series HPLC coupled to an Agilent G1315B diode array detector (DAD) was used for UV/Vis HPLC analysis. Separation was performed on a Phenomenex ROA Organic Acid H+ (8%) column (300 x 7.8 mm) with an injection volume of 10 µL. The mobile phase consisted of isocratic 0.01 M H_2SO_4 at a flow rate of 0.5 mL / min. Column temperature was 70 °C, with a total run time of 20 minutes. Synthesized sulfanegen was derivitized with

monobromobimane (MBB) by heating at 70 °C for 15 minutes in deionized water. All analytes were detected by absorption at 212 nm.

2.6 Calibration, Quantification, and Limit of Detection

For validation of the method, the FDA bioanalytical method validation guidelines were followed.[123] The initial calibration curve was prepared with 0.5 µM-10 mM 3MP-NEM standards in plasma (0.5, 1, 2, 3, 4, 5, 7.5, 10, 25, 50, 100, 250, 500, 1000, 2000, 3000, 4000, 5000 µM) to determine linear range. 3MP-NEM calibration curves were created in both plasma and water to determine effects of the biological matrix. Quality Controls (n=5) were prepared at 3 concentrations that were not on the calibration curve: 250 µM (high QC), 75 µM (mid QC), and 25 µM (low QC). Both internal standard and QC samples were prepared fresh daily for intra and inter-assay accuracy and precision studies. Precision was determined as %RSD.

Limit of detection was defined as the lowest concentration that regularly gave a signal to noise ratio of at least 3, with baseline noise directly adjacent to the 3MP-NEM peak. 3MP is naturally present in the blood of humans, but is below the limit of detection for this assay.

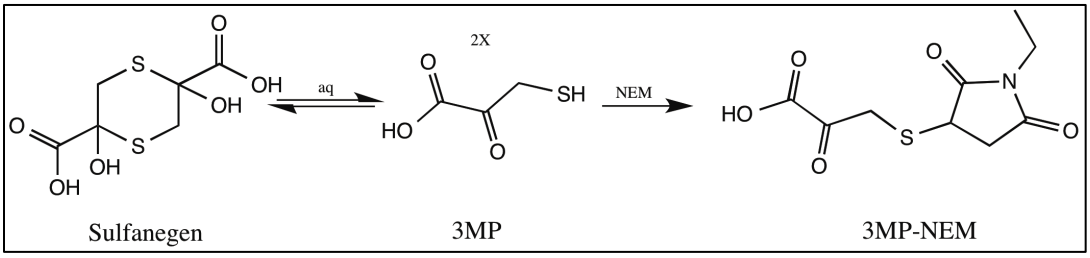


Figure 11. Derivitization of 3MP prevents reformation of the sulfanegen dimer. Without derivitization, reversible dimer formation creates chromatographic peaks unfit for detection. The NEM derivitization reaction is shown here.

2.7 Recovery

Low (25 μM), mid (50 μM), and high (250 μM) concentration 3MP quality controls (n=3) were added to plasma prior to sample preparation. Percent recovery of 3MP was determined by dividing the method determined plasma concentration by the known concentration added.

3. Results and Discussion

3.1 HPLC analysis of 3MP in human plasma

Irreversible derivitization of the 3MP monomer is required to trap the 3MP monomer therefore preventing reformation of the sulfanegen dimer. Commercial reagents are readily available for thiol derivitization, and are reviewed elsewhere.[124] Multiple thiol-specific derivitization compounds were tested, and NEM was selected for biological samples because it gave the best separation from endogenous peaks in the blank human plasma controls. **Figure 12** shows representative chromatograms of IS alone, IS spiked plasma, and 3MP-NEM + IS spiked plasma. The 3MP-NEM derivative eluted at 12.9 minutes, maleic acid IS eluted at 11.0 minutes, and any excess unreacted NEM eluted at 27 minutes. The method has good selectivity and no endogenous plasma peaks co-elute with peaks of interest.

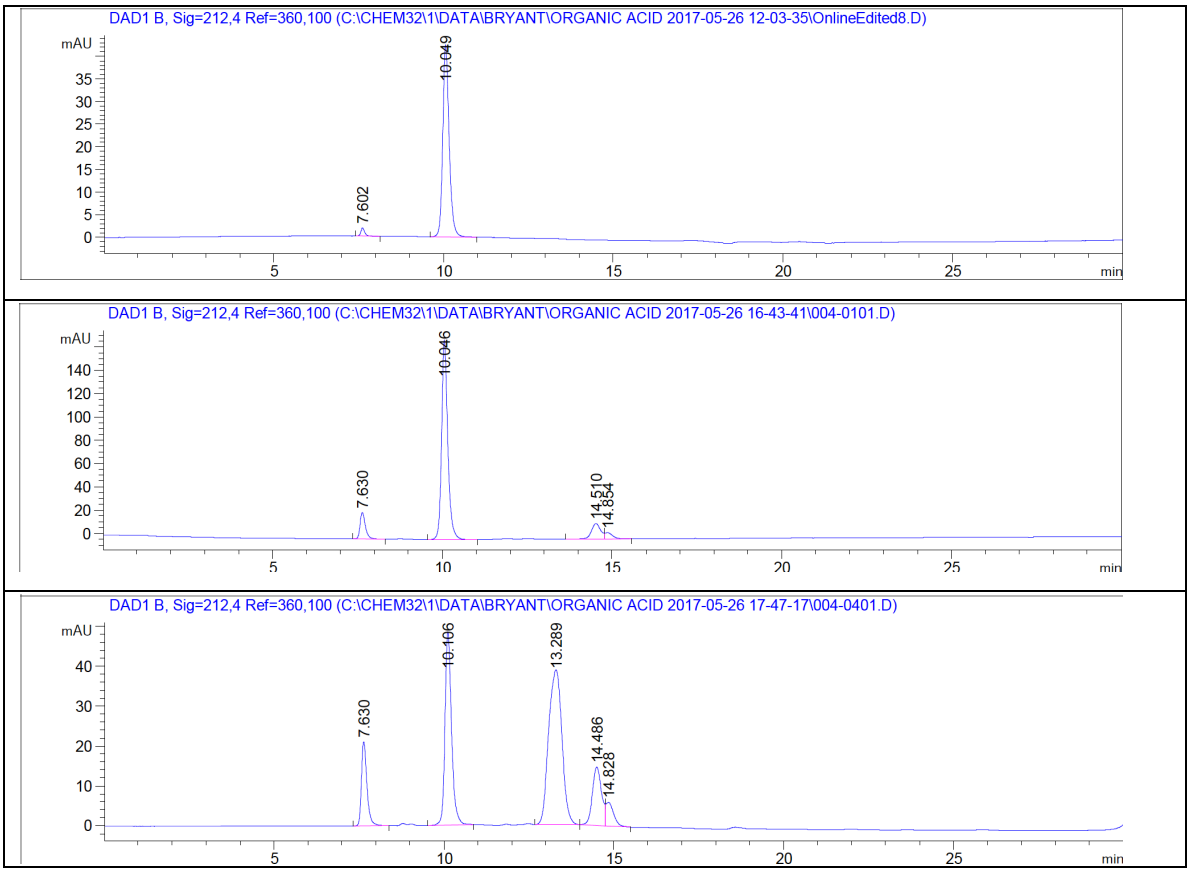


Figure 12. Representative chromatograms. Top: Maleic acid (IS) alone. Middle: IS spiked human plasma. Bottom: 3MP-NEM IS spiked human plasma. Maleic acid elutes at 10.0 minutes, while 3MP-NEM elutes at 13.2 minutes.

3.1.1 Linear range, limit of detection, and sensitivity

Calibration curves of 3MP were prepared in the range of 0 to 5,000 µM in human plasma. The signal ratio (peak area / IS peak area) was used as the corrected signal. The linear range for this method was determined to be 5 µM to 1,000 µM with an R^2 of 0.99997. The limit of detection for the method was 5 µM 3MP-NEM. Matrix effects between calibration curves compared in plasma and water were minimal.

3.1.2 Accuracy and precision

Accuracy and precision were determined by analysis of low, mid, and high quality controls (n=5) on three consecutive days (**Table 3**). The intra-assay accuracy (<5%) and precision (<3) as well as the inter-assay accuracy (<5%) and precision (<6) were sufficient for detecting small molecules in biological matrices.

Concentration (μM)	Intra-assay accuracy	Intra-assay precision	Inter-assay accuracy	Inter-assay precision
25	-0.79%	0.12	+2.9%	3.96
75	+4.3%	0.36	+0.26%	5.81
250	+4.9%	2.8	+4.7%	3.03

Table 3. Accuracy (%) and precision (RSD) of method for detecting 3MP in spiked human plasma.

3.1.3 Recovery

Recovery from biological samples was poor (<40%), but consistent. 3MP is inherently unstable at biological pH, and although the same sample preparation method was used as Stutelberg *et al* in rabbits, recovery from human plasma samples were lower than in that method (75%). Incomplete recovery can be explained by MST conversion in the plasma, and represents an area for improvement. Human plasma MST activity from chapter 1 was 114 +/- 5.0, while NZW rabbit MST activity for males was 72.3 +/- 6.0 and for females was 32.0 +/- 1.5. The sex of the individual rabbits in the Logue study was not provided with the samples; this difference may explain the recovery differences seen in the present study if samples from both male and female rabbits were included.

3.2 HPLC analysis of 3MP Purity and Stability

For the stability and purity indicating method, the derivitization reagent of choice was MBB. MBB yielded the best limit of detection while shifting the elution time of 3MP far from either β-hydroxypyruvic acid, bromopyruvic acid **(Figure 13)**. 3MP-MBB elutes at 8.1 minutes, while β-hydroxypyruvic acid and bromopyruvic acid elute at 11.1 and 12.9 minutes respectively. The limit of detection of 3MP-MBB was 50 nm, while the limits of β-hydroxypyruvic acid and bromopyruvic acid were 2.4 and 50 μM respectively. With sufficient distance between elution time of 3MP derivitized with MBB and either β-hydroxypyruvic acid or bromopyruvic acid, the relatively low limits of detection of bromopyruvic acid can be overcome by injection of larger sample volume to the column.

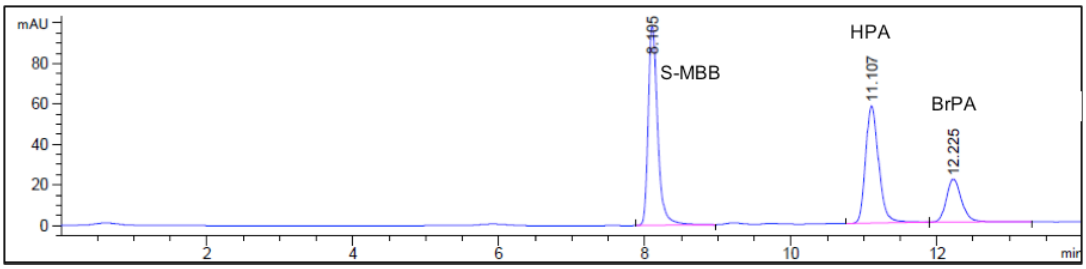


Figure 13. Representative chromatograms for stability and purity indicating method. Chromatogram is 100 μM 3MP-MBB (S-MBB), 500 μM β-hydroxypyruvic acid (HPA) and bromopyruvic acid (BrPA).

4. Conclusions

An HPLC method was developed for the quantification of 3MP with applications to both biological quantification and quality control. This simple method employs UV detection and is readily amenable to many laboratories without the need for more expensive detection equipment. The low temperature of the NEM derivitization reaction is also ideal for the quantification of 3MP, due

to 3MP's low aqueous stability at high temperatures. The linear range is also appropriate for biological studies, and plasma concentrations determined from previous studies would require minimal dilutions for analysis. There is room for improvement in regards to recovery of the biological method. Because 3MP increases in stability with decreasing pH beginning at pH 5, acidification before protein removal should yield better results. Additionally, the limit of detection of the quality control method could be improved with further derivitization of the carboxylic acid. This was not pursued further as the column chosen is specific to carboxylic acids, and derivitization would likely require selection of a different column. Moreover, the goal of a reasonable method for quality assurance was achieved.

CHAPTER IV:

SECONDARY MECHANISM ELUCIDATION

1. Introduction

The primary mechanism of action of sulfanegen in cyanide detoxification is MST catalyzed conversion of cyanide to thiocyanate, with 3MP/sulfanegen as the sulfur donor. This mechanism is supported by the observed conversion of cyanide to thiocyanate described Chapter 1, as well as previous studies detailing the selectivity of substrates between MST and rhodanase.[118,125,126] Previously published works from our lab have shown sulfanegen is more efficacious when administered 5 minutes before cyanide in the righting reflex assay than either of the currently approved cyanide antidotes: either a combination of thiosulfate and sodium nitrite (nithiodote™) or hydroxycobalamin (cyanokit™).[101,113] Additionally, when administered 5 minutes after a toxic yet sub-lethal dose of cyanide, sulfanegen was still highly effective, while other cyanide antidotes showed only minimal protection when compared to controls **(Figure 14)**.[101] At twenty minutes post cyanide, sulfanegen reduces recovery time whereas nithiodote™ and cyanokit™ are ineffective at reducing recovery time.[101]

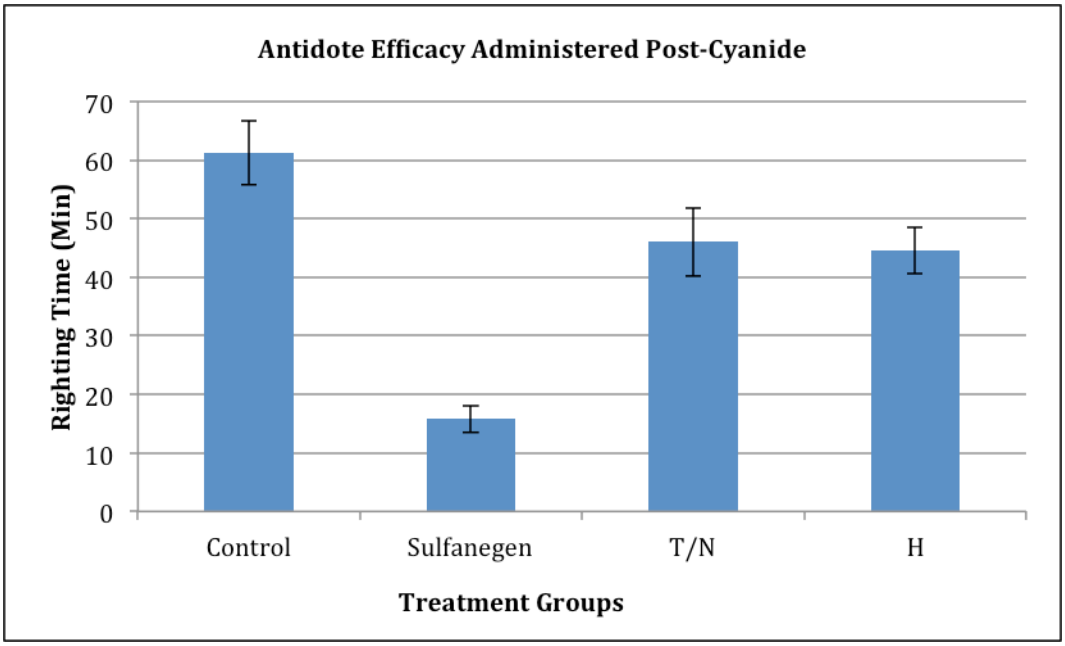


Figure 14. Effects of cyanide antidotes on righting reflex time. Antidotes administered 5 minutes after sub-lethal cyanide dose. Adapted from [101] T/N=Thiosulfate/Nitrite combination H=Hydroxycobalamin

Therefore, sulfanegen appears to be more efficacious and may have a longer treatment window than currently available antidotes. Additionally, since cyanokit™, a chelator, was ineffective when administered 20 minutes after cyanide, it is likely there is no longer free circulating cyanide at that time. This raises the question: why is sulfanegen effective at time points where other antidotes have little or no effect. The stark difference in righting reflex time could be attributed to the ubiquitous distribution of MST, which provides a combination of benefits: 1) being distributed in both the cytosolic and mitochondrial fractions 2) being expressed in the most sensitive tissues to cyanide exposure such as the CNS.[127] However, the possibility of secondary mechanisms of action contributing to overall efficacy has not been ruled out. Elucidating these possible secondary mechanisms and establishing their relative contributions are the focus of this

chapter, and could open the door to designing antidotes that are even more efficacious in the future.

