Methods for Assessing Cytochrome *c* Oxidase Inhibitors and Potential Antidotes

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Abstract

The Countermeasures Against Chemical Terrorism (CounterACT) Program, sponsored by the U.S. Department of Homeland Security, seeks to promote and support research aimed at finding new (therapeutics) pharmaceuticals that are antidotal toward toxicants considered likely to pose significant terrorist threat. More specifically, this means countermeasures to toxicants that can be easily prepared from readily available precursors in quantities suitable for inflicting mass casualties on civilian and/or military targets. The challenge to Public Health issued by the CounterACT program is to identify and develop antidotes to any such toxicants deemed to be of particular concern, including the mitochondrial poisons sulfide, cyanide and azide. Ideally, in addition to efficacy, the antidotes should be stable enough for stockpiling and safe enough for self-administration. Herein, various biological systems mimicking some relevant aspects of acute poisonings in humans have been tested for their usefulness as experimental "models" suitable for examining toxic mechanisms and assessing the efficacies of putative antidotes. Larvae of the greater wax moth (*Galleria mellonella*) are shown to be a convenient and inexpensive invertebrate model for investigating the action of some mitochondrial poisons and their antidotes. The acute toxicities of sulfide, cyanide and azide have been studied together with the ameliorating effects of sodium nitrite and a cobalt-based scavenging agent. The results obtained with the larvae are

compared to findings employing a cultured mammalian cell line (bovine pulmonary artery endothelial cells) and rodents (Swiss-Webster mice). The *Galleria mellonella* larvae are argued to be an extremely useful intact organism for (i) pre-screening putative antidotes for efficacy and (ii) circumventing any confounding effects that can arise in some studies of intracellular processes due to the presence of blood in intact vertebrates.

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Preface

Before you lies the results of the most intense, difficult, and amazing journey ever. It would not have been possible without the help of my mentors, Jim Peterson and Linda Pearce. I sincerely doubt I can ever thank them enough for taking a chance on me and making me a part of their lab in 2013. I have grown so much as a scientist in the time I have spent under their guidance. Their mentorship and friendship mean the world to me and I am very excited to take all I have learned to my future endeavors. A very special thanks to my committee members, Dr. Aaron Barchowsky and Dr. Joel Haight, I never dreamed it would be so hard to schedule a day for the defense, but we did it. Thank you so much for making time for me.

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Thank you to my family, they are my rock. The words of encouragement were always heard, and always appreciated. It may have seemed that you were last on my list of priorities, but I promise you are always in my heart, and you were a big part of what kept me moving forward.

Last but not least, I would like to thank my friends and colleagues for sharing your stories, making me laugh, listening to me practice, providing advice, and going out to lunch with me. I feel so fortunate to have known such amazing, intelligent, and passionate people. You all made the journey worth it. Thank you!

Finally, thank you to Joanne Pegher for all of her help with my dissertation.

1.0 Introduction

1.1 Mitochondria

Human and environmental exposure to toxic chemicals can happen through accidental or intentional means and depending on the dose received, the exposures can pose a potential risk to human life. Currently, there are more than 80,000 chemicals registered for use in the United States that remain untested for potentially toxic exposures.[1] According to the Environmental Protection Agency (EPA) each year approximately 2,000 new chemicals[2] are introduced and the average person is exposed to more than 100 chemicals[3] before getting to work in the morning. Unlike the extensive research that goes into pharmaceuticals, there are tens of thousands of chemicals (environmental, personal hygiene, cosmetics) that are not as extensively tested for toxicity and this includes their effect on mitochondria in humans.[1, 4]

Mitochondria are the key organelles producing cellular energy in the form of adenosine triphosphate (ATP) through oxidative phosphorylation (OXPHOS).[5-7] The Krebs cycle is the third step in the cellular respiration pathway, after glycolysis and pyruvate oxidation[7], providing the electrons equivalents needed for oxidative phosphorylation.[8] OXPHOS is the pathway by which nutrients are oxidized and energy, in the form of ATP, is produced.[8] The electron transport system or ETS (see Figure 1) is made up of a collection of membrane-embedded proteins and organic molecules.[2] Electrons produced by the transfer of NADH to NAD in complex I and electrons transferred from the reduction of FAD to FADH2 in complex II, flow sequentially into Q (ubiquinone) and on through complex III, cytochrome *c* and finally, into the terminal electron acceptor, cytochrome *c* oxidase,[9 5, 6, 10] also known as complex IV. During OXPHOS, the flow of

electrons (Figure 1) through the ETS pumps protons across the inner membrane and into the intermembrane space[5, 7, 10, 11] forming a gradient in the intermembrane space. During chemiosmosis, the energy stored along the gradient is used to make ATP.[5] Protons are passed back through the inner mitochondrial membrane via a membrane protein called ATP synthase[5, 9, 12] The flux of protons across this gradient generated by electron transfer causes inorganic phosphate (Pi) to bind ADP, forming ATP. [9, 12]

Healthy mitochondria under normal physiological conditions account for more than 90% of cellular ATP production.[8, 13] The physiological functions and mechanisms of the complexes involved in OXPHOS, as well as their conformations and the inhibition sites by various inhibitors, are well known. Mitochondria are not only responsible for energy production; they are involved in several cellular processes, such as apoptosis, and control of the cell cycle[5, 8, 14].

Healthy mitochondrial function is critical to our survival and problems that arise from events such as chemical toxicity can adversely affect mitochondrial pathways, leading to dysfunction of the cell.[15] If the mitochondria cease to function properly and oxygen turnover is inhibited, then death is the outcome. This highlights the need for a rapid, effectual, and reliable screening procedure for evaluating any threat to public health posed by new chemical agents.

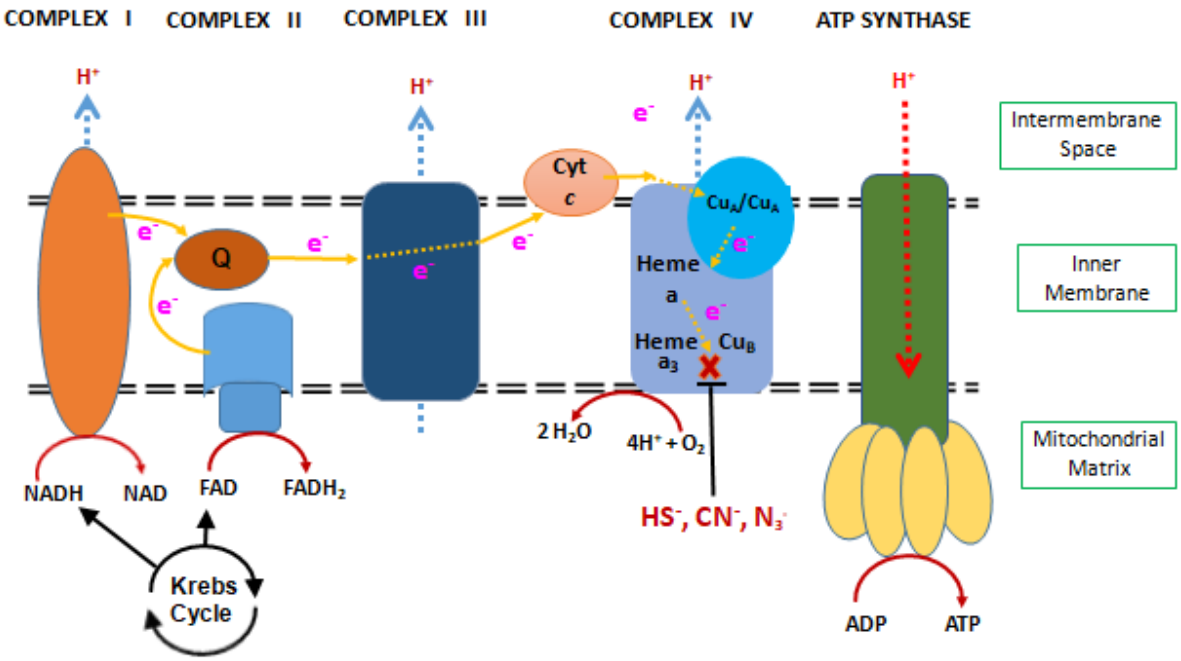

Figure 1. Electron Transport System.

The electron transport system which is embedded in the mitochondrial inner membrane transfers electrons, from Krebs cycle products, NADH and FADH2, to the carrier molecules and uses them to concentrate hydrogen ions in the intermembrane space. The hydrogen ions then produce an electrochemical gradient that flows into the matrix through ATP synthase which then converts ADP to ATP. The flow of electrons through cytochrome *c* oxidase produces oxygen. This process can be inhibited by the toxicants binding the active site.

1.2 Cytochrome *c* Oxidase

It can be argued that one of the most essential components of the mitochondrial electron transport system is complex IV or cytochrome *c* oxidase[16]. In eukaryotes the enzyme is located in the inner mitochondrial membrane and serves as the terminal electron acceptor in the ETS[16]. In mammals, cytochrome *c* oxidase is made up of 13 subunits with the catalytic core containing three mitochondrial DNA encoded subunits (I, II, III) that are synthesized in the mitochondrial matrix.[9, 12, 13] Of the three, two play a key role in oxygen turnover (I and II); the function of subunit III is not entirely known[12]. The three subunit core is surrounded by smaller subunits encoded by the nuclear genome, that have been suggested to modulate the catalytic activity of the enzyme and to protect it from oxidative damage.[12] Different organisms contain a variable number of cytochrome *c* oxidase subunits ranging in number from 4 to 13.[12] The catalytic core subunits (I-III) are rather evolutionarily conserved and carry the heme and Cu^{+2} redox centers.[12, 13, 17] It is significant to recognize that the cytochrome *c* oxidase enzyme is found in all organisms that utilize oxygen. Popovic *et al*,[17] studied the similarities of cytochrome *c* oxidase in different organisms, and found that they are structurally similar, sharing the same key amino acid properties.

Internal electron transport in cytochrome *c* oxidase is the rate limiting step of the respiratory chain under normal physiological conditions.[13] Cytochrome *c* oxidase contains two heme a groups (a and a_3) and three copper ions, arranged as two copper centers (Cu^{2+} A and Cu^{2+} B)[12, 13]. Electrons are transferred from the Cu_A center to $heme_b$, and then to the active oxygen binding site, consisting of heme a_3/Cu_B [5, 12, 13]. This active site is a binuclear center for oxygen binding where electrons reduce molecular oxygen (the terminal electron acceptor) to water ($4e^- + O_2 + 4H^+ \rightarrow 2\ H_2O$).[18] Four electrons ($4e^-$) transferred from Cu_A in subunit 2 to the heme a_3/Cu_B binding site in subunit 1, and $4H^+$, "scalar protons" are required to react with an oxygen molecule

to produce 2 water molecules[13, 18]. Cytochrome *c* oxidase activity directly affects mitochondrial function[10] and this activity is an indicator of the oxidative capacity of the cell[14, 16]. Many xenobiotics have been shown to inhibit cytochrome *c* oxidase activity in a dose dependent manner leading to mitochondrial stress.[19-21] The heme a_3/Cu_B active site is the target of sulfide, cyanide and azide inhibition. If an inhibitor binds this site, it stops oxygen turnover, with potentially lethal consequences.

1.3 The Inhibitors and the Putative Antidotes

1.3.1 Sulfide, Cyanide and Azide

To start, come clarification of terms "sulfide", "cyanide" and "azide" is needed. Sulfide is a 50/50 combination of both H_2S and the anion HS^-,[22] cyanide is almost all (98%) HCN,[23] and the anion CN^-, finally, azide is composed almost entirely of the anion N_3^-.[24] All three are dangerous toxins with potential public health implications. It is widely accepted that the mechanism of action of these three toxicants is similar and the primary target is cytochrome *c* oxidase within the central nervous system.[25-29] These mitochondrial toxicants bind the heme a_3/Cu_B binding site, blocking oxygen turnover[25-29]. Upon inhibition of cytochrome *c* oxidase, death results from respiratory paralysis.[25, 27, 30] Electron transfer activities of different cytochrome *c* oxidases are essentially independent of source tissue and or species.[13] Inhibition constants (K_i) have been calculated for various inhibitors including the three mentioned above, sulfide: 0.45μM,[27] cyanide: 0.2μM and azide: 22μM.[31] The K_i is an indicator the inhibitors potency, the smaller the K_i the greater the binding affinity, and therefore, the smaller the amount needed to inhibit enzyme activity.[32]

Hydrogen sulfide poisonings mostly occur as occupational accidents, but there are other public health concerns. For years hydrogen sulfide has been infamous for its use in "detergent suicides" in both Japan and the United States[33] with 75 cases of chemical suicide between 2008 and 2015,[34] 80% of these cases resulted in first responder injury.[34] In Japan, residents of an apartment building had to be treated for exposure when an individual committed suicide in an apartment and the hydrogen sulfide gas entered the ventilation system.[33] The danger lies not only with the victims, but with first responders entering the scene. Even more serious than the chemical suicides is the potential of hydrogen sulfide to be used in a nefarious capacity, it has been mentioned in the Mujahedeen Poisons Handbook,[35] where extremists provide information on its use in scenarios expected to lead to mass casualties.

Hydrogen sulfide results from the breakdown of organic matter in the absence of oxygen; such is the case in swamps and it occurs naturally in natural gas, well water and volcanic gasses.[33] Most exposures stem from occupational means such as manure storage tanks and sewers[33] (Table 1). Sanitation workers are often exposed when cleaning or maintaining municipal sewers and septic tanks [36]. This danger is also found in farm workers who can be exposed when cleaning manure storage tanks, and oil and natural gas drilling and refining employees who may be exposed when hydrogen sulfide is present in oil and gas deposits.[36]

Sodium hydrosulfide (NaHS) has a distinct rotten egg smell. At 0.1 ppm sulfide quickly blocks the sense of smell (anosmia).[37] At concentrations higher than 500 ppm the gas causes irritation to nose, throat and lung tissue resulting in pulmonary edema, and eventually, (at concentrations >800 ppm) death.[35, 37, 38] Sulfide reacts with a wide variety of biological materials including hemoglobin making working with this toxin difficult.[35, 38] Currently, there are no approved antidotes for hydrogen sulfide toxicity.

Sodium azide is a rapid acting, potentially deadly, compound used as a chemical preservative in laboratories and hospitals, explosives detonators, and as a pest control agent and soil fumigant in agriculture.[37] Most notably azide is used as a fuel in automobile airbags.[37] In airbags, a sodium azide pellet with an oxidizing agent is heated to about 300°C upon impact, decomposing to nitrogen, inflating the airbag and leaving a residue of sodium oxide.[37] The potential impact of having azide in the inflators of vehicle airbags will be felt in the next few decades when these cars age. Accidental release of the azide into the environment could happen due to a lack of regulations requiring the detonation of airbags when a car is destroyed.[39] Sodium azide was linked to the suicide of a UC Berkley professor in 2014.[40] In 2010, sodium azide was added to an iced tea dispenser to poison customers of a local Dallas County, Texas restaurant.[41] (CDC). Sodium azide is also mentioned in the Mujahedeen Poisons Handbook.[35]

In 1954 sodium azide's toxicity was explained by Robertson and Boyer as the inhibition of heme-type enzymes such as catalase, peroxidase and cytochrome *c* oxidase.[42] Hypotension is the most common health affect from exposure to low levels of sodium azide.[37] Other effects include hypothermia, loss of vision, clonic seizures, pulmonary edema, coma, and death.[43] Onset of symptoms can occur onset anywhere from an hour to several days depending on route of exposure.[37] Both azide and cyanide are on the list of agents of concern with the Department of Homeland Security.[44, 45]

Throughout history, cyanide (CN^-) has been successfully used as a poison and as a warfare agent.[46] The highly reactive cyanide salts are used for industrial applications, chemical synthesis, electroplating, agriculture, manufacturing, fumigants and pesticides.[19] Cyanide can be found in cyanogenic food, [47, 48] and in waste products from mining. Most infamously, cyanide was used by the Germans in World War II in the gas chambers (Zyklon B) to carry out genocide.[44] [49-52] In

addition, the Japanese made frangible hydrocyanic acid grenades. In 2010, a dam at the Baia Mare gold mine overflowed and caused a flood of toxic cyanide 40km long down the River Tisza in northern Yugoslavia. This accident caused a tide of dead fish, spreading the poison further downstream into the Danube River.[53]

Though occupational exposure is rare, there is a growing concern for fire fighters, as cyanide is a by-product of the combustion of polyurethanes.[54] Cyanide readily enters the bloodstream, rapidly diffusing into tissues where it irreversibly inhibits cytochrome *c* oxidase, causing death within minutes[55] (The estimated lethal dose in adults is 50-200 mg NaCN or 100-300 ppm HCN[55]). This rapid action is what makes cyanide so lethal. While cyanide is a potent toxicant (at pH 7 NaCN is 98% HCN[23]), there are mechanisms in the body to detoxify small amounts of CN^-, for example, rhodanese is a mitochondrial enzyme that detoxifies cyanide (CN^-) by catalyzing the cyanide-dependent cleavage of thiosulfate to form thiocyanate (SCN^-) and sulfite (SO_3^{2-})[56] (Rhodanese $S_2O_3^{2-} + HCN \rightarrow SCN^- + HSO_3^-$). The thiocyanate is then excreted in the urine over a period of days.[56] Unlike sulfide and azide, there are available antidotes for cyanide poisoning.

Table 1. Cytochrome *c* Oxidase Inhibitors.

	Sulfide	Cyanide	Azide
Molecular Weight	56.06 g/mol[c]	65.116 g/mol[f]	66.0099 g/mol[i]
Appearance	Yellow chips[c]	White crystalline solid[f]	White solid[i]
pK_a	7[c]	9[f]	4.8[i]
Mode of Exposure	Oil and natural gas drilling and refining, agriculture, sanitation, decomposition[a]	Cyanogenic foods, cigarette smoke, combustion, mining, manufacturing, pesticides[d]	Automobile airbags, chemical preservative (hospitals, laboratories), detonators, agriculture (pest control)[h]
Symptoms of Exposure	Pulmonary edema, respiratory arrest, death[b]	Heart, brain and nerve damage, death[e]	Heart, brain damage, pulmonary edema, death[g]
Public Health Concerns	Suicides, Terrorism[b]	Health, Terrorism[e]	Accidents, Terrorism[h]

[a] Sulfide: Malone-Rubright, S., *et al, Nitric Oxide* 17 (2017) 1-13
[b]. ATSDR Agency for Toxic Substances and Disease Registry, Hydrogen Sulfide Carbonyl Sulfide, Last updated: March 3, 2011, https://www.atsdr.cdc.gov/substances/toxsubstance.asp?toxid=67
[c] PUBCHEM Hydrogen Sulfide. U.S. National Library of Medicine National Center for Biotechnology Information. https://pubchem.ncbi.nlm.nih.gov/compound/hydrogen_sulfide
[d] Cyanide: Malone, S. *et al, Toxicology of Cyanides and Cyanogens: Experimental, Applied and Clinical Aspects* (2015) 368
[e] ATSDR Agency for Toxic Substances and Disease Registry, Cyanide, last updated: March 3, 2011. https://www.atsdr.cdc.gov/substances/toxsubstance.asp?toxid=19
[f] PUBCHEM Sodium Cyanide. U.S. National Library of Medicine National Center for Biotechnology Information. https://pubchem.ncbi.nlm.nih.gov/compound/Sodium-cyanide
[g] Azide: Chang, S. *et al, Int J Toxicol* (2003) 22, 175-186
[h] CDC Centers for Disease Control and Prevention. The National Institute for Occupational Safety and Health (NIOSH). SODIUM AZIDE : Systemic Agent. Last updated: May 12, 2011 https://www.cdc.gov/niosh/ershdb/emergencyresponsecard_29750027.html
[i] PUBCHEM Sodium Cyanide. U.S. National Library of Medicine National Center for Biotechnology Information. https://pubchem.ncbi.nlm.nih.gov/compound/Sodium-azide

1.3.2 Putative Antidotes

Two antidotes are available for use against cyanide poisoning in the United States. First, Nithiodote™,[57] which consists of a combination of sodium nitrite/sodium thiosulfate, is an off

label antidote that has been approved, but not FDA labeled, for use in humans.[58] Nitric oxide (NO) is a messenger molecule that regulates physiological processes in mammals[26] and multiple studies have shown that NO is an antagonist of cyanide, ameliorating its effect on cytochrome *c* oxidase enzyme activity [59, 60 61, 62]. NO produces methemoglobin in this reaction: $HbO_2 + NO \rightarrow Hb^+ + NO_3^-$.[63] Then, methemoglobin can bind the toxicant of interest (e.g. $Hb^+ + CN^- \rightarrow HbCN$).[63] In addition, the enzyme rhodanese[64] acts in concert with a sulfur donor, to convert cyanide to the less toxic metabolite, thiocyanate (SCN^-). Pearce *et al*,[59, 65] proposed that nitrite acts as a NO donor and not a methemoglobin former in the mechanism of antidotal action toward cyanide toxicity.

The second available antidote is Cyanokit™,[66] an FDA approved antidote that is a form of vitamin B_{12}, or hydroxocobalamin, which binds cyanide and sequesters it for excretion, acting as a decorporation agent.[66] Hydroxocobalamin was FDA Approved in 2006.[66, 67] It has 6 ligands around cobalt atom, and only 1 available site (OH^-) to substitute HS^-, CN^- or N_3^- ligands (Figure 2A). It is a very large, complex molecule with a molecular weight of 1,346 g/mol.[68] In addition to being large it is expensive, the average cost per gram is $180/g (making it nearly $1,000 for single adult intravenous dose)[23], and patients may need a second dose. Because of the expense many ambulances that carry it, usually only carry one, most will carry Nithiodote™ instead. Again, it is effectively antidotal toward cyanide, but the antidotal effect for azide and sulfide remained inconclusive and until recently there are no approved antidotes for sulfide or azide.[35] Due to similar mechanisms of toxicity between sulfide, azide and cyanide, however, existing cyanide therapies could be expected to be beneficial for sulfide and azide toxicity.

Our group has sought to develop new (simpler) cobalt-containing compounds that were smaller, less complex structures that would increase solubility, reduce costs, and offer equal or better antidotal effect toward toxicants than hydroxocobalamin, especially in a mass casualty

situation. A potential antidotal compound is CoN_4[11.3.1] (a complex of cobalt (II) with the ligand2,12-dimethyl-3,7,11,17-tetraazabicyclo-[11.3.1]heptadeca-1(17)2,11,13,15-pentaene (CoN_4[11.3.1])[69] (Figure 2B) that, can bind two molecules of cyanide in a cooperative fashion with an association constant of 2.7 (±0.2) × 10(5).[70] CoN_4[11.3.1] or "the Busch compound", was first synthesized by Darryl Busch in Ohio in 1967. ([69] It is smaller, cheaper ($180/g), and might be easier to deliver in mass casualty applications. It provided good antidotal results when used in sub-lethal cyanide intoxication assays on mice.[23]

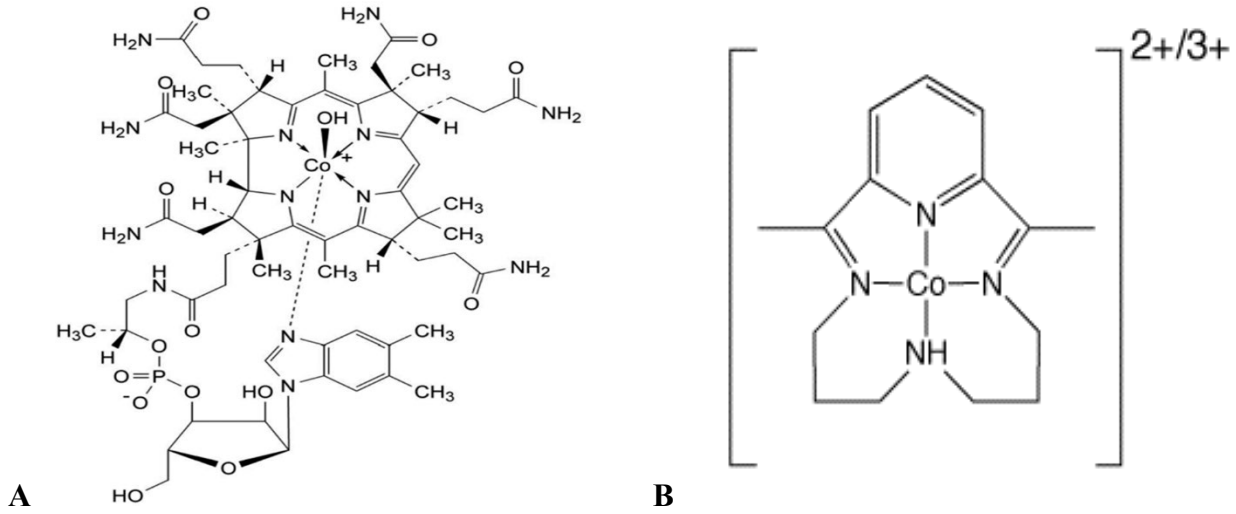

Figure 2. Structures of cobalt containing compounds.
A) hydroxocobalamin (vitamin B12); and B) CoN4[11.3.1](cobalt(II/III)(2,12-dimethyl-3,7,11,17tetraazabicyclo-[11.3.1]heptadeca-1(17)2,11,13,15-pentaene)

1.4 Typical Models Used for Toxicological Experiments

The reality is that animal studies and *in vitro* models are always going to be used for screening of toxic agents in order to identify and predict potential ill effects to humans, wildlife and the environment.[71] We need an efficient method of screening dangerous chemical, prior to

their release into the community, before these chemicals impact public health while using fewer animals in toxicological testing.

There are many models used for preliminary toxicological experiments, with mammals such as mice and rats being among those traditionally preferred.[72] However, the use of mammals in research is expensive and requires ethical and regulatory oversight.[73] Russell and Burch introduced the 'Three R's" in 1959, Replacement, Reduction and Refinement.[74] These principles encourage the use of alternative models (i.e. invertebrate and cell models) in research investigations in place of mammals to reduce the number of mammals used in an investigation, thereby decreasing the incidence or severity of inhumane procedures applied to vertebrate animals that still need to be used. It is important to keep in mind that models used in these studies should mimic some aspects of human poisoning and/or treatment.[74] Zebrafish, fruit flies and mammalian cells are just a few of the models that are currently utilized in preliminary investigations,[71] all have pros and cons but all of these models serve as worthy alternatives to mammals in toxicological experiments.

1.4.1 Cell Models

Cells offer researchers many advantages for preliminary toxicological investigations (Table 3). *In vivo* assays are, in some cases, an excellent tool for these investigations and can provide quick, reliable data for proof of concept experiments.[71] Investigations can be carried out in a controlled environment, studying just a part of an organism in great detail.[71] Though one of the most important advantages of cell culture is that you can control the physiochemical environment,[71] results from cells that have been isolated and grown in an artificial environment outside of the organism may not accurately predict the conditions inside of a living organism where

mechanisms of the toxicity may be acted on by other pathways.[71] As well, the use of cells prevents knowledge of the behavioral patterns affected by these mechanisms.

1.4.2 *Drosophila melanogaster*

Invertebrate models, have proven very useful in the study of bacterial, fungal and parasitic pathogenesis and treatments thereof,[43, 71, 73, 75-78] as well as advantageous candidates for preliminary chemical testing[71]. *Drosophila melanogaster* (Table 3), or the fruit fly, has been a very important model in many biological and biomedical studies and gained recognition as a tool to identify molecular genetic mechanisms of toxic substances[71, 77]. Many genes correspond to human genes and control the same biological functions; about 60% of genes are conserved between fruit fly and humans.[77] Several fly models have been developed to study mechanisms of complex diseases such as Parkinson's and Alzheimer's due to the ease and cost effectiveness of producing transgenic flies to generate models of human diseases.[71] Though the anatomy of the brain and other major organs within *Drosophila* differ from that of humans,[77] fundamental cellular processes, genes and signaling pathways are conserved between them. The insect models not only save money (Table 3), they require very few ethical or regulatory restrictions (Table 3).[78] These models have a rather short life cycle, and produce a large number of offspring in the lab setting. Multiple generations can be observed in a very short time span. Many larvae can be used in each experiment making data easy to obtain, more reliable and repeatable. However, *Drosophila* larvae are very tiny and, for instance, injections are almost impossible to accurately administer.[77] Using fruit flies highlights some issues such the lack of an adaptive immune system, and different drug effects when compared to human studies.[77]

1.4.3 Zebrafish

Zebrafish (*Danio Rerio*) have been used in many studies, evaluating the toxicity of agrochemical agents,[71, 79, 80] but more recently, they have been used to assess toxicity of pharmaceutical compounds.[71, 79, 80] Zebrafish are used to screen for effects of exposures on the cardiac system, central nervous system, the intestinal tract, auditory and visual functions, pro-convulsant potential and bone formation.[79, 80] Zebrafish are not only small, and easy to breed quickly, they share similar molecular pathways and physiology to humans.[71] The zebrafish eggs are transparent (Table 3) and remain so throughout embryonic development. Because of the lack of pigment, little magnification is needed to view adverse effects of chemical exposure on development of the brain, notochord, and heart. Unlike mammalian embryological development the zebrafish embryo can be continually followed in live individuals rather than harvested embryos and fetuses. They are a powerful *in vivo* model used in high-throughput screens for toxicity testing, small-molecule screening, and drug discovery[80]. Their genome has been sequenced and they share 70% of genes with humans[79] (Table 3).