It has previously been demonstrated that in addition to transferring sulfur to cyanide, MST also produces hydrogen sulfide (H_2S).[128] Concentrated H_2S is a potent neurotoxin, but more recent studies have shown lower levels of H_2S act as an endogenous neuromodulator in the brain.[129,130] H_2S induces calcium release,[130] can protect neurons from oxidative stress,[131–134] is a smooth muscle relaxant,[135] vasodilator,[136] and has antioxidant roles in reducing inflammation.[137–140] Of primary interest to us is the ability of H_2S to provide neuronal protection from oxidative stress, which has been postulated to be a downstream effect of cyanide toxicity.[59,141,142] Therefore, if significant amounts of H_2S are also produced by MST following sulfanegen administration, quenching oxidative stress via MST may be a previously unaccounted for mechanism of action that could help explain the differences in efficacy between sulfanegen and other enzyme based antidotes.

In addition to H_2S production, sulfanegen may have other means of quenching cyanide-induced oxidative stress. Thiols are reducing agents and are therefore known to have antioxidant properties of their own,[143] with glutathione being the most common and widely studied.[144–146] During cyanide toxicity, there is a marked decrease in glutathione levels due to the increase in ROS generation.[58] Therefore, it is possible sulfanegen is able to directly decrease ROS since it is a prodrug of a low molecular weight thiol.

Finally, some α-keto acids such as α-ketoglutarate have shown efficacy in protection from cyanide exposure, even though they lack the thiol group required for reduction of ROS or utilization by MST.[58,88,147] Such ketones (and aldehydes) react with cyanide, forming cyanohydrins. 3MP is an α-keto acid, and therefore may form a cyanohydrin.

We hypothesized that sulfanegen efficacy is partly due to secondary mechanisms such as cyanohydrin formation or quenching cyanide-induced reactive oxygen species (ROS) - either indirectly through MST generation of H_2S or directly as a reducing thiol. These mechanisms are summarized in **Figure 15.**

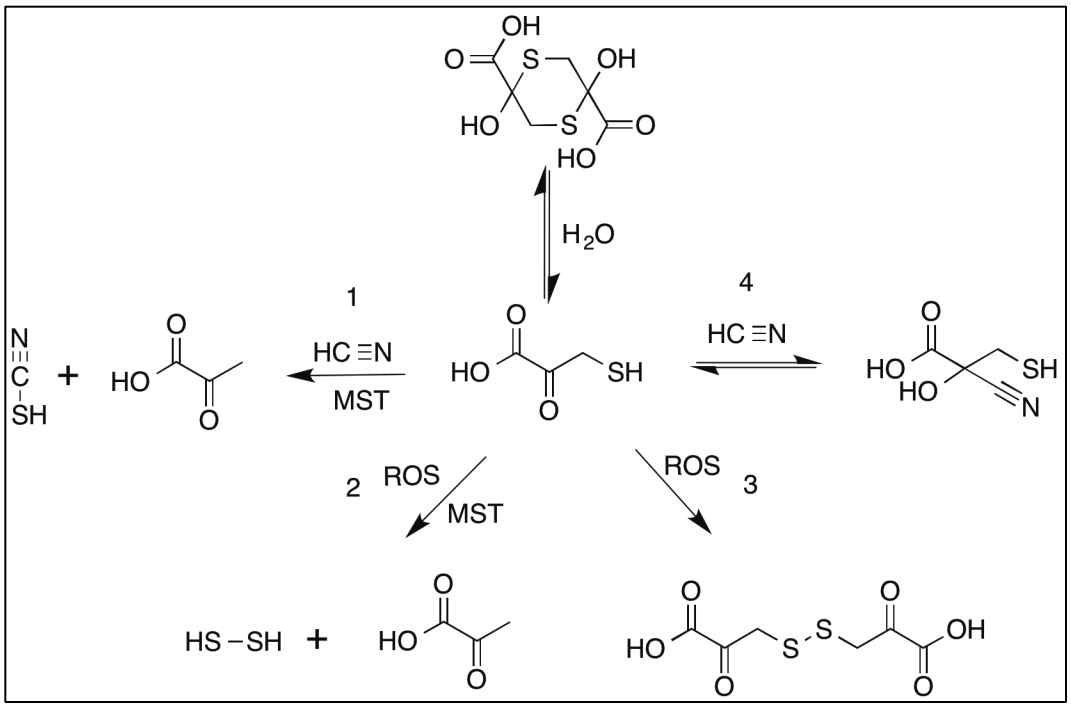


Figure 15. Potential mechanisms of sulfanegen. Sulfanegen disassociates into two molecules of 3MP, which are then able to: (1) act as a substrate for the enzymatic conversion of cyanide to thiocyanate by MST, (2) indirectly quench cyanide induced ROS via MST production of H_2S, (3) directly quench cyanide induced ROS by acting as a reducing thiol, (4) directly detoxify cyanide via reversible cyanohydrin formation.

2. Methods

2.1 Cell Culture

To test the hypothesis that sulfanegen quenches cyanide-induced ROS through generation of H_2S, an immortalized rat mesencephalic cell line, $1RB_3N_{27}$ (N27), was used. This dopaminergic cell line was chosen because it has been used extensively in mechanistic studies of cyanide and other neurotoxin poisoning,[148–151] as well as being the cell type most likely involved in the pathology of Parkinsonian-like symptoms associated with sub-lethal cyanide exposure.[152–154] Cells were plated in Roswell Park Memorial Institute (RPMI) media containing 10% fetal bovine serum (FBS) and 1% penicillin/streptomycin. Cells were grown to 80% confluency as previously described by Adams *et al.*[155]

2.2 ROS Quenching: DDPH Assay

To determine sulfanegen's ability to act as a reducing thiol, the 2,2-diphenyl-1-picrylhydrazyl (DPPH) assay was used to test ROS quenching in-vitro. DPPH exists in solution as a stable free radical with a deep purple coloration (515 nm). This color becomes pale yellow upon quenching of the stable radical by antioxidants, and is therefore a useful indicator of antioxidant efficacy and kinetics by determining changes in absorbance at 515 nm over time.[156–158] The DPPH assay was performed as described by Xie et all.[158] DPPH was freshly dissolved in 50:50 water/methanol to prepare a 0.0913 mM DPPH solution and protected from light with aluminum foil. 3MP, GSH, NAC, or Ascorbic Acid (10 µL of 0.5-500 mM) were added to 1mL of 0.0913 mM DPPH containing cuvette (0.5-500 µM final concentration antioxidant) and the

absorbance at 515 nm was recorded every second for 10 minutes using a SpectroMax M5e spectrophotometer.

2.3 ROS Quenching: DCFH-DA Assay

To determine the in-vivo potential of sulfanegen to quench cyanide induced ROS, 2,7-dichlorofluorescin diacetate (DCFH-DA), which is commonly used as an ROS probe, was employed.[58] DCFH-DA is able to freely pass through the cellular membrane, where it is deacetylated by cellular esterases and trapped within the cell. ROS then oxidizes the non-florescent compound into a florescent product detected at 495_{ex} / 529_{em}. N27 cells were plated 15,000 cells/well in a 96 well plate and incubated overnight. The next day, cells were washed in PBS, and pre-incubated with 100 μM DCFH-DA for 30 minutes. After the pre-incubation, cells were washed with PBS and 0-400 μM 3MP, GSH, or NAC were added along with 500 μM H_2O_2. Florescence was measured with a microplate reader every 5 minutes for 2 hours at 495_{ex} /529_{em} using a SpectroMax M5e spectrophotometer.

2.4 Determination of GSH Concentration

To determine the effects of sulfanegen in the presence of ROS on cellular redox state, GSH concentrations were determined using the Abcam GSH Ratio Detection Assay Kit II (ab205811). Confluent N27 cells were plated in 6 well plates at 1 million cells/well and incubated overnight. The following day, 0-200 μM 3MP were added along with 500 μM H_2O_2 and incubated for 4 hours at 37 °C. After 4 hours, cells were washed in ice cold PBS and lysed with mammalian lysis buffer (Abcam) and protease inhibitor cocktail (Sigma). Following lysis, cells were

centrifuged at 1,500 x g for 5 minutes to pellet cellular debris. The supernatant was assayed according to kit protocol.[159]

2.5 Determination of H_2S Generation

To determine the effects of sulfanegen administration on MST H_2S generation, the H_2S sensitive probe 4-chloro-7-nitrobenzofurazan (NBD-Cl) was utilized. Human liver cytosolic fractions were prepared according to Moeller et al.[118] For the H_2S assay, all solutions were sparged with nitrogen to remove dissolved oxygen. To determine H_2S production following sulfanegen administration, 50 μM NBD-CL was added with 50 μL lysate and 0-500 μM 3MP in 50 mM PIPES, 0.1% ascorbic acid, and 100 mM KCL either in the presence or absence of sodium cyanide or ROS and incubated at 37 °C for 15 minutes. NBD-Cl reacts with H_2S and the resulting fluorescent product was monitored at 534 nm using a SpectroMax M5e spectrophotometer.

2.6 Cyanohydrin formation – analytical chemistry approaches

A 3MP solution (aqueous, 50 mM) was treated with 50 mM aqueous NEM by incubating 15 minutes at room temperature to prevent reformation of the 3MP dimer. This 25 mM stock solution of 3MP-NEM was diluted (water) to 1 mM and incubated with an equimolar concentration sodium cyanide in water at room temperature for 30 minutes. Following incubation, the reaction mixture was analyzed using mass spectrometry (MS) in MMI Mixed Negative mode.

2.7 Cyanohydrin Formation – Cytochrome C Oxidase

To determine if cyanohydrin formation plays a biological role in preventing cyanide inhibition of cytochrome c oxidase, the cytochrome C oxidase assay kit

(CYTOCOX1, Sigma Aldrich) was used. NaCN (0-40 μM in water) was added to the aqueous reaction mixture containing cytochrome and cytochrome c oxidase to quantify inhibition. This was repeated with cyanide being first incubated at room temperature with an equimolar concentration of 3MP-NEM to complex cyanide. Oxidation of cytochrome c was monitored at 550 nm every second for 1 minute using a SpectroMax M5e spectrophotometer.

3. Results

3.1 DPPH Assay

The DPPH assay was performed on NAC, GSH, 3MP, and ascorbic acid in 50:50 methanol/water. There were no discernable differences in the absorbance for any antioxidant tested at 0.5 μM or 1 μM. At higher concentrations, ascorbic acid acted the fastest, reaching equilibrium before measurements were performed. Every ascorbic acid concentration tested above 50 μM fully quenched the DPPH radical (**Figure 16**). 3MP demonstrated the 2nd fastest reaction kinetics; with both 500 μM and 250 μM concentrations reaching complete quenching of DPPH by the end of the reaction time (**Figure 17**). Both NAC and GSH lagged behind 3MP, with even the highest concentrations test not fully quenching the DPPH radical during the time allotted (600 s) (**Figures 18 & 19**). These results are most readily apparent when overlaid (**Figure 20**).

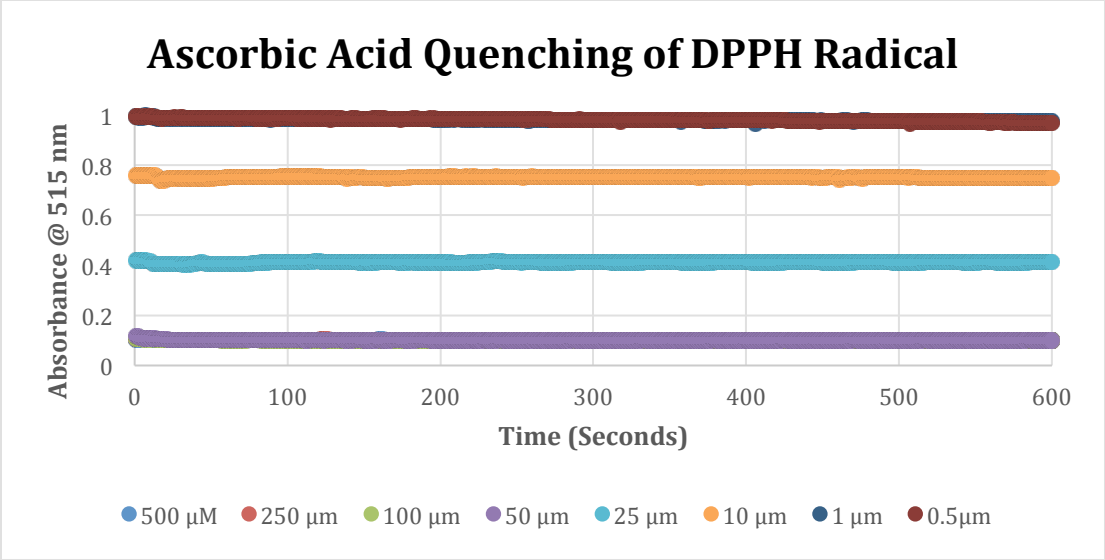


Figure 16. Dose response curve of ascorbic acid quenching the DPPH radical. The reaction was nearly instantaneous and all concentrations tested above 25 µM fully quenched the radical.

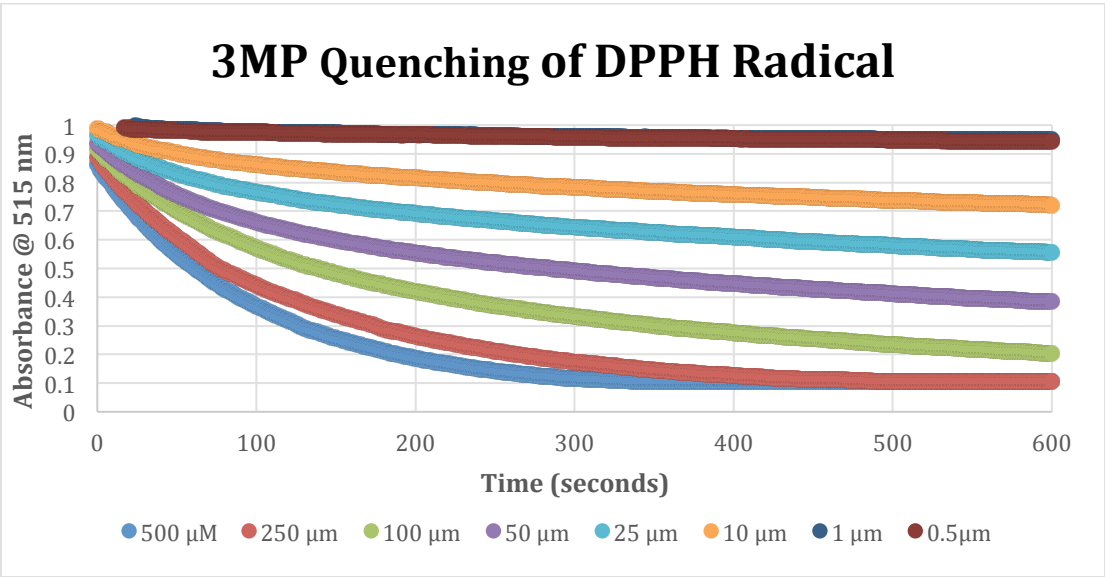


Figure 17. Dose response curve of 3MP quenching the DPPH radical.