Some drawbacks of zebrafish research include: they need to be kept in a clean tank, they have temperature and food requirements (Table 3) and aquatic vertebrate species are subject to existing regulatory oversight according to the *Guide for the Care and Use of Laboratory Animals*[80]. Most significantly, in relation to the present worn, zebrafish are not air breathing.

1.4.4 Mouse Model

Mice[82] are the gold standard for toxicological studies because they have biological, physiological and behavioral characteristics that closely resemble those of humans, and many

symptoms of human conditions can be replicated in mice. They are indispensable in contributing to our understanding of the functions of genes, the etiology and mechanisms of different diseases, and the effectiveness and the toxicities of medicines and chemicals.[72] Swiss-Webster mice are advantageous in toxicology because they are an outbred model, exhibiting variability between animals whereas inbred mice are effectively genetically identical, unlike humans. [72, 81]

Adult mice reproduce quickly (as often as every three weeks), and have a very short generation time (about 10 weeks) allowing for multiple generations to be observed at once.[81] The life span is approximately two years which makes it easy to measure the effects of aging. Mice are a preferred model for genetic research because they share 92% of their genes with humans[72] (Table 3) and their genome can be easily modified to present desired characteristics to simulate human disorders.[72] Mouse models have aided in the progress of research and enabled the development of important new drugs and therapies in humans. They have been pivotal in studying naturally occurring diseases that affect complex biological systems found in humans, such as the immune, nervous system, and cardiovascular diseases. [72]

In toxicological studies, whole animals are often required to compare the effects of a toxic chemical in a living organism to that of a known human response. Laboratory tools and cells simply cannot duplicate these complicated phenomena. Whole animals are complicated and therefore simple systems are often required. There are obvious differences between vertebrates, invertebrates and cells such as blood and biological structures (i.e. lungs, liver, and brain) that still require that a comparison be drawn between at least two models to glean a full understanding of any exposure. [71]

1.4.5 *Galleria mellonella*

Figure 3. Different developmental stages of *Galleria mellonella*.
Approximately 30-day-old caterpillar (last instar larval stage), pupae, shed chrysalis and adult moth.

Intact mammals are often too complicated for detailed niche studies where a different model is needed. There are over two million insects available for development as models for use in preliminary toxicological testing,[82] and unlike vertebrate models, insects do not come with a high price tag or require special ethical and legal requirements associated with their use in the laboratory setting (Table 3). *Galleria mellonella* have been successfully used for the study of bacterial, fungal and parasitic pathogenesis[83, 84] and treatments thereof, as well as for evaluating the efficacy of antimicrobial agents,[75, 78] food additives[85] and pesticides[78]. Along with *Drosophila melanogaster, Galleria mellonella* (L.) (Lepidoptera: Pyralidae) has emerged as a desirable model for such studies.[86]

After employing *G. mellonella* larvae in bacterial infection investigations, the question arose as to whether or not the larvae could be beneficial in preliminary *in vivo* toxicological studies. *G. mellonella* larvae displayed many advantages over other models. They can be administered

toxins through routes similar to *Drosophila* (i.e., inhalation, feeding or topical applications)[43] (Table 3). Unlike vertebrate models such as mice and zebrafish, larvae have no special care requirements (Table 3), and they can be reared in temperatures ranging from 15°C through 37°C allowing researchers to mimic *in vivo* conditions. Research has established that there is a strong structural and functional similarity between the insect immune response and that of mammals, allowing the use of insects in place of, or in comparison to common toxicological models, such as mice[87] in preliminary toxicity studies.

Physiologically there are many similarities between insect and mammalian anatomy. *Galleria mellonella* have a heart with an aorta and a nervous system. Unlike mammals, the larvae do not have a brain, instead they have ganglia which are located in several places along the nerve cord, with a main ganglia acting as the primary brain-like structure.[88]

The insect fat body is an organ that functions in a metabolically similar fashion to the mammalian liver and adipose tissue.[10, 88, 89] Consisting of a network of lobes, it is a delicate connective-tissue membrane that stores fat reserves, glycogen and protein. The cells are laden with mitochondria and are as diverse in enzymes as the mammalian liver.[88] A number of antioxidant enzymes produced by the fat body such as superoxide dismutase, catalase and glutathione-s-transferase are highly conserved between the species.[90]

The Malpighian tubes function similar to the mammalian kidney.[10, 88] These simple tubular glands which open at the junction between the midgut and hindgut are the main excretory organs. The cytoplasm of the Malpighian tubes is rich in mitochondria.[88] Finally, the digestive system of *G. mellonella* is a relatively simple, straight-through type of gut, consisting of a foregut, midgut, hindgut and anus.[88, 89] Where they are similar to humans, is in the midgut, which is lined with epithelial cells similar to the intestinal cells of the mammalian digestive system.[89]

The *G. mellonella* circulatory system differs from that of humans as it is open.[10, 88, 89] There is little distinction between hemolymph and interstitial fluid, and the hemolymph circulates freely throughout the body cavity,[89] the closed tubular heart circulates hemolymph primarily by the contractile action of a dorsal vessel, which is divided into an abdominal heart and a thoracic aorta[88]. Dissolved substances circulate by diffusion.[10] The insect respiratory system does not include lungs; instead spiracles are located on the lateral sides of the larvae. These spiracles are openings that allow for gas (O_2, CO_2) exchange through the trachea. The trachea branch off into tracheole, which branch to touch every cell, allowing for oxygen transport within the body without an oxygen carrier molecule such as hemoglobin.[89]

The hemolymph is a functional analogue of mammalian blood,[91] (Table 2) though it does not function in the transport of gasses, primarily oxygen, the hemolymph contains hemocytes which function in the immune defense of the larvae.[10, 89] Additionally, hemolymph bathes all cells in the body and accounts for 50% total body water. It produces a high pressure hydrostatic skeleton giving the larvae its body shape and allowing it to move. Hemolymph has pH of 6.4-6.8 (Table 2) and a buffering capacity (bicarbonate, inorganic phosphate, carboxylic and amino acids). Human neutrophils and insect hemocytes exhibit many similarities[92] including the ability to produce superoxide by a functional NADPH oxidase complex.[93]

Table 2. Difference between mammalian blood and larval hemolymph.

	HEMOLYMPH	BLOOD
COMPOSITION	90% water / 10% solids	95% water / 5% solids
pH	6.4 – 6.8	7.35 to 7.45
CELLS	Hemocytes, platelets	RBC, WBC, platelets
COLOR	Yellow/ green	Red
OXYGEN TRANSPORT	None	Hemoglobin
CONTENTS	Water, inorganic salts, organic compounds	Water, dissipated proteins, mineral ions, carbon dioxide

Insect Physiology and Biochemistry, second edition, 2008

Insect electron transport is similar to that of many vertebrates and invertebrates.[94] On the other hand, glycolysis in insect flight muscles is always aerobic and can persist for prolonged periods of time.[10] This is due to the extensive tracheole system which is capable of supplying sufficient oxygen for totally aerobic oxidation during flight.[10, 88] While glycolysis is a reasonable source of energy for cellular metabolism, electron transport is where most of the ATP production, specifically oxidative phosphorylation is generated in the insect larvae.

Like mammals, the NADH dehydrogenase enzyme-CI complex is bound to the inner membrane, however, the glycerol-3-phosphate dehydrogenase, (located on the outer portion of the inner membrane) is linked to a flavoprotein other than NAD^+.[10] The metabolic water that is formed at the end of the chain is an important source of water for insects,[10, 88] especially for the larvae that are not eating in the last instar. It has been noticed that after being exposed to sulfide, cyanide, and azide the larvae would eat the paper towel circle in the petri dish and there would be an increase

in excrement left in the dish. This was not observed in the sentinels or in larvae treated with only PBS, cobalt, or nitrite.

Table 3. Current Toxicological Study Models.

	Mouse	Zebrafish	Fruit Fly	Wax Worm	Mammalian Cells
Scientific name	*Mus musculus*	*Danio rerio*	*Drosophila Melanogaster*	*Galleria mellonella*	*SHSY-5Y BPAEC*
Stages	Any stage of development	Embryos, adults	Larvae, flies	Larvae	n/a
IACUC	Yes	Yes	No	No	No
Special Housing Requirements	Yes	Yes	Yes	Bait container	Incubator, 5% O_2
Temperature requirements	18-23°C	24-27°C	18-25°C	15-37°C	37° C
Food requirements	Yes	Yes	Yes	No	Media
Veterinary care	Yes	Yes	No	No	No
Blood/ Hemolymph	blood	blood	hemolymph	hemolymph	n/a
Route of exposure (accurate)	Inhalation, topical, injection, feeding	Injection, waterborne	Inhalation, injection, feeding	Inhalation, topical, injection, feeding	Direct exposure
Genome sequenced	Yes	Yes	Yes	No	n/a
# of genes	23,000	26,000	14,000	14,602	n/a
% genes shared with humans	92%	70%	60%	Not yet available	n/a
Benefits	Similar physiologically to humans	Transparent eggs, embryonic development in living model	Many genes correspond to human genes, control same biological functions	Used in bacterial studies, easy to inject	Interaction between molecules within Eukaryotic system

1.5 Overall Objective of the Dissertation

In addition to the thoughtful identification of candidate compounds, the rational design of improved toxicant-specific antidotes requires at least some understanding of the molecular mechanisms of both the toxicant and potential antidotes. In obtaining this information, it was necessary to conduct studies involving synthesized compounds, isolated biochemicals, cultured cells, excised tissues, and intact organisms. For some time our research group has focused on mitochondrial (electron transport system) toxicants and their amelioration utilizing these experimental systems.

It is widely accepted that the mechanism of action of sulfide,[95, 96] cyanide,[19] and azide [37] is similar, and that cytochrome *c* oxidase located primarily within the central nervous system is their primary target for inhibition, resulting in death from respiratory paralysis. Cyanide is apparently the only one of the three toxicants for which there are known antidotes. Following the earlier work of our group concerning the discovery and improvement of antidotes to cyanide, we began attempts to evaluate the significance of the possible antidotal effects of the nitric oxide donor, sodium nitrite,[59, 60] and the anionic ligand scavenger, CoN_4[11.3.1],[23, 69] on sulfide and azide, toxicities using mice. [22]

While useful information was obtained, some experimental limitations became apparent during these studies. First, there was the confounding/interfering role of hemoglobin in the biochemistry being studied led to some ambiguity in interpretation of the results. Second, the mice exhibited a variation in response to the three toxicants, resulting in different experimental paradigms for exposure and recovery assessments having to be developed for each toxicant. This prompted a need for multiple behavioral assessments to be developed and implemented, making straightforward comparison of the effectiveness of any given potential antidote toward multiple

toxicants extremely challenging. Initially, in an effort to circumvent these issues, a study with cultured mammalian endothelial cells was undertaken (Section 2). Unfortunately, some toxicants of interest to our research group, as well as some of the potential antidotes to be tested, interfered directly with many of the molecular (indicator) probes that are typically used to monitor cellular functions. For this, as well as other reasons, the cultured cells presented practical experimental difficulties that were at least comparable to those observed with the intact mice.

Our group was interested in finding a non-hemoglobin invertebrate organism that would be a useful toxicological model system for preliminary screening of antidotes and some early-stage mechanistic investigations. The already widely used fruit fly (*Drosophila melanogaster)* and zebra fish (*Danio rerio*) approaches were quickly discounted as inadequate for intended application so we turned to another readily available organism, the larval (pre-pupation) stage of the greater wax moth (*Galleria mellonella*) commercially available as "wax worms", commonly used as fishing bait, reptile treats, and commonly used, roasted, as the "protein" content in some commercial bird feeds.

Since their immune systems seemingly share some similarities with ours, the wax worms have started to become accepted as a model for bacterial infections.[43] In some preliminary investigations (not published) we had looked at the effect of antidotes such as sodium nitrite on *Escherichia coli* infections in *G. mellonella*. Knowing that the wax worms are able to be injected accurately and display no negative effects when inoculated with nitrite made us hypothesize that maybe these larvae would be a suitable non-hemoglobin model to investigate the mechanism of sodium nitrite amelioration in sulfide, cyanide and azide toxicities. In a similar manner, we wanted to investigate amelioration of the effects of the three toxicants in the *G. mellonella* caterpillars by CoN_4[11.3.1]. We anticipated that comparison of

the findings with the cultured cell and mouse data might be of assistance in the further interpretation of the results from the mammalian systems. To date, there is only one other published study exploring the use of *G. mellonella* as a viable model for preliminary toxicological studies – also from our group.[97]

2.0 Sulfide Toxicity and Its Modulation by Nitric Oxide in Bovine Pulmonary Artery Endothelial Cells

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2.1 Abstract

Bovine pulmonary artery endothelial cells (BPAEC) respond in a dose-dependent manner to millimolar (0–10) levels of sodium sulfide (NaHS). No measurable increase in caspase-3 activity and no change in the extent of autophagy (or mitophagy) were observed in BPAEC. However, lactate dehydrogenase levels increased in the BPAEC exposed NaHS, which indicated necrotic cell death. In the case of galactose-conditioned BPAEC, the toxicity of NaHS was increased by 30% compared to that observed in BPAEC maintained in the regular glucose-containing culture medium, which indicated a link between mitochondrial oxidative phosphorylation and the mechanism of toxicant action. This is consistent with the widely held view that cytochrome *c* oxidase (complex IV of the mitochondrial electron-transport system) is the principal molecular target involved in the acute toxicity of "sulfide" (H_2S/HS^-). In support of this view, elevated NO (which can reverse cytochrome *c* oxidase inhibition) ameliorated the toxicity of NaHS and, conversely, suppression of endogenous NO production exacerbated the observed toxicity. Respirometric measurements showed the BPAEC to possess a robust sulfide oxidizing system, which was able to out-compete cytochrome *c* oxidase for available H_2S/HS^- at micromolar concentrations. This detoxification system has previously been reported by other groups in several cell types, but notably, not neurons. The findings appear to provide some insight into the question of why human survivors of H_2S inhalation frequently present at the clinic with respiratory insufficiency/pulmonary edema, while acutely poisoned laboratory animals tend to either succumb to cardiopulmonary paralysis or fully recover without any intervention.

2.2 Introduction

There is still no approved antidote for hydrogen sulfide poisoning and, unhelpfully, a better clarification of the mechanism(s) of acute toxicity remains a stumbling block to the rational development of effective therapies. While it is widely accepted that the principal molecular target for the acute toxicity of hydrogen sulfide (H_2S) and/or its mono-anion hydrosulfide (HS^-) is mitochondrial cytochrome *c* oxidase (complex IV of the electron-transport system)[38, 96, 98-101] the evidence for this *in vivo* remains circumstantial and, consequently, ambiguous, or even contradictory. For instance, it is not clear how to reconcile the observation that free H_2S/HS^- seemingly only persists for a matter of seconds in the bloodstream[102-105] yet onset of symptoms presumably associated with cytochrome *c* oxidase inhibition by H_2S/HS^- occurs at 2 minutes after injection of the toxicant dose.[38] Confounding matters further, it is noteworthy that significant numbers of human victims of H_2S inhalation arrive at the clinic exhibiting compromised respiratory function 30 minutes or more after exposure and frequently succumb hours later.[96, 100, 106-108] We began the present study with the expectation that employing cultured cells as the experimental system would enable us to at least demonstrate inhibition of cytochrome *c* oxidase by H_2S/HS^- in the intact cellular environment, free of any potential experimental interference from factors in the bloodstream and, subsequently, go on to investigate possible reversal of this inhibition by putative antidotes to the toxicant. Since the lung is reported to be particularly sensitive to H_2S/HS^- [100, 109-113] we selected bovine pulmonary artery endothelial cells (BPAEC) as the principal experimental system. BPAEC seem additionally appropriate because pulmonary edema is a lethal consequence of H_2S inhalation[106, 114, 115] and most non-cardiogenic pulmonary edemas (of varying underlying cause) appear to be associated with endothelial barrier dysfunction.[116, 117]

The first acid dissociation constant (pK_{a1}) for H_2S is 7.04, and the second (pK_{a2}) is not accessible in water.[118] Consequently, irrespective of whether inhaled as H_2S gas or injected as a NaHS solution, *in vivo*, the toxicant is ~30% H_2S and ~70% HS^- prior to any biochemical modification – in keeping with common practice in the biochemical/toxicological literature, we subsequently refer to this aqueous mixture as "sulfide." In a previous study, we showed that the NO-donor species sodium nitrite could significantly ameliorate acute sulfide toxicity in mice if given prophylactically.[38] These earlier findings are at least consistent with nitrite-derived NO reversing the inhibitory effect of sulfide bound to cytochrome *c* oxidase as we have previously argued in the case of cyanide poisoning.[61, 62] Here, we begin by showing that slow infusion of sulfide solution directly into the bloodstream of mice through the tail vein leads to a syndrome in which death results from respiratory collapse seemingly due to cardiopulmonary paralysis. The results indicate that this acute pattern of intoxication does not conform to Haber's law and suggest a plausible explanation of why, if the administration of toxicant is slow enough, some laboratory animals (and human poisoning victims) reportedly succumb to a post-acute syndrome involving pulmonary edema rather than cardiopulmonary failure. We then go on to examine the effects of sulfide on mitochondrial function in cultured BPAEC and reversal of sulfide-dependent inhibition by the nitric oxide (NO) donor sodium nitrite. The findings provide further insight into the possible role of endothelial dysfunction/pulmonary edema in post-acute sulfide toxicity and suggest an approach to developing practical antidotal protocols.

2.3 Experimental Procedures

2.3.1 Chemicals

Unless stated to the contrary, reagents were ACS grade, or better, and used without further purification. Sodium hydrogen sulfide was obtained as NaHS•xH$_2$O (Sigma) where x was determined to be 2.5 essentially following titration procedures described by Koltoff *et al.*[119] Briefly, concentrations of HS^- were determined by quantitative reaction with excess iodine ($2HS^- + I_2 \rightarrow S + 2H^+ + 2I^-$) followed by titration of the liberated iodide[119] (as $I^- + I_3^-$) with silver nitrite (precipitating $AgI + AgI_3$) using an Ag^+ sensitive ion-selective potentiometric electrode (Accumet Silver/Sulfide Combination SIE 13-620-511) to detect the end point.[38] Sodium hydrogen sulfide solutions were freshly prepared for all experiments by dissolving NaHS in septa-sealed (Suba-Seal) tubes with minimal head space. All volumetric transfers were made with gas-tight syringes.

2.3.2 Animals and Sulfide Exposure

All animal procedures were approved by the University of Pittsburgh Institutional Animal Care and Use Committee (Protocol Number 13092637). Veterinary care was provided by the Division of Laboratory Animal Research of the University of Pittsburgh. Male Swiss Webster (SW) mice weighing 35-40 g were purchased from Taconic, Hudson, NY. Adult animals were 7-8 weeks old and were housed four per cage. The mice were allowed access to food and water *ad libitum*. Experiments commenced after the animals were allowed to adapt to their new environment for one week. All sulfide solutions were prepared in septa-sealed vials, by dilutions into sterilized saline, using gas-tight syringes for any transfers. Mice were placed in a Tailveiner

Restrainer for mice (Braintree Scientific, Inc., Braintree, MA) for the duration of tail vein injection using a mechanically driven Hamilton syringe to introduce sulfide at a rate of 10-6 µL/min (dependent on mouse weight) to achieve the desired dose of sulfide. To monitor the progress of injections, a small amount of Evans blue dye was added to sulfide solutions to induce darkening of the tail vein. Blue coloration in the ears and nose confirmed efficient delivery of the solution over the times indicated. In general, a group of at least 3 mice were tested for each experimental point. At the end of exposures and tests, mice were euthanized with CO_2 and subsequent cervical dislocation.

2.3.3 Cells and Cell Culture

Bovine pulmonary artery endothelial cells (BPAEC) were purchased from Cell Applications, Inc. (San Diego, CA 92121) and used at passages 5-8. BPAEC were grown in Opti-MEM complete media supplemented with 10% fetal bovine serum, 5 mM L-glutamate, 100U/mL penicillin and 100 µg/mL streptomycin under 5% CO_2 and 3% oxygen (92% N_2) and minimally handled. Cells were grown to ~80% confluence prior to being plated and were harvested by trypsinization. Cell numbers were determined using a hemocytometer. The culture media and supplements were purchased from Life Technologies (Carlsbad, CA) or Sigma (St. Lois, MO).

2.3.4 Hydrogen Sulfide Toxicity in BPAEC

Hydrogen sulfide solutions were prepared by dissolving NaHS in tubes sealed with minimal head space and septa-seal caps to minimize leakage. All volumetric transfers were made with Hamilton gas-tight syringes. Hydrogen sulfide toxicity in BPAEC was measured by

employing cells grown in 6 well plates, seeded at a density of 4 x 10^5 cells/well, reaching ~80% confluence in two days in Opti-MEM media. On test day, Parafilm was placed over the wells to reduce the loss of hydrogen sulfide during exposures. Sulfide solutions were prepared in media and were injected into the wells while lightly shaking the plate. After injections, a second layer of Parafilm was placed over the plate and smoothed down onto the plate surface. The plate was then incubated at 37°C in 3% oxygen for one hour.

2.3.5 Cellular Assays

Propidium Iodide was used to detect cell death in BPAEC exposed to sulfide. Briefly, cells were treated with NaHS (see above) and subsequently, propidium iodide (15 μM final concentration) was added to each well in order to determine cell death. SYBR Gold (1:10,000 dilution) was used according to the manufacturer's (Life Technologies) instructions to determine the live cell count. The plate was incubated for 15 minutes (3% oxygen, 37°C) and cell fluorescence (PI, λ_{ex} = 535 nm; λ_{em} = 617 nm; SYBR Gold, λ_{ex} = 495 nm; λ_{em} = 537 nm) was observed. Annexin V-Cy3 assays were performed using a kit from BioVision (Milpitas, CA) where the manufacturer's protocol was followed. BPAEC were seeded at passage 6-8 at a density of approximately 4 x 10^4 cells/mL in Opti-MEM in 6 well plates (2mL/well). To compare the influence of glucose versus galactose on cells exposed to NaHS, the media was removed from all plates and replaced with either 5mM glucose or 10 mM galactose media and incubated (3% oxygen, 37°C) for 3 hours prior to addition of NaHS. Cells were then scraped, spun down and counted, the pellet resuspended in binding buffer and finally, the annexin antibody was added. For comparison, other BPAEC were exposed to L-NAME (N (ω)-nitro-L-arginine methyl ester), nitrite and sulfide prior to annexin V-Cy3 assay. L-NAME (0.5 mM) was prepared using nano-pure

water, added to wells and incubated for 1 hour at 37°C. Plates were covered in Parafilm prior to 5 mM NaHS solution being injected into wells while shaking the plate slightly. The plate was covered again with a second layer of Parafilm and returned to the incubator for a second 1 hour incubation. Sodium nitrite solutions (0.5 mM) were prepared using phosphate-buffered saline (PBS). The sodium nitrite solutions were injected into the cell media of wells while shaking the plate slightly. After 2 minutes, 5 mM sodium hydrogen sulfide solution was injected into the wells through Parafilm. Fluorescent cells were counted using a fluorescent microscope, divided by the number of total cells in the field of view and the percent cell death for the sample was calculated.

Caspase activity was determined using the protocol for the Molecular Probes (Eugene, OR) EnzChek caspase 3 assay kit from Invitrogen (ThermoFisher Scientific, Waltham MA). Staurosporine (1μM) was used as a positive control for apoptosis. Prior to the assay, protein concentrations of the samples were determined using the Pierce BCA assay kit (ThermoFisher Scientific, Waltham MA).

Nitrotyrosine levels were determined using the ELISA protocol from Millipore (Temecula CA). A 3-nitrotyrosine standard curve was generated for each assay.

Lactate dehydrogenase (LDH) activity of the BPAEC supernatant was measured in the presence of NAD^+ and lactate following exposure to 5 mM NaHS. The increase in absorbance, due to the appearance of NADH, at 340 nm was measured over a 10 minute time span and compared to controls.

2.3.6 Western Blotting

BPAEC were lysed in radio immunoprecipitation assay buffer (150 mM NaCl, 0.5% sodium deoxycholate, 0.1% SDS, 50 mM Tris, 1 mM EDTA) containing a protease inhibitor

cocktail mixture (Roche). Protein concentrations of the samples were determined using the Pierce BCA Protein Assay Kit (ThermoFisher Scientific, Waltham, MA) and probed, subsequently, for β-actin and/or LC3-β (goat polyclonal IgG#SC-1615/#SC-16755; Santa Cruz Biotechnology, Inc., Santa Cruz CA). Proteins were separated on 14% tris-gycine gels and transferred to a polyvinylidene difluoride membrane blocked with 3% nonfat milk according to a protocol by Tanida *et al*.[120] Blots were incubated with primary antibodies (1:1000) overnight at 6°C, rinsed and then incubated with horseradish peroxidase (donkey anti-IgG HRP conjugated; Santa Cruz Biotechnology, Inc., Santa Cruz CA) (1:5000) for one hour at room temperature. Photolytic ladder (Ladder generously provided by Karla Wasserloos, University of Pittsburgh) was used to visualize standards.

In an analogous manner, Western blots were carried out to detect mitophagy in BPAEC using β-actin and/or PINK1 primary antibodies (H-300 rabbit polyclonal Ig3, Santa Cruz Biotechnology, Inc. #SC-33796). (Beta Actin and chemiluminescence detection reagents were generously provided by Alexis Carter, University of Pittsburgh).

2.3.7 Respirometric Experiments

An Oxygraph O2k polarographic instrument (Oroboros, Innsbruck, Austria), equipped with a Clark-type electrode for high-resolution respirometry was used to measure oxygen fluxes and concentrations. BPAEC, at an average density of 2.5 x10^6 cells/mL, suspended in DMEM media were loaded into the sample chambers and equilibrated (sealed from the atmosphere) at 37°C for 20 minutes. Solutions for additions (10-50 μL volumes) of 1 mM sodium nitrite and/or 2 mM hydrogen sulfide were prepared in nano-free water and were added by gas-tight syringes into the sealed sample chambers. Data analysis was carried out with the DatLab software provided by

Oroboros. (Note: the sensitivity of this system is such that adding a deoxygenated solution to the more oxygenated Oxygraph chambers can erroneously register as an increase in oxygen consumption. Helmy *et al.*[121] have described this artifact as a dilution effect. Conversely, of course, adding an oxygenated solution to the Oxygraph chambers containing less oxygenated solutions can register as a decrease in oxygen consumption (see Supplemental Data). In practice, one usually avoids such effects by adding small reagent volumes of a few microliters to the chambers (2.0 mL total volumes) and ignoring any minor erratic signals in the traces obtained during the first couple of minutes after any additions.)

2.3.8 Instrumentation

Fluorescence measurements were carried out using a BMG Labtechnology FLUOstar Galaxy microplate reader, a Zeiss IM 35 Fluorescent microscope with Infinity Analyze camera software or the Shimadzu RF-5301 PC spectrofluorophotometer. Electronic absorption measurements were performed using Shimadzu UV-2501PC and UV-1650PC spectrophotometers.

2.3.9 Data Analysis

Statistical comparisons were carried out using one way analysis of variance (ANOVA) and student t-tests. Values are expressed as means +/- standard errors. A p-value ≤ 0.05 was considered significant and statistical analyses were performed with KaleidaGraph.