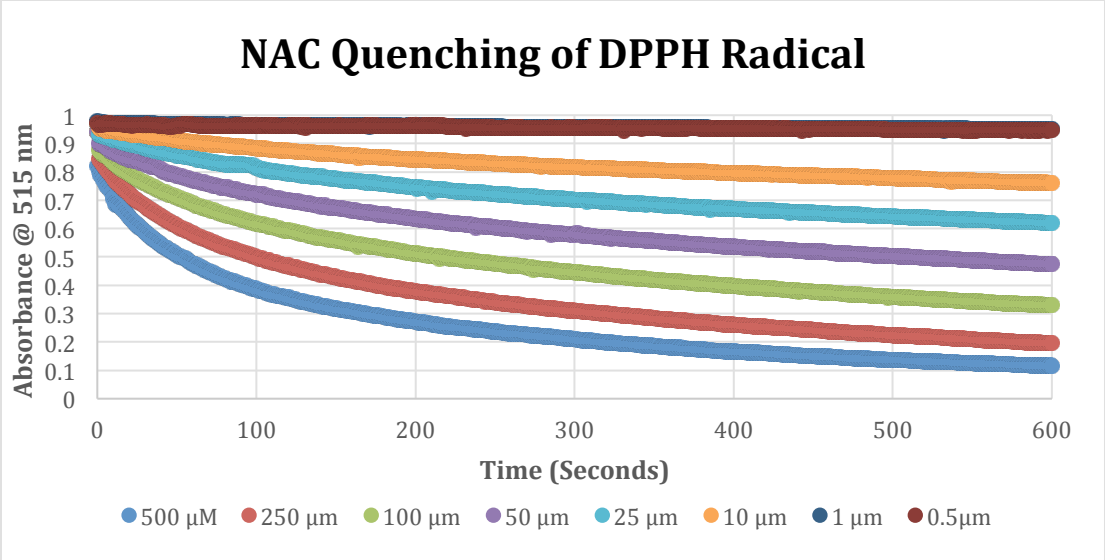


Figure 18. Dose response curve of NAC quenching the DPPH radical.

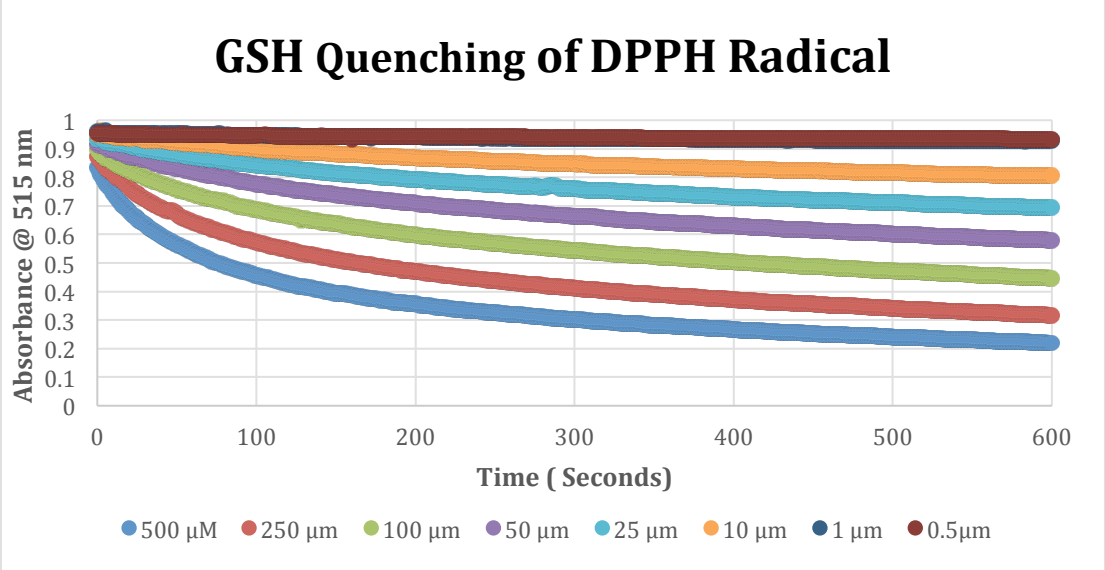


Figure 19. Dose response curve of GSH quenching the DPPH radical.

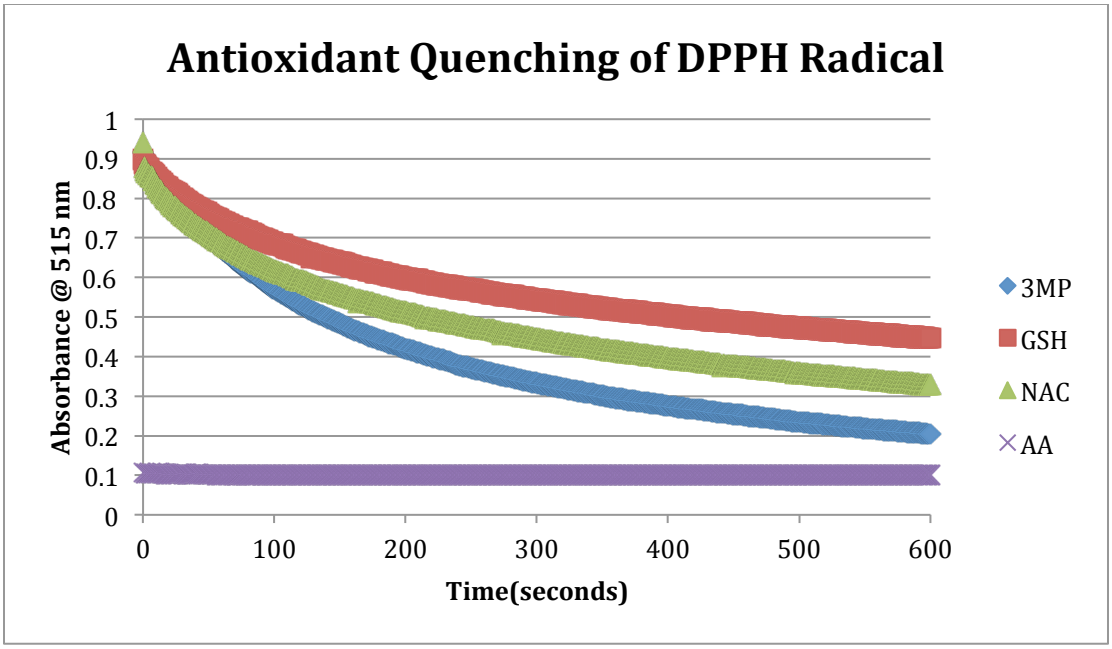


Figure 20. Antioxidant quenching of DPPH radical where concentration of antioxidant are roughly equivalent to DPPH radical (91.3 μM DPPH 100 μM antioxidant). 3MP = 3-mercaptopyruvate, GSH = glutathione, NAC = N-acetylcysteine, AA = ascorbic acid.

3.2 DCFH-DA Assay

Biological relevance of the DPPH radical results was tested in vivo using the DCFH-DA assay to determine intracellular ROS generation. The same general trend of antioxidant activity was seen in the DCFH-DA assay as the DPPH radical scavenging assay. Out of the three compounds tested, 3MP demonstrated the greatest antioxidant activity, followed by NAC and GSH (~80%, 60%, and 40% reduction in relative florescence at 100 μM respectively) as shown in **Figure 21**. This is consistent with 3MP's antioxidant activity in vitro being enhanced by MST's ability to produce H_2S.

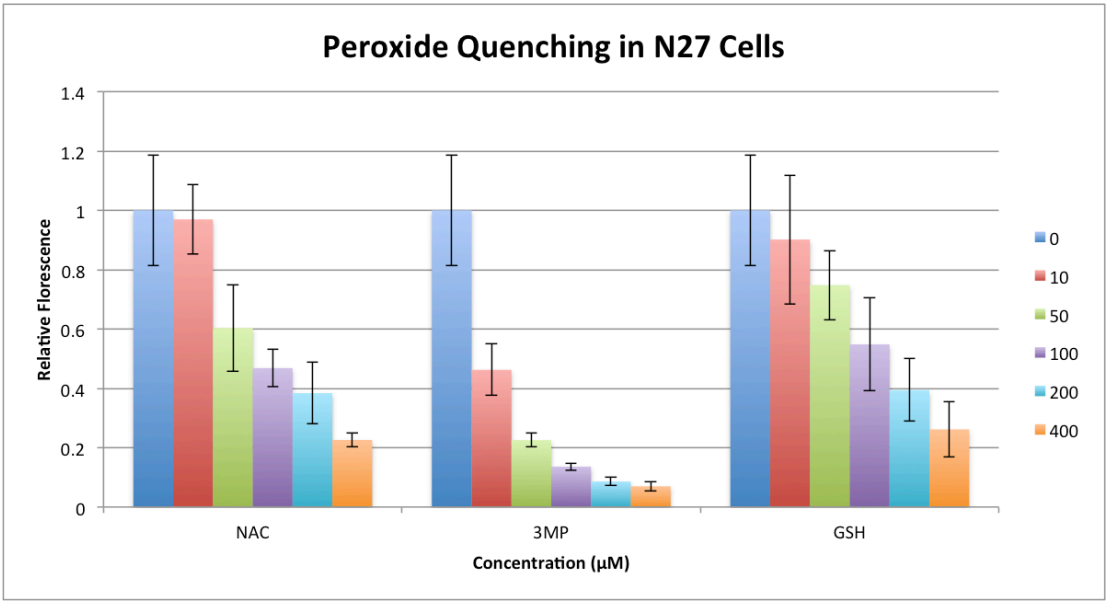


Figure 21. Dose response of ROS quenching by NAC, 3MP and GSH in N27 cells using the DCFH-DA assay.

3.4 GSH Concentration

Sulfanegen's antioxidant ability resulting in quenching ROS was evaluated by measuring GSH concentration in N27 cells. As sulfanegen concentration increased from 0 or 50 to 200 µM intracellular GSH increased **(Figure 22)**. While 200 µM 3MP was unable to fully recover GSH levels to those in the negative control (no H_2O_2 added), higher concentrations of 3MP may yield full recovery. Overall, higher concentrations of 3MP correlated to higher concentrations of GSH, demonstrating lower overall oxidative damage to the cells as 3MP quenched ROS. This is consistent with antioxidant properties of 3MP contributing to 3MP's mechanism of cyanide antagonism.

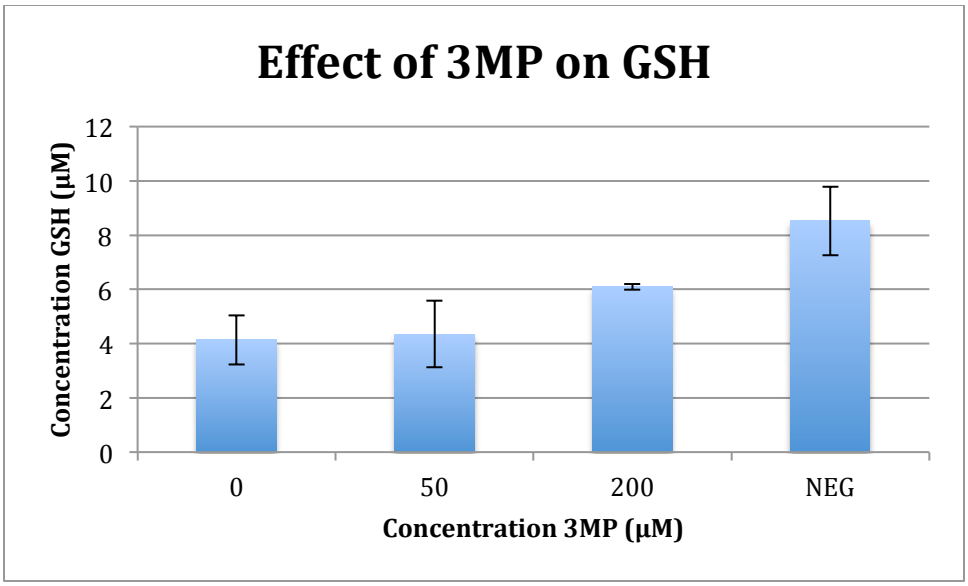


Figure 22. The effect of 3MP administration on intracellular GSH concentration. Negative control is lacking ROS and 3MP.

3.3 H_2S Generation

Generation of H_2S was monitored using NBD-Cl and measuring UV absorbance at 534 nm. H_2S formation increased in the presence of MST, and in the presence of peroxide absorbance nearly doubled **(Figure 23)**. Therefore, when challenged by ROS, available 3MP likely quenches ROS, preventing or limiting cellular damage. NBD-CL reacts slowly with 3MP alone, and in the absence of MST the decrease in absorbance seen in the 3MP + H_2O_2 assay provides indirect evidence of 3MP's antioxidant effect, as the resulting disulfide does not react with NBD-CL.

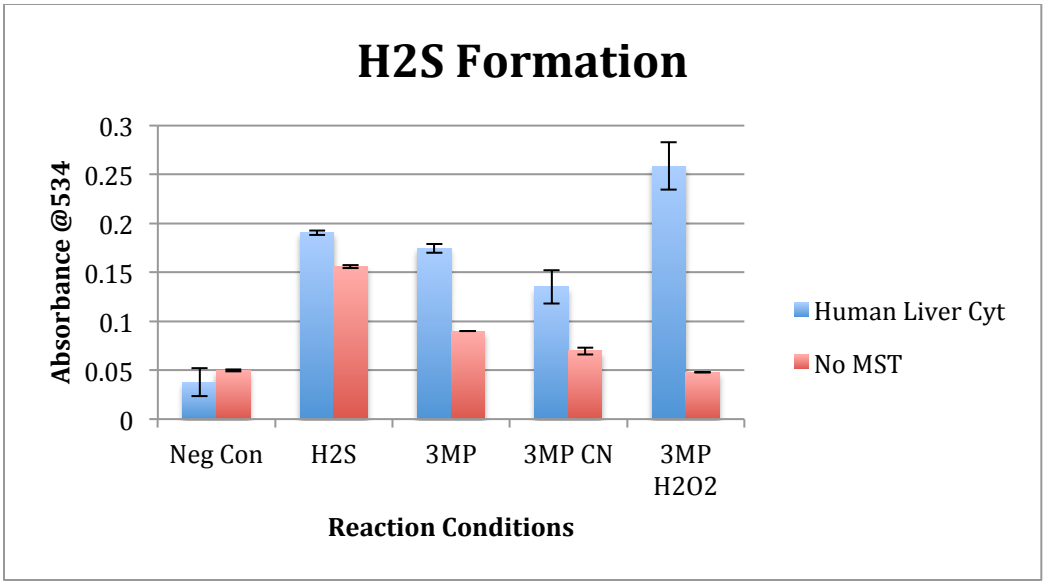


Figure 23. H_2S formation in the presence or absence of MST.