2.4 Results and Discussion

2.4.1 Intact Mice

In previous experiments with mice we have employed single-shot intraperitoneal injections to administer sulfide solutions.[38] This experimental paradigm, in which the animals either significantly recover or succumb within 4 minutes, does not reflect the consequences of real-world poisonings in at least two important aspects. First, unlike human victims, none of the surviving animals exhibited any symptoms persisting longer than a few minutes following cessation of the toxicant dose. Second, upon autopsy, the tissues of non-surviving animals did not show any of the green discoloration due to the formation of sulfhemoglobin reported in the case of human fatalities.[122-125] In the few cases where this information is available, the durations of human exposures (by H_2S gas inhalation) resulting in unconsciousness and some deaths are from a few minutes up to about 15 minutes.[126-129] Consequently, anticipating some possible experimental advantage to administering the toxicant dose over several minutes rather than by single-shot injection, we began to explore slower intravenous infusion through the tail vein.

Injection methods (as opposed to inhalation) allow for more precise control of dose delivered, but importantly, the animals must not be sedated, since there are some well-documented (if incompletely understood) confounding effects associated with anesthesia.[62, 130, 131] Briefly, physically restrained Swiss-Webster males (6-8 weeks old, 25-35 g) were infused through catheters inserted into their tail veins with NaHS dissolved in saline (10-30 mg/kg in maximally 0.1 mL solution per mouse) employing mechanically-driven Hamilton-type syringes and times until death (cessation of breathing) were observed. The results turned out to be highly reproducible with a remarkably linear relationship between lethal dose and the duration required for its infusion

(Figure 4). Clearly, the present data do not obey what has become known as Haber's law[132] in which the lethal dose would be a constant given by the concentration administered times the duration of the dose. Instead, the lethal dose is highly variable (Figure 4) being proportional to the duration of the dose, or inversely proportional to the concentration administered. Deviations from Haber's law are not that unusual, but in the context of the present study, it is noteworthy that earlier authors administering inhaled H_2S to rats (at much lower concentrations than here) did observe adherence to Haber's law when the dose durations were several hours.[114] Immediately, after absorption, the composition of the sulfide toxicant is the same (~30% H_2S and ~70% HS^-) irrespective of whether it is delivered as H_2S or NaHS. Therefore, observation of adherence to Haber's law at relatively slow delivery of low levels of toxicant as opposed to non-adherence to Haber's law at faster delivery of the same toxicant at higher concentrations is strongly indicative of there being two quite distinct mechanisms of toxicity and/or antagonistic (*i.e.* protective) tissue responses under the two regimens.

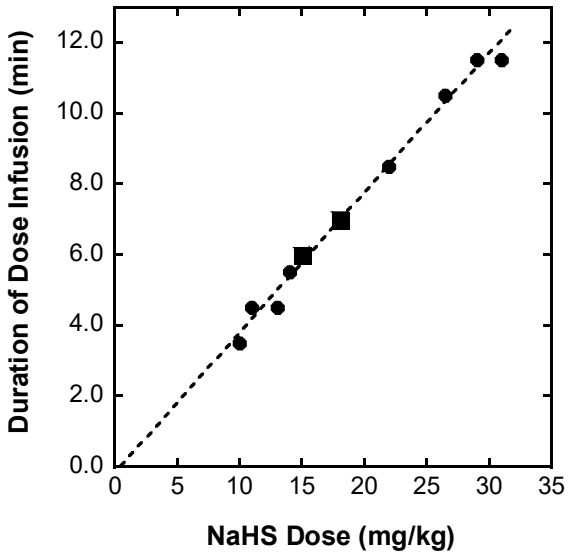

Figure 4. Slow intravenous infusion of NaHS into mice.
Un-sedated, but physically restrained, Swiss Webster males (6-8 weeks old) were injected through catheters inserted into their tail veins with NaHS dissolved in saline (maximally 0.1 mL per mouse, employing mechanically-driven

Hamilton-type syringes) and time until death (cessation of breathing) recorded. Broken line: linear regression fit to data (R = 0.995); (solid black circle) indicates individual data points; (solid black square) indicates two superimposed data points.

At all dose rates examined (Figure 4) the mice were rendered unconscious as the lethal doses were approached; but if the dose was terminated before death, the animals recovered righting ability within 30-60 seconds. Full recovery of normal neuromuscular function – as determined by the ability of animals to remain on a rotating cylinder (RotaRod testing, data not shown) – was evident by 15 minutes following cessation of the toxicant infusion. These surviving animals exhibited normal behavior for up to 3 days after their intoxications, at which time they were sacrificed. In our previous study employing intraperitoneal injections of sulfide solutions[38] the mice began to show signs of intoxication (slow, irregular movement and raised hackles) about 2 minutes after receiving the dose. For purposes of comparison, extrapolating the data of Figure 4 to the 2-minute dose duration indicates a lethal dose of ~5 mg/kg, whereas the lethal dose determined in the earlier study was actually greater than 20 mg/kg.[38] Together, these observations seem to indicate that only about 25% of the sulfide given intraperitoneally ends up entering the bloodstream, presumably with the majority of the toxicant dose having been metabolized within the peritoneum. This observation is suggestive of at least one way in which slow delivery of low H_2S doses by inhalation[114] might present such a different toxic response to infusion of higher sulfide concentrations directly into the bloodstream (Figure 4) namely, metabolism of the toxicant within the pleural membrane might present a significant barrier to sulfide entry into the bloodstream. Since functionally similar endothelial cells are present in both membranous structures, the decision was made to further investigate sulfide metabolism in a cultured pulmonary endothelial cell line since pulmonary cells are presumably those most impacted during actual (H_2S gas) poisonings.

Curiously, while we have verified (data not shown) that purified mouse hemoglobin can readily be manipulated to undergo the same conversion to green sulfhemoglobin as the human protein, the tissues of mice lethally exposed to sulfide have (to date) never exhibited any evidence of green discoloration, irrespective of whether the toxicant were given by single-shot intraperitoneal injection[38] slow tail vein infusion (as in Figure 4) or by H_2S inhalation (data not shown). This marked difference in outcome between human victims[122-125] and the animal models remains to be understood.

2.4.2 Cultured Cells

Proliferating (sub-confluent) bovine pulmonary artery endothelial cells (BPAEC) provide a means of investigating changes in mitochondrial function within the cellular environment – as we have previously shown in studies examining the effects of ionizing radiation[133] and acute cyanide toxicity[60] – without any potentially confounding effects due to the presence of hemoglobin and other bloodstream constituents. Culturing cells and performing experiments at 3% (v/v) oxygen ensures that any "oxidative stress" experienced by the BPAEC is not artificially elevated beyond that to be expected *in vivo*. In the past, we have successfully employed the metabolic indicator dye AlamarBlue® to monitor cell proliferation[60, 133] but, more recently, have found this to undergo direct reduction by sulfide warranting a switch to a propidium iodide-based method for assaying cell death.[38] As equilibrated aqueous sulfide is about 30% H_2S at pH 7.4, it is rapidly lost to the atmosphere from unsealed containers. To prevent this, cell cultures were covered with Parafilm and inoculations of reagents made by injections through the covering. Undoubtedly, however, there were some losses and the concentrations of sulfide administered to the BPAEC in the following experiments should be read as the instantaneous sulfide concentrations just after the

inoculations. To obtain a reproducible dose-response after 1 hour, it was found necessary to employ millimolar concentrations of sulfide in the cell media, irrespective of whether death was estimated by the propidium iodide method (Figure 5A) or by annexin V binding (Figure 5B). The extent of cell death determined by either method never exceeded ~25%, most likely attributable to H_2S (g) being lost under the conditions employed, with a maximum dose being achieved at ~5 mM NaHS in solution.

BPAEC treated with toxic levels of sulfide appeared to be blebbing (not shown) which can be associated with a number of phenomena, including cell death by apoptosis and/or necrosis. The annexin V-Cy3 assay is sensitive to the translocation of phosphatidyl serine to the cell exterior, or the presence of plasma-membrane fragments;[134] hence it may be indicative of both apoptosis and necrosis. The appearance of lactate dehydrogenase (LDH) activity in the medium following sulfide treatment, however, suggests the cell death to have been mostly necrotic (Figure 5C). While the propidium iodide and annexin V dose response curves are not completely superimposable, they are the same within the experimental uncertainty, again indicating necrosis to have been the predominant mechanism of cell death.

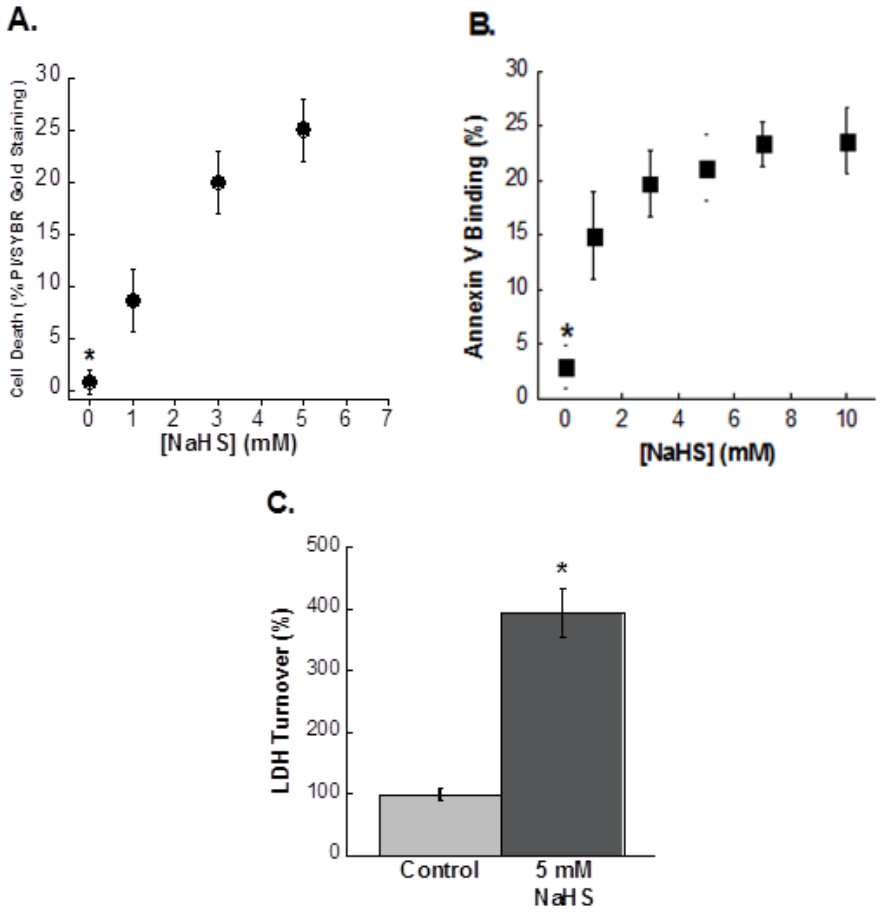

Figure 5. Hydrogen sulfide toxicity in BPAEC assessed by propidium iodide, annexin V Cy-3 and lactate dehydrogenase.

BPAEC (at 80% confluency, grown in 3% oxygen at 37°C, in OptiMEM media) were exposed for 1 hr to increasing levels of NaHS (1-10 mM). BPAEC were subsequently treated with either propidium iodide (A) or annexin V Cy-3 (B) (see Experimental Procedures for details). * $p < 0.01$ for BPAEC treated with 1-10 mM NaHS compared to the control cells. Lactate Dehydrogenase (LDH) activity (C) was determined in BPAEC with and without NaHS exposure (5 mM, 1 hr) * $p < 0.05$. All p-values were determined by one way ANOVA using KaleidaGraph.

Interestingly, there was clearly increased death in the case of cultures grown in a galactose-containing medium compared to those grown in a glucose-containing medium (Figure 6A). Cells conditioned to galactose rely more heavily on mitochondrial oxidative phosphorylation for their ATP production compared to cells maintained in glucose which rely more on glycolysis for their energy requirements.[135, 136] Therefore, the present data clearly support the consensual viewpoint suggesting the principal target for sulfide toxicity to be mitochondrial. In both the galactose-

conditioned cells and the glucose-maintained cells, the addition of the NO-donor species[55, 137] sodium nitrite ameliorated sulfide-induced cell death and the addition of the NO synthase inhibitor L-NAME exacerbated the sulfide-induced cell death. These findings concerning the effects of NO were confirmed in the case of glucose-maintained cells employing the propidium iodide assay (data not shown). In summary, the results (Figure 6A) suggest that delivery of NO reverses the mitochondrial-linked toxicity of sulfide, while suppression of endogenous NO production exacerbates the toxicity. We have previously demonstrated that NO is able to reverse the inhibition of cytochrome *c* oxidase by cyanide[59, 60] and shown that this seemingly results in sodium nitrite being an effective cyanide antidote in mice.[61, 138] Similarly, we have more recently shown sodium nitrite to have significant antidotal activity in mice toward sulfide intoxication.[38] Consequently, the present findings (Figure 6A) are entirely consistent with the idea that inhibition of cytochrome *c* oxidase by sulfide is the principal molecular mechanism of toxicity and that the inhibition can be antagonized by NO.

To further clarify the mechanism of sulfide-induced cell death, we performed a caspase-3 activity assay (Figure 6B). Irrespective of whether the BPAEC were galactose-conditioned or glucose-maintained, there was no measureable caspase-3 activation, demonstrating that sulfide does not induce apoptosis in this particular cell line and, consequently, the cell death observed in the other experiments (*i.e.* Figures 5A & B, 6A) was, unambiguously, almost entirely necrotic. Staurosporine causes apoptosis by an extrinsic mechanism and was used here as a positive control (Figure 6B).

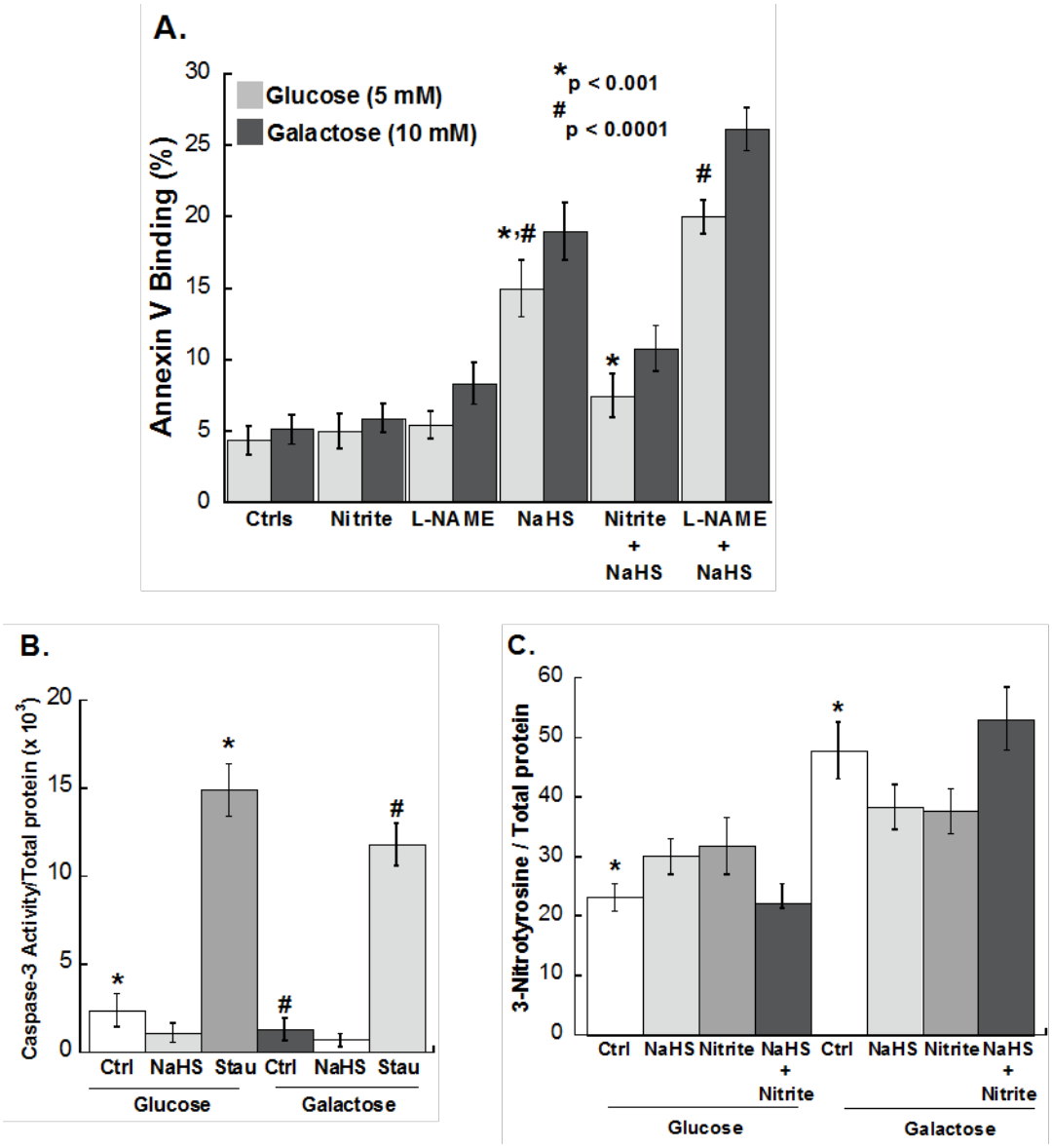

Figure 6. Autophagy and 3-nitrotyrosine levels in glucose/galactose-conditioned BPAEC exposed to NaHS and/or $NaNO_2$.

BPAEC were grown in 3% oxygen at 37°C and either maintained in 5 mM glucose media (DMEM) or conditioned in 10 mM galactose-media (DMEM) for 3 hours prior to further treatments. A: Annexin V-Cy3 binding. BPAEC were treated either with 5 mM NaHS (1 hr), 0.5 mM sodium nitrite (added 5 min prior to NaHS), 0.5 mM L-NAME (added 1 hr prior to NaHS), or combinations thereof. p –values measured between *sulfide treated and sulfide/nitrite treated BPAEC grown in 10 mM glucose by one way ANOVA. p –values were also measured between #sulfide treated and sulfide/L-NAME treated BPAEC grown in 10 mM glucose by one way ANOVA. Similar p-values were observed between sulfide, nitrite/sulfide and L-NAME/sulfide treated galactose-conditioned (5 mM) BPAEC. B: Caspase-3 activity. BPAEC were treated with either 5 mM NaHS (1hr) or 1μM staurosporine (STAU) (30 min) and analyzed for caspase-3 activity (normalized to protein concentrations) $p < 0.01$ for the BPAEC controls versus the *glucose-maintained and #galactose-conditioned cells. C: 3-Nitrotyrosine levels. 3-Nitrotyrosine levels (normalized to protein concentrations) in BPAEC treated with similar concentrations of 0.5 mM nitrite (10 min) and 5 mM NaHS (1 hour) were determined using an ELISA (see Experimental Procedures for details). *p <0.01 between the glucose- and galactose-conditioned controls. All p-values were determined by one way ANOVA using KaleidaGraph

NO is clearly a modulator of sulfide toxicity in BPAEC (Figure 6A) and sulfide inhibition of cytochrome *c* oxidase should lead to oxygen accumulation and accompanying elevated production of superoxide (O_2^-). Since NO in the presence of superoxide results in the diffusion-limited generation of peroxynitrite ($NO + O_2^- \rightarrow ONO_2^-$) we should consider whether peroxynitrite might be involved here. Evidence for the production of peroxynitrite is most readily obtained through determination and quantification of its biomarker species 3-nitrotyrosine.[139-141] Three-nitrotyrosine levels were increased in galactose-conditioned BPAEC compared to glucose-conditioned cells – to a greater extent than the effects of adding sulfide, nitrite, or both (Figure 6C). Note in particular that addition of nitrite alone did not result in any measurably significant increase in 3-nitrotyrosine levels, consistent with superoxide being the limiting reagent in the peroxynitrite formation. Compared to glucose-maintained BPAEC, the galactose-conditioned cells have a higher rate of oxygen turnover due to a greater reliance on mitochondrial oxidative phosphorylation to generate ATP (see below). Consequently, the present results (Figure 6C) are consistent with the widely held view that mitochondria can be a major source of superoxide in cells. Compared to untreated controls, neither galactose-conditioned nor glucose-maintained BPAEC exhibited any significant increase in peroxynitrite production in response to sulfide, nitrite, or a combination of the two. Thus, there does not appear to be any role for peroxynitrite in the antagonistic interplay between NO and sulfide within these cells.

A Western blot analysis (Figure 7A) showed that cells conditioned in 10 mM galactose for 3 hours prior to harvesting exhibited increased levels of LC3 II (lane 1) compared to those grown in 5 mM glucose (lane 3). A decreased level of actin in the galactose-conditioned cells (lane 1) compared to those maintained on glucose (lane 3) is consistent with increased cellular degradation through autophagy in the former. Interestingly, the addition of 5 mM NaHS to the galactose-

conditioned cells 1 hour before harvesting resulted in decreased autophagy (lane 2) compared to the toxicant-free controls (lane 1). Addition of 5 mM NaHS to the glucose-maintained cells did result in a slight increase in autophagy (lane 4) compared to the toxicant-free controls (lane 3). Overall, however, it is clear that the net rate of autophagy observed in BPAEC exposed to NaHS is similar in both media (compare lanes 2 and 4) and cannot account for the increased sulfide toxicity evident in galactose-conditioned BPAEC (Figure 6A).

The mitochondrially targeted serine/threonine kinase, PINK1, has been shown to provide protection against mitochondrial dysfunction during cellular stress by helping to clear depolarized mitochondria via selective autophagy (*i.e.* mitophagy).[142] Changes in PINK1 levels of galactose-conditioned and glucose-maintained BPAEC following exposure to NaHS were determined by Western blot analysis (Figure 7B). Again, actin levels were also followed as a check on cellular integrity. Compared to the galactose-conditioned cells (lanes 1 and 2) the glucose maintained BPAEC showed clearly elevated PINK1 levels (lanes 3 and 4) indicative of increased mitophagy. This result is fully consistent with the glucose maintained cells being more dependent on glycolysis, rather than oxidative phosphorylation, to meet their ATP requirements. Addition of NaHS does not appear to have much impact on the observed results in either growth medium and, therefore, mitophagy does not contribute to the increased sulfide toxicity evident in galactose-conditioned BPAEC (Figure 6A).

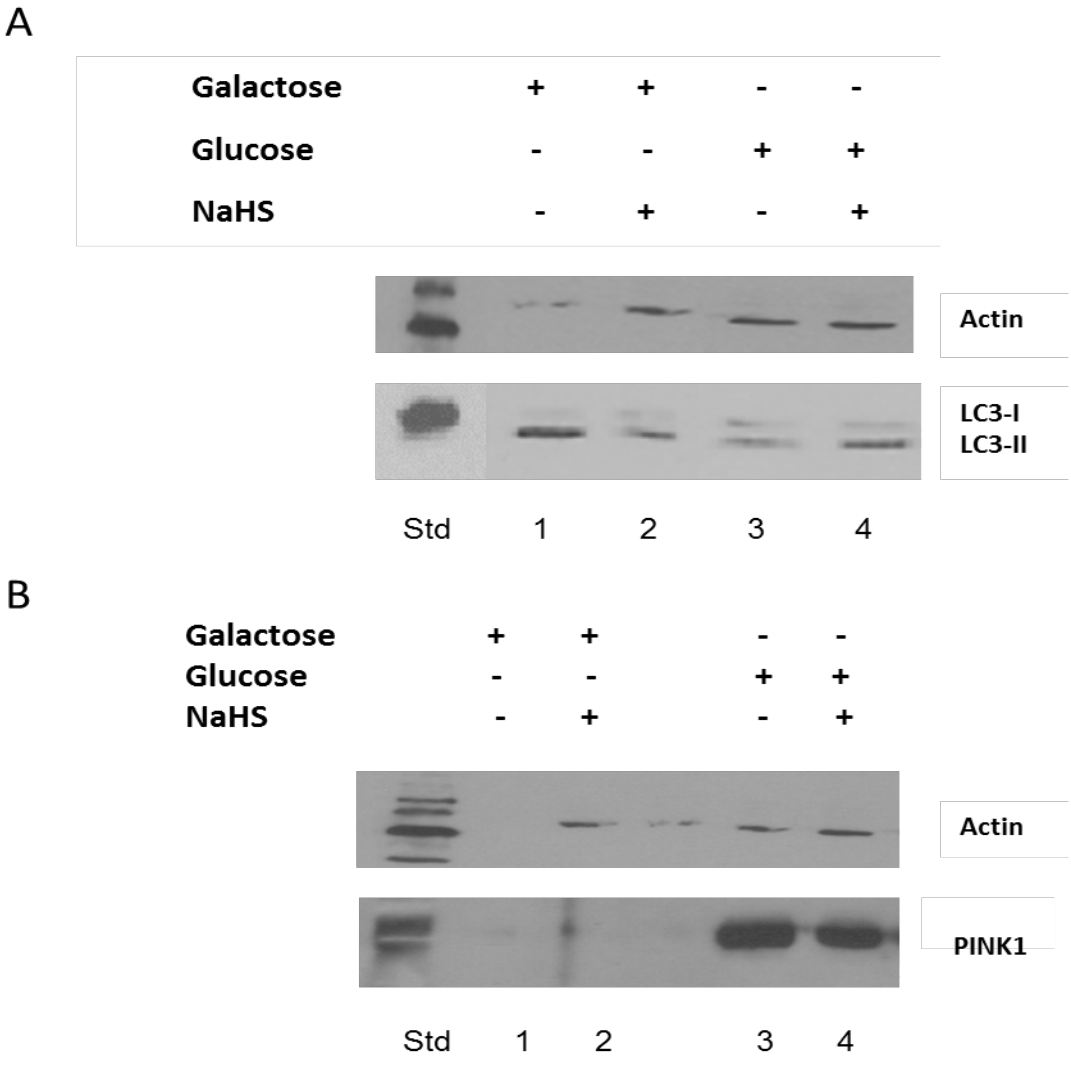

Figure 7. Western Blot analysis of LC3-I/II and PINK1.

BPAEC were grown in 3% oxygen at 37°C and either maintained in 5mM glucose media (DMEM, lanes 3&4) or conditioned in 10mM galactose-media (DMEM, lanes 1&2) for 3 hours prior to treatment with NaHS (lanes 2&4). Either LC3 –I/II or PINK1 levels are shown with actin levels for comparison and normalized by protein concentration. Protein standards are shown on the far left-hand side of both blots. A: LC3-I/II. BPAEC were poisoned with 5 mM NaHS for 1 hour (in either glucose or galactose) prior to harvesting for western blot analysis. B: PINK1. BPAEC were poisoned with 5 mM NaHS for 1 hour (in either glucose or galactose) prior to harvesting for western blot analysis (See Experimental Procedures for details of the Western blots).

2.4.3 Respirometric Measurements

High-resolution respirometry was performed with an Oroboros Oxygraph O2k equipped with a Clark-type oxygen-sensing electrode for polarographic measurements. Using analogous equipment, Bouillaud and colleagues have shown[143, 144] that rat heart mitochondria have a robust set of oxidizing enzymes that are able to detoxify sulfide (Figure 8) and that this system is lacking in neuronal mitochondria. We took the trouble to briefly confirm these observations using minced mouse ventricular tissue and whole brain (data not shown). The first enzyme in this sulfide oxidizing unit is associated with the mitochondrial inner membrane and passes two electrons into the electron-transport system while converting hydrosulfide anion (HS^-) to protein-bound persulfide (cys-S-SH).[58, 145] This is a crucial step in the overall process and important for interpretation of the respirometric data; specifically, for sulfide to be catabolically eliminated through sulfide oxidation, the electron-transport system must be functioning. If sulfide is bound and, therefore, inhibiting cytochrome *c* oxidase, then formation of the persulfide intermediate by the sulfide-quinone reductase and the subsequent oxygen-dependent steps cannot occur. Administration of aqueous sulfide into the medium of galactose-conditioned BPAEC (4 and 8 μM) led to a rapid but transient increase in the rate of oxygen consumption (Figure 9A, increased "O_2 flux") commensurate with protection of mitochondrial function through oxidation of the sulfide. In this respect, the BPAEC appear to be more like cardiomyocytes than neurons. [143, 144] At 16 μM NaHS there was a more prolonged elevation in the oxygen consumption rate, suggesting that the sulfide-oxidizing unit may have been saturated for several minutes, but there was never any detectable net inhibition of electron transport (i.e. no sulfide-dependent decrease in oxygen consumption rate was observed). Therefore and, most importantly, the sulfide-oxidizing system was able to efficiently outcompete cytochrome *c* oxidase for the available sulfide! Repeating these

experiments with glucose-maintained BPAEC yielded unsurprising results (Figure 9B). Using samples normalized by cell count (10^6 cells in each case), the overall oxygen-consumption rates were slower in the glucose-maintained BPAEC (~40%) and the increased oxygen uptake rates in response to sulfide additions were more modest. Again, there was no evidence for inhibition of electron transport and, accordingly, addition of the NO-donor species sodium nitrite did not impact on the respirometric results (data not shown). Note, however, that the sulfide concentrations used in these experiments were about two orders of magnitude less than the concentrations used to demonstrate the mitochondrial link to sulfide-induced death (Figure 6A). Unfortunately, at sulfide concentrations approaching 50 μM and in the absence of any cells in the sample chambers, the Clark electrodes exhibited a variable (sulfide-dependent) basal oxygen flux (see Supplemental Data) and this interference prevented meaningful respirometric studies at higher sulfide concentrations where inhibition of electron transport might be observable.