3.4 Cyanohydrin Formation

Under the conditions outlined above, cyanohydrin formation was detected via mass spec, albeit in smaller than expected ratios. The peak with a mass/charge ratio (m/z) of 244 corresponds to 3MP-NEM, while the peak with (m/z) 271 corresponds to the resulting 3MP-NEM cyanohydrin. A representative mass spectrum is given in **Figure 24**. Peaks corresponding to double the expected mass charge ratios are present, which is a common artifact of that spectrometer.

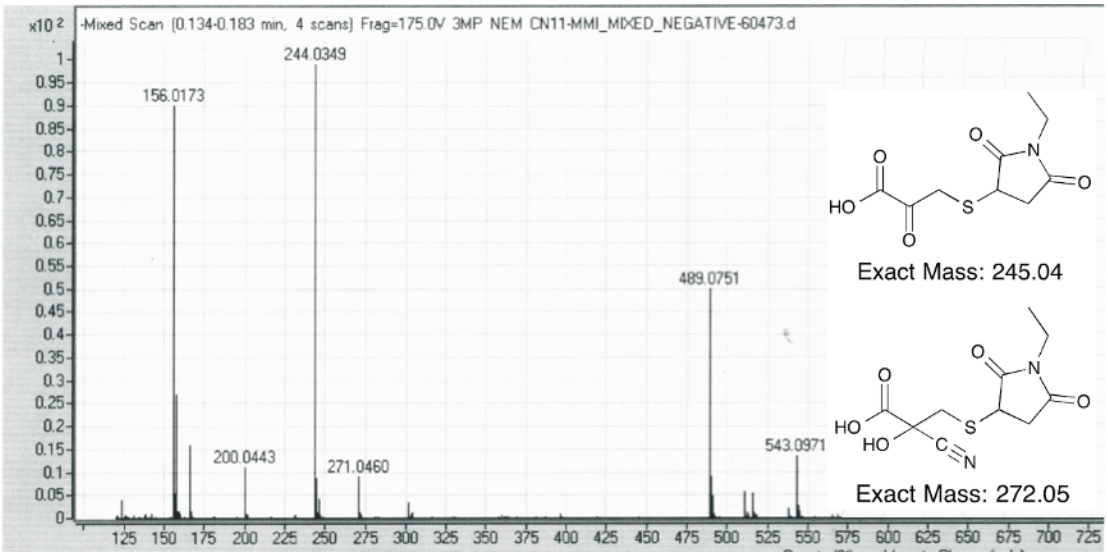


Figure 24. Mass Spectroscopy Results of Cyanohydrin formation. M/Z 244 is 3MP-NEM, while 271 peak corresponds to resulting cyanohydrin.

3.5 Cytochrome C Oxidase Assay

Under the conditions described above, 40 μM concentration of sodium cyanide fully inhibited cytochrome c oxidase, matching the negative control devoid of cytochrome c oxidase. Half of that dose (20 μM) of sodium cyanide produced a 50% inhibition when compared to the positive control containing no cyanide. When 20 μM cyanide was incubated with 20 μM 3MP for half an hour before the assay, cytochrome C oxidase activity closely resembled the activity of 20 μM NaCN alone **(Figure 25)**. This is inconsistent with cyanohydration formation contributing to the mechanism of cyanide antagonism.

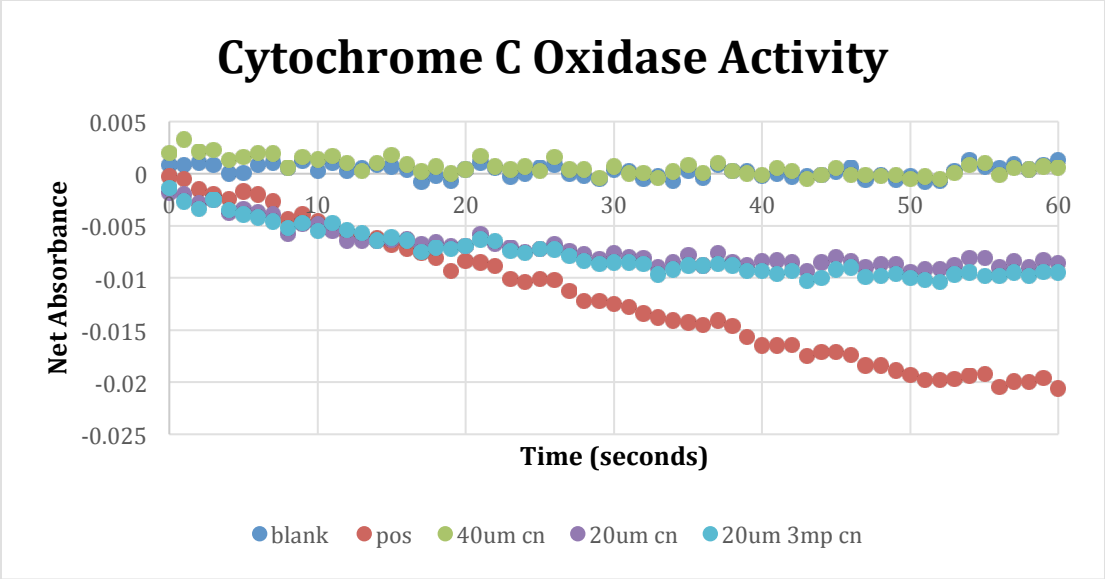


Figure 25. Cytochrome C oxidase activity in the presence or absence of 3MP & cyanide. Blank samples contained no cytochrome C oxidase, while the positive control had cytochrome C oxidase without cyanide present.

4. Discussion

The potential mechanisms of sulfanegen administration are summarized below in **Table 4**. Enzymatic conversion of cyanide to thiocyanate is the primary mechanism of action of sulfanegen, and therefore responsible for most of the efficacy. However, the demonstrated efficacy in pigs, even in the absence of high MST activities, are evidence of biologically relevant secondary mechanisms. Sulfanegen's ability to directly reduce ROS was first examined in the DPPH radical assay, where it was more effective at quenching ROS than NAC or GSH. These results were confirmed in cell culture, where it was more effective than NAC or GSH at quenching ROS in the N27 cell line. Analysis of reduced glutathione after administration of sulfanegen and ROS showed less oxidative stress on the cells.

The ability of sulfanegen to non-enzymatically detoxify cyanide via the reversible cyanohydrin reaction was examined using both analytical chemical and biochemical techniques. Cyanohydrin formation was detected at low levels in the mass spectrum, but this is not a direct demonstration of biological relevance. A better approach would be to demonstrate sulfanegen's ability, or lack thereof, to restore Cytochrome C oxidase activity after cyanide administration in the absence of MST. In the assay, 20 μM of 3MP incubated with cyanide were statistically the same as 20 μM cyanide alone, demonstrating that cyanohydrin formation, even if formed in detectable amounts by mass spectrometry, had no measurable effect on cytochrome C oxidase activity. This is likely due to both the small amounts of free cyanide being removed from the system via cyanohydrin formation, as well as the formation of cyanohydrin being reversible. It is also possible that cyanohydrin formation observed by MS was an artifact of the conditions in the mass spectrometer i.e. the cyanohydrin formed in the source chamber of the spectrometer but not necessarily in solution. Thus, it appears cyanohydrin formation does not contribute to sulfanegen's antagonism of cyanide toxicity.

Quenching of cyanide induced ROS via enzymatic conversion of 3MP to H_2S, was the final mechanism examined. In the presence of MST, 3MP produced higher levels of H_2S than 3MP incubation with the probe alone. At this time, the biological significance of this reaction is unknown, but it is expected to help ameliorate cyanide induced ROS. These mechanisms are likely to contribute to the overall efficacy of sulfanegen.

Potential Mechanism	Order	Mechanism	Ex-vivo Detection?	Biologically Relevant?
Detoxification of Cyanide	Primary	Enzymatic Conversion of Cyanide to Thiocyanate	Yes	Yes
	Secondary	Cyanohydrin Formation	Yes	No
Quenching Cyanide-Induced ROS	Secondary	Enzymatic Conversion of 3MP to H_2S	Yes	Suspected
	Secondary	Direct Antioxidant Quenching of ROS	Yes	Yes

Table 4. Summary of data for potential mechanisms of sulfanegen.

5. Conclusions

The ubiquitous distribution of MST makes it an ideal target for enzyme-catalyzed detoxification of cyanide to thiocyanate using 3MP. However, cyanide exposure has wide ranging effects beyond inhibition of cytochrome C oxidase. Cyanide induced NMDA receptor activation results in an influx of Ca^{2+} and an increase in inducible nitric oxide synthase (iNOS) activity. The resulting ROS has devastating downstream effects unless quenched.[59] The experiments above demonstrate that sulfanegen is a potent antioxidant, and as a result is able to quench cyanide induced ROS both directly as a thiol as well as indirectly though enzymatic production of H_2S. The work demonstrates cyanohydrin formation probably is not a significant contributor to cyanide antagonism for sulfanegen. However, sulfanegen's MST catabolized metabolism of cyanide to thiocyanate is likely augmented by sulfanegen's antioxidant properties.

CHAPTER V:

MISCELLANEOUS STUDIES

1. 3MP-NEM Indicator Studies

1.1 Introduction

A test to rapidly determine cyanide exposure in the field does not currently exist. Upon arriving at a suspected cyanide exposure, first responders must administer medical countermeasures before a causative agent can be determined. Therefore many methods for detecting and quantifying cyanide have been developed over the years, but most require time intensive steps, non-aqueous solvent systems, expensive laboratory equipment, or suffer from selectivity issues with other anions.[160,161] As a result, specific cyanide indicators that can be used in biological systems have been lacking. Colorimetric methods are particularly advantageous, as they can provide rapid detection of cyanide with the naked eye. Work on developing new cyanide detectors has been previously reviewed by Ma and Dasgupta, and the Logue laboratory has made recent progress on such a field-based test.[161,162] Here, we report a novel cyanide indicator based on sulfanegen, an IM injectable, preclinical cyanide antidote that our lab has developed. The indicator can be quickly synthesized from sulfanegen using commercially available materials and works in 100% aqueous media for visual detection of cyanide.

During the course of secondary mechanism elucidation studies covered in chapter 4, it was discovered that a solution of 3MP, after derivitization with NEM, produced a bright pink coloration upon treatment with a sodium cyanide solution. Here, we describe the characterization of the 3MP-NEM reaction with cyanide, and its applications to colorimetric detection of cyanide.

1.2 Methods

1.2.1 Structure Activity Relationship Studies

3MP, Cysteine, or 3-mercaptopropionic acid (100 mM) and 100 mM NEM in deionized water and were incubated at room temperature for 15 minutes and briefly mixed every 5 minutes using a VWR vortex mixer. Then, a solution of NaCN was added such that final concentrations were 50 mM for both indicator and cyanide respectively. The resulting mixture was monitored until observation of a color change, or in absence of a color change, for 30 minutes.

1.2.2 Sensitivity, Selectivity, and Specificity Studies

To determine sensitivity of the indicator observable by the human eye, 3MP-NEM (50 mM) and cyanide (0-50 mM) were added to 96 well plates. The reaction was monitored at 300, 405, and 515 nm.

Selectivity of the indicator was examined by adding 50 mM 3MP-NEM and 50, 100 or 500 mM sodium or potassium salts of I^-, F^-, NO_3^-, AcO^-, SO_4^-, Cl^-, SCN^-, or CO_3^- to each well of a 96 well plate. The sodium or potassium counterions did not have a discernable effect on the assay. The reaction was started by addition of 50 mM NaCN and monitored at 300, 405, and 515 nm.

Specificity studies were determined by adding 50 mM 3MP-NEM to a 96 well plate and adding 50, 100, or 500 mM sodium, potassium, or chloride salts of I^-, F^-, NO_3^-, AcO^-, SO_4^-, Cl^-, SCN^-, CO_3^-, SO_3^{2-}, Br^-, CLO_4^-, Cu^{2+}, Mg^{2+}, Fe^{2+}, NH_3^+, Ca^{2+}, PO_4^{3-}, HPO_4^{2-}, or $H_2PO_4^-$ to each well. Monitoring the reaction at 515 nm for 30 minutes identified anions that caused an increase in absorbance without cyanide present.

1.2.3 Analytical Chemistry Analysis: Mass Spec and NMR

3MP (50 mM) was derivitized with 50 mM NEM as described above. The resulting 50 mM stock solution of 3MP-NEM was diluted to 1 mM and incubated with an equimolar concentration of aqueous sodium cyanide at room temperature for 30 minutes. The final reaction volume was 1 mL. Following incubation, the reaction mixture was analyzed using mass spectrometry (MS) in MMI Mixed Negative mode, which performs electrospray and atmospheric chemical ionization modes simultaneously. NMR studies were carried out by Dr. Rui Ding in our lab.

1.3 Results

1.3.1 Structure Activity Relationship Studies

To determine if cyanohydrin formation could be the mechanism of action of the 3MP-NEM indicator, the α-keto acid moiety was examined by replacing 3MP for either cysteine, in which the α-keto acid is substituted for NH_2, or 3-mercaptopropionic acid, in which the α-keto acid is removed entirely (**Figure 26**). Both modifications of the α-keto acid resulted in no color formation upon derivitization with NEM and addition of NaCN. Upon addition of excess hydroxide (OH^-), 3MP-NEM turned a bright pink, while Cysteine-NEM turned faintly pink, and 3-mercaptopropionic-NEM remained colorless (**Figure 27**). However, the color change associated with OH^- was brief, and the solution returned to colorless within 5 minutes. This was not the case for 3MP-NEM + NaCN, whose color persisted for hours.

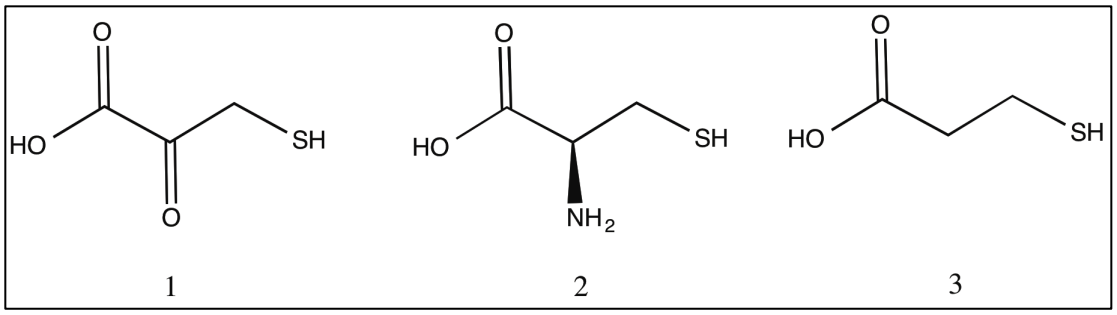


Figure 26. Summary of SAR studies on α-keto acid substitutions. (1) 3MP, (2) Cysteine, (3) 3-mercaptopropionic acid.