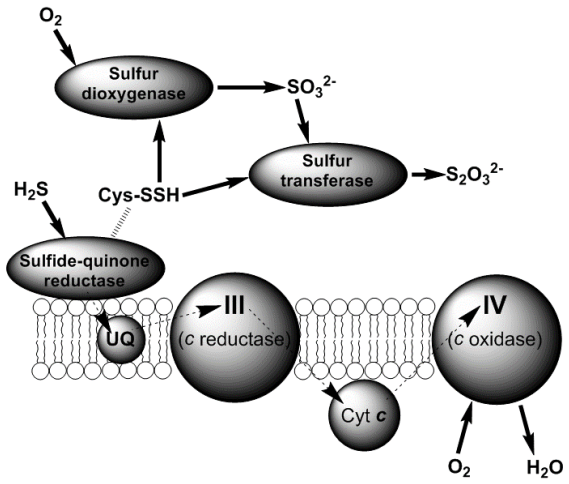

Figure 8. Sulfide Oxidizing Unit (SOU) in Mitochondria.
Adapted with permission from John Wiley and Sons, Inc. from FEBS J. Three enzymatic activities catalyze the oxidation of sulfide to thiosulfate in mammalian and invertebrate mitochondria; Hildebrandt, T.M. and Grieshaber, M.K.; Vol. 275; Copyright 2008; permission conveyed through Copyright Clearance Center, Inc. [57]

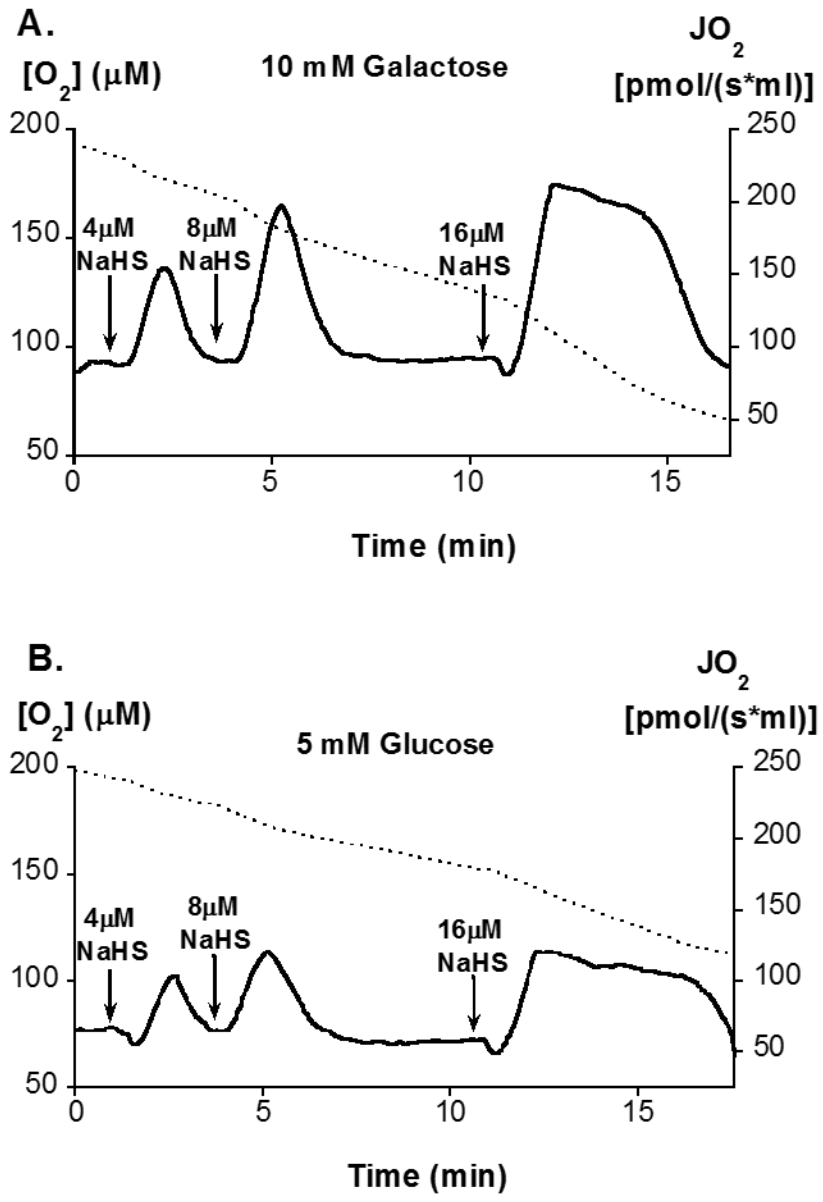

Figure 9. Respirometric Analyses.

Oxygen consumption was assessed in BPAEC (3 x 106 cells/mL) conditioned in either (A) 5 mM glucose or (B) 10 mM galactose 3 hours prior to testing. BPAEC were allowed to equilibrate for ~10 minutes prior to sequential additions of NaHS (final concentrations of 4, 8 and 16 μM). Oxygen concentration is shown on the right axes (dashed lines) and oxygen flux on the left axes (solid lines). Galactose treated BPAEC turned over oxygen at a rate of 90(±10) pmole/(s mL) versus 60(±10) pmole/(s mL) for the rate of the glucose-condition cells. The default settings of block temperature, 37°C; stir bar speed, 400 rpm and data recording, 2 s were used.

2.5 Conclusion

2.5.1 Post-Acute Toxicity

As we previously observed with mice given single-shot intraperitoneal injections of NaHS in saline[38] slower infusion of the toxicant solution into the tail vein (Figure 4) also resulted in death through cessation of respiration. While in a rat model it appears that death is due to cardiac failure,[105] in both cases the animals recover quickly if infusion of toxicant is stopped before they succumb. In keeping with the widely held view[96, 101, 146] that the central nervous system is the critical target for acute sulfide toxicity (at least in these experimental systems) we suggest that it is probably reasonable to think of all these findings falling under the single category of death through some kind(s) of cardiopulmonary paralysis. Certainly, this seems reasonable given the observation, with which we concur, that neuronal mitochondria are less able to detoxify sulfide than myocardial mitochondria.[143, 144] Some of the reported cases of human poisonings reflect these experimental findings – death can be very rapid, or unconscious individuals can recover without medical intervention. Significant numbers of human victims of H_2S inhalation, however, arrive at the clinic exhibiting compromised respiratory function, most frequently with pulmonary edema, 30 minutes or more after exposure and may only succumb hours later.[96, 100, 106-108]

It now seems quite clear that surviving human victims of sulfide poisoning, who do not spontaneously recover and present with respiratory insufficiency, cannot possibly be subject to the same mechanism(s) of toxicity as experimental animals that have been infused/injected with the toxicant. In particular, we suggest the following scenario that plausibly explains the critical differences. Virtually all cases of human sulfide poisonings involve inhalation of H_2S gas, which necessarily means that systemic levels of the toxicant species must be lower than that experienced

by the pulmonary tissue. Given the ability of endothelial cells to oxidize sulfide (Figure 9) it appears that the lung may be able to protect other tissues, especially those of the central nervous system, from the inhaled toxicant. Therefore, victims exhibiting rapid knockdown and/or death around the time of exposure to the gas must have experienced H_2S levels high enough to result in saturation of the sulfide detoxifying capabilities of their pulmonary tissue and/or received some of the dose through nasal tissues by-passing the lung. At lower levels of H_2S exposure, the central nervous system may never be enough affected to result in cardiopulmonary paralysis, but if the dose continues for at least several minutes, then pulmonary tissue damage including necrosis of endothelial cells (Figures 5 and 6) will ensue. Since death in response to sulfide exposure has also been demonstrated in pulmonary smooth muscle cells,[147] it is to be anticipated that the sulfide-induced endothelial barrier dysfunction leading to lung edema may involve at least these two cell types. It follows that lethality in the "post-acute" timeframe (*i.e.* an hour or more after the toxicant exposure) appears to be due principally to respiratory insufficiency secondary to the lung edema.

2.5.2 An Approach to Antidotes?

A virtue of the above discussion is that it does offer an explanation as to why application of cyanide antidotes to the treatment of sulfide intoxication might be ineffective. Cyanide and sulfide have generally been thought to be similar mitochondrial poisons, exhibiting essentially indistinguishable inhibitory effects towards cytochrome *c* oxidase.[98] However, while cyanide antidotes like sodium nitrite (protective of mitochondrial function) are effective if given after the toxicant dose[61, 62] they tend to only work prophylactically as sulfide antidotes.[38] Whether by inhalation of HCN, ingestion of cyanide salts, or other cyanogenic compounds, in acute cases significant toxicant reaches the central nervous system and death results from cardiopulmonary

paralysis.[148, 149] Therefore, one is drawn to conclude that in order to be effective the available cyanide antidotes must ameliorate these toxic effects on the central nervous system. As discussed above, victims of sulfide poisoning reaching the clinic alive are probably no longer experiencing any acute toxicity where cardiopulmonary paralysis is a significant problem and, consequently, one should not necessarily expect cyanide antidotes to be of much use for therapeutic application to sulfide poisoning cases.

In developing new therapies for sulfide poisoning, it seems most reasonable to assert that the focus should be on treating the post-acute pulmonary edema. Our present findings show that NO is antagonistic towards sulfide-induced damage in endothelial cells (Figure 6A) and others have previously shown that NO donors are cytoprotective towards sulfide damage in pulmonary smooth muscle cells.[147] While there are probably many more examples in the literature of deleterious or even causative effects of NO in relation to lung injury, during the past decade or so, there have been multiple reports of the efficacious application of NO delivery in the treatment of a variety of pulmonary edemas in both animal models[150-152] and human subjects.[153-155] Undoubtedly, the extent of the edemas in question and the precise dose of NO to be employed are critical matters that remain to be satisfactorily explored. Nevertheless, in our opinion, there is now already enough evidence to suggest that an NO-inhalation therapy approach to the treatment of sulfide poisoning should be considered a promising area for further research.

2.6 Supplemental Materials and Figures

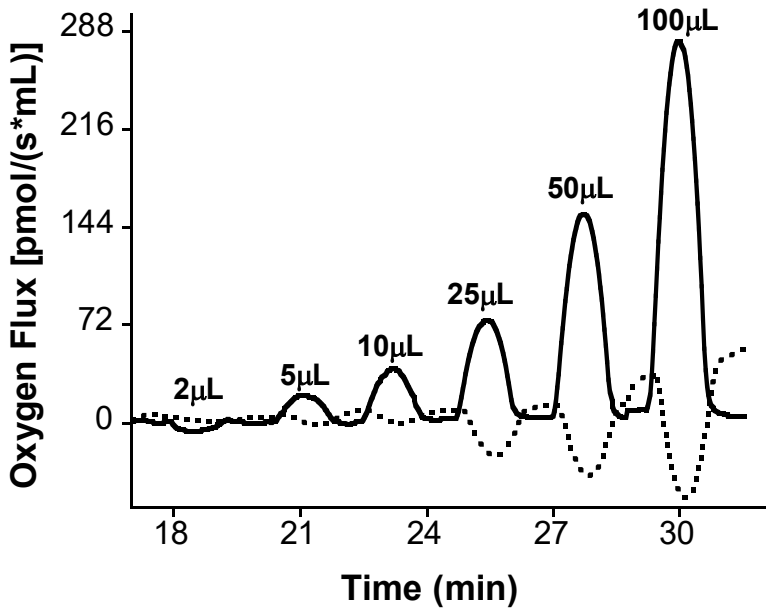

Figure 10. Cultured cell-free respirometric data.
Solid trace: To initially aerobic PBS (2.0 mL in the sample chamber, at 25°C) additions of anaerobic buffer (2, 5, 10, 25, 50, 100 μL at 25°C) were made with gas-tight syringes at two-minute intervals. The trace shows the response of the system to *decreasing* levels of oxygen following the introduction of anaerobic solution. If cells were present, this type of response can mistakenly be taken for oxygen consumption (increased respiration). Dotted trace: 2.0 mM aerobic NaHS was prepared in a septum-sealed container and titrated (2, 5, 10, 25, 50, 100 μL at 25°C; each 1 μL addition resulting in a 1 μM increase in NaHS concentration) into initially aerobic PBS (2.0 mL in the chamber, at 25°C) with a gas-tight syringe. The trace shows the response of the system to *increasing* levels of oxygen following introduction of aerobic solutions – indicating that there must have been a net lowering of the oxygen level in the sample chamber during the course of the experiment that was probably due to slow oxidation of added sulfide. In addition, however, following the additions of 25-μL and greater volumes of the NaHS solution, the apparent basal oxygen consumption began to rise, also probably due to slow oxidation of added sulfide. Again, if cells were present, this type of response could be misinterpreted as cellular oxygen consumption (increased respiration). It is not clear if the observed oxidation of sulfide was occurring at the electrode surface, or in free solution; but there was no irreversible change in the system's behavior, simply rinsing out the sample chamber resulted in a return to initial behavior. There is, however, clearly a potentially serious artifact detected during the collection of respirometric data at 25 μM and higher added NaHS.

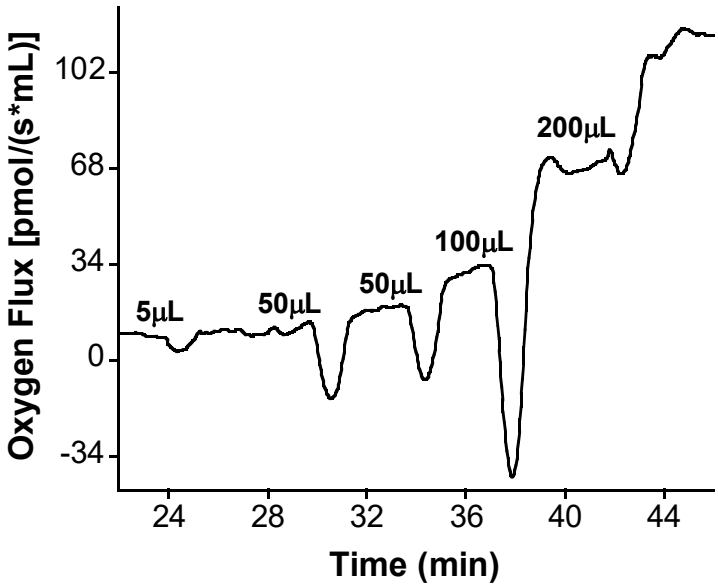

Figure 11. SHSY-5Y neuronal cells exposed to successive concentrations of NaHS.
SHSY-5Y neuronal cells were exposed to successive concentrations of NaHS, injected into the chamber in µL additions (5, 50, 100, 200). NaHS was prepared in an airtight container with a suba-seal and injections were done with a Hamilton gastight syringe, at 37°C. Final NaHS concentration in the chamber, 1 µL = 1 µM.

3.0 Results of Toxicant/Antidote Testing in a Mouse Model

3.1 Introduction

The following results offer an abridged review of the antidotal activity in Swiss-Webster mice exposed to each of the three toxicants (sulfide, cyanide or azide) and treated either prophylactically or therapeutically with $NaNO_2$ or CoN_4[11.3.1] through ip injection. For each of the studies reviewed, recovery of righting ability in the mice was measured following a simplification of a procedure originally used by Crankshaw *et al*,[156] defined as the time it took from the initial administration of the toxicant until the mouse flipped from a supine position (on back) to a prone position (on feet) in a plastic, critter tube. Treatment efficacy was established by a decrease in the timed recovery of righting ability after receiving injections of both the antidote and toxicant, compared to mice treated with the toxicant alone. Interestingly, we were able to produce a similar unconscious or "knockdown" response in the *Galleria mellonella* larvae exposed to the three toxicants. The duration of said recovery of righting (minutes) differed among the two species; as did the dose that elicited the knockdown response. It was interesting to our group to draw a comparison of the *G. mellonella* larvae data to that of the mouse data.

3.1.1 Sulfide Toxicity Testing

Sulfide poisoning and amelioration in mice was examined by Cronican *et al*, 2018[38] (Appendix A) in both 16–18 week old adult, male Swiss-Webster mice (40-59 g) and 6-8 week old juvenile male Swiss-Webster mice (25-35 g). Briefly, the mice were exposed to an LD_{40} (16

mg/kg (adult) or 18 mg/kg (juvenile) aqueous dose of NaHS through an intraperitoneal (ip) injection resulting in 57% survival in adult and a 67% survival in juvenile male mice (Table 4). In the case of sulfide, the knockdown/recovery of righting ability was variable and short. The mice that did not die within 5 minutes of receiving the toxicant were fully recovered within 15 minutes. Since a significantly larger number of animals would have been required to demonstrate the effects of the antidotes through righting recovery, efficacy of the $NaNO_2$ amelioration of NaHS toxicity had to be validated through percent survival. (Appendix A)

We observed a different response in the *G. mellonella* poisoned by sulfide. We were able to administer an intrahaemocoel (ih) injection of NaHS (72mg/kg) into the *G. mellonella* larvae and produce a repeatable 10 minute unconscious state (Section 4.4.4; FIGURE 16A). During the unconscious state the larvae were placed on their backs, similar to the mice, and the recovery of righting was able to be timed (from the initial administration of the toxicant until the larvae turned onto their feet).

In the Swiss-Webster mice, the prophylactically administered $NaNO_2$ (24 mg/kg dose), five minutes before, was highly protective against acute toxic effects of the NaHS (LD_{40}) in both adult (16 mg/kg) and juvenile mice (18 mg/kg) increasing the percent survival from 57-67% to 93–94% (Table 4), respectfully. Not surprisingly, in a preliminary study using juvenile mice, CoN_4[11.3.1] (26 mg/kg) was administered prophylactically 2 minutes before NaHS (18 mg/kg), this dose was antidotal toward the sulfide poisoning (92% survival) (Table 4). *G. mellonella* larvae were also treated with both $NaNO_2$ (5 mg/kg) and CoN_4[11.3.1] (8 mg/kg) therapeutically, one minute after NaHS. The length of the knockdown was cut in half when the antidotes where administered, from 10 minutes to ~5 minutes (Section 4.4.4; Figure 16A).

The dose given to mice (16/18 mg/kg) was much smaller than the dose given to *G. mellonella* (72 mg/kg). The apparent difference in the response to sulfide (pK_a ~7.1) could be due to the difference in pH of the two systems. In mice hemoglobin, (pH 7.4) NaHS is a 30:70 mixture of H_2S/HS^-.[38] Whereas, in the larvae hemolymph (pH 6.8), the NaHS is a 70:30 mixture H_2S/HS^- [33]. In the *G. mellonella* larvae system, more sulfide is present in the gaseous form (H_2S) (Section 4.5.2) so small gaseous molecules like H_2S could easily be lost from the small larval body by passive diffusion. This explanation may account for the larger dose of NaHS having to be used in *G. mellonella*.

Table 4. Antidotal activity of sodium nitrite and CoN_4[11.3.1] against sulfide toxicity in Swiss-Webster mice.

Experimental Conditions	Number of Mice	% Survival	References
Juvenile Mice (7-8 weeks old)			
18 mg/kg NaHS (t = 0)	24	67%	[23]
24 mg/kg $NaNO_2$ (t = 0) + 18 mg/kg NaHS (t = 5)	16	94%	
26 mg/kg Co_4[11.3.1] (t = 0) + 18 mg/kg NaHS (t = 2)	12	92%*	
Adult Mice (16-20 weeks old)			
16 mg/kg NaHS (t = 0)	28	57%	[23]
24 mg/kg $NaNO_2$ (t = 0) + 16 mg/kg NaHS (t = 5)	16	93%	

All agents given in saline solutions by ip injection. Antidotes administered prophylactically
* Data not published

3.1.2 Cyanide Toxicity Testing

In the next study, Cambal *et al*[157] exposed adult, male Swiss-Webster mice to 5 mg/kg doses of NaCN by ip injection. The percent survival of the cyanide exposed mice was 66% and after a subsequent dose of $NaNO_2$ (12 mg/kg), 2 minutes after NaCN (5 mg/kg), the percent survival increased to 82% and recovery time was dropped from 24 minutes to 6-9 minutes (Table

5). Expanding on the Cambal cyanide study, Andrea Cronican, et.al[23], (Appendix B) went on to examine the ameliorative effects of four cobalt-containing complexes, including CoN_4[11.3.1], on cyanide poisoning in 7-8 week old juvenile, male Swiss-Webster mice. The percent survival of the juvenile mice exposed to NaCN (5 mg/kg) only, was greater than that of the adults in the Cambal *et al*[157] cyanide study, 80% versus 66% (Table 5), however, the recovery of righting time was similar (24 minutes). When the mice received an ip dose of CoN_4[11.3.1] (50 µmol/kg) one minute after the ip cyanide, survival was increased to 92% and the recovery of righting time decreased to 4 minutes (Table 5). In the juvenile mice cyanide study, [23] the mice administered CoN_4[11.3.1] exhibited a noteworthy improvement compared to other cobalt-containing compounds tested, but did not offer the same measurable impact when administered 5 minutes after. This study suggests that within five minutes after the cyanide dose, the toxicant had bound the active site of cytochrome c oxidase and was unable to be removed.

A comparable study was completed in the *G. mellonella* larvae (Section 4.4.4.). CoN_4[11.3.1] (8 mg/kg) was given one minute after NaCN similar to the mouse cyanide data (Section 4.4.4). *G. mellonella* were injected intraheamocoelly (ih) with 7.5 mg/kg NaCN, a dose similar to the 5 mg/kg dose the mice received. In the larvae, the recovery time for the toxicity of cyanide was cut in half with both the $NaNO_2$ and the CoN_4[11.3.1] when administered one minute after the toxicant (from 11 minutes to ~5 minutes) (Figure 16B). The similarity here suggests that insect and mammalian cyanide toxicity models may not be very different.

Table 5. Antidotal activity of sodium nitrite and CoN_4[11.3.1] against cyanide toxicity in Swiss-Webster mice.

Experimental Conditions	Number of Mice	Recovery Time (min)	% Survival	References
Juvenile Mice (7-8 weeks old)				
5 mg/kg NaCN (t = 0)	82	24 ± 7	80%	[43]
5mg/kg NaCN (t = 0) + 26 mg/kg Co_4[11.3.1] (t = 1)	12	4 ± 3	92%	
Adult Mice (16-20 weeks old)				
5 mg/kg NaCN (t = 0)	28	24 ± 1	66%	[40]
5 mg/kg NaCN (t = 0) + 3-12 mg/kg $NaNO_2$ (t = 2)	105	8 ± 0.5	82%	

All agents given in saline solutions by ip injection. Antidotes administered prophylactically

3.1.3 Azide Toxicity Testing

Finally, a preliminary study was done by our group to examine the effects of $NaNO_2$ (not published) and CoN_4[11.3.1] [24] on 16-20 week old adult, male Swiss-Webster mice poisoned by azide. The mice exposed to a 26 mg/kg (adult) ip dose of sodium azide had an 88% survival, with an approximate 40 minute righting recovery time (Table 6). A 24 mg/kg dose of $NaNO_2$ was not particularly effective at ameliorating NaN_3 therapeutically in adult mice, only decreasing recovery time by 2 minutes with similar survival (Table 6). However, therapeutic injection CoN_4[11.3.1] five minutes after NaN_3 produced a 100% survival, and a decrease in the righting recovery time from 40 minutes to 12 minutes (Table 6). Next, *G. mellonella* were treated with 5 mg/kg $NaNO_2$ or 8mg/kg CoN_4[11.3.1] (Figure 16C) one minute after a 14 mg/kg dose of NaN_3 (Section 4.4.4). In *G. mellonella* treated with $NaNO_2$ the recovery of righting ability improved from 33 minutes in larvae treated with azide alone to ~19 minutes; even more impressive, the larvae that received the CoN_4[11.3.1] one minute after showed an improvement in righting recovery from 33 minutes to 11 minutes (Figure 16C). Recovery with the Busch compound was more significant than the $NaNO_2$ ($p \leq 0.001$).

After reviewing the results in the *G. mellonella* study (Section 4.4.4), we made the decision to re-examine the $NaNO_2$ in the mice. (Submitted to Chem Res Toxicol) This time we investigated the effects in juvenile, male Swiss-Webster mice (6-8 weeks old; data not published). The mice received a therapeutic dose of 24 mg/kg $NaNO_2$ or a 70 μmol/kg ip dose of CoN_4[11.3.1], five minutes after a 27mg/kg dose of NaN_3. Of the mice that received $NaNO_2$, 2 of the 6 mice knocked down and recovered righting ability within ~23 minutes of receiving the azide injection (Table 6). We went on to inject CoN_4[11.3.1] five minutes after the NaN_3, and again there was a 100% survival in these mice and recovery time was cut in half from 38 minutes to 16 minutes (Table 6). Again, we injected a small sample of the adult mice (12 weeks old) and were surprised to see that the adult mice did not regain recovery of righting ability faster than mice receiving azide alone (data not shown). This supported the adult mouse data reported earlier where the $NaNO_2$ was not shown to be helpful in adult mice.

Table 6. Antidotal activity of sodium nitrite and CoN_4[11.3.1] against azide toxicity in Swiss-Webster mice.

Experimental Conditions	Number of Mice	Recovery Time (min)	% Survival	References
Juvenile Mice (7-8 weeks old)				
27 mg/kg NaN_3 (t = 0)	32*	38 ± 19*	84 %*	Frawley, 2019*
27 mg/kg NaN_3 (t = 0) + 26 mg/kg Co_4[11.3.1] (t = 5)	16*	27 ± 12*	100 %*	
27 mg/kg NaN_3 (t = 0) + 24 mg/kg $NaNO_2$ (t = 5)	6*	15 ± 2*	100 %*	
Adult Mice (16-20 weeks old)				
26 mg/kg NaN_3 (t = 0)	8	40 ± 8	88 %*	Praekunatham 2019*
26 mg/kg NaN_3 (t = 0) + 26 mg/kg Co_4[11.3.1] (t = 5)	7	12 ± 4	100 %*	
26 mg/kg NaN_3 (t = 0) + 24 mg/kg $NaNO_2$ (t = 5)*	9*	38 ± 8*	88 %*	Frawley, 2019*

All agents given in saline solutions by ip injection. Antidotes administered prophylactically
* Data not published

3.2 Summary

We were able to observe a comparable ameliorative response to both the $NaNO_2$ and CoN_4[11.3.1] in the mouse and *G. mellonella* models. It was of interest to compare the results of mouse and *G. mellonella* studies conducted in our laboratory since the similarities in response to the toxicants/antidotes suggests that insects and mammalians may not be very different. We know that the cytochrome *c* oxidase is conserved between the species[17] and there is a significant difference related to blood and hemolymph in the two organisms. Due to the promising nitrite data in the cell (Section 2.4.2), *G. mellonella* (Section 4.4.4) and mice, this putative antidote appears to be promising for future studies. Finally, CoN_4[11.3.1] significantly reduced the righting recovery in both *G. mellonella* (Section 4.4.4) and mice. The similarity found in this data suggests that for comparative purposes, in preliminary screening, *G. mellonella* larvae may be an acceptable model for prescreening antidotes ahead of mammalian studies.

4.0 Assessing Modulators of Cytochrome *c* Oxidase Activity in *Galleria mellonella* Larvae

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4.1 Abstract

Caterpillars of the greater wax moth, *Galleria mellonella*, are shown to be a useful invertebrate organism for examining mitochondrial toxicants (inhibitors of electron transport) and testing putative antidotes. Administration of sodium azide, sodium cyanide, or sodium (hydro) sulfide by intra-haemocoel injection (through a proleg) results in a dose-dependent paralyzed state in the larvae lasting from <1 to ~40 min. The duration of paralysis is easily monitored, because if turned onto their backs, the larvae right themselves onto their prolegs once they are able to move again. The efficacy of putative antidotes to the three toxicants can routinely be assessed by observing shortened periods of paralysis with larvae given toxicant and antidote compared to larvae administered only the same dose of toxicant. The validity of the approach is demonstrated with agents previously shown to be antidotal towards cyanide intoxication in mice; namely, sodium nitrite and CoN_4[11.3.1] (cobalt(II/III) 2,12-dimethyl-3,7,11,17-tetraazabicyclo-[11.3.1]-heptadeca-1(7)2,11,13,15-pentaenyl cation). These same compounds are shown to be antidotal towards all three toxicants in the *G. mellonell*a caterpillars; findings that may prove important in relation to azide and sulfide poisonings, for which there are currently no effective antidotes available. The observation that sodium nitrite ameliorates cyanide toxicity in the larvae is additionally interesting because it unambiguously demonstrates that the antidotal action of nitrites does not require the involvement of methemoglobin, contributing to the resolution of an ongoing controversy.