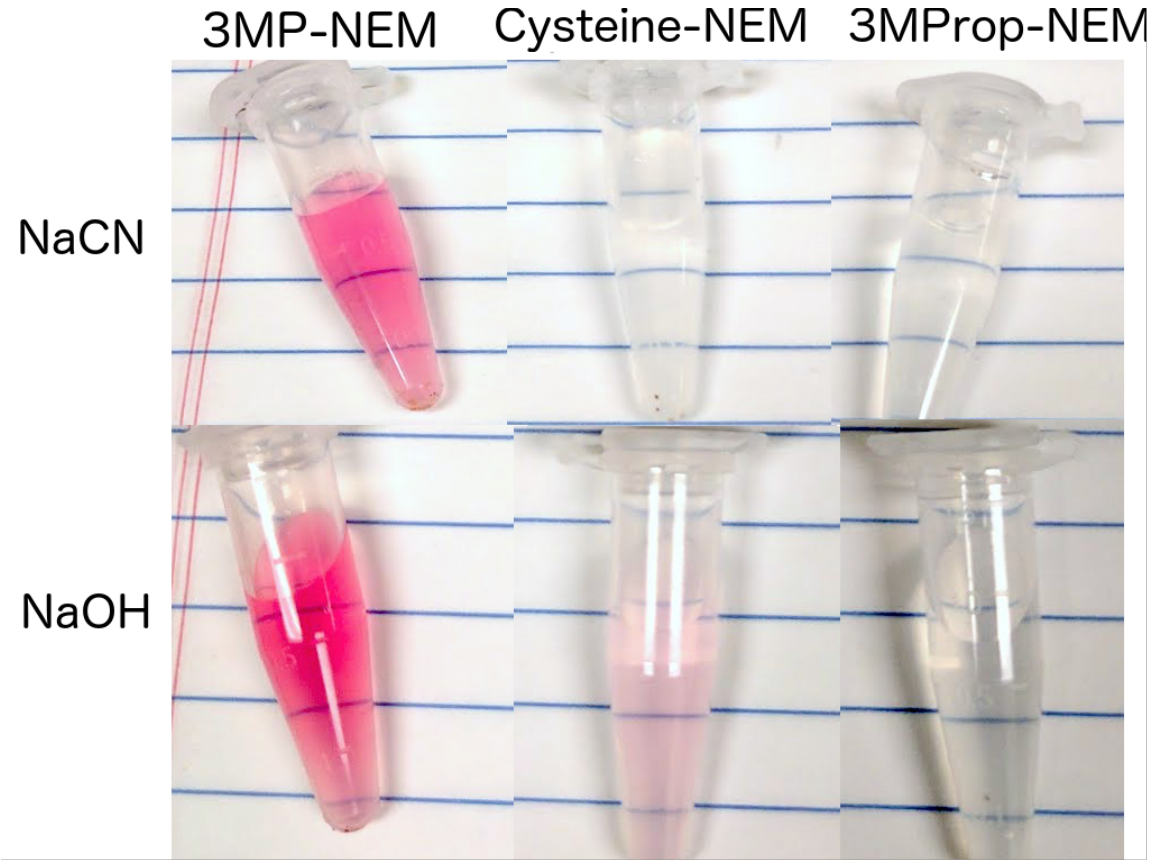


Figure 27. Representative results of SAR studies on α-keto acid substitutions. Only 3MP-NEM changed color upon addition of CN, while both cysteine-NEM and 3-mercaptopropionic-NEM (3MProp-NEM) remained colorless.

Additionally, N-ethylsuccinimide was examined determine if the resulting structure of the NEM tag after derivitization was responsible for color formation.

N-ethylsuccinimide did not visibly change color upon addition of CN^- or excess OH^-, and the corresponding UV spectra were unchanged from N-ethylsuccinimide alone.

1.3.2 Sensitivity, Selectivity, and Specificity Studies

Sensitivity was examined using 50 mM 3MP-NEM and 0-50 mM sodium cyanide. As cyanide concentration increased, pink color formation was easily seen by the naked eye after 2 mM and intensified with additional cyanide (**Figure 28**). UV absorbance data shows a cyanide concentration dependent decrease in absorbance at 305 nm, and an increase in absorbance at both 410 nm and 515 nm **(Figure 30).**

Inhibitors of the indicator were examined in the presence of 50 mM 3MP-NEM, 50 mM cyanide, and 50, 100, or 500 mM other salts. Of the potential inhibitors tested, high concentrations (500 mM) of nitrate (as $NaNO_3$) and bicarbonate ($NaHCO_3$) delayed the onset of color formation **(Figure 28)**. This inhibition is quantified via UV absorbance at 515 nm in **Figure 29**.

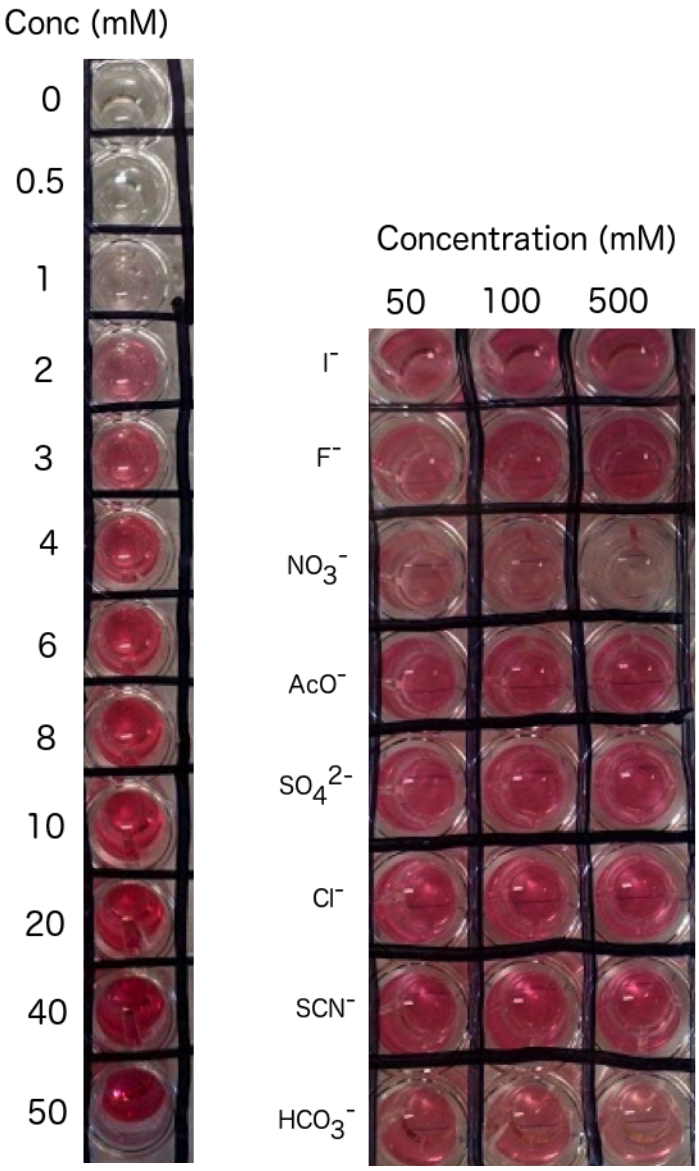


Figure 28. Sulfanegen (50 mM) sensitivity to cyanide (0-50 mM) (left), and selectivity in the presence of common anions inhibition (right).

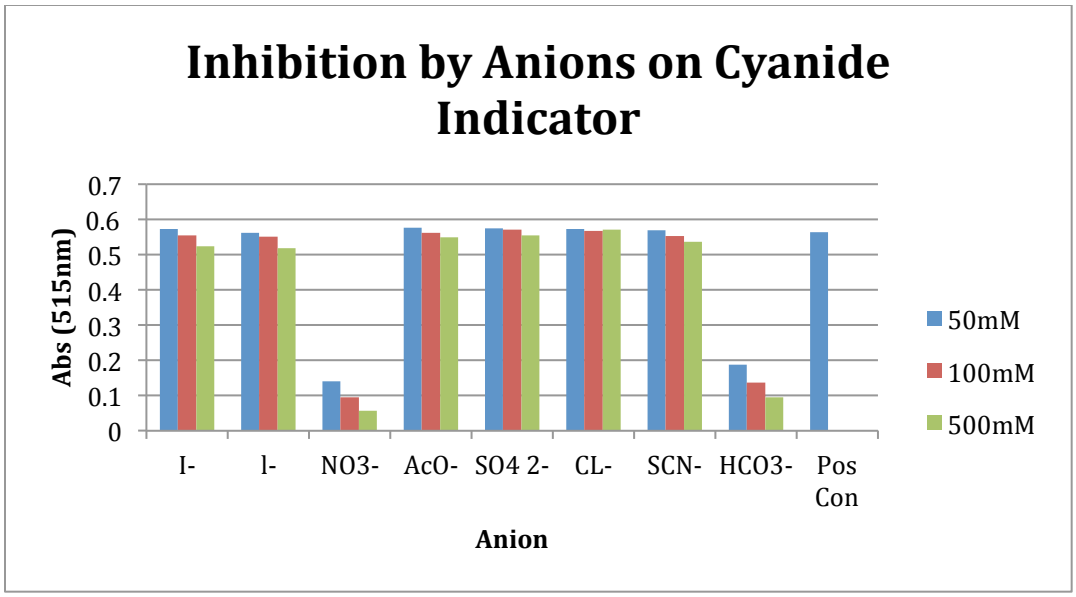


Figure 29. Quantification of inhibition by anions on cyanide indicator.

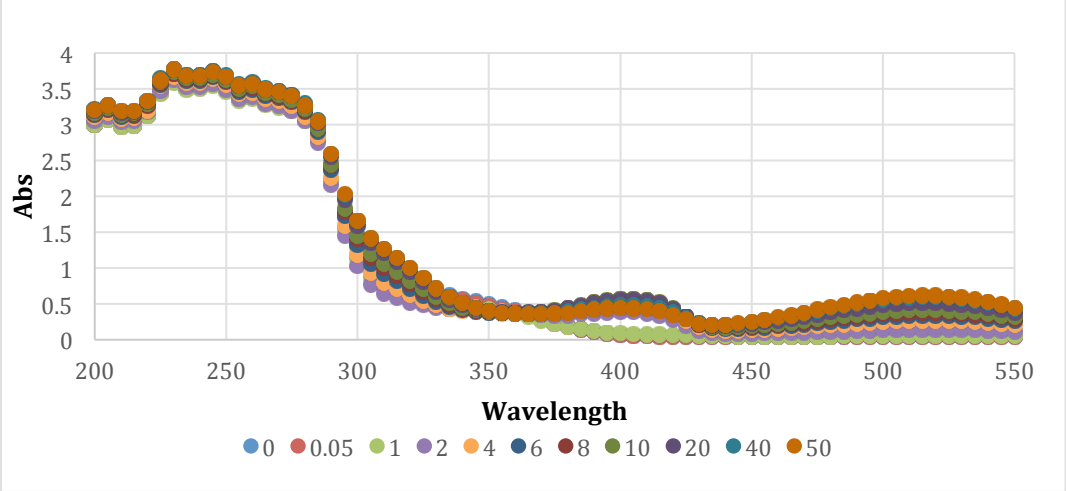


Figure 30. Absorbance spectra changes as increasing levels of cyanide are added (0-50 mM). With increasing cyanide concentration there is a dop in absorbance at 305 nm, and increase in absorbance at both 410 and 515 nm

From the UV/VIS absorbance specrum data, a stark difference can be seen between 3MP-NEM samples reacted with cyanide or base. In the cyanide treated samples, we see the characteristic drop at 305 nm, and increases at 410 and 515 nm. However, in samples treated with base there is an increase at 305 nm, no change seen at 410 nm, and an increase at 515 nm **(Figure 31)**. The

increase at 515 nm in samples where NaOH was added instead of cyanide clears within minutes, while in the cyanide treated sample persists for hours (**Figure 31**).

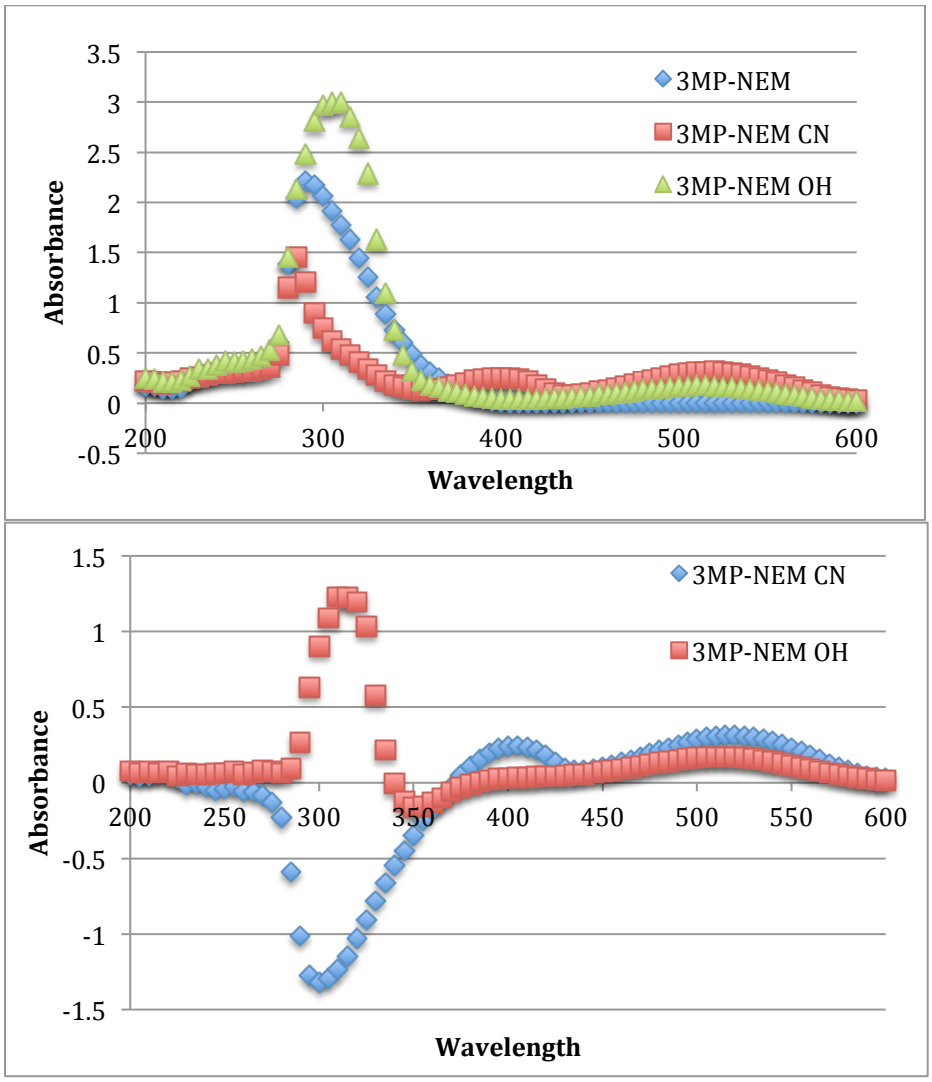


Figure 31. UV absorbance spectra of the 3MP-NEM indicator. Top: Net change in absorbance after subracting 3MP-NEM. Bottom: 3MP-NEM in the presence or abense of cyanide and base.