4.2 Introduction

Any compounds entering mammalian blood, whether deliberately introduced in the diet, as pharmaceuticals, or inadvertently absorbed environmental agents, will encounter millimolar concentrations of blood proteins, particularly hemoglobin and serum albumin. From an experimental perspective, this can be problematic, because the broader effects that a xenobiotic may have on the other systemic cells/tissues can be masked by overwhelming interactions with the blood proteins present at anomalously high levels compared to most non-structural biochemical molecules. In addition, reactive small bioinorganic molecules, secondary derivatives of oxygen and nitric oxide competent to modify many organic compounds, can be generated in the bloodstream. The comparative experimental use of invertebrates to investigate the mechanistic biological effects that chemical agents may have in the absence of a confounding mammalian vasculature is, therefore, of some value.

The larvae (caterpillars) of the greater wax moth *Galleria mellonella* (commonly called "wax worms") (Figure 12) have been proposed as a model organism for screening potential therapeutics for microbial infections [73, 75, 77] and investigating the safety of food additives [158]. More generally, of course, the application of such larvae could reduce considerably the number of mammals otherwise required for preliminary toxicological testing of any new commercial compounds. For many experimental applications, the *G. mellonella* larvae (average length: 2 cm; average weight: 250 mg) display advantages compared to the common insect model *Drosophila melanogaster* (average length: 3 mm; average weight: 0.5 mg). For instance, while administration of reagents through routes similar to those used with *Drosophila* such as inhalation, feeding, or topical application [159] is possible, the larger *G. mellonella* larvae can also routinely be given reagents through intra-haemocoel injection (ih) allowing precise control of the dose administered.

The inexpensive *G. mellonella* larvae are available commercially in large numbers, facilitating good statistical analyses. Unlike vertebrate models, such as mice and zebra fish, the larvae have no special care requirements, do not need feeding, can be reared at temperatures ranging from 15°C through 37°C and are easily housed in plastic containers with perforated lids and some wood shavings. *G. mellonella* larvae are also devoid of any of the ethical and legal requirements associated with the experimental use of mammals.

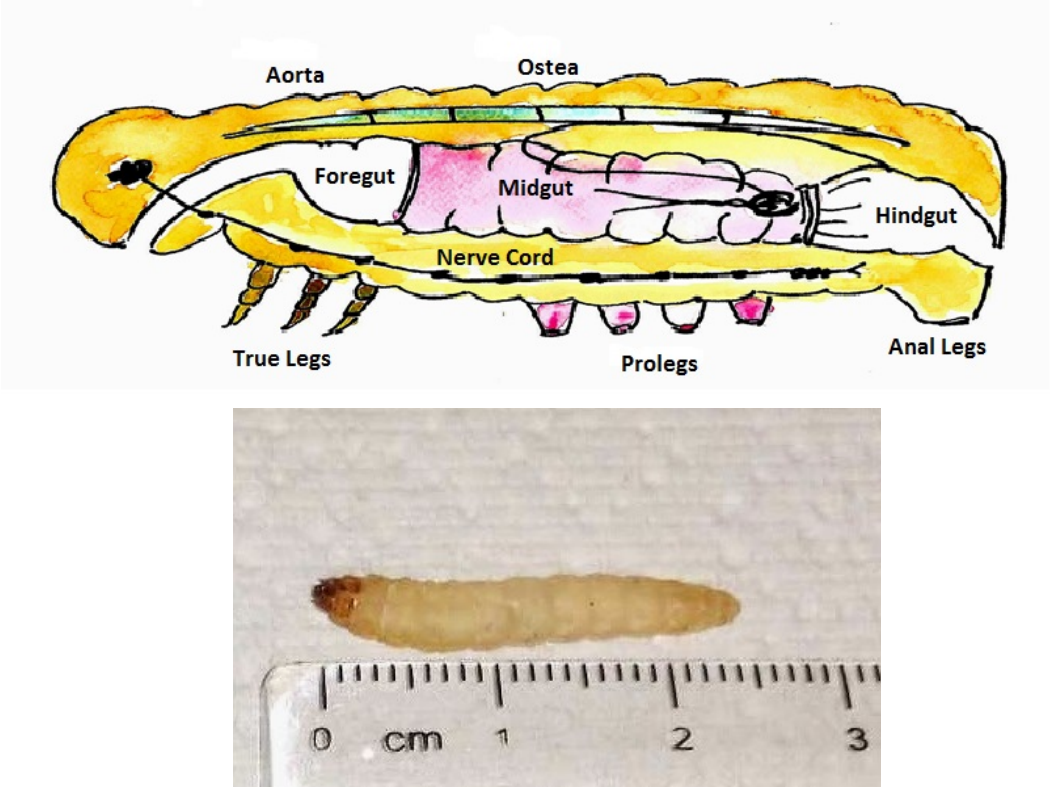

Figure 12. *G. mellonella.*
Diagram and scaled picture of the *Galleria mellonella* (Lepidoptera: Pyralidae, the greater wax moth)

In our laboratory, interest in *G. mellonella* larvae arose in relation to screening potential antidotes to mitochondrial toxicants. Most importantly, for this purpose, the larvae contain functioning mitochondria and, since they are air breathing, exposure to gaseous compounds of

interest is possible. Physiologically, the fat body contained in *G. mellonella* larvae is an organ that functions in a metabolically similar fashion to the mammalian liver and adipose tissue, containing a number of cytochrome P450, glutathione- and sulfo- conjugation enzymes which are involved in drug detoxification.[76, 158] The hemolymph is an analogue of mammalian blood [91] though it does not function in the transport of gasses and has no hemoglobin, or other oxygen carrier. The hemolymph does, however, participate in the immune defense of the larvae [89, 160]. The insect larval midgut epithelial cells also share similar physiological phenotypes to the intestinal cells of mammalian digestive systems [73, 161].

In the current study, we have examined the susceptibility of *G. mellonella* larvae to the mitochondrial toxicants azide, cyanide and sulfide. All three seem to inhibit the insect cytochrome *c* oxidase, the terminal electron acceptor of the mitochondrial electron transport system, in a manner similar to the results previously reported for the mammalian enzyme. At this time, there is no available antidote and/or reliable protocol for treating acute azide or sulfide poisoning and only a single FDA-labeled treatment for cyanide toxicity (Cyanokit®, containing hydroxocobalamin). Another off-label cyanide antidote is available (Nithiodote®, containing nitrite and thiosulfate) but this two-component cocktail continues to be a source of controversy. Specifically, it was believed for decades that sodium nitrite functioned as a cyanide antidote by virtue of its methemoglobin generating capability, resulting in cyanide scavenging through formation of cyanomethemoglobin. Quite recently, the validity of this hypothesis has been seriously challenged [61, 62, 162, 163], but the older erroneous explanation for the efficacy of sodium nitrite continues to persist in the literature [164-166]. Herein, we seek to further discredit the cyanomethemoglobin-formation hypothesis by demonstrating the effectiveness of sodium nitrite as a cyanide antidote in *G. mellonella* larvae which do not contain any hemoglobin. In total, we

have studied the ameliorative capabilities of sodium nitrite and a cobalt-containing hydroxocobalamin mimic towards azide, cyanide and sulfide in the larvae. Through comparison with the reported analogous data for mammalian systems, the results provide valuable insights into the mechanisms of action and proof-of-concept data that will assist in the design of improved antidotes to these and similar mitochondrial poisons for use in humans and livestock.

4.3 Materials and Methods

4.3.1 Reagents

Unless stated to the contrary, all reagents were ACS grade or better, purchased from either Sigma-Aldrich or Fisher Scientific and used without further purification. The composition of the sodium hydrosulfide (NaSH•*x*H2O) as supplied was determined by a previously described titration method.[38] Sodium azide (NaN_3), sodium cyanide (NaCN) and sodium hydrosulfide solutions were prepared immediately prior to use for injection into *G. mellonella* in phosphate-buffered saline (PBS) followed by filtration (22 μm) into septum-sealed vials with minimized headspaces. Subsequent volumetric transfers were made with gas-tight syringes. At pH 7.4, a mixture of ~30% H_2S and ~70% HS^- exists in aqueous solution. In keeping with what seems to be a common convention, we refer to this mixture at physiological pH as "sulfide." Although greater than 98% of HCN is present at physiological pH, the combination of HCN + less than 2% CN^- is referred to as "cyanide." Very little HN_3 is found at physiological pH and, therefore, the term "azide" for solutions prepared from the sodium salt is unambiguously appropriate. Cobalt(II/III)2,12-

dimethyl-3,7,11,17-tetraazabicyclo-[11.3.1]-heptadeca-1(7)2,11,13,15-pentaenyldibromide (CoN_4[11.3.1]) was synthesized and characterized as previously reported.[70]

4.3.2 *G. mellonella* Larvae Exposures

Sixth instar *G. mellonella* larvae (order Lepidoptera, family Pyralidae, the greater wax moth) were purchased from Vanderhorst Wholesale, Inc. (Saint Mary's, Ohio). The larvae were shipped overnight and, upon arrival, incubated in their shipping containers, at 30°C, in complete darkness. The larvae were allowed to acclimate for two days from the date of delivery and were then used within 10 days. Larvae were handled minimally and carefully to avoid stress; feeding was unnecessary. Preceding intra-haemocoel injections, gastight syringes were washed with 10% bleach solution (2x), followed by 100% ethanol (2x), water (3x) and finally PBS (1x); repeating the washing after every 10 larvae and again between each group. Larvae were counted, measured (2-2.5 mm), weighed (250-300 mg) and placed in a holding dish. During manipulation, the larvae were held between thumb, forefinger and index finger, cleaned with 70% ethanol using a cotton swab and injected into the hemocoel through the last left pro-leg-using a 10 µL syringe. Each subsequent injection into the same larva was into a different leg by rotating to the right and the larvae were then transferred to a Petri dish containing a piece of paper towel, to observe time until knockdown (in the case of toxicants) and recovery. Once larvae recovered and began crawling around again they were relocated to a clean Petri dish with a new piece of paper towel. The dishes were taped, labeled and incubated at 30°C overnight.

4.3.3 Righting-Recovery Testing

Following toxicant injections of sodium azide (5.7-22.5 mg/kg), sodium cyanide (1.5-20 mg/kg), or sodium hydrosulfide (18-140 mg/kg), the larvae exhibited a state of paralysis (became motionless) and were able to be placed on their backs in the supine position. The duration of time required for the larvae to turn back over onto their legs/prolegs to the prone position, and remain so, was measured and recorded. For each individual, the time between the onset of paralysis and the larva flipping back onto its legs/prolegs was taken as the righting-recovery time, similar to the procedure we have previously employed with mice.[38, 61] The caterpillars were then returned to the Petri dish and observed for 48 hours for any adverse reactions, of which there were none.

4.3.4 Antidote Testing

Solutions of antidotes (5 mg/kg sodium nitrate or 8 mg/kg CoN_4 [11.3.1]) were prepared fresh for each experiment in sterilized PBS. The potential toxicity of the putative antidotes was tested by injecting the larvae (n = 20/group) with a 5 μL intra-hemocoel injection through the proleg. Larvae were placed in a Petri dish within an incubator at 30°C observed for 48 hours, during which time no adverse reactions were observed. Subsequently, nitrite or CoN_4[11.3.1] solution was injected into *G. mellonella* larvae 5 min prior to 14 mg/kg NaN_3, 7.5 mg/kg NaCN, or 72 mg/kg NaHS and the righting recovery times were measured.

4.3.5 *G. mellonella* Tissue Collection

Approximately 20 larvae were put into a Petri dish and placed in the refrigerator (4°C) for twelve minutes. The larvae were then transferred to a weighing boat on ice with 1 mL cold mitochondrial isolation buffer (154 mM KCl, 1 mM EDTA; pH adjusted to 6.8) [159] and chopped with scissors. The minced tissue was poured into an ice-cold tissue grinder with 5 mL cold mitochondrial particle extraction buffer and gently homogenized using a 15-mL conical tissue homogenizer with a glass pestle (80 strokes up and down). The homogenate was poured into a 15 mL conical tube through cheesecloth to collect the liquid, which was centrifuged (1500 x g; 4°C) for 8 minutes (Beckman Coulter Allegra X-12R centrifuge). The supernatant was discarded and the pellet washed with 200 µL mitochondrial isolation buffer (1500 x g; 4°C) for 2 minutes. The pellet was finally suspended in 100 µL mitochondrial isolation buffer plus 100 µL 10% dodecyl maltoside solution and incubated on ice for 30 min prior to cytochrome *c* oxidase activity assays. Alternately, for respirometric measurements, the final tissue pellet was resuspended in 200 µL mitochondrial isolation buffer prior to being stored on ice subsequent to use (see below).

4.3.6 Cytochrome *c* Oxidase Assays

Ferrocytochrome *c*:O_2 oxidoreductase activity was spectrophotometrically determined by employing a method similar to Sinjorgo *et al.*[167] After incubation, the *G. mellonella* tissue was diluted 1:10 with mitochondrial assay buffer (115 mM KCl, 10 mM KH_2PO_4, 2 mM MgCl, 3 mM HEPES, 1 mM EGTA, BSA 0.2%; pH adjusted to 6.8)[159], ferrocytochrome *c* (5-30 µM) was added and the change in absorbance at 550 nm was measured over time in 0.25 mM oxygen at 25°C. All kinetic time courses for ferrocytochrome *c* oxidation were essentially linear in the range 10−60 s.

Where required, rates were estimated from the linear-region slopes of ferrocytochrome *c* concentration versus time plots. Azide and cyanide inhibition of cytochrome *c* oxidase was determined using 0.1 mM azide, 1.0 mM azide, 7 μM cyanide and 14 μM cyanide, varying the initial concentration of ferrocytochrome *c*. The assays were performed using Shimadzu UV-1650PC and UV-2501PC spectrophotometers.

4.3.7 Respirometric Experiments

An Oxygraph O2k Polarographic instrument (Oroboros Instruments, Innsbruck, Austria), equipped with a Clark-type electrode for high-resolution respirometry was used to measure oxygen fluxes and concentrations. Mitochondrial respirometer solution, MiR05,[168](0.5 mM EGTA, 3 mM $MgCl_2$, 60 mM lactobionic acid, 10 mM KH_2PO_4, 20 mM HEPES, 110 mM D-Sucrose, 1g/l BSA) [35] (2.1 mL) was added to each chamber and equilibrated for 20 minutes prior to the addition of ~50 μL tissue homogenate (prepared as described above) into the Oxygraph chambers (sealed from the atmosphere) at 25°C. Mitochondrial electron transport was observed with the addition of NADH oxidase substrate precursors, pyruvate (final concentration: 5 mM), malate (final concentration: 0.5 mM) and glutamate (final concentration: 10 mM) to the respirometer. A determination of the state of coupling of the mitochondrial tissue was evaluated by the addition of CCCP (final concentration: 0.05 μM) and cytochrome *c* (final concentration: 10 μM) to the respirometric chamber. Oxygen turnover was examined by the addition of succinate (final concentration 10 mM) and NADH (0.5 mM). Rotenone (final concentration: 0.5 μM) and antimycin A (final concentration: 2.5 μM) were added to inhibit complex I and complex III, respectively. Each of the following cytochrome *c* oxidase inhibitors were added as small volumes of unbuffered solutions in deionized water: sodium azide (pH 7.0, final concentration: 1.25 mM), sodium

cyanide (pH 11.3, final concentration: 15 μM) or sodium hydrogen sulfide (pH 10.0, final concentration: 15 μM) by gas-tight syringe into the sealed Oxygraph chambers. Respirometric data analysis was carried out with DatLab 7 software provided by Oroboros.

4.3.8 Numerical Analysis

Statistical comparisons were carried out using one-way analysis of variance (ANOVA) and student t-tests. Values are expressed as means +/- standard errors. A p-value ≤ 0.05 was considered significant and statistical analyses were performed with KaleidaGraph® (Synergy Software).

The subunit sequences (CO1, CO2 and CO3) encoded in the mitochondrial DNA of cytochrome *c* oxidase from *H. sapien, M. musculus, B. taurus* and *G. mellonella* species were compared using UniprotKB (https://www.uniprot.org/). A BLASTp search was done to find sequence similarity between *H. sapien* (H9RLZ4) and *G. mellonella* (A0A0S1YCX8) protein sequences for subunits 1-3 (CO1, CO2, CO3), based on E-value data. The E-values for the comparison of the *G. mellonella* to *H. sapiens* cytochrome *c* oxidase subunits 1-3 were the following: CO1 gene (D8VVZ2_GALME vs H9RLZ4_HUMAN) was 6.1e-47, for CO2 (COX2_GALME vs COX2_HUMAN) 4.7e-87 and for CO3 (A0A0S1YD76_GALME vs COX3_HUMAN), 1.6e-139. These E-values showed a significant relationship between the two sequences compared in all subunits. BLAST also provided a quantitative measurement of the similarity between the two sequences with related species having a higher percent identity than more distantly related species. For CO1 there was an 83% identity, for CO2 a 55% and CO3 a 65.8% respectfully. Finally, Clustal Omega Alignment was used to view the characteristics of the

four species alongside each other to observe the relationships between ligands for prosthetic groups within the CO1 and CO2 subunits of cytochrome *c* oxidase.

4.4 Results

4.4.1 Cytochrome *c* Oxidase Turnover and Inhibition Kinetics in Tissue from *G.mellonella*

Homogenized *G. mellonella* tissue extracted with lauryl maltoside was examined for cytochrome *c* oxidase activity using a method similar to Sinjorgo *et al,* [167] but working at pH of 6.8, the *in vivo* pH of *G. mellonella*. Inhibition of the reaction in the presence of azide (at 0.1 and 1.0 mM, Figure 13A) and cyanide (at 7 and 14 μM, Figure 13B) was measured (Figures 13A and 13B, respectively). This same turnover approach could not be used in the case of sulfide inhibition, as it directly reduces ferricytochrome *c* back to ferrocytochrome *c*, interfering with the assay. The azide inhibition constant, $K_{i(azide)}$, determined from the turnover kinetics was found to be 22(±4) μM, the identical result to that originally reported by Petersen [31] for the beef heart cytochrome *c* oxidase (monitoring oxygen-consumption kinetics). The close similarity of the present result and earlier findings is not surprising as several groups, including our own, have previously shown that the electron-transfer activities of different cytochrome *c* oxidases are essentially independent of source tissue and/or species [167, 169]. The cyanide inhibition constant, $K_{i(cyanide)}$, was determined in the present study to be 4(±1) μM for the *G. mellonella* enzyme, 20 times larger than the 0.2 μM previously reported for the beef enzyme [31]. It follows from the observations with azide, that we probably should not necessarily simply attribute this 20-fold discrepancy to any intrinsic difference in the cyanide-inhibition characteristics of the insect larval and mammalian cytochrome *c* oxidases.

A more plausible explanation might be that some insects have significant levels of enzymatic activities that deactivate cyanide, such as rhodanese and β-cyanoalanine synthase, making them unusually resistant to cyanide toxicity.[170-172] Additionally, it is to be noted that the cyanide content of neutral aqueous solutions is ~99% molecular HCN, which can rapidly be lost by partitioning into the gas phase. The extent of loss depends upon the ratio of head space to solution volume and the rate of loss on the surface tension, which in turn depends upon a complicated set of experimental variables including ionic strength and detergent/lipid content.

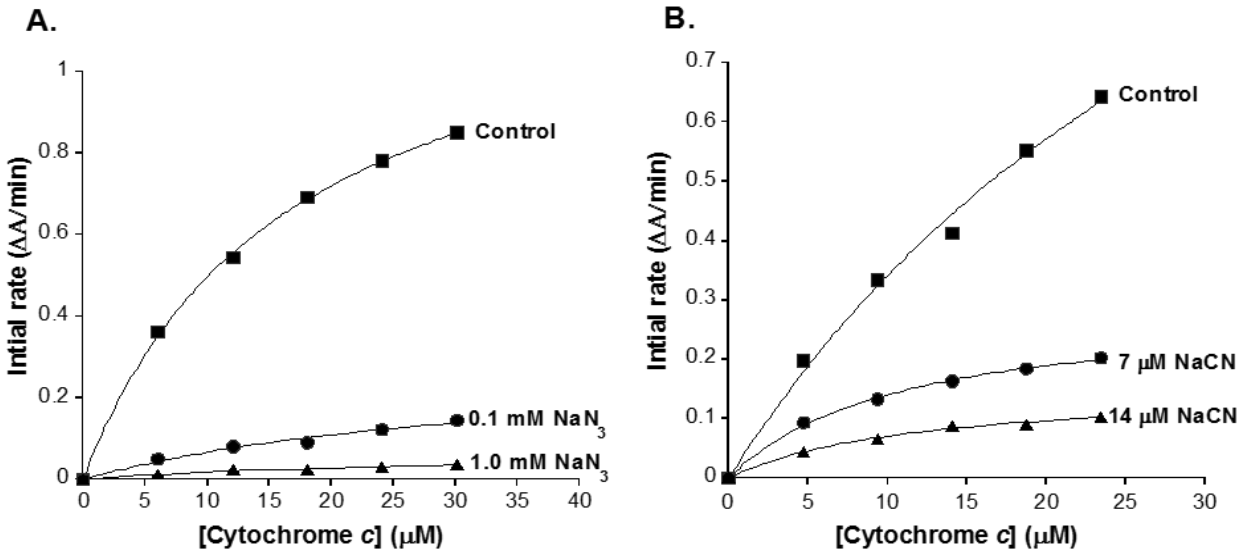

Figure 13. Turn-over analysis: cyanide and azide inhibition of cytochrome c oxidase extracted from *G. mellonella* tissue.

Sodium azide (A) and sodium cyanide (B) inhibition of cytochrome c oxidase during turnover at 25°C, in mitochondrial assay buffer (115 mM KCl, 10 mM KH_2PO_4, 2 mM MgCl, 3 mM HEPES, 1 mM EGTA, BSA 0.2%; pH adjusted to 6.8). The concentration of cytochrome *c* was varied from 5-30 μM. Twenty five μL of extracted cytochrome *c* oxidase (see Materials and Methods for details) was used in each assay.

4.4.2 Respirometric Analysis of *G. mellonella* Tissue

In addition to the cytochrome *c* oxidase (complex IV) assays, mitochondrial electron transport in the *G. mellonella* tissue extracts was also examined by high-resolution respirometry (Figure 14). Any observed variations in oxygen consumption rates (broken traces) are typically slight and difficult to follow, so the slope of the raw data (oxygen flux) is also shown (solid traces). Since the flux as presented is actually $-d[O_2]/dt$, increases in the ordinate values of the solid traces should be interpreted as indicating increased oxygen consumption rate. It is also to be understood that the Oroboros system is quite sensitive, enough that >5 μL additions of fully aerated (or fully de-aerated) reagent solutions register as changes in measured oxygen flux.[35] Any troughs appearing in the solid traces during the first minute following reagent additions (Figure 14) are the result of fully aerated additions detected as decreases in oxygen consumption (*i.e.* increases in oxygen concentration) – the experimentally meaningful consequences of reagent additions follow these artifacts. Using high salt to isolate mitochondrial particles all but insured that the electron transport system would be disrupted and hence, uncoupled. The following titrations of substrates and inhibitors into the mitochondrial tissue during respirometric experiments generated a series of observations (data not shown) indicating that the isolated particles were indeed uncoupled but contained the competent electron-transport system: additions of an uncoupler, CCCP, to the *G. mellonella* tissue did not affect the oxygen flux, while ADP gave only a very modest increase in oxygen consumption; additions of cytochrome *c* (replacing that lost through disruption) measurably increased the oxygen flux and was therefore added to all other experiments; additions of succinate to this cytochrome *c*-replenished preparation resulted in a sharp increase in the rate of oxygen consumption confirming the presence of complex II

(succinate dehydrogenase) and inhibition of oxygen consumption following addition of antimycin A confirmed the presence of complex III (cytochrome *c* reductase).

Addition of NADH to a cytochrome *c*-replenished preparation also resulted in a sharp increase in the rate of oxygen consumption (Figure 14A) confirming the presence of complex I (NADH dehydrogenase) – as expected, this could be reversed by the addition of either rotenone (complex I inhibitor) or antimycin A (data not shown). NADH-driven electron transport was readily blocked by inhibition of the terminal oxygen acceptor, cytochrome *c* oxidase, with either azide (Figure 14B) or cyanide (Figure 14C) in keeping with the spectrophotometric results (Figure 13). The findings for the inhibition of electron transport by added sulfide were more complicated (Figure 14D) as following a temporary decrease in oxygen flux (the large "dip" at ~35 min, a previously described artifact due to direct interaction of sulfide with the electrode system [35]) a non-transient decrease in oxygen flux was observed (from 36-40 min) discernibly slower than in the case of azide or cyanide. Mammalian cytochrome *c* oxidase is known to undergo several reactions with sulfide including turnover and slow inhibition [27, 173-175] that are never observed for reactions of the same enzyme preparations with azide or cyanide. Therefore, in the present case, the findings are still commensurate with cytochrome *c* oxidase being the site of inhibition of electron transport by sulfide in the *G. mellonella* mitochondria.

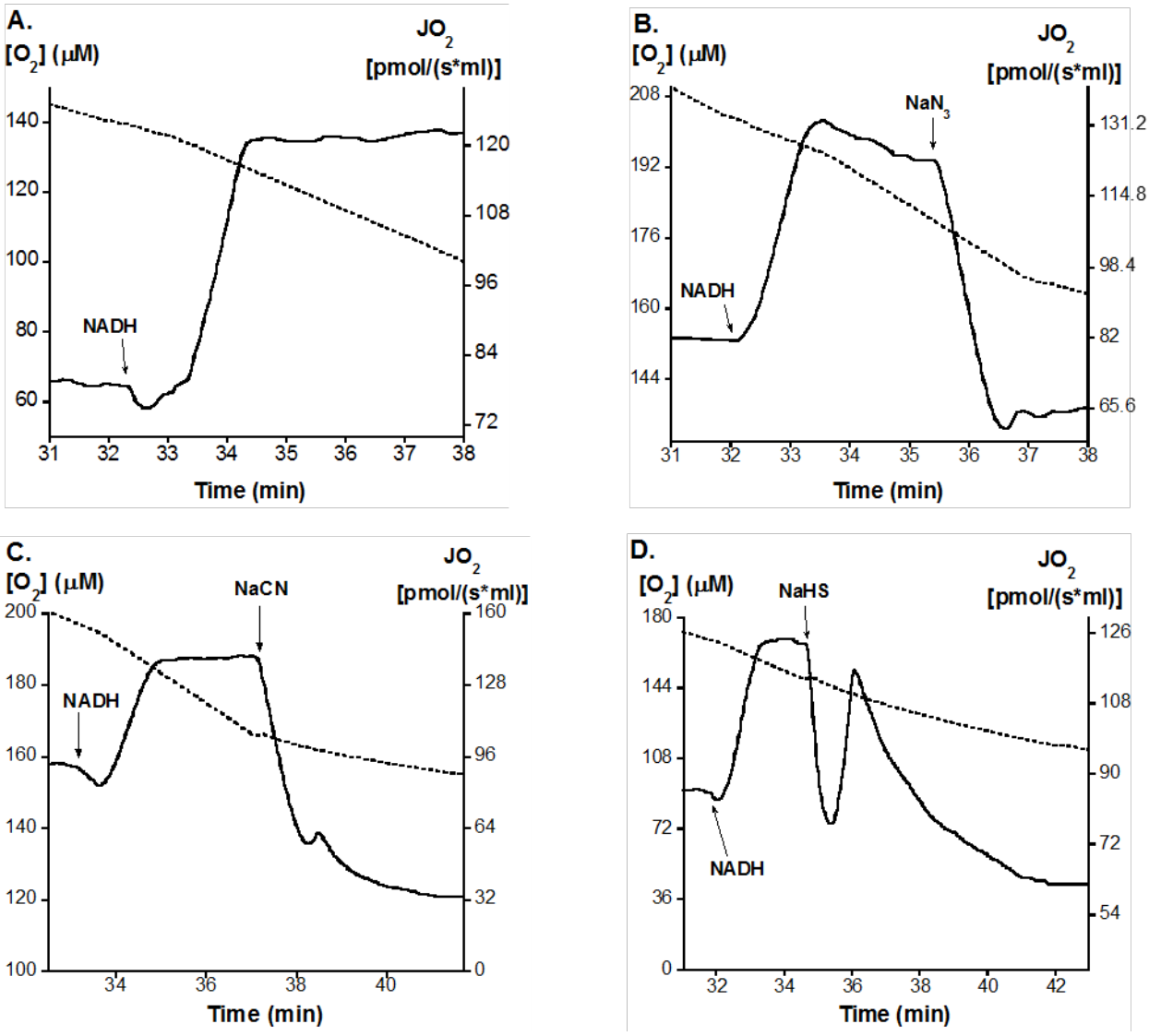

Figure 14. Respirometric Analysis from Ground-up *G. mellonella* Tissue.
Oxygen consumption was assessed in homogenized *G. mellonella* tissue diluted in MiR05 respirometric solution (see Materials and Methods for details). The tissue (2.1 mL) was allowed to equilibrate in chamber for ~10 minutes prior to measuring electron flow. Default respirometric settings of block temperature, 25°C; stir bar speed, 400 rpm and data recording, 2 s were used. All regents/substrates amounts are given as final concentrations. Oxygen turnover (oxygen concentration, left y-axis; oxygen flux right y-axis) by *G. mellonella* tissue was followed after additions of cytochrome *c* (10 µM) and NADH (0.5 mM): (A) control, (B) plus NaN_3 (1.25 mM), (C) plus NaCN (14 µM) and (D) plus NaHS (15 µM).