Selectivity studies were carried out by adding aqueous 50 mM 3MP-NEM and 50,100, or 500 mM salts to identify false positives. Of the non-hydroxide

salts tested, only phosphate (as the trisodium salt i.e. Na_3PO_4) showed color formation without the presence of cyanide (**Figure 32**). Fe^{2+} interacted with the 3MP-NEM indicator, but the color change was a very dark green and not easily confused with the pink color seen in cyanide positive samples.

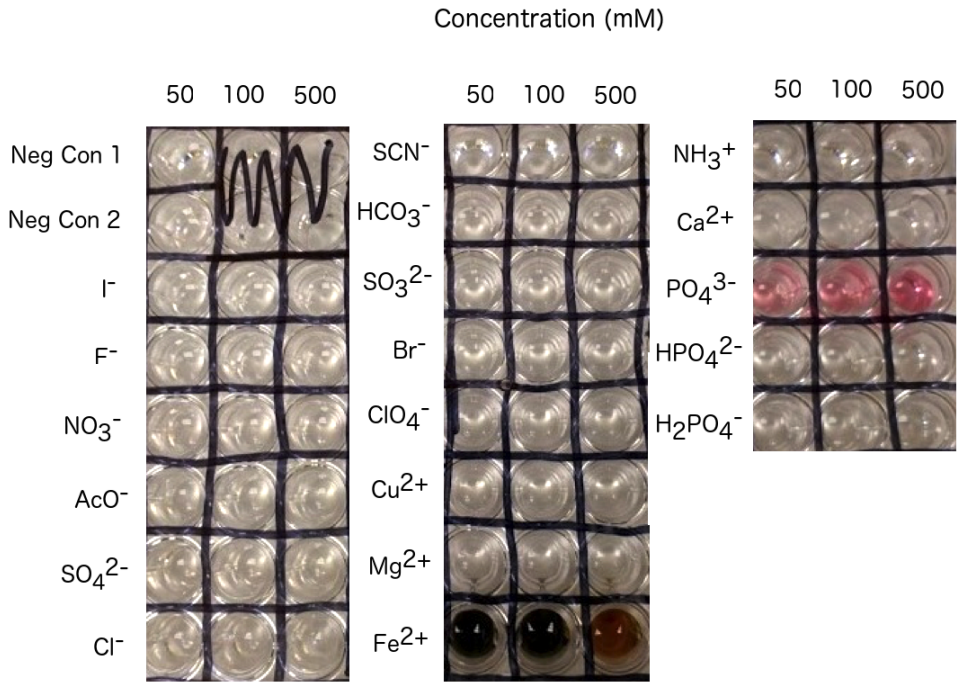


Figure 32. Specificity studies of 3MP-NEM in presence of other ions.

1.3.4 Mass-Spec Analysis

Reactions of 3MP-NEM with either CN^- or OH^- were examined via mass spectrometry. Addition of NaCN yielded the same 3MP-NEM cyanohydrin M/Z as presented in Chapter 4. Adding an equimolar amount of hydroxide to 3MP-NEM yielded peaks corresponding to formation of a ketone hydrate at the α-keto acid moiety (**Figure 33**).

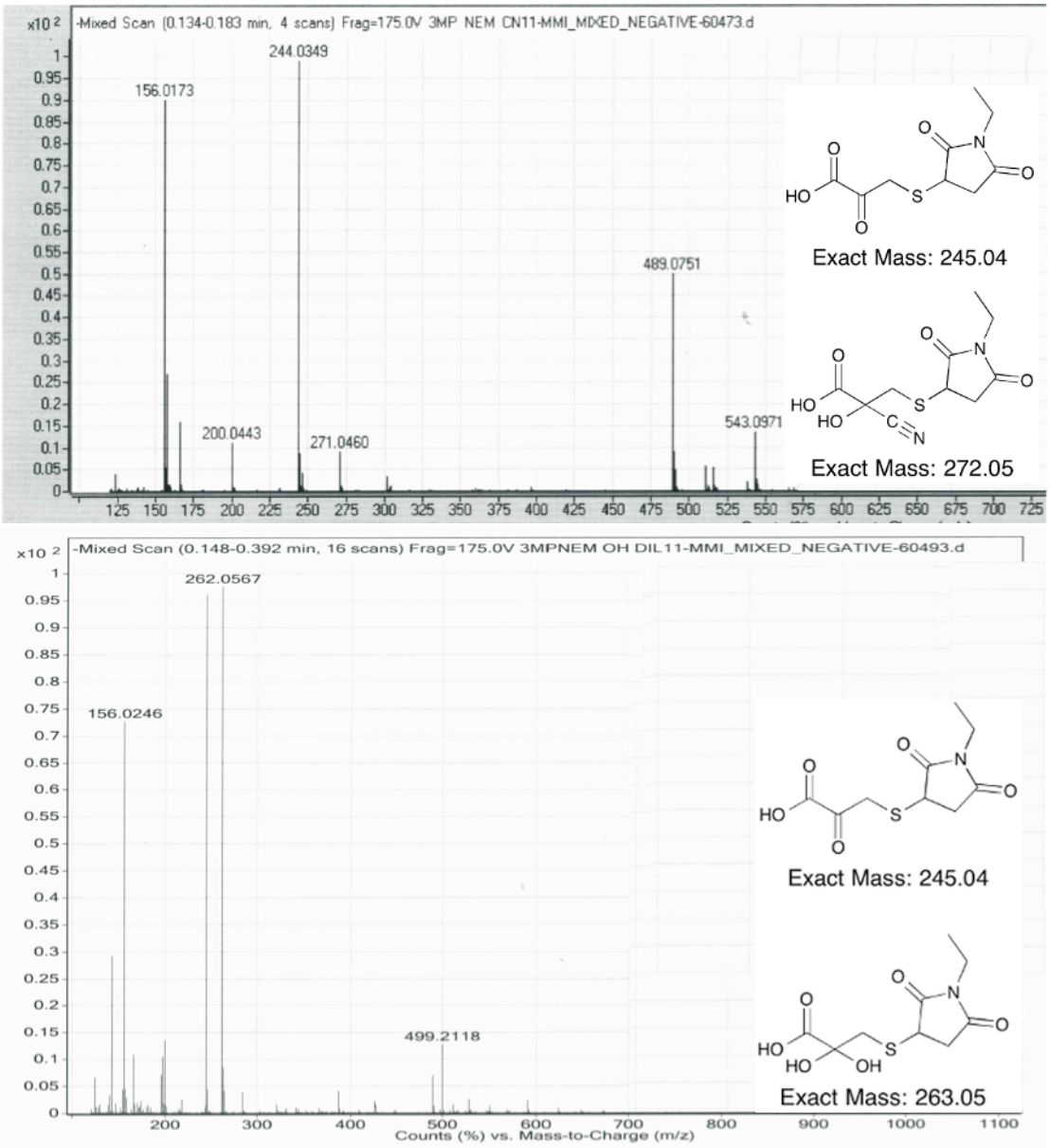


Figure 33. Mass Spectrometry results of the 3MP-NEM reaction with cyanide (top), and hydroxyl (bottom). M/Z 244 is 3MP-NEM, while M/Z 271 and M/Z 262 correspond to resulting cyanohydrin and hydroxide addition respectively.

1.3.5 NMR Analysis

Dr. Rui Ding in our lab analyzed 3MP-NEM alone, 3MP-NEM + CN^-, and 3MP-NEM + NaOH reactions via NMR. NMR results for the 3MP-NEM compound show that the molecule undergoes a ring closing reaction resulting from an intramolecular condensation of the imide enolate moiety with the ketoacid moiety

in solution (**Figure 34**). This molecule was isolated and fully characterized by Dr. Rui Ding. NMR data for the NaOH reaction indicates formation of many products that were likely the result of an aldol-type polymerization. None of these products were isolated and characterized.

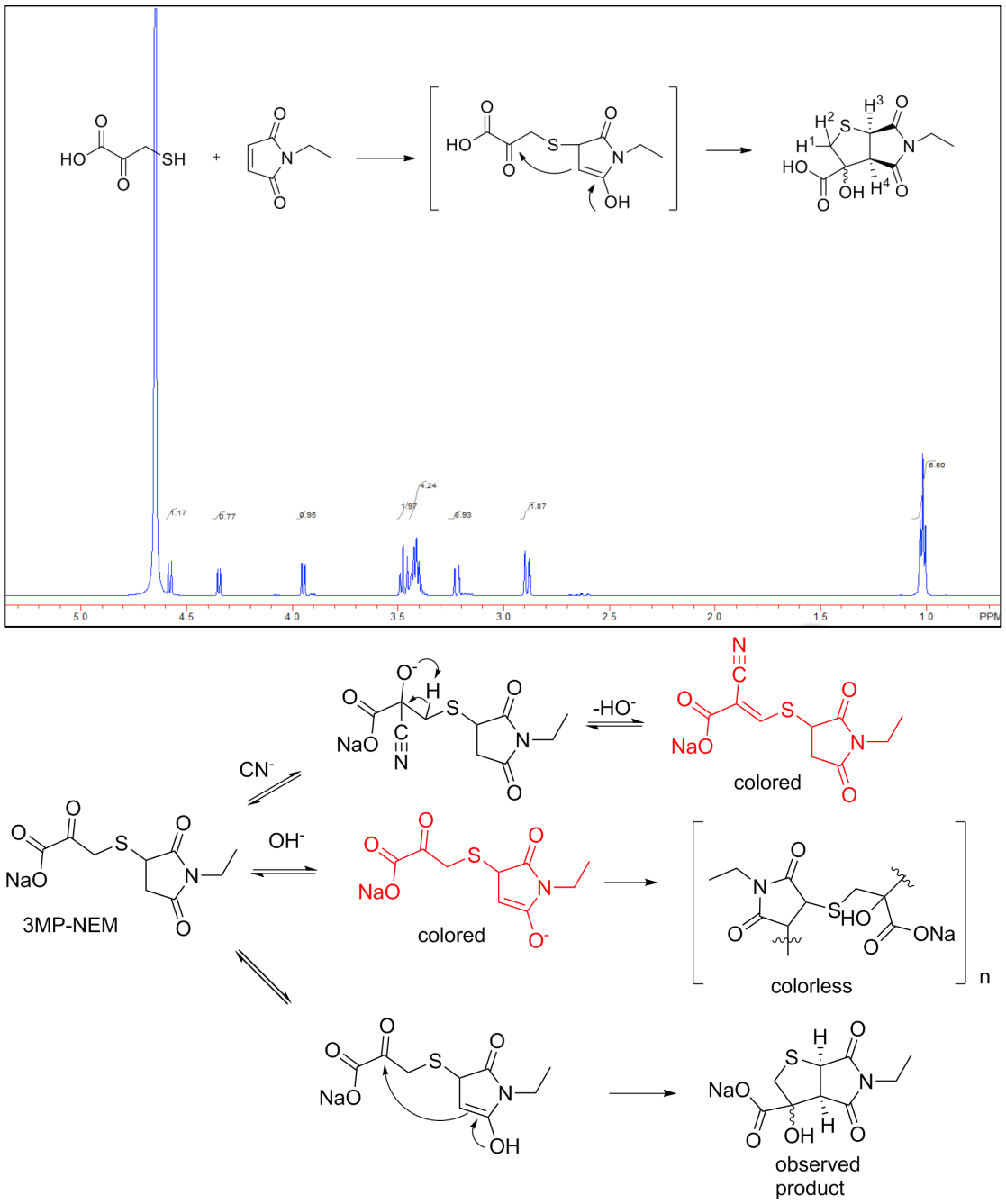


Figure 34. Proposed structure of colored species observed upon addition of sodium cyanide (top right in red), the colored intermediate under basic conditions (middle in red) and the observed product that was isolated and characterized (bottom right).

1.4 Conclusions

Cyanide addition to 3MP-NEM produces a color shift in the visible range. While this indicator is useful for milimolar quantities of cyanide, sensitivity is insufficient for most applications, as the WHO guideline for the maximum concentration of cyanide in drinking water is 1.7 μM.[163] The proposed mechanism is a reversible dehydration of the cyanohydrin that gives the resonance stabilized transient intermediate (**Figure 35**).

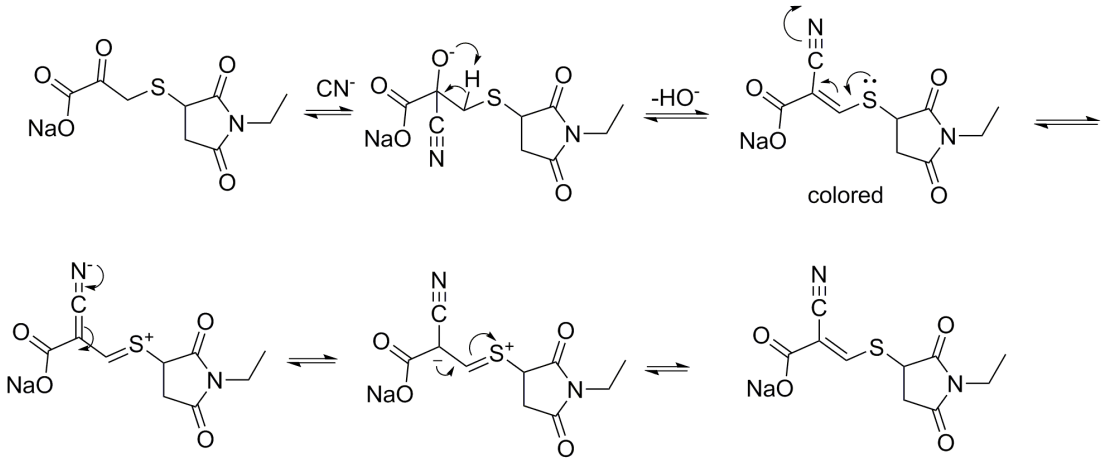


Figure 35. 3MP-NEM cyanohydrin formation and resulting resonance structures.

The colored species upon treatment with cyanide was not isolated. This proposed intermediate probably exists only transiently in solution, being in equilibrium with the more thermodynamically stable 3MP-NEM and corresponding cyanohydrin. Such transient intermediates or transition states are consistent with known mechanism of the benzoin condensation, the mass spectrometry data, and this scheme represents a novel cyanide indicator.

2. Ames Testing

2.1 Introduction

As part of preliminary toxicity testing for sulfanegen, the Ames test was used to determine and characterize potential mutagenicity of the compound. The Ames test uses specific strains of *Salmonella Typhimurium* that have mutations in histidine synthesis genes. As a result of these mutations, the bacteria are unable to grow in media that is not supplemented with histidine. Application of a compound with mutagenic properties may result in a gain-of-function mutation, allowing the bacteria to produce their own histidine and grow in histidine free media.[164] For these studies, both TA98 and TA100 strains of *Salmonella Typhimurium* were utilized, containing frameshift and base pair substitution mutations respectively.

2.2 Methods

Sulfanegen was synthesized in our lab by Dr. Alexandre Monteil as previously described.[97] The Mod-ISO 2 strain kit was purchased from EPBI (Ontario, CA). The directions for the kit were followed with respect to both the TA98 and TA100 kits using dilutions of Sulfanegen Lysine ranging from 5 mM to 2.5 μM. The upper concentration was based on rabbit PK data, and was more than double the C_{max} seen during those tests. The positive control for TA98 was 4-Nitro-*ortho*-phenylenediamine, and for TA100 was sodium azide. After treating the bacteria with positive controls, various concentrations of Sulfanegen Lysine, or blank negative control for 48 hours, reversion wells were counted and compared to controls.