4.4.3 Cytochrome *c* Oxidase Inhibitors Induce a "Knockdown" State in *G. mellonella*

The cytochrome *c* oxidase inhibitors, sodium azide, sodium cyanide and sodium hydrosulfide, were independently administered by intra-hemocoel (ih) injections through the proleg (see Experimental Methods for details) of the *G. mellonella* larvae. The initial dose of each toxicant given to the caterpillars was the same dose that we had previously found to induce "knockdown" (unconsciousness) in mic.[38, 61] Subsequently, the doses were increased/ decreased in 2-5 mg/kg increments to obtain dose-response curves and find final working doses that produced a state of paralysis (larvae became motionless) for a reproducible and suitable time period (>10 minutes) without causing death. The reported recovery times are the durations from the initial injection of toxicant into the larvae until they "righted" themselves and began to move again when touched following the period of "knockdown" (or state of paralysis).

When sodium azide was administered to *G. mellonella* larvae (5-25 mg/kg) and the recovery time monitored, a dose-response curve was obtained exhibiting saturation at a recovery time of 80 min using the LD_{40} dose of 25 mg/kg (Figure 15A). Increasing the dose to 28 mg/kg sodium azide resulted in 100% deaths. A "knockdown" or unconscious response of 38 min to sodium azide was observed in the *G. mellonella* larvae at the IC_{50} dose (ih) of 15(±1) mg/kg (fit to the data in Figure 15A). In comparison, a brief preliminary study with mice showed that a 27-28 mg/kg (ip) sodium azide dose (~LD_{40}) induced a "knockdown" state of 40(±8) min in surviving animals (see Supplemental Material) and 30 mg/kg (ip) sodium azide proved lethal. After administration of azide doses, the larvae immediately became rigid and could then be placed on their backs where they remained motionless until they turned over. This was in contrast to cyanide and sulfide *G. mellonella* intoxications, where approximately

15 seconds elapsed prior to the larvae showing any signs of succumbing to the does. After the larvae recovered from azide intoxication, they seemed unusually sensitive if touched along their backs with a cotton swab, but remained lethargic for hours if not disturbed.

The dose-response (recovery time) curve obtained for sodium cyanide exposures in *G. mellonella* larvae also exhibited saturation, but with maximum recovery time of ~40 min at the LD_{40} dose of 20 mg/kg (Figure 15B). An 8 mg/kg (ih) dose of sodium cyanide induced a state of paralysis within fifteen seconds with a recovery time of 20(±3) min and a 100% survival rate. In comparison, mice given a 5 mg/kg (ip) dose of NaCN exhibit a "knockdown" state with a 24(±7) min recovery time and an 80% survival rate.[61, 62] The similarity of dose-response findings for the larvae and the mice is intriguing – suggesting that, *in vivo*, the insect and mammalian electron-transport systems cannot be very different, contrary to the 20-fold variation in the $K_{i\ (cyanide)}$ values between the insect and mammalian enzymes found above.

Sodium hydrogen sulfide administered to *G. mellonella* larvae exhibit a dose-response curve without any apparent asymptote in the practically accessible dose range (Figure 15C). A dose of 108 mg/kg (ih) NaHS caused a state of paralysis lasting 25(±3) min with an LD_{30}. In contrast, a dose of 16 mg/kg of NaHS created a "knockdown" state lasting 4 (± 1) min with only a 57% survival rate in a mouse model.[176] Thus, there is an apparent large difference (~7-fold) between the response of the *G. mellonella* larvae and mice for the mitochondrial toxicant sulfide.

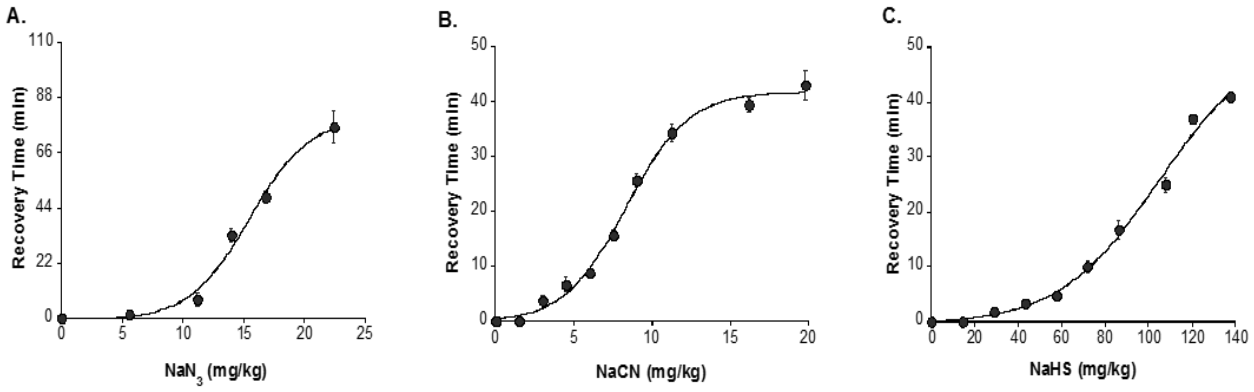

Figure 15. Dose-response data for azide, cyanide and sulfide.
Toxicants (azide, cyanide and sulfide) or phosphate buffered saline (PBS) (5-10 µL) were injected into the proleg of *G. mellonella* larvae using a gas-tight syringe. The time from which the larvae ceased movement until movement began again was recorded (recovery time). Larvae injected with PBS (5 µL) were used as controls and showed no inhibition of movement. (A) Sodium azide (NaN_3) was injected at the following doses: 5.6, 11.2, 14, 16.8, 22.4 mg/kg, and the recovery time observed. The IC_{50} was determined to be 15(±1) mg/kg with an average time until recovery of 40(±10) min. (B) Sodium cyanide (NaCN) was injected at the following doses: 1.5, 3, 4.5, 6, 7.5, 9, 11.3 mg/kg, and the recovery time observed. The IC_{50} determined to be 8.3(±0.3) mg/kg with an average time until recovery of 20(± 3) min. (C) Sodium hydrogen sulfide (NaHS) was injected at the following doses: 14.4, 28.8, 43.2, 57.6, 72, 86.4, 108, 120, 140 mg/kg, and the recovery time observed. The IC_{50} determined to be ~108 mg/kg with an average time until recovery of 24(± 3) min.

4.4.4 Evidence for Amelioration of Cytochrome c Oxidase Inhibition in *G. mellonella* Larvae by Putative Antidotes

Previous work has shown that both sodium nitrite and several cobalt complexes, including CoN_4[11.3.1], can act through different mechanisms to ameliorate cyanide toxicity in mice.[61, 62, 70, 176-178] These same compounds are not significantly antidotal towards sulfide in mice unless given prophylactically [35, 38] and the limited available literature suggests they might be ineffective as azide antidotes [179]. *G. mellonella* larvae were administered (ih) sub-lethal doses of either sodium azide (8 mg/kg), sodium cyanide (7.5 mg/kg), or sodium hydrosulfide (72 mg/kg), resulting in mean knockdown durations of 33 min, 11 min and 10 min respectively (Figures 16A, 16B & 16C). When given either sodium nitrite (5 mg/kg, i.h.) or $Co(II)N_4$[11.3.1] (8 mg/kg), 1

min after the toxicant, the azide injected larvae recovered significantly more quickly (~19 min & 11 min respectively) than the controls given no antidote (Figure 16A). In the case of larvae injected with cyanide or sulfide solutions, administration of either sodium nitrite or Co(II)N_4[11.3.1] 1 min later resulted in an approximate halving of the recovery durations compared to controls given no antidote (Figures 16B & 16C respectively). In all groups treated with either nitrite or Co(II)N_4[11.3.1], the larvae survived (100%) post injections with no side effects noted, some going on to pupate within 96 hours similar to sentinel-type controls.

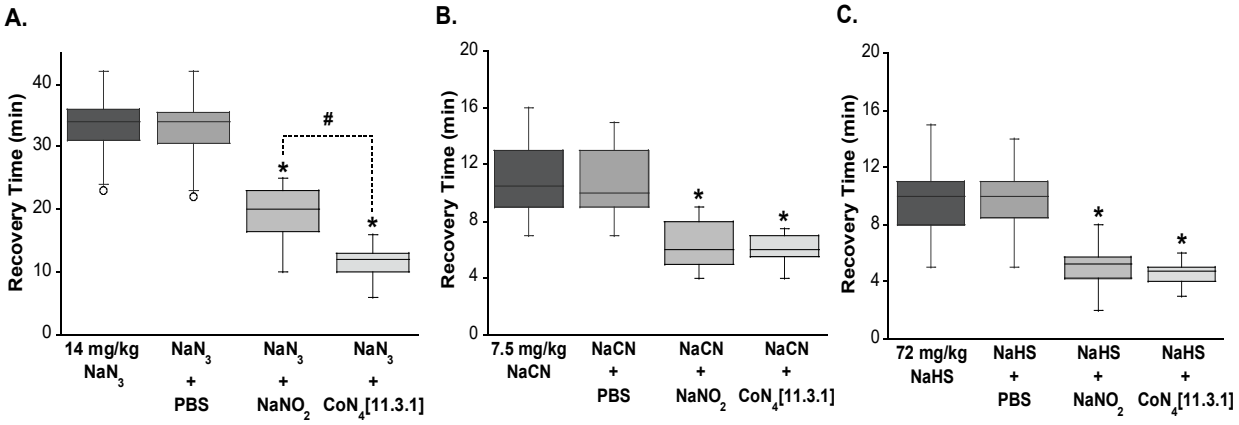

Figure 16. Amelioration of toxicants (sulfide, cyanide and azide) by CoN_4[11.3.1] or sodium nitrite. Larvae were injected with each of the three toxicants either alone, or received subsequent injections of 5µL PBS, 5µL sodium nitrite (5mg/kg) or 5µL CoN_4 [11.3.1] (8 mg/kg) one minute after the initial injection. The time until recovery was significantly decreased by the injection of both the sodium nitrite and the cobalt compound (* $p \leq 0.001$). *(Graph shows the distribution of the samples, specifically, the median of each sample (line) within the interquartile range or "box" containing 50% of the sample, with the "whiskers" extending from the top (Q3) and bottom (Q1), these values show the maximum and minimum of the sample.

4.4.5 The Conserved Nature of the Cytochrome *c* Oxidase Active Site

Since we are interested in the possible use of *G. mellonella* as an invertebrate model for modulation of the activity of mammalian cytochrome *c* oxidase by various inhibitors and their potential antidotes, a comparison of the subunits encoded by mitochondrial DNA (CO1, CO2 and

CO3) from the human, bovine, murine and *G. mellonella* species seemed highly appropriate. Subunit 1 (CO1) contains the active site prosthetic groups, heme a_3 and Cu_B, where oxygen is reduced to water. The first subunit also contains heme a, which functions to funnel electrons to the heme a_3/Cu_B active site. Subunit 2 (CO2) contains Cu_A, a copper complex that transfers electrons to heme *a*. The third subunit (CO3) encoded by mitochondrial DNA is thought to maintain the structural integrity of the first and second subunits [180]. These 3 subunits form the functional core of the enzyme, including the ability to pump protons. A comparison of the protein sequences of *G. mellonella* CO1, CO2 and CO3 subunits [181] with those from human showed that these subunits were quite similar (see Materials and Methods). Most importantly, all the ligands to the prosthetic groups (heme a, heme a_3, Cu_A and Cu_B) were identical between species (Figure 17) indicating the highly conserved nature of the essential functional unit.

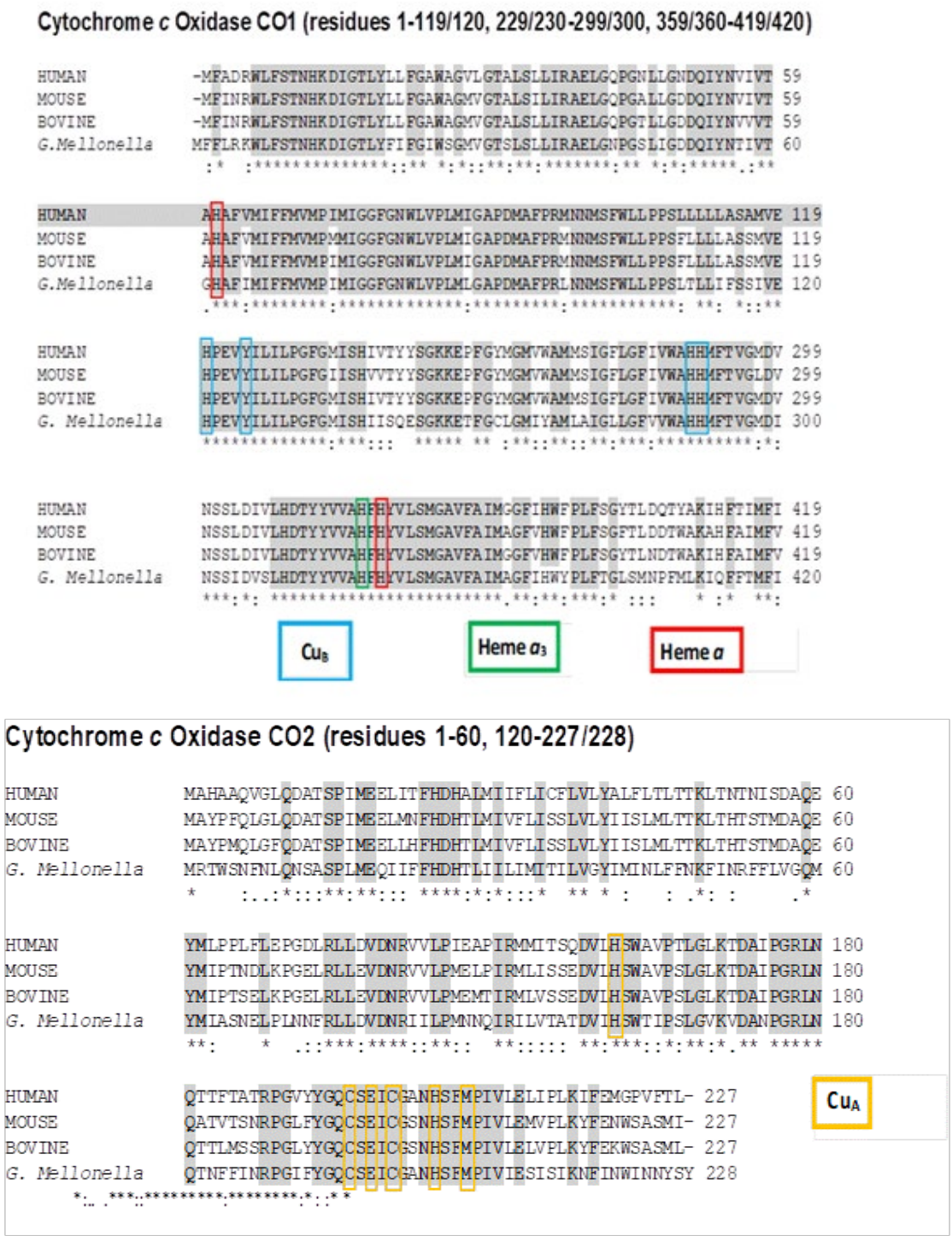

Figure 17. Partial comparison of cytochrome *c* oxidase amino acid residues in subunits CO1 and CO2 from human, bovine, mouse and *G. mellonella*.

Conserved amino acid sequences (*) are represented by amino acid residues, highlighted in grey. Similar amino acid residues (for example, V and L) are marked with double dots (:) and dissimilar residues are marked with a single dot (.). The ligands for heme *a* and heme a_3 are marked with red and green boxes, respectively. The ligands for Cu_A and Cu_B are marked with blue and gold boxes, respectively. See Material and Methods for details.

4.5 Discussion

4.5.1 Comparison of Structure and Function in Cytochrome *c* Oxidases

It has been known for some time that, except for one non-physiological reaction with carbon monoxide, the functional (electron-transfer) properties of eukaryotic cytochrome *c* oxidases do not measurably vary between tissues [167] or species [169]. Furthermore, this strong similarity even seems to extend to bacterial examples [17] and so, it is not surprising that the enzyme from *G. mellonella* also contains essentially the same active site characteristics (Figure 17). These observations provide a solid foundational argument for the further development of the larval system as a functional model for the mammalian enzyme in a variety of experimental circumstances.

4.5.2 Observed Paralysis in *G. mellonella* Larvae Secondary to Cytochrome *c* Oxidase Inhibition

The ligands azide (N_3^-), cyanide (CN^-) and sulfide (HS^-) are all known inhibitors of mitochondrial respiration, blocking the heme a_3/Cu_B oxygen-reduction site of cytochrome *c* oxidase.[31, 98] Accordingly, the respirometric experiments showed that in mitochondrial particles extracted from the *G. mellonella* larvae, oxygen turnover was inhibited by all three of these heme ligands (Figures 13 & 14). In mammals, intoxication with azide, cyanide, or sulfide typically results in loss of consciousness and, if the toxicant dose is lethal, death is widely accepted to follow through collapse of the nervous system supporting cardiopulmonary function. The response of the larvae to the toxicants appears to be somewhat analogous, with the observed paralysis (Figure 14)

clearly implicating dysfunction of the nervous system, presumably due to depletion of available ATP following inhibition of oxidative phosphorylation.

With the *G. mellonella* larvae, all three toxicants act rapidly, resulting in paralysis within about 15 s of injection (ih). In contrast, while the action of cyanide and sulfide are rapid in mice, knockdown/death occurring within 3-4 min of injection (ip), the onset of symptoms following azide administration is significantly slower – knockdown after 10 min and deaths at 30 min to several hours post intoxication (ip). Also, the larvae seem to exhibit measurable effects due to azide at lower doses than the mice, whereas in the case of cyanide and sulfide the larvae require higher doses than mice for comparable effects. In short, the larvae were observed to be unexpectedly sensitive to azide compared to cyanide and sulfide. The vertebrate blood-brain barrier might provide a plausible explanation for these observations. At physiological pH, azide exists almost exclusively in its anionic form (N_3^-) and, consequently, is probably restricted from crossing the blood-brain barrier. Cyanide and sulfide on the other hand exist as anions in equilibrium with molecular forms (HCN and H_2S) at physiological pH and are, therefore, able to cross the blood-brain barrier by passive diffusion. Thus, the slower onset of azide toxicity in mice can readily be understood. Insects also possess protective neural barriers, but it presently remains unclear exactly how functionally similar they are to the vertebrate systems [182, 183]. The rapid onset of azide effects we report here may suggest that, at least in the case of *G. mellonella* larvae, the neural barrier must be significantly more permeable to azide anions than its vertebrate counterpart.

The IC_{50} for sulfide (~108 mg/kg NaHS) was ten times larger than that found for cyanide (11 mg/kg NaCN) in the *G. mellonella* larvae (Figure 14) indicating the sulfide to be an order of magnitude less toxic. In mice, we have previously found sulfide to be only about three-to-four times less toxic than cyanide [38]. The caterpillars contain no oxygen carrier/storage molecule in the

hemolymph, gas exchange being accomplished through spiracles located on the sides of the larval body, these in turn attached to a tracheole system delivering oxygen directly to all cells [88, 161]. Thus, even when paralyzed, small gaseous molecules like H_2S could conceivably be efficiently lost from the small larval body by passive diffusion. In the mice, although compromised, pulmonary function does continue prior to death, so H_2S can be exhaled during intoxication. A plausible explanation for the lower toxicity of sulfide in the caterpillars compared to mice may be attributable to the different pH characteristics of blood compared to hemolymph. The normal pH of murine blood is 7.4, just to the alkaline side of the first acid dissociation constant of H_2S (pK_a ~7.1) while the pH of the *G. mellonella* hemolymph is 6.8. This dictates that "sulfide" in mammalian blood is an approximately 30:70 mixture of H_2S/HS^- [33] whereas in the hemolymph "sulfide" will be a 70:30 mixture of H_2S/HS^-. Thus, in the *G. mellonella* larvae, 70/30 times more administered sulfide will be present in the easily lost gaseous molecular form than in the mice. So, if sulfide is 70/30 times more easily lost from the larvae and is 3-4 times less toxic than cyanide in mice, we can most simply predict that sulfide should be ~8 times (*i.e.* 70/30 x 3.5) less toxic than cyanide in the *G. mellonella* larvae. The predicted value of 8 compares rather favorably with the factor of 10 that we actually found. Such simple considerations, however, appear misleading in relation to the observed toxicity of cyanide. In activity studies of isolated preparations of cytochrome *c* oxidase, the inhibitory characteristics of cyanide and sulfide tend to be experimentally indistinguishable [98] and, with a pK_a of ~9.3, "cyanide" is >98% HCN throughout the pH 6.8-7.4 range. Consequently, it is unclear why cyanide is not more readily lost and, therefore, of lower toxicity than sulfide in both the caterpillars and mice. Thus, not surprisingly, it is apparent that satisfactory quantitative comparisons of the relative toxicities require detailed

knowledge of the toxicodynamics/kinetics (*i.e.* delivery and elimination rates) in addition to the intrinsic inhibition characteristics at the target enzyme site.

4.5.3 *G. mellonella* Larvae as a Potential Model for Screening Antidotes

An objective of this study was to demonstrate the usefulness of *G. mellonella* larvae as a model for screening potential antidotes for mitochondrial toxicants, specifically cytochrome *c* oxidase inhibitors. To this end, the practical approach of finding an easily monitored toxic response (righting recovery) and establishing a convenient experimental time frame on a trial-and-error basis, thereby avoiding any need to rigorously understand the mechanisms of toxicity and antidotal action, has been achieved (Figure 16). The two antidotes selected, sodium nitrite and CoN_4[11.3.1], have previously been tested in animals for efficacy against cyanide, so the inclusion of these results here (Figure 16B) is, in part, as positive controls for proof of principle. While the two antidotes are both clearly effective against sulfide intoxication in the larval experiments (Figure 16C) they are unlikely to ever become of any practical value in real poisonings. This is because surviving victims of sulfide poisoning typically reach the clinic presenting pulmonary injury, including edema, but no longer experiencing potentially reversible sulfide inhibition of mitochondrial function[33]. The findings regarding azide intoxication and its amelioration by both sodium nitrite and CoN_4[11.3.1] (Figure 16A) are the most interesting of this particular set. There is presently no available antidote for azide poisoning and the very limited available literature suggests that at least sodium nitrite might be of no therapeutic benefit. The present results clearly indicate that the possible efficacy of sodium nitrite as an azide antidote should be more fully re-investigated and that CoN_4[11.3.1] represents a candidate lead compound for further development as a potential therapeutic. More generally, these results lend support to the assertion that *G.*

mellonella caterpillars offer an inexpensive alternative to rodents for simple preliminary investigations identifying harmful chemical exposures and approaches to their amelioration.

4.5.4 Methemoglobin Formation is not Required for the Antidotal Action of Nitrites

The belief that nitrites are antidotal towards cyanide intoxication primarily by virtue of their methemoglobin-forming ability continues to persist in the literature.[164-166]. It has even been suggested that enough nitrite to result in 20-30% methemoglobin formation might be optimum for amelioration of cyanide toxicity [184] – an alarming recommendation, given that the patients in question will likely already be presenting with cyanide-compromised respiratory function. Strong evidence has already been reported showing that neither inorganic [61, 62] nor organic [162, 163] nitrites depend on methemoglobin formation to account for their efficacy as cyanide antidotes. The present findings unambiguously confirm this fact, since the *G. mellonella* larvae contain no hemoglobin, yet sodium nitrite is clearly antidotal in the caterpillars (Figure 16B). Our group has previously postulated [61, 62] that the nitric oxide (NO) donor capability of nitrites is primarily responsible for their antidotal activity towards cyanide, as NO is an efficient antagonist of cyanide inhibition at cytochrome *c* oxidase [59, 60]. Further support for the critical role played by NO in amelioration of cyanide inhibition of cytochrome *c* oxidase has recently emerged during studies in which administration of a neuronal NO synthase inhibitor attenuated the protective effect of hyperbaric oxygen treatment on cyanide-intoxicated rats [185].

4.6 Supplemental Materials and Figures

Approval for the mouse protocol was obtained from the University of Pittsburgh Institutional Animal Care and Use Committee (Protocol Number 16088947). The Division of Laboratory Research of the University of Pittsburgh provided veterinary care. All animal experiments complied with the National Institutes of Health guide for the care and use of Laboratory animals (NIH Publications No. 8023, revised 1978). Swiss-Webster (CFW) mice weighing 35-45 g from Charles River Laboratories, Wilmington, MA, were 10-12 weeks old and housed four per cage. The mice were allowed access to food and water *ad libitum*. Experiments commenced after the animals were allowed to adapt to their new environment for one week.

The righting recovery method for determining the effectiveness of azide antidotes has been routinely used in our laboratory for almost a decade. Upon loss of consciousness ("knockdown"), approximately 10-12 min following administration of sodium azide (NaN_3, 26-30 mg/kg ip), mice were placed in a transparent but dark colored plastic tube in the supine position. The time duration from the NaN_3 injection until the mouse flipped from the supine to the prone position in the plastic tube was taken as the end point ("recovery time"). Toxicant and Antidotal experiments with G. mellonella caterpillars are described in the Materials and Methods.

Table 7. Caterpillar *(Galleria mellonella)* Toxicant Dose Response Data.

Larva #	Righting Recovery Time (min)																				
	Sodium azide (mg/kg)					Sodium cyanide (mg/kg)								Sodium hydrosulfide (mg/kg)							
	5.6	11.2	14	22.4	28	3	4.5	6	7.5	9	11.3	16.2	19.8	28.8	43.2	57.6	72	86.4	108	120	138
1	1	5	18.5	80	*	4	5	8.5	13	24	36	36	43	1.5	3	5	8	20	24.5	40	45
2	1	8	31.5	40	*	3	6	8	12	26	34	37	43	1.5	3.5	5	12	14	22.5	41	40
3	0.5	6	31	80	*	3	8	7	15	25	33	38	44	2	3.5	4.5	11	18	28	38	42
4	3	7	36	69	*	5	6	8.5	15	27.5	35	36	45	1.25	3	4.5	10	13.5	22	*	*
5	2	12	33	72	*	4	8	7.5	12	25.5	34	34	50	2.5	3	5	12	17	24.5	*	*
6	1	6	34	75	*	3	7	8	13	26	33	39	43	1.5	3.5	4.5	10	15.5	25	*	*
7	1	12	35	50	*	3	7	8	12	25	35	*	*	1.5	3.5	4.5	12.5	16	26	*	*
8	2	5	24	*	*	5	8	8	15	27	*	*	*	2	3	4.5	8	15	*	*	*
9	1	7	35	*	*	5	6	8.5	15	25.5	*	*	*	1.5	2.5	5	10	19.5	*	*	*
10	1	8	35	*	*	4	6	7	15	25.5	*	*	*	2	3.5	5	13	*	*	*	*
11			42						12								15				
12			37						10								14				
13			20						10								16				
14			36						11								12				
15			34						10								15				
16			35						10								12				
17			43						10.5								14				
18			37						12								12				
19			48						10.5								13				
20			50						11								12				
Mean	1.5	7.6	34.8	66.6	0	3.9	6.7	7.9	12.2	25.7	34.3	36.7	44.7	1.73	3.2	4.75	12.1	16.5	24.6	39.7	42.3
Std Dev	0.75	2.55	7.84	15.5	0	0.88	1.06	0.57	1.9	1.01	1.11	1.75	2.73	0.38	0.35	0.26	2.17	2.3	2.04	1.53	2.52
Std Error	0.24	0.81	1.75	5.87	0	0.28	0.34	0.18	0.43	0.32	0.42	0.71	1.12	0.12	0.11	0.08	0.48	0.77	0.77	0.88	1.45
*** Caterpillar succumbed to the dose**																					

Table 8. Mouse *(Mus musculus)* Sodium Azide Dose Response Data.