2.3 Results

2.31 TA100

TA100 results are summarized in **Table 5**. Nonreversion wells remain purple, while those wells that regained histidine synthesis change color from purple to yellow **(Figure 36)**. No significant mutagenicity was found for any concentration of sulfanegen lysine examined under the reaction conditions described. Based on the number of reversions in the negative control, an average of 11 reversions per concentration are needed to have significant mutagenicity with significance set at p=0.05. The 5 mM concentration approached significance, but did not cross the threshold for base pair substitution mutagenicity.

Sample	Plate 1	Plate 2	Plate 3	Average
Sterile Control	0			0
Positive Control	38	36		37
Negative Control	7	4	3	4.6
5 mM	10	8	13	10.3
1.25 mM	6	12	9	9
310 µM	9	3	6	6
38.75 µM	10	7	11	9.3
2.42 µM	8	12	8	9.3

Table 5. Summary of Ames testing results for TA100 strain for base pair substitutions.

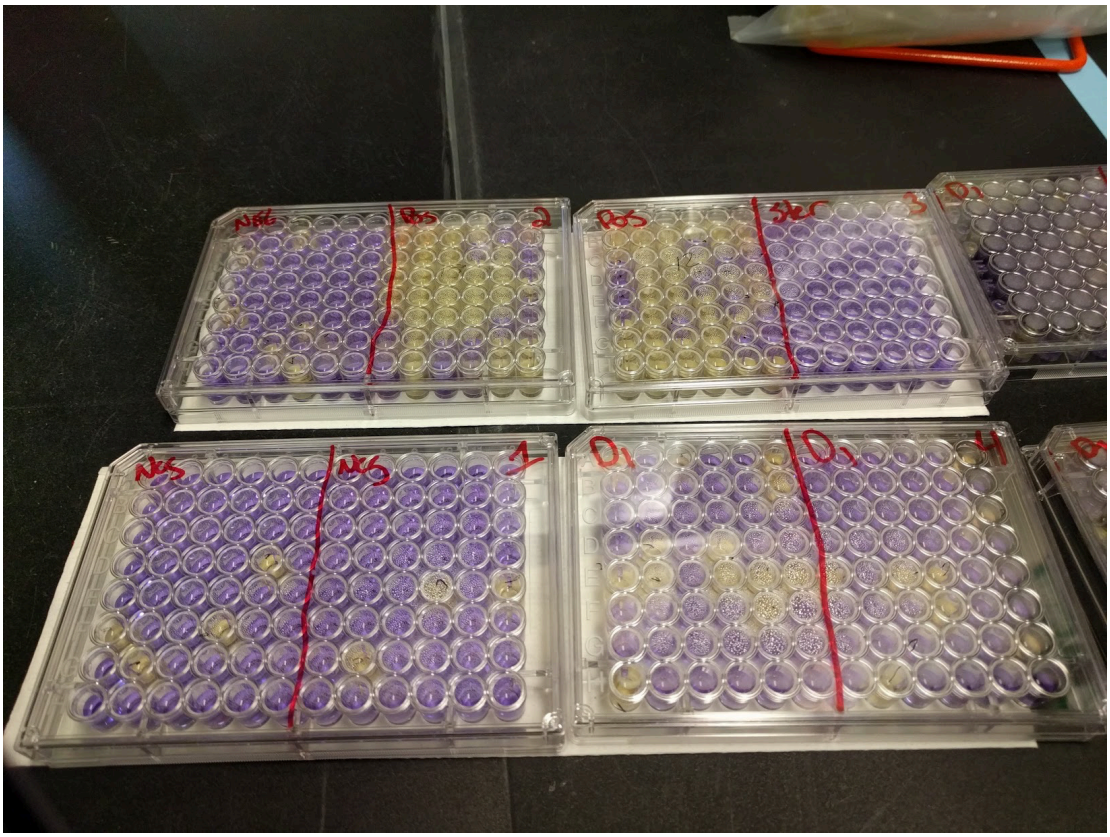


Figure 36. Ames testing results for TA98 strain. Reversion wells are yellow while non-reversion wells remain purple.

2.32 TA98

The results for TA98 are summarized in **Table 6**. No significant mutagenicity was found for any concentration of sulfanegen lysine examined under the reaction conditions described. Based on the number of reversions in the negative control, an average of more than 7 reversions would be needed in order to have significant mutagenicity at p = 0.05.

Sample	Plate 1	Plate 2	Plate 3	Average
Sterile Control	0			0
Positive Control	41	40		40.5
Negative Control	3	4	0	2.33
5 mM	2	4	6	4
1.25 mM	0	2	0	0.666
310 µM	10	2	1	4.3
38.75 µM	3	4	4	3.66
2.42 µM	0	0	2	0.666

Table 6. Summary of Ames testing results for TA98 strain for frameshift substitutions.

2.4 Conclusions

Sulfanegen Lysine formulation was not found to be significantly mutagenic at any dose tested. Doses were as biologically relevant as possible by doubling the C_{max} seen in rabbit studies as the max dose. Ames testing is only a small part of the battery of toxicological testing required to demonstrate safety for drug approval, but these preliminary results, which will need to one day be completed under good laboratory practice (GLP) conditions, are promising at the biologically relevant doses tested. High levels of mutagenicity were not expected, as all components of the proprietary formulation are endogenous.

3. Non-Enzymatic Oral Antidotes

3.1 Introduction

Cyanide intoxication can occur by multiple of routes of administration. Inhalation of HCN gas is one of the most dangerous, with death occurring within minutes. Oral ingestion of cyanide leads to a slower onset of toxicity, and this lag may present an opportunity for chemical countermeasure administration. Oral antidotes have not received much attention, as it is believed that most cyanide is converted into HCN upon reaching the gastric stomach acids. HCN is electrostatically neutral, and as a result is able to freely pass through cellular membranes. However, the stomach pH is not static, and variations may allow time for oral antidotes to detoxify cyanide before it is able to show effects systemically. Here we present the results of proof of concept studies designed at testing the efficacy of non-enzymatic trisulfide antidotes to detoxify cyanide into thiocyanate in acidic environments.

3.2 Methods

3.2.1 Chemicals

Trisulfide compounds for potential antidotes were synthesized by Dr. Johnathan Cohen in our laboratory. Cysteine-S-Cysteine (Cys-S-Cys) and Glutathione-S-Glutathione (GS-S-SG).

3.2.2 Non-Enzymatic Thiocyanate Production

Safety warning:

Beware of working with cyanide in acidic environments. Take proper safety precautions and work in a fume hood. Neutralize reactions with excess bleach after assay completion in to prevent further toxic HCN gas formation.

Thiocyanate production was measured using the same methods as in MST thiocyanate production studies (Chapter 2), i.e. by addition of ferric nitrate and UV absorption as previously described.[118,165] In short, 15 mM Cys-S-Cys or GS-S-SG was prepared in 450 µL 0.01M HCL (pH 2.0) in a micro-centrifuge tube. HCN (50 µL, 500 mM) was added to the reaction, and the vial immediately capped. After 10 minutes, the vial was opened, 500 µL ferric nitrate was added to the solution and a 20 µL aliquot was diluted to 200 µL. The vial was then immediately closed. Absorbance was read at 450 nm.

3.2.3 Kinetics

Kinetics of the reaction were monitored using the assay above, except total reaction volumes were increased to 1000 µL. Every 2 minutes for 40 minutes, a 50 µL aliquot of the reaction is mixed with 50 µL ferric nitrate solution and absorption read at 450 µL. Results were confirmed with multi-vial kinetic reactions designed to prevent overall cyanide concentration decreasing via HCN gas leaking from multiple openings of the reaction vial.

3.3 Results

Non-enzymatic trisulfide antidotes were successful at producing thiocyanate in the 10 minutes allotted for the reaction time (**Figure 37**). However, reaction yield was poor, as Cys-S-Cys and GS-S-SG produced only 750 µM (5%) and 370 µm (2.5%) thiocyanate respectively. Following these results, Cys-S-Cys reaction kinetics were more closely examined. Using 2.2 mM Cys-S-Cys and a reaction time of 40 minutes, thiocyanate production ceased after only 4 minutes

to 120 μM thiocyanate, representing once again ~5% total SCN production **(Figure 38)**.

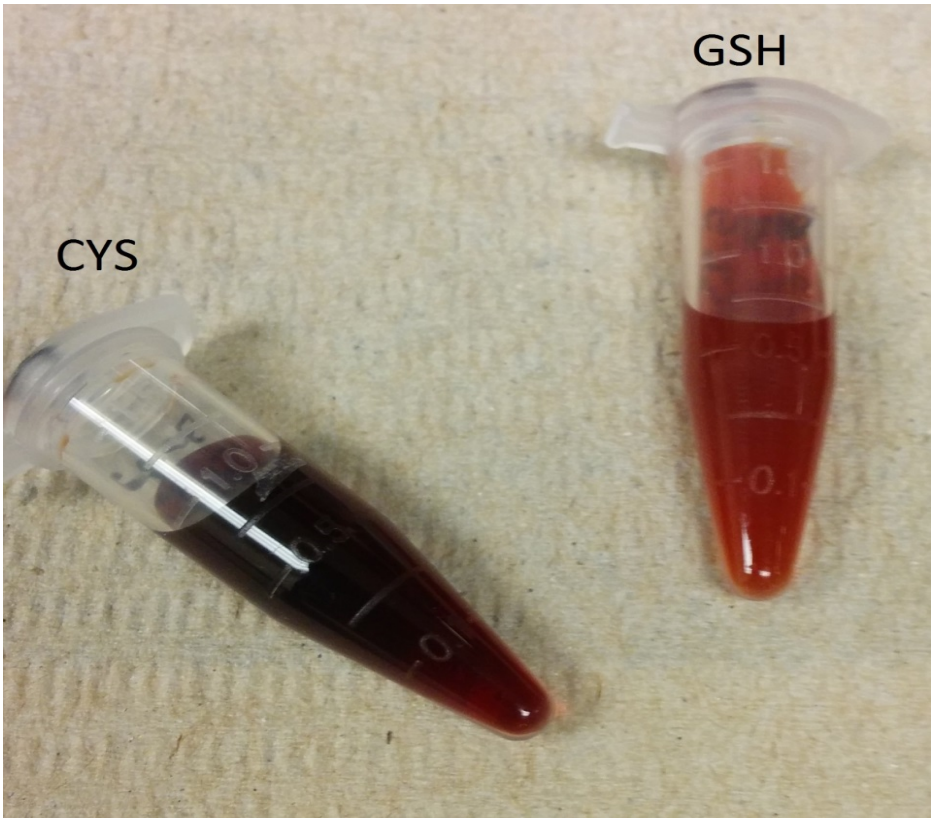


Figure 37. Representative thiocyanate production of trisulfate antidotes. Darker solution equals more thiocyanate produced. CYS = Cys-S-Cys GSH= GS-S-SG

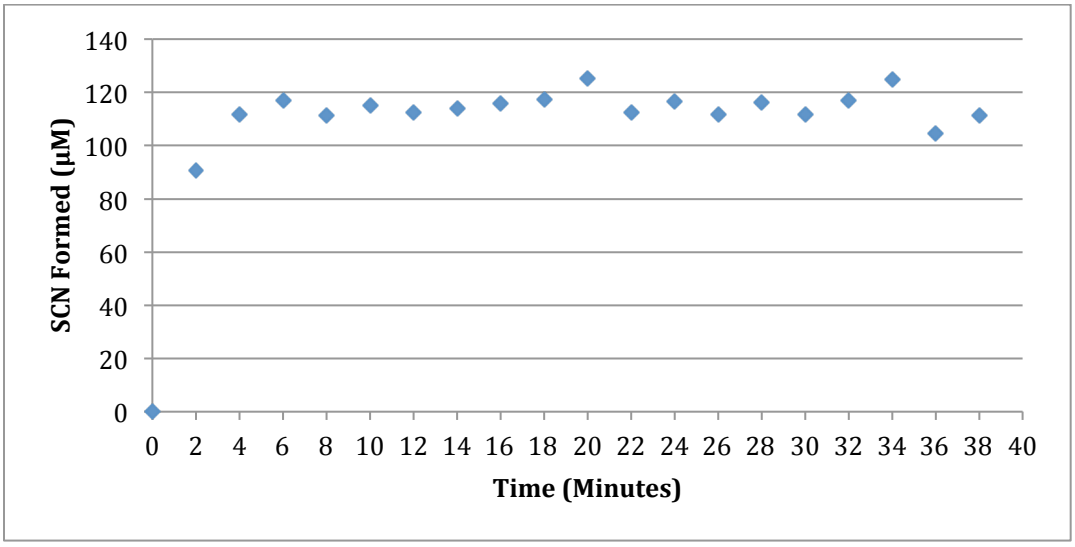


Figure 38. Cys-S-Cys multivial reaction kinetics. Thiosulfate reached its maximal concentration at around 6 minutes after reagents were mixed, with a 5% yield.

3.4 Conclusions

Although the trisulfide antidotes were capable of detoxifying cyanide by conversion into thiocyanate non-enzymatically, the small reaction yields prevent application to oral cyanide toxic exposure. In these instances, patients would likely have better outcomes if either of the currently approved therapies were administered. It is possible thiocyanate production could be improved if the pH of the reaction conditions were neutralized, but those experiments were outside of the scope of the current investigation. It is unknown to what extent low SCN yields were due to outgassing of HCN, though the tests were performed in closed vessels, HCN could have accumulated in the headspace above the reaction volume. Further pursuit of trisulfide oral antidotes was abandoned, as outgassing would also occur in the stomach, limiting utility.

4. Expanding the Scope of Diseases – Obliterative Bronchiolitis and Thioredoxin Mimetics

4.1 Introduction

Obliterative bronchiolitis (OB) is a rare condition that results in progressive and irreversible airway restriction.[166] OB is most commonly seen in lung transplant patients, where the disease is often deadly. OB is responsible for up to 40% of patient deaths after the first year in lung transplant patients, and affects up to 50% of patients who survive at least 5 years post transplant.[167] The exact cause of OB has not been delineated, but fibroblasts, lymphocytes, and graft antibodies have all been implicated.[166,168–172] Ultimately, some unknown upstream trigger of epithelial injury leads to an antibody and lymphocyte immune response, whose oxidative damage and inflammation causes progressive fibrosis and airflow limitations.[166]

The antioxidant capability of sulfanegen, which was characterized in Chapter IV, was tested in OB with mild efficacy. Lack of a robust response to halt progression of the disease in animal models is likely due to sulfanegen's pharmacokinetic (PK) properties, previously studied in rabbits.[122] Sulfanegen's short half-life of 114 minutes (in the absence of cyanide) is adequate for acute poisonings, but likely insufficient for chronic conditions like OB without frequent administration. With proof-of-concept studies for antioxidant efficacy in OB demonstrating potential benefits, Dr. Marzena Bahran in our lab performed synthesis of thioredoxin mimetics as potential antioxidants with the goal of increasing half-lives.