Mouse #	Righting Recovery Time (min)				
	Sodium azide (mg/kg)				
	30	28	27.5	27	26
1	*	30	30.5	30.5	20
2	*	*	60	52	20.5
3	*	26	45	*	20
4	*	*		45	10
5	*	25			13.5
6	*	*			11
7	*	*			*
8		*			10
9		16			43
10		79			30
11					20
12					16
13					21
14					17
15					
16					
17					
18					
19					
20					
Mean	0	35.2	45.167	42.5	19.385
Std Dev	0	25.014	14.751	10.966	9.0073
Std Error	0	11.187	8.5163	6.3311	2.4982
*** Mouse succumbed to the dose**					

Table 9. Caterpillar *(Galleria mellonella)* Antidote Data

Larva #	Righting Recovery Time (min)								
	Sodium azide (8 mg/kg)			Sodium cyanide (7.5 mg/kg)			Sodium hydrosulfide (72 mg/kg)		
	Control	$NaNO_2$ (5mg/kg)	CoN_4[11.3.1] (8 mg/kg)	Control	$NaNO_2$ (5mg/kg)	CoN_4[11.3.1] (8 mg/kg)	Control	$NaNO_2$ (5mg/kg)	CoN_4[11.3.1] (8 mg/kg)
1	10	5	5	9	6	4	18.5	16.5	12.5
2	11	5	3.5	11	6	7	31.5	14.5	9.5
3	10	5.5	6	11	12	4	31	20	14
4	12	7	4	9.5	7.5	7	36	12	11.5
5	11	4	3	12.5	11	7.5	33	20	11
6	12	5.5	4	10	5	7	34	20.5	10.5
7	15	8	5	10.5	6	4	35	16	6.5
8	10	3	5	9	8	5.5	24	23	14
9	10	4	6	10	10	7	35.5	21.5	6
10	7.5	2.5	4.5	8	9	5.5	35	20	8.5
11	7	5.5	6	7	4	5	42	23	13
12	10	5.5	5.5	12	4	6	37	24	9
13	11	6	4.5	8	9	7	20	25	12
14	7	6	4.5	8	5	6	36	20	14
15	11.5	5	3.5	14	6	8	34	20	12.5
16	11.5	7.5	3	12	10	6.5	34	16	12
17	9	5	5	10	7	7.5	28	12	16
18	10	5.5	5	7	14	5.5	34	24	24
19	5	4.5	5.5	16	6.5	6	60	24	13
20	9	2	3.5	10	4	5.5	19	23	12
21	7			10			23	26	
22	8			9.5			24	16.5	
23	10			16			25	20	
24	8			8			30	10	
25	9			14			30	17	
26	8			15			38	16	
27	12			16			41		
28	14			7			37		
29	9			16			42		
30	7			11			34		
31	8			15					
32	7			12					
33	10			13					
34	13			11					
35	11			12					
36	8			11					
37	11			14					
38	10			7.5					
39	12			10.5					
40	8			11					
41	8			10					
42	10			10.5					
43	8			12					
44	9			9					
45	9			8					
46	9			9.5					
47	10			15					
48	10			8.5					
49	8			8.5					
50	15			9					
51	7			11.5					
52	8			12					
53	8			13					
54	15			13					
55	9.5			10					
56	10			15					

Table 9 Continued

57	9			13					
58	10			8.5					
59	7			9.5					
60	13			10.5					
61				10					
Mean	9.7000	5.1000	4.6000	10.984	7.5000	6.0750	32.717	19.250	12.075
Std Dev	2.1613	1.5183	0.96791	2.4949	2.8238	1.2061	8.2990	4.3064	3.7811
Std Error	0.27902	0.33950	0.21643	0.31944	0.63141	0.26969	1.5152	0.84455	0.84548

5.0 Conclusions

Improving toxicant-specific antidotes is even more important in today's world where people are becoming increasingly concerned about the numerous chemicals agents that are "thought-to-be-safe" making news. Some examples include, the weed killer Roundup™ (glyphosate),[186] beauty product additive, triclosan,[187, 188] and about twelve ingredients in sunscreens[189] that are harming humans and the environment. Consumers are approaching the government to mandate better screening of dangerous products prior to their release into the community where the chemicals can impact public health.[4, 82, 190, 191] Additionally, the release of toxicants into the public domain, either accidentally or through nefarious means, necessitates the need for the development of antidotes for protection from poisonings. Finding a reliable, practical organism that can be used to screen ahead of testing in rodents and other mammals, cutting down the number of animals used, and thereby simplifying assessments is a very worthwhile, but considerable task. Ideally, the organism must be inexpensive, fairly robust, not as complex as a mouse, yet have a more developed physiology than a cell model for simple, preliminary, *in vivo* toxicity studies. There are a number of non-mammalian organisms that fit some of these criteria.

Cells, mice, and *G. mellonella* larvae are the primary systems used by our group for assessing cytochrome *c* oxidase inhibitors and putative antidotes. Bearing in mind Russell and Burch 's Three R's, (replacement, reduction and refinement[74]), all of our models mimicked some aspects of human poisoning and/or treatment, and each model introduced advantages and disadvantages. The advantages of cell and mouse studies (Section 1.4) have been widely documented in many peer reviewed journals. Unfortunately, for the purpose of our assessments, cell and mouse studies take considerable time and were quite costly. BPAEC needed to be

purchased, cultured, treated and maintained in precise conditions. When using sulfide in the cell model, it was difficult to control the loss of H_2S to the environment even with Parafilm™ covering the wells, thereby affecting the reproducibility of the toxicant dose taken up by the cell. Like the cells, there are disadvantages to animal studies; first, we are limited to the minimal sample size necessary to achieve significance for each condition in our preliminary study due to the expense involved with purchasing, housing and care of the mice. The most important drawback to animal studies is that the mice require regulatory oversight and protocols must be carefully followed, limiting experimental flexibility.

While these are not the only models used for toxicological screening, others also offer drawbacks, for instance, zebrafish (*Danio rerio*) and fruit flies (*Drosophila melanogaster*). Typically, zebrafish embryos are placed into the wells of plates and measured aliquots of reagent solutions are added to the media, as in cultured cells, the dosage is merely an estimation. The embryos are very small and a microscope must be used to visualize changes. Prior to beginning any experiments, unhealthy embryos must be removed from the sample, emerging problems are sometimes not clearly apparent. Reproducibility of dose between individuals is a particular problem with fruit flies. *Drosophila* larvae are so small it is difficult to accurately inject them, even with experience, and very time consuming. The alternate approach of feeding the larvae with toxin also results in significant variation in the individual doses. This is the same issue as quantifying the dose that was broached in the cell studies with sulfide and cyanide toxicity (Section 2). Zebrafish, fruit flies and mammalian cells are just a few of the alternative models that are currently utilized in preliminary toxicological investigations and are worthy alternatives to the more expensive mouse model but all demonstrate issues with the final concentration of the dose taken up into the system.

In addition to being useful organisms for bacteriological studies, our non-mammalian, invertebrate model demonstrated many advantages in toxicological assessments. *G. mellonella* allowed us to by-pass many of the burdens we faced with the cells and mice used in our laboratory. They require no regulatory oversight, after a 48 hour acclimation they are ready to use, and a hundred or more larvae can be easily and accurately injected in a day at very minimal cost (500 larvae for $14, plus shipping), increasing the statistical significance of the study. They are delivered in a bait container filled with wood shavings which was placed in a box with air holes allowing the larvae a dry, dark area to acclimate; since they are in their last instar, they require no food or water. *G. mellonella* breathe air, though they have no lungs they take in air through the spiracles and diffuse the oxygen through a vast tracheole system that delivers oxygen to each cell, and removes CO_2. Using the larvae allowed us to observe the toxicants without significant loss of the gasses to the air, as seen in the cells. *Galleria mellonella* are not only commercially available and a robust and economical model, that can be easily and accurately injected, they can also be used at or near human physiological temperatures. We were able to assure an accurate dose in a similar fashion to the ip injection of the mouse by injecting into the hemocoel (ih injection) of the *G. mellonella* larvae. The larvae are a whole organism and exhibit behaviors, such as the "knockdown" response that was used to quantify the toxicity and produce dose response relationships, as well as demonstrate amelioration of the toxicants by the antidotes. Respirometry data for *G. mellonella* generated for this study was easily obtained from crushing the larvae and using the isolated mitochondrial homogenate proving that cytochrome *c* oxidase was inhibited by each of the toxicants.

In the past, many procedures for investigating cytochrome *c* oxidase in our laboratory have involved isolation from sources such as beef heart. This is a complicated preparation that is both

labor and time intensive. The procedure developed for isolating the mitochondria from the larvae was considerably easier and less time consuming than the procedure for isolating mitochondria from beef heart. Cytochrome *c* oxidase turnover in the *G. mellonella* mitochondrial homogenate exhibited the same inhibition constant (K_i) for azide that Petersen *et al* found (22 ± 4 μM) for azide in beef heart cytochrome *c* oxidase. Though the K_i for cyanide (4 ± 1 μM) was 20 times larger than the 0.2 μM reported for beef enzyme,[31] insects tend to have higher levels of rhodanese and β-cyanoalanine synthase, perhaps, making them more resistant to cyanide. Although I could not find any data on the *G. mellonella* specifically, β-cyanoalanine synthase activity has been detected in gut protein extracts, and rhodanese is present in detectible quantities in both larvae and adults, of multiple Lepidoptera species. Also, sulfide was not able to be tested in the turnover analysis because it is such a strong reductant, it directly reduced ferrocytochrome *c* back to ferricytochrome *c* interfering with the assay. Using the Oroboros O2k high- resolution respirometer we were able to run assays on the *G. mellonella* mitochondrial homogenate confirming the toxicants were inhibiting cytochrome *c* oxidase in real time. Sulfide was a problem in many aspects of our studies with the cells, including respirometry. The inability to add enough sulfide (millimolar concentrations) to the cells in the chamber to see inhibition without interacting with the electrode was a complication. However, in the larvae mitochondrial homogenate we were able to see inhibition at micromolar concentrations (Figure 14). In the past we have employed the metabolic indicator dye AlamarBlue, however, we were unable to use the indicator dye to assess viability in cells poisoned with sulfide as it reduced the indicator dye immediately. This was also the case with the lactate dehydrogenase assay, the indicator dye used was reduced by the sulfide. The wax worms circumvented issue with the indicator dyes and offered another example of the advantages of this invertebrate in testing the effectiveness of antidotes in a whole organism. Again, there are obvious

differences between mice, cells and larvae such as blood and biological structures (i.e. lungs, liver, and brain) that still facilitate a comparison be drawn between at least a couple of models to glean a satisfactory understanding of any exposure.

The usefulness of this particular invertebrate system as a model for prescreening was that we were able to confirm that all three of the toxicants inhibited cytochrome *c* oxidase, and both sodium nitrite and the Busch compound were antidotal to all three toxicants in the wax worms. It was exciting that the sodium nitrite worked so well in the *G. mellonella* with azide, as this was previously not the case with the 16-20 week old Swiss-Webster mice that were originally tested. There is presently no available antidote for azide poisoning and the very limited literature suggests that sodium nitrite might be of no therapeutic benefit. Because of the *G. mellonella* data, we went back and examined the sodium nitrite in both 6-8 week old male juvenile and 12 week old adult, mice. We discovered that the sodium nitrite was antidotal in both the larvae and the juvenile mice, but not in the adult mice. This study illustrated that effects can easily be found in the *G. mellonella* that may initially be missed in the mice.

It was previously thought that the antidotal effect of nitrite toward cyanide was through the production of methemoglobin (Hb^+), perhaps by secondary NO: $HbO_2 + NO \rightarrow Hb^+ + NO_3^-$. The methemoglobin produced then binds the toxicant of interest, i.e., cyanide (e.g. $Hb^+ + CN^- \rightarrow HbCN$). As already stated previously, (section 2; page 48) our group has hypothesized [61, 62] that NO has been shown to be an efficient antagonist of cyanide by displacing CN^- from the heme a_3/Cu_B binding site of cytochrome *c* oxidase, restoring its oxygen binding capability thus restoring oxygen turnover.[59, 60] We suggest that nitrite acts as a NO donor but not a methemoglobin former in the antidotal mechanism antagonizing cyanide toxicity. Again, our current results support this

hypothesis, since the *G. mellonella* larvae do not have any hemoglobin, and yet sodium nitrite was antidotal in the larvae (Figure 16).

So far *G. mellonella* have been shown to be a useful organism for preliminary toxicological screening, mimicking vertebrate toxicology in certain respects. Our group began a study looking at potential phosphine antidotes.[97] We implemented the *G. mellonella* in a preliminary inhalation study examining if Au (I) complexes could be beneficial phosphine-scavenging compounds. Three Au (I) compounds were studied and auro-sodium bis-thiosulfate (AuTS) proved to be the best therapeutic agent in the *G. mellonella* and was subsequently tested in mice. Mice given AuTS (50 mg/kg) 1 min after a similar phosphine exposure, demonstrated a modest improvement in behavioral testing over untreated phosphine exposed mice. Similar to azide, we were able to test antidotal effects in the larvae prior to beginning an animal study decreasing the amount of mice needed, saving time and money.

The importance of toxicological testing is vital, and no doubt, mice will remain the gold standard for toxicological investigations because of the genetic, biological, physiological and behavioral characteristics they share with humans. However, the data shared here establishes that *G. mellonella* are useful for pre-screening mitochondrial toxins and a solid tool in the toxicological model arsenal.

Appendix A Antagonism of Acute Sulfide Poisoning in Mice by Nitrite Anion Without Methemoglobinemia

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Abstract

There are currently no FDA-approved antidotes for H_2S/sulfide intoxication. Sodium nitrite, if given prophylactically to Swiss Webster mice, was shown to be highly protective against the acute toxic effects of sodium hydrosulfide (~LD_{40} dose) with both agents administered by intraperitoneal injections. However, sodium nitrite administered after the toxicant dose did not detectably ameliorate sulfide toxicity in this fast-delivery, single-shot, experimental paradigm. Nitrite anion was shown to rapidly produce NO in the bloodstream as judged by the appearance of EPR signals attributable to nitrosylhemoglobin and methemoglobin, together amounting to less than 5% of the total hemoglobin present. Sulfide-intoxicated mice were not helped by the supplemental administration of 100% oxygen, nor were there any detrimental effects. Compared to cyanide-intoxicated mice, animals surviving sulfide intoxication exhibited very short knockdown times (if any) and full recovery was extremely fast (~15 min) irrespective of whether or not sodium nitrite was administered. Behavioral experiments testing the ability of mice to maintain balance on a rotating cylinder showed no motor impairment up to 24 hr post-sulfide exposure. It is argued that antagonism of sulfide inhibition of cytochrome *c* oxidase by NO is the crucial antidotal activity of nitrite rather than formation of methemoglobin.

Introduction

Methods for generating hydrogen sulfide gas (H_2S) from household chemical products obtainable at multiple retail outlets have been publicized through the Internet and suicide by H_2S inhalation is an emerging trend in some countries.[192, 193] Significantly, it appears that neighbors not in the immediate vicinity of the release site, or emergency responders, were sometimes affected/injured[194, 195] leading to a growing realization that H_2S might find application as a weapon.[192] Additionally, there are approximately 10^2 known commercial sources of H_2S, resulting in thousands of occupational exposures per year in the US alone, including individuals engaged in paper pulping, tanning, vulcanizing, management of animal waste, sewer maintenance, heavy water production[196, 197] and, especially, natural gas mining.[198] Preventable deaths of inadequately protected would-be rescuers, both coworkers and emergency personnel, have been reported. [127] It is, therefore, of concern that no FDA-approved antidote, or reliable protocol, for treating acute H_2S intoxication is currently available.[96] Emergency medicine bulletins/pamphlets issued by several international, federal and state authorities [146, 199-204] suggest the use of cyanide antidote kits containing nitrite-thiosulfate, or cobalamin, but the basic science that would justify this approach is lacking. Moreover, there are conflicting anecdotal case reports attesting to both the success and failure of cyanide therapeutics administered in situations where H_2S was known or suspected to be the toxic agent.[205] More seriously, we have recently shown that the production of hydrosulfide (HS^-) in the blood from thiosulfate administered as a cyanide antidote is measurably toxic.[61] Therefore, the suggested use of the nitrite-thiosulfate combination as a therapy for H_2S poisoning is alarming. Certainly the toxicology of H_2S shares features in common with that of cyanide; for instance, both toxins are highly efficient disruptors of mitochondrial electron-transport chain

function [95, 99, 174] with approximately identical inhibition constants (K_i) for cytochrome *c* oxidase.[61, 98] It follows that in developing potential therapies for treating acute H_2S intoxication, initial efforts should be directed toward overcoming inhibition of cytochrome *c* oxidase and the associated rapid cardiopulmonary collapse.[96]

The first pK_a of H_2S is 6.9-7.0 (37-25 ºC), the second pK_a being inaccessible in water [206, 207] which results in aqueous solutions of approximately 30-25% H_2S and 70-75% HS^- at pH 7.4, irrespective of whether initially introduced as the molecular gas, or as a salt (*e.g.* NaHS). In keeping with what seems to be a common convention, we refer to this mixture at physiological pH as "sulfide" (and do not mean to imply S^{2-} at any time herein). Haouzi *et al*[102] have recently shown that, at sub-lethal levels, intravenously administered aqueous sulfide becomes converted to other forms in the bloodstream of sheep and rats within 1 minute of infusion. This seems to be incompatible with the findings of Truong *et al*[208] who reported that cobalamin given intraperitoneally greatly increases the survival rate of sulfide toxicity in mice when administered 2 min after the toxicant. Curiously, these latter authors report death following sulfide intoxication in a zero-to-24-hr window with no mention of the rapid effects that we describe below. To further confound matters, suspected human victims of H_2S inhalation reaching the clinic have been reported to succumb to the poisoning hours after the exposure[95, 106] possibly indicating additional slower mechanisms of toxicity subsidiary to cytochrome *c* oxidase inhibition. In this study, we attempt to resolve these puzzling observations with regard to the acute toxicity of sulfide.

Sodium nitrite has periodically been mentioned as a possible antidote for H_2S for over 30 years,[95, 209-211] based upon the assumption that detoxification would result from nitrite-induced generation of methemoglobin (metHb) followed by binding of sulfide to form sulfidomethemoglobin (metHbSH).[96] However, we propose this widely held mechanistic belief to

be erroneous. The matter is a critical issue to be overcome, because concerns that increased metHb levels (methemoglobinemia) would further challenge the oxygen utilization capabilities of already sulfide-compromised individuals have almost certainly hindered the rational investigation of nitrite as a possible frontline antidote. In the related case of acute cyanide intoxication, we have shown that there need be no significant methemoglobinemia following administration of sodium nitrite (< 2% metHb[61]) as the principle antidotal action is to generate nitric oxide (NO) that ameliorates inhibition of cytochrome *c* oxidase function.[59, 60] Herein, we have examined the antidotal activity of sodium nitrite towards sulfide toxicity and the potential ameliorating effects of supplemental oxygen, employing a combination of behavioral assessments on mice and spectroscopic (EPR) measurements on drawn blood. A variety of sequelae, secondary to sulfide poisoning, have been anecdotally reported in humans, including neurological defects. Accordingly, we have performed some neuromuscular assessments on mice following recovery from sulfide intoxication to determine if any improved survivability observed with nitrite administration might also be associated with undesirable neurological consequences.

Experimental Methods

Chemicals. All reagents were ACS grade, or better, used without further purification and unless stated to the contrary, were purchased from Fisher or Sigma-Aldrich. Argon, carbon dioxide, nitric oxide, nitrogen and oxygen gases were purchased from Matheson Incorporated and with the exception of nitric oxide (see Enzyme Preparation and Cell Culture) used without further purification. Sodium hydrosulfide or sodium cyanide solutions were prepared in septa-sealed vials with minimized headspaces immediately prior to use and volumetric transfers made with gas-tight

syringes. Sodium hydrosulfide was obtained as NaHS•xH$_2$O (Sigma) where x was determined to be 2.5 essentially following procedures described by Koltoff *et al.*[119] Briefly, concentrations of HS^- were determined by quantitative reaction with excess iodine ($2HS^- + I_2 \rightarrow S + 2H^+ + 2I^-$) followed by titration of the liberated $2I^-$ (as $I^- + I_3^-$) with silver nitrate (precipitating AgI + AgI_3) using an Ag^+ sensitive ion-selective potentiometric electrode (Accumet Silver/Sulfide Combination ISE 13-620-551) to detect the endpoint.

Animals, Exposure and Blood Collection. All animal procedures were approved by the University of Pittsburgh Institutional Animal Care and Use Committee (Protocol Number 13092637). Veterinary care was provided by the Division of Laboratory Animal Research of the University of Pittsburgh. Male Swiss Webster (CFW) mice weighing 40-50 g were purchased from Taconic, Hudson, NY. Adult animals were 16-18 weeks old and were housed four per cage. The mice were allowed access to food and water *ad lib*. Experiments commenced after the animals were allowed to adapt to their new environment for one week. A series of experiments testing the efficacy of sodium nitrite as a sulfide antidote were performed. All solutions were prepared by dilutions into sterilized saline in septa-sealed vials using gas-tight syringes for all transfers and administered through ~0.1 mL intraperitoneal (*ip*) injections. In general, a group of at least 6 mice were tested for each experimental point. At the end of exposures and tests, mice were euthanized with CO_2. Juvenile male Swiss-Webster mice 6-8 weeks old, 25 – 35 g, supplied by Taconic, were treated in the same fashion.

For collection of blood samples, mice were first euthanized in an atmosphere of carbon dioxide and blood drawn by cardiac puncture into a 1-mL syringe containing 25 μL of 500 mM EDTA. The blood was expelled into the bottom of a quartz EPR tube containing an argon atmosphere, then frozen by immersion in liquid nitrogen. This entire process could comfortably

be completed in 2 min. The cryogenically preserved sample was stored in liquid nitrogen and subsequently transferred to the EPR spectrometer without ever having been thawed. Samples of heart tissue were also collected. Euthanized animals were perfused with 5ml of PBS by cardiac puncture, then the hearts were removed and immediately frozen in liquid nitrogen. After storage at -80°C, individual hearts were thawed, homogenized in 1 mL PBS and a 200-μL aliquot of this homogenate was introduce into an EPR tube and frozen in liquid nitrogen before subsequent transfer to the spectrometer.

Righting-Recovery Testing. Lengths of time required for recovery of righting ability in mice were determined based on some of the recommendations of Crankshaw *et al*[212] regarding their measurement of the righting reflex, but adopting a simpler procedure. Following *ip* administration of toxicant (±$NaNO_2$) mice were placed in a transparent but dark green-colored plastic tube (Kaytee CritterTrail, available from pet stores) in a supine position. The time duration from the toxicant injection until the mouse flipped from the supine to a prone position in the plastic tube was taken as the endpoint.

RotaRod Testing. To assess motor skill learning and recovery following intoxication, we used the accelerating RotaRod™ (Coulbourn Instruments, Whitehall, PA) – a rotating cylindrical apparatus (4 to 40 r.p.m.) on which the mice were placed. The animals were evaluated for 3 trials per time point on three consecutive days, with a resting time of 30 s between each trial. An individual trial was considered ended when the mouse either fell off or had remained on the rotating cylinder for 60 s. Latency to fall, and highest speed reached were recorded for each trial. Mice were trained for 8 trials on the first day by placing them on an accelerating RotaRod for 60 s, during which time the rotation rate was varied linearly from 4 to 11.2 r.p.m. On the day of the toxicity experiments (day 2), animals were tested for a single set of three trials, with the same

parameters as during training to establish a baseline performance, before injection. Trained animals were tested at fifteen min intervals after toxicant administration for 2 hrs and an additional time 24hrs after injection, by placing them on an accelerating RotaRod for 60 s, accelerating from 4 to 22 r.p.m. Motor performance was determined to be the highest rotation speed reached before the animal fell off the apparatus, determined from the mean r.p.m. in three trials for each mouse at each experimental time point. For comparison between groups the mean performance in pre-injection testing for a group was used to normalize all the other experimental points of that group.

Enzyme Preparation and Cell Culture. Bovine pulmonary artery endothelial cells (BPAEC) were purchased from Lonza and used at passages 4-8. Cells were grown in Opti-Mem media supplemented with 10% fetal bovine serum, 5 mM glutamate, penicillin and streptomycin under 5% CO_2. Cytochrome *c* oxidase was prepared as previously described[59] from intact bovine heart mitochondria using a modified Harzell-Beinert procedure (without the preparation of Keilin-Hartree particles). The enzyme was determined to be spectroscopically pure if the 444 nm to 424 nm absorbance ratio for the reduced enzyme was 2.2 or higher. Derivatives were prepared in 50 mM potassium phosphate, 1 mM in sodium EDTA and 0.1% in lauryl maltoside, pH 7.4-7.8, to concentrations of 10-80 μM (in enzyme). Enzyme concentrations were determined as total heme *a* using the differential extinction coefficient of $\Delta\varepsilon_{604} = 12\ mM^{-1}cm^{-1}$ for the reduced minus oxidized electronic absorption spectra.[213] Concentrations throughout are given on a per enzyme concentration basis (NOT per [heme *a*]). Ferrocytochrome *c*:O_2 oxidoreductase activity was determined spectrophotometrically employing the high ionic strength method of Sinjorgo *et al.*[214] Electronic absorption spectra were measured and photometric determinations made using Shimadzu UV-1650PC and UV-2501PC spectrophotometers. Nitric oxide (for reactions with cytochrome *c* oxidase) was scrubbed with water and KOH pellets prior to use, bubbled through anaerobic buffer (prepared by bubbling argon through the solution) and added to enzyme samples volumetrically with gas-tight syringes. Buffered solutions never exhibited any significant change of pH (*i.e.* < 0.05 pH units) following NO additions.

Electron Paramagnetic Resonance (EPR). X-band (9 GHz) EPR spectra were recorded on a Bruker ESP 300 spectrometer equipped with an Oxford Instruments ESR 910 flow cryostat for ultra-low-temperature measurements. Access to this instrument and the software (SpinCount) used to analyze the EPR spectra were provided by Professor Michael Hendrich, Carnegie Mellon University. Quantification of EPR signals was performed by simulating the spectra using known (or determined) parameters for each sample in question. Simulations employed a least-squares fitting method to match the lineshape and signal intensity of a selected spectrum. Simulated spectra were expressed in terms of an absolute intensity scale, which could then be related to sample concentration through comparison with a Cu^{II}(EDTA) spin standard of known concentration.

Data Analysis. Statistical data was analyzed using Graph Pad Prism 6 software by t-test. A *p*-value of ≤ 0.05 was considered statistically significant.

Results

Comparison of Sulfide and Cyanide Toxicities. It is frequently noted that there are parallels between the acute toxicities of H_2S versus HCN and it will be instructive to explore this comparison further (*e.g.* Table 10). In our procedures, where the toxicants are given to mice as *ip* injections of sodium salts in saline solutions at approximately LD_{50} doses, those animals that succumb typically do so within 2-4 min, in keeping with the well-documented rapid action of these poisons. In the particular case of sulfide, we have to date observed deaths in excess of 200 animals – more than 98% of these occurring within 5 min of the toxicant dose, 2 individuals died in the 5-10 min period, and only 1 individual in the 10 min to 24 hr window. Consequently, we are very

surprised by the methodology and observations of Truong *et al*[208] who report returning mice to their cages after giving similar *ip* doses of aqueous NaHS to record deaths 24 hr later, with no mention of any fatalities in the first few minutes following administration of the toxicant. The antidotal regimen adopted by these same authors involved *ip* administration of hydroxycobalamin solutions at 2 min following the toxicant dose and not at any later times in the 24-hr experimental window before deaths were recorded. A time delay of only 2 min between the toxicant and antidote doses for efficacy suggests to us that these authors were probably dealing with the same kind of acute response as we now report (*i.e.* the majority of deaths within a few minutes of the toxicant dose) but for some reason left this unclear.