Thioredoxins (TRX) are a ubiquitous class of protein that play roles in maintaining the cellular redox state.[173] The mechanism of their antioxidant capability stems from their cysteine-x-x-cysteine structure, where "X" represents an amino acid. Close proximity of the cysteine residues permit reduction of oxidized proteins, forming cys-cys disulfide bonds.[173–175] Oxidized thioredoxin is then reduced by thioredoxin reductase (TRXR), which is regenerated by reduction via NADPH (**Figure 39).** [173–175]

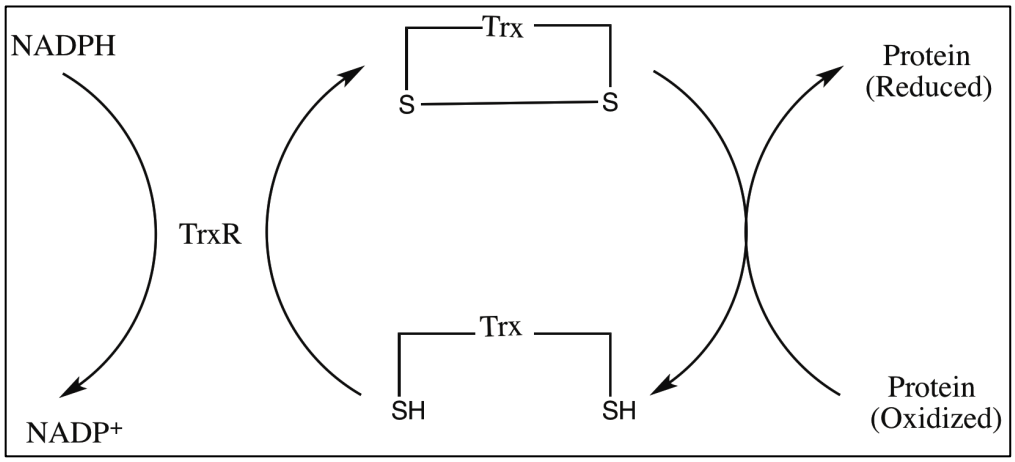


Figure 39. Thioredoxin reaction scheme. Thiorexoxin (Trx) alleviates cellular oxidative damage by forming a disulfide. Trx is reduced by thioredoxin reductase (TrxR), which is regenerated by NADPH.

Cyclophosphamide, a chemotherapeutic and immune suppressant, is often administered following organ transplantation to prevent rejection. Cyclophosphamide administration leads to DNA alkylation and oxidative stress.[176,177] Cyclophosphamide is converted into its active metabolite 4-hydroxycyclophosphamide (4HC) by cytochrome P450 2B1.[177] 4HC then spontaneously breaks down into phosphoramide mustard, which alkylates the guanine residue of DNA, forming cross-links.[177,178] The oxidative cell damage 4HC causes is especially concerning in lung transplant patients, who are already

at increased risk of fibrosis and OB. Therefore, it is hypothesized that thioredoxin mimetics will be able to ameliorate 4HC induced oxidative stress in human bronchial epithelial cells (HBECs).

4.2 Methods

4.2.1 Chemicals

Prodrug thioredoxin mimetics (the oligopeptides MB-11, MB-13, and MB-16) were synthesized by Dr. Marzena Bahran. 4HC was purchased from Toronto Research Chemicals (Toronto, ON) DCFH-DA was purchased from Abcam (Cambridge, UK).

4.2.2 Cell Culture

Immortalized human bronchial epithelial cells (HBEC) were grown to 80% confluency in bronchial epithelial growth medium (BEGM). Confluent cells were plated in 96 well plates at 15,000 cells / well and incubated overnight before assaying.

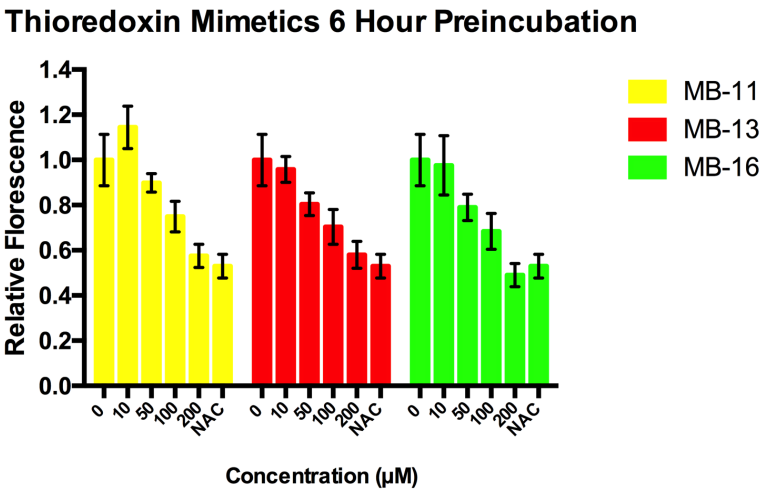
To examine thioredoxin mimetic efficacy on quenching 4HC induced ROS, the DCFH-DA assay was employed. After overnight incubation, the cells were washed in PBS and 0-400 μM MB-11, MB-13, MB-16, or NAC were added to HBEC cells and incubated for 0, 6, or 18 hours. Media containing the compound of interest were collected 30 minutes prior to the end of incubation, cells were washed in PBS, and 100 μM DCFH-DA was added for 30 minutes. Following the DCFH-DA incubation, cells were re-washed with PBS and media containing the compounds of interest were re-added. 50 μM 4HC was then added, and florescence was monitored at $485_{em}/530_{ex}$ for 2 hours.

Cytotoxicity studies were carried out using a Vybrant cytotoxicity assay kit (Molecular Probes, Eugene OR) by measuring glucose-6-phosphate (G6P) leakage. After 15,000 cells / well were plated overnight, 0-400 μM MB-11, MB-13, or MB-16 were added to the wells and incubated for 18 hours. Protocol for the kit was then followed and absorbance was read at $560_{em}/590_{ex}$ for 30 minutes. Detergent was added to the positive control wells to lyse the cell membranes, yielding the total amount of G6P in the wells.

4.3 Results

4.3.1 DCFH-DA Assay

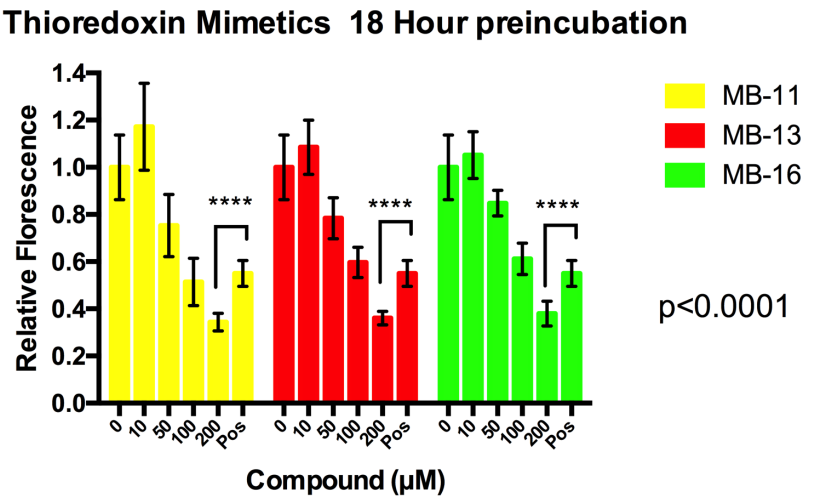
Without pre-incubation of the thioredoxin mimetics before 4HC addition, the test compounds were. However, pre-incubating the thioredoxin mimetics for either 6 or 18 hours showed interesting results. At 6 hours, 200 μM of MB-11, MB-13, and MB-16 were approximately equipotent to 200 μM NAC at quenching 4HC-induced ROS **(Figure 40)**. MB-11, MB-13, MB-16, and NAC all decreased relative florescence to around 60% at the 200 μM concentration.



Thioredoxin mimetic efficacy at quenching ROS after 4-HC challenge in HBEC cells. Positive control is 200 μM NAC

Figure 40. Thioredoxin mimetic efficacy in HBEC after 6 hour pre-incubation. Positive control is 200 μM NAC.

This trend of increasing efficacy with longer pre-incubation times when compared to NAC continued at the 18 hour preincubation time point for all compounds tested. NAC (200 μM) efficacy remained the same, decreasing relative florescence once again to around 60%, but MB-11, MB-13, and MB-16 outperformed NAC at these longer incubation times. NAC (200 μM) was roughly equivalent to the 100 μM thiorexodin mimetic concentrations **(Figure 41)**.



Thioredoxin mimetic efficacy at quenching ROS after 4-HC challenge in HBEC cells. Positive control is 200 μM NAC

Figure 41. Thioredoxin mimetic efficacy in HBEC after 18 hour pre-incubation. Positive control is 200 μM NAC.

4.3.2 Cytotoxicity Assay

Cytotoxicity of the thioredoxin mimetics was determined by measuring glucose-6-phosphate leakage from the cellular membrane. Concentrations from the DCFH-DA efficacy assay were doubled (0-400 μM) and incubated for 18 hours. No significant toxicity (representing more than a 5% increase in cellular death) was seen **(Figure 42)**.

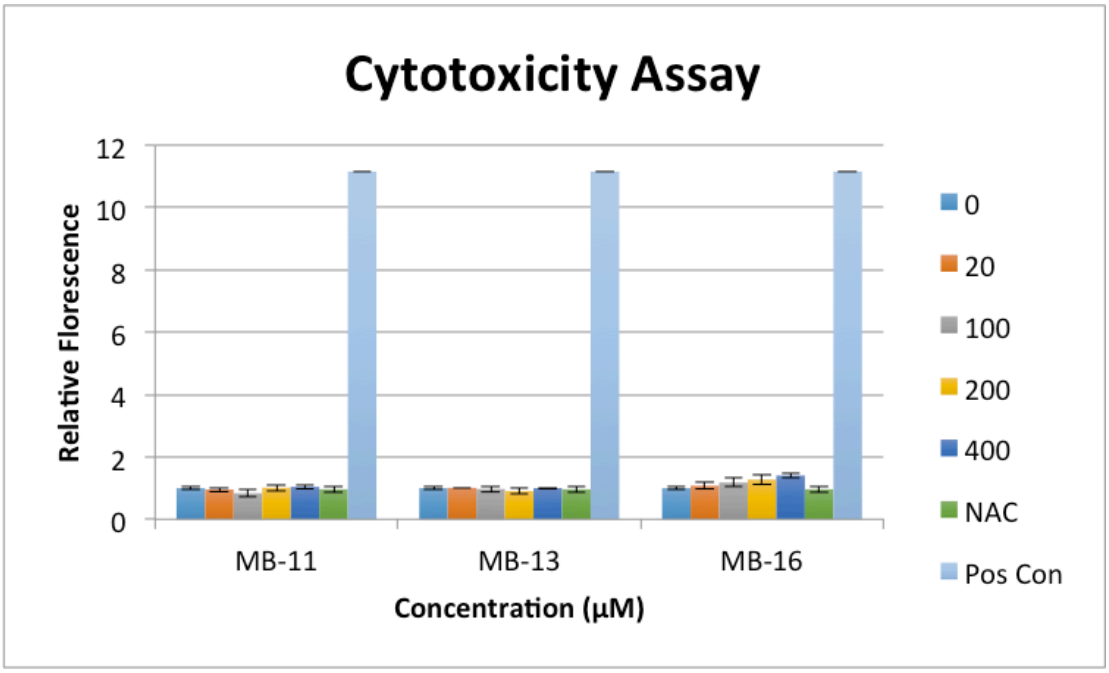


Figure 42. Cytotoxicity of thioredoxin mimetics as measured by G6P leakage. NAC was 400 µM, and positive control represents 100% G6P leakage.

4.4 Conclusions

The thioredoxin mimetics MB-11, MB-13, and MB-16 were ineffective when added concurrently with 4HC, yet all showed efficacy after preincubation with HBEC cells. This preincubation was likely required to allow the deacetylation of the prodrug compounds into their active form. Additionally, the compounds did not show significant cytotoxicity at the concentrations tested. At this time, it is not known if MB-11, MB-13, or MB-16 are substrates for thioredoxin reductase. Elucidation of this potential mechanism of action will be carried out in due course along with studies to confirm disease modification in transplant animals.

Chapter VI:

Conclusions and Future Directions

The IM route of administration of sulfanegen is well suited for mass-casualty cyanide exposure scenarios. Studies in murine, pig, and rabbit models of cyanide toxicity demonstrate suflanegen's efficacy at detoxifying cyanide. However, the FDA's "Animal Rule" necessitates additional conditions be met for approval because ethical concerns prevent efficacy evaluation in humans. The focus of this dissertation is elucidation of sulfanegen's mechanism as a cyanide antidote and addressing the additional hurdles to clinical translation resulting from the animal rule.

Chapter 1 reports both a blood and tissue-specific MST species comparison to demonstrate which animal models of cyanide toxicity are most appropriate for predicting human efficacy. Murine and rabbit models flanked humans for MST specific activity and thus, when combined, represent reasonable models of human activity. In our judgment, these species should suffice as reasonable models of efficacy for humans. An unexpected finding from this study was the difference in MST specific activity that smoking status had in human liver samples. Smokers had statistically significant higher MST specific activities in the liver mitochondria than their non-smoking counterparts. Increased activity could be an adaptation to chronic, low-level cyanide exposure found in cigarette smoke and indicate that mitochondrial MST may be inducible in humans. Although we only addressed MST location and activity here, these data should be consolidated with thiocyanate elimination data to more strongly justify their choice as species for MST efficacy. Comparative thiocyanate elimination kinetics will be addressed in due course.

Chapter 2 reports a UV HPLC method suitable for detecting 3MP, with applications to both quality control and biological quantification. UV detection makes the analysis readily amenable to most laboratories, without the need for specialized detection methods. Previous pharmacokinetic studies by the Logue and Patterson labs yielded a C_{max} of 1775 μM 3MP in rabbits, and if concentrations of 3MP are similar following administration in humans, samples require minimal dilution for analysis. Previously, quality control methods were unavailable.

Chapter 3 reports the possible secondary mechanisms of sulfanegen administration, both in the presence and absence of MST. Sulfanegen showed biologically relevant efficacy as an antioxidant. As a reducing thiol, sulfanegen can quench cyanide induced ROS without a catalyst. Moreover, in the presence of MST sulfanegen administration leads to the production of H_2S, which can also quench cyanide induced ROS. Finally, we examined sulfanegen's ability to directly detoxify cyanide by forming a cyanohydrin. However, even though this mechanism was detectable via mass spectrometry, in the absence of MST sulfanegen was ineffective in reversing cyanide-induced inhibition of cytochrome C oxidase. We therefore conclude cyanohydrin formation is probably not therapeutically relevant for this drug candidate, although sulfanegen's antioxidant actions as a thiol and MST-mediated H_2S generation contribute to overall efficacy.

In addition to the IM route of administration, sulfanegen has previously shown efficacy when administered orally. Therefore, future directions for this

project could include an oral sulfanegen formulation for preventing acute or chronic cyanide toxicity in cases of either contaminated food or drinking water. Moreover, Konzo, a debilitating paralysis condition resulting from chronic, low dose cyanide exposure, may be preventable by taking sulfanegen prophylactically. Additionally, oral sulfanegen could have veterinary applications as a food additive protecting livestock from cyanogenic glycosides in the field.

Further studies required for clinical translation of sulfanegen as an IM injectable antidote include: A battery of GLP safety studies, including mutagenicity, reproductive and development toxicity, definitive efficacy studies in justified animal models as well as an extended phase 1 safety study in humans. The work covered in this dissertation establishes groundwork that should enable studies carried out in GLP laboratory conditions, both for safety evaluations, mutagenicity and definitive animal models under the animal rule. In addition, these studies here should help satisfy the FDA requirement for reasonable attempts to elucidate the mechanism of action for sulfanegen.

CHAPTER VII:

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