Table 10. Summary of HCN and H_2S Toxicological Observations in Mice.

	NaCN/HCN[39] (~99% HCN at pH 7.4, 37°C)	**NaHS/H_2S** (~25% H_2S at pH 7.4, 37°C)
Strain/Sex	Swiss-Webster/Males	Swiss-Webster/Males
Supplier	Charles River	Taconic
Age	17 weeks	17 weeks
Median weight	40 g	43 g
Mean weight	39.8±6.6* g	43.0±4.0* g
Toxicant dose	5.0 mg/kg (LD_{33} – LD_{50}) ip	16 mg/kg (LD_{40}) ip
Total knockdowns	100%	71% (20 of 28)
Knockdown duration	30.5±8* minutes	3.5±1.4* minutes
Survival with knockdown	50 – 67%	40% (8 of 20)
Mode of death	Respiratory paralysis	Respiratory paralysis
Time to full recovery	< 2 hours	~15 minutes

*The quoted uncertainties are standard deviations

A period of unconsciousness, or "knockdown," is a common acute symptom of exposure to both sulfide [96] and cyanide.[148] With a simplified modification of a procedure developed by Crankshaw *et al*[212] we have previously assessed sub-lethal cyanide intoxication in mice and its antagonism using the observed duration of knockdown to indicate extent of incapacitation and

recovery [61, 62, 177]. Injection of mice with 0.1 mmol/kg (5 mg/kg, *ip*) NaCN in saline results in loss of consciousness, with clear indication of the onset of narcosis (animals stagger or are motionless) beginning at around 1 min following administration of the toxicant. Shortly thereafter, the animals may be placed on their backs (supine position) and observed until they regain consciousness, at which time they turn themselves to an upright (prone) position. The observed "righting-recovery time" generally lasts around 30 min with about 60% survival in the case of cyanide intoxication at the stated dose. The method has proven suitable for demonstrating the efficacy of putative antidotes given before, or up to 20 min after the cyanide.[61] Unfortunately, however, the same method proved impractical for use with respect to sulfide as the toxicant. Mice that experienced sulfide-induced knockdowns were more likely to die than to survive and, in fact, only about one quarter of all surviving animals experienced knockdown (Table 10). Furthermore, using 0.29 mmol/kg (16 mg/kg, *ip*) NaHS in saline we found that a 16 mg/kg dose (57% survival) caused a knock-down duration of only 3.5 ±1.4 min (n = 28). Increasing the dose incrementally did not lead to any significant lengthening of knockdown times observed and at 20 mg/kg all the mice injected (n = 6) died within 5 min. That is, in the case of sulfide intoxication, knockdown/righting-recovery was the atypical response, variable and short – necessitating that an unreasonably large number of animals would have been needed to demonstrate any beneficial effects of putative antidotes by this method. Therefore, we reluctantly investigated the efficacy of $NaNO_2$ toward antagonism of NaHS toxicity using death or survival (observed at 15 min and 24 hr) as the endpoint.

In addition to the clear differences in frequency of response and knockdown duration noted above with sulfide- and cyanide-dosed mice, the recovery times were also found to be quite distinct. Following a toxicant dose (~LD_{40}) with no antidote given, sulfide-intoxicated mice took

about 15 min to recover essentially normal behavior, whereas almost 2 hr had elapsed before cyanide intoxicated mice exhibited similar recovery (Table 10) and RotaRod experiments described below). It appears from these observations that the part(s) of the central nervous system dealing with consciousness is (are) significantly more deeply affected by sub-lethal cyanide intoxication than is the case with sulfide. The experimentally-established LD_{40} doses of NaHS and NaCN, 16 mg/kg and 5.0 mg/kg, respectively, represent a molar ratio of ~3:1 (total sulfide: total cyanide). As the relevant pK_as are 6.9 and 9.2 respectively,[206, 207] this implies $[H_2S] \cong [HCN]$ circulating in the bloodstreams at pH 7.4; that is, the toxicant species expected to be most readily membrane-permeable were administered at the same level. This makes sense, given that we can anticipate the rate of diffusion of the toxicants from the bloodstream to depend upon mass action and the principle target molecule, cytochrome *c* oxidase, is similarly inhibited by sulfide and cyanide.[98] In summary, while the pattern of sub-lethal narcosis clearly differs between the two toxicants, our present data are fully consistent with the notion that the two mechanisms of lethality are essentially analogous, with pulmonary function principally affected.

Potential Antidotes to Sulfide Poisoning. Doubts and conflicting reports have continued to persist regarding the usefulness of sodium nitrite as a sulfide antidote for almost 40 years.[96, 209, 215-217] Based on our recent experience with regard to cyanide and nitrite, we suspected that some of the confusion in the literature might stem from an incorrect understanding of how the antidotal action of nitrite might best be mechanistically explained. The general consensus is that cyanide exerts its acute toxicity primarily on the central nervous system through inhibition of cytochrome *c* oxidase, with death primarily the result of interruption to the pulmonary nervous supply.[130, 148] We have shown that nitric oxide (NO) relieves the inhibition of cytochrome *c* oxidase by cyanide,[59, 60] leading to the suggestion that the antidotal action of nitrite towards cyanide poisoning

involves the anion functioning as an NO donor to alleviate electron-transport chain inhibition by cyanide.[61, 62] Consequently, we set out to investigate whether sodium nitrite might also be antidotal toward sulfide poisoning through a mechanism in which the anion acts as an NO donor. We began with a prophylactic paradigm (Figure 18A). Previously, 12 mg/kg $NaNO_2$ was established as the optimal antidotal dose in the case of acute cyanide toxicity.[61, 62] This same dose, however, resulted in only a modest improvement in survival of 79% (Figure 18B) for sulfide toxicity. When 24 mg/kg $NaNO_2$ was administered 5 min before a 16 mg/kg NaHS dose, survival was significantly increased to 93% (Figure 18B) compared to 57% in the case of controls given no nitrite (Figure 18B). Several groups of authors have suggested that delivery of supplemental oxygen during treatment for sulfide poisoning may be beneficial[218-221] and, indeed, while the supporting evidence is anecdotal, the idea can be rationalized on the basis that the known detoxification pathway of sulfide uses oxygen.[96, 205] We investigated the matter experimentally. Following prophylactic doses of $NaNO_2$, mice were maintained under normoxic conditions for 5 min, then placed in a 100% oxygen environment immediately after injections of NaHS. Control animals were just given NaHS and placed in the 100% oxygen chamber. Exposures to 100% oxygen were discontinued after 15 min or at time of death (< 15 min). There was no detectable improvement in survival of mice provided with the supplemental oxygen following the toxicant dose (Figure 18B) compared to those maintained under normoxic conditions throughout (Figure 18B). While this result could be considered negative, it is, nevertheless, important. Faced with patients in respiratory distress, it is quite normal practice for emergency responders to provide supplemental oxygen if available. Therefore, it is comforting that our data show *no effect* of supplemental oxygen, neither good *nor bad*, suggesting that the protocol should at least do no harm in cases of sulfide intoxication.

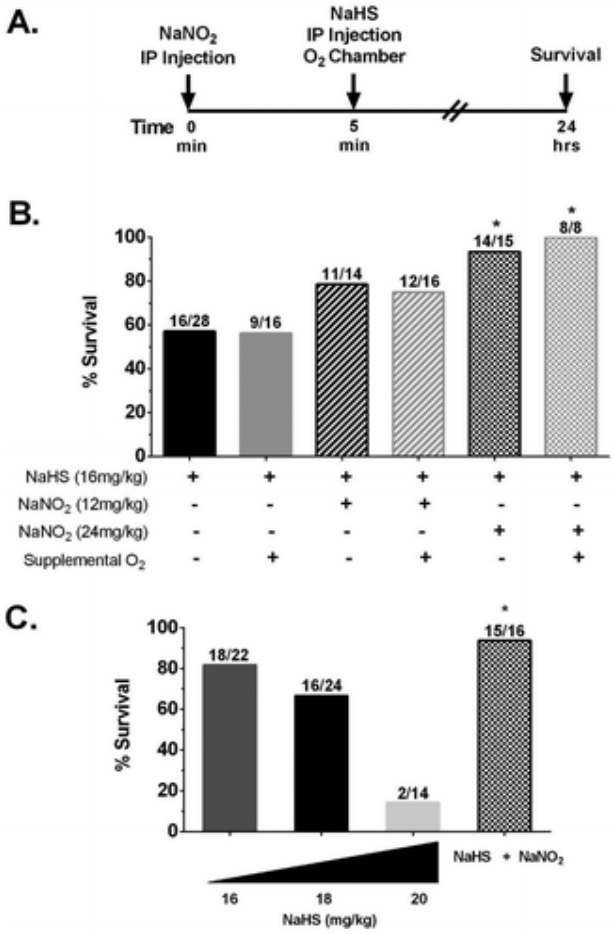

Figure 18. Prophylactically administered $NaNO_2$ ameliorates NaHS toxicity in mature and juvenile mice. **A:** Injection paradigm. **B:** Mice (Swiss-Webster males, 16-18 weeks of age) were given NaHS in saline (16 mg/kg, ip) and times until death recorded. The duration of survival (breathing cessation) was measured from the time of the sulfide injection (t = 0). Survival quotients are shown with surviving mice/total mice written above the bar. $NaNO_2$ (12, or 24 mg/kg, ip) was given 5 min prior to NaHS injection. Supplemental oxygen (100% O_2) was administered for either 15 min, or until death, immediately after NaHS injections (* $p \leq 0.05$ vs NaHS injection alone). **C:** Juvenile mice (Swiss-Webster males, 6-8 weeks old) were injected (ip) with either 16, 18, or 20 mg/kg NaHS and survival recorded as for adults. In addition, 24 mg/kg $NaNO_2$ was given 5 min before 18 mg/kg NaHS (* $p \leq 0.05$ vs 18mg/kg NaHS injection).

Unfortunately, in experiments where $NaNO_2$ (24 mg/kg) was administered 1-2 min *after* the toxicant (NaHS, 16 mg/kg), there was no significant improvement in survival observed (data not shown). While $NaNO_2$ was not beneficial when administered after sulfide doses, we were able to confirm the results of Truong *et al*[208] in which administration of hydroxycobalamin acetate (*ip*) at 2 min after the NaHS dose (*ip*) increased survival to 80% (data not shown). We interpret these

observations to reinforce the idea that cobalamin binds sulfide circulating in the bloodstream, slowing the passage of the toxicant to the tissues in the critical 2-4 min period during which the majority of deaths were observed. Nitrite, on the other hand, does not exert its primary action in the bloodstream (see below) and the NO released must reach inhibited mitochondria within tissues to reverse the effect of the toxicant. As the maximal release of NO occurs about 5 min after ip injection[61], this method of $NaNO_2$ delivery is too slow for post-NaHS use in the current scenario and, ultimately, alternate methods (*e.g.* aerosol inhalation) will probably have to be considered.

Sulfide Toxicity in Juvenile Mice. It is of interest to determine if juveniles are more susceptible to the effects of sulfide. To this end we carried out a series of experiments examining the dose response to sulfide and the efficacy of nitrite as an antidote in 6-8 week old mice. As shown in Figure 18C, juveniles proved slightly more resistant to sulfide, as a dose of 18 mg/kg (Figure 18C) was needed to obtain a similar level of survival compared to adult mice exposed to 16 mg/kg. It is of interest to note that at 2 mg/kg more (20 mg/kg) survival dropped precipitously from 67% to 14%. This kind of steep relationship was also observed in preliminary testing with adults (data not shown). Survival was improved significantly upon the prophylactic administration $NaNO_2$ (Figure 18C) at the same dose (24 mg/kg) as in the adults. Also like the adults, those sub-lethally intoxicated juvenile subjects recovered quickly (~15 min) and all deaths occurred within 5 min of receiving the toxicant dose; none were observed in the 5 min to 24 hr time window.

Recovery of Neuromuscular Function Following Acute Sulfide Intoxication. There is anecdotal evidence in the occupational medicine literature for neurological dysfunction in humans following acute exposures to H_2S.[96, 106] To address this possibility experimentally, we employed an approach based on the RotaRod device – primarily a determinant of neuromuscular coordination, but there is also assessment of muscle strength, with a more limited cognitive

component. The mice were trained 24 hours before the intoxication procedures and baseline peformance was established at 1 hr prior to administration of toxicant. Following toxicant injections, mice were then tested every 15 min, up to 1 hr, and subsequently at 24 hrs (Figure 19A). There was no significant difference in RotaRod performance of mice (Figure 19B) given 16 mg/kg NaHS (●) 24 mg/kg $NaNO_2$ (□) or $NaNO_2$ + NaHS (▲). In general, the animals improve with practice, so the increased performance between 15 min and 45 min post-intoxication should be taken to indicate a learning curve as it has a slope similar to that observed during the training period (Figure 19B). These data show that all the animals have essentially recovered neuromuscular coordination at 15 min after administration of NaHS, irrespective of whether nitrite was given or not. A comparison (Figure 19C) of the RotaRod performace of mice given 16 mg/kg NaHS (●) 5.0 mg/kg NaCN (◆) or $NaNO_2$ + NaCN (□) treated mice showed that the cyanide intoxicated mice had a longer recovery time (~2 hr) compared to sulfide (15 min). Furthermore, while administering nitrite shortened the recovery time, the performance of these nitrite-treated and cyanide-intoxicated mice still clearly lagged behind that of the sulfide-intoxicated animals (Figure 19C). Nevertheless, it is to be noted that, in the case of both toxicants, there was no indication of persistent impairment of neuromuscular coordination or readily apparent cognitive (learning/memory) issues as reported for humans – all the surviving animals rapidly made full recoveries as judged by the RotaRod testing and did not develop any latent problems at 24 hr.

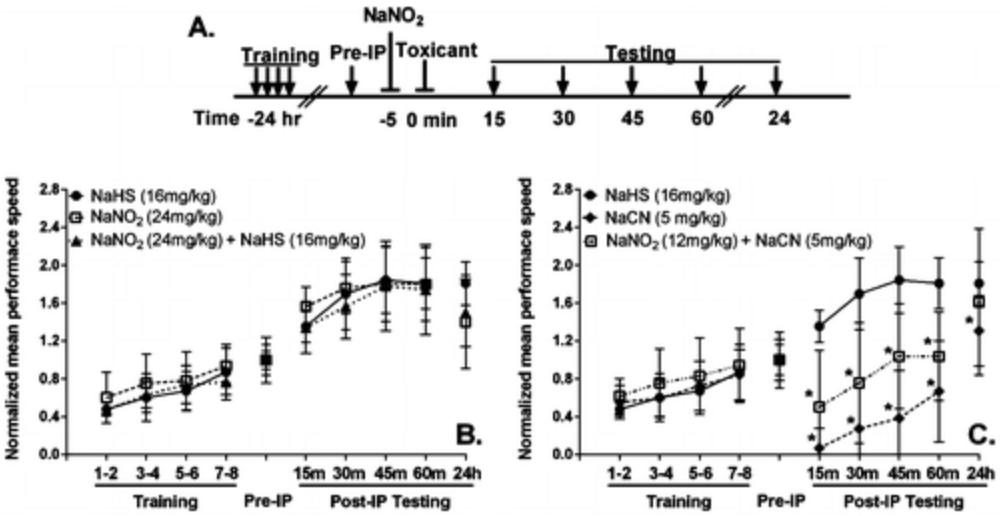

Figure 19. RotaRod^{TM} testing of neuromuscular coordination following NaHS/NaCN/$NaNO_2$ exposures in adult Swiss Webster mice.

A: RotaRod testing paradigm: Arrows indicate RotaRod testing times, lines with bars indicate injection times (all ip). Mice were trained on the RotaRod 24 hr before injection and a baseline performance was obtained 1 hr before injection (Pre-ip). Mice were tested every 15 min after injections for 1 hr to assess recovery. **B:** Comparison of performance for injections of 16 mg/kg NaHS (●), 24 mg/kg, $NaNO_2$ (□) and 24 mg/kg $NaNO_2$ injected 5 min prior to 16 mg/kg NaHS dose (▲). **C:** Comparison of performance for injections of 16 mg/kg NaHS (●), 6.4 mg/kg NaCN (◆), 24 mg/kg NaNO2 injected 5 min prior to 6.4 mg/kg NaCN (□). Numbers of animals (in parentheses) used in each set of experiments: NaHS (6), $NaNO_2$ (8), $NaNO_2$ + NaHS, (8), NaCN (9), $NaNO_2$ + NaCN (10). (* $p \leq 0.05$ vs controls)

Nitrite-Dependent Release of NO in Blood and Heart Muscle. We have previously shown that minority hemoglobin species in blood samples (HbNO, metHb and metHbS) can be quite accurately determined using EPR spectroscopy.[61, 62] The addition of sodium nitrite to mice, followed by euthanasia with CO_2 and sample preparation (see below) led to dose dependent (0-24 mg/kg) EPR signals, identified as HbNO and metHb. In general, the amount of HbNO observed in the blood (a maximum of 0.5 mM, or ~ 6% of the total hemoglobin) was roughly 2-3 times the amount of metHb (0.15 mM, or ~2% of the total hemoglobin). Followed over time, the signal intensities peaked at 10-15 minutes and measurably persisted up to 1 hour after administration of the nitrite dose.[61, 62] The presence of both signals could be construed as evidence for the presence of NO in the blood rather than nitrite (HbO_2 + NO → metHb + nitrate; Hb + NO → HbNO) but as the signals were not present in the spectra of blood samples taken from control animals, they

clearly arose in a nitrite-dependent manner. In the present study, mice were given $NaNO_2$ (12-24 mg/kg in saline, *ip*) then later euthanized in an atmosphere of CO_2 starting at 7 minutes after the nitrite dose. We chose this delay because previously the maximal level of NO-dependent EPR signals were found between 5 and 15 minutes following $NaNO_2$ administration. Within 2 min of starting euthanasia, blood had been withdrawn by cardiac puncture, 250 μL dispensed into an EPR tube and the sample cryogenically preserved by immersion in liquid nitrogen. When required, NaHS (16 mg/kg in saline, *ip*) was administered either alone or 5 min following the nitrite dose (*i.e.* 2 min before commencing euthanasia).

The EPR spectra of control blood samples, from animals given neither sulfide nor nitrite, exhibit only weak signals arising from transferrin at ~1600 gauss (not shown). In samples drawn from mice given $NaNO_2$, both metHb (~1100 gauss) and HbNO (~3400 gauss) EPR signals were readily observed (Figure 20A). The HbNO exhibits a three-line hyperfine due to the interaction of the nuclear spin of the nitrogen atom in NO with the electron spin.[222, 223] Most of the HbNO probably accumulates during euthanasia, as the animal will rapidly become systemically anaerobic before the blood sample can be drawn and cryogenically preserved. Consequently, while the HbNO level contributes to the quantitative estimation of effective NO concentration at time of sacrifice, no other useful information can be deduced from these particular signals. The blood of animals treated with NaHS alone, yielded EPR spectra that were essentially the same as controls, containing only very weak signals and certainly nothing that could be associated with the toxicant dose (Figure 20B). On the other hand, the blood of mice administered sulfide 5 min after the nitrite exhibited EPR spectra that were interesting in a couple of respects. First, the known EPR signal associated with metHbSH (rhombic in nature with features at 2736, 3057 (crossover) and 3715 gauss[61]) was routinely observed (Figure 20C). There are, however, three overlapping sets of EPR

signals present: metHb (~1100 gauss), HbNO (~3400 gauss) and the metHbSH signal. To quantitate these signals we simulated the spectra using the program SpinCount (M. T. Hendrich, Carnegie Mellon University – see Methods) and the resulting signal intensities are presented in Table 11. The concentration of metHbS detected was 0.13 mM, or < 2% of the total hemoglobin, which equates to 0.5 μmol in a mouse of total blood volume < 4 mL (0.13 mM x 4 mL = 0.52 μmol). Now, a 16 mg/kg dose of NaHS in a 45 g mouse equates to 12.9 μmol (16/56 x 40/1,000 = 12.9 μmol). Thus, only 4% of the total sulfide dose given was scavenged by the blood as metHbSH. Interestingly, if nitrite was given to mice 2 min after administration of the sulfide, both reagents provided at the same doses as above, any EPR signals associated with the binding of HS^- to metHb were weaker and in the majority of cases none were detected (*e.g.* Figure 20D) seemingly consistent with the rapid elimination of HS^- from the bloodstream before nitrite can release NO and generate metHb. Examination of samples of juvenile mouse blood drawn from animals subjected to the same nitrite/sulfide treatments as adults revealed no significant differences in the EPR signals observed (not shown).

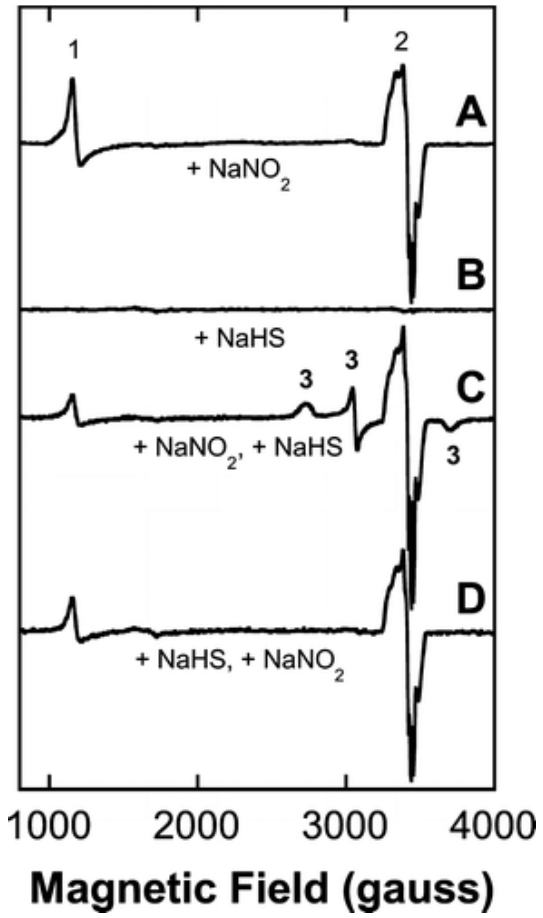

Figure 20. EPR spectra (x-band, 10 K) of whole mouse blood.
A: EPR spectrum of drawn blood following dose of 24 mg/kg $NaNO_2$ administered ip 5-10 min prior to sacrifice showing clear evidence for the generation of nitric oxide. Signal (1) at ~1100 gauss: metHb; signal (2) at ~3300 gauss: HbNO. The combined intensities of the metHb plus HbNO signals represents < 5% of the total heme present in the blood (~9 mM). B: Spectrum of blood following dose of 16 mg/kg NaHS. This dose of NaHS roughly amounts to a maximal concentration of ~2.8 mM in the blood. C: Spectrum of blood following dose of 24 mg/kg of $NaNO_2$ (t = 0) and 16 mg/kg of NaHS at 2 min. The signals at ~2700, 3060 and 3700 gauss all arise from metHbSH (designated signal 3) and represent < 3% (~0.2 mM) of the total Hb (~200 mM). D: Spectrum of blood following NaHS injection 2 min prior to $NaNO_2$. No metHbSH signals were detected.

Table 11. Quantitation of EPR signals (x-band, 10 K) observed in mouse tissue.

	Whole Blood			Minced Heart	
	metHb (μM)	HbNO (μM)	metHbSH (μM)	metMb (μM)	MbNO (μM)
Control	< 5	0	0	< 2	0
NaHS (16mg/kg)	< 5	0	0	< 2	0
$NaNO_2$ (24 mg/kg)	90 (30)	190 (30)	0	15 (3)	50 (5)
$NaNO_2$ + NaHS* (*5 min delay)	60 (30)	190 (40)	130 (30)	7 (2)	54 (3)

EPR samples were made 7 min after injections (ip) to the mice (Swiss-Webster, 16-18 week old males) by either cardiac puncture (2 min after euthanasia) or by mincing the heart tissue and quickly freezing samples in EPR tubes for later analysis. Values are means (3-6 samples) of signal concentrations determined either by integration or by signal simulation, with standard errors quoted in parentheses (see Methods).

Mouse hearts were removed from animals exposed to either 24 mg/kg $NaNO_2$ alone, 16 mg/kg NaHS alone, or the nitrite/sulfide combinations immediately after euthanasia, flash frozen in liquid nitrogen and stored at -80 °C prior to preparation of EPR samples as described in the Methods. EPR signals consistent with metmyoglobin (metMb) and nitrosylmyoglobin (MbNO) formation were observed in animals treated with nitrite (Figure 21A). EPR signals of MbNO lack the three-line hyperfine pattern of HbNO and, thus, the two spectra are readily distinguishable (see Figures 20A and 21A). There was very little sulfidometmyoglobin (metMbSH) signals present in any of the spectra obtained from the blood of animals given NaHS (Figures 21B-21D). The level of metMb was diminished in samples where $NaNO_2$ had been given before the NaHS (Figure 20C, Table 11) but any explanation of this could only be speculative in the absence of detectable metMbSH formation. For practical purposes, the detection limit of single-scan EPR measurements is a few μM, so it is certainly the case that biologically significant levels of potentially EPR detectable species can arise, but remain below detection. Thus, these data are not very informative regarding the movement of sulfide species into cells, but they do confirm significant trafficking of NO/nitrite from the bloodstream into the heart muscle. Presumably, this also applies to other soft

vascularized tissues, but since most do not contain high levels of constitutive traps (like myoglobin) the assertion cannot be verified by our EPR methodology in such cases. While tissues of the central nervous system are of prime importance in the present context, our EPR findings certainly do not exclude their efficient uptake of NO/nitrite and at least NO is known to cross the blood-brain barrier.[224]

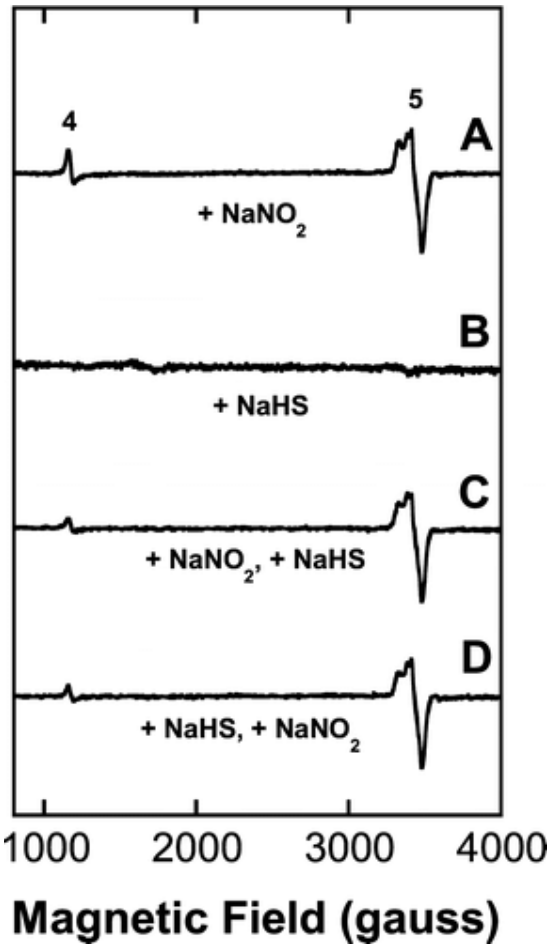

Figure 21. EPR spectra (x-band, 10 K) of mouse heart tissue.
A: EPR spectrum of minced heart tissue following dose of 24 mg/kg $NaNO_2$ administered ip 5-10 min prior to sacrifice showing clear evidence for the generation of nitric oxide. Signal (4) at ~1100 gauss: metMb; signal (5) at ~3300 gauss: MbNO. The combined intensities of the metMb plus MbNO signals approximates to 100% of the total heme (~260 μM) present in the heart. **B:** Spectrum of heart tissue following dose of 16 mg/kg NaHS. **C:** Spectrum of heart tissue following dose of 24 mg/kg of $NaNO_2$ (t = 0) and 16 mg/kg of NaHS at 2 min. No signals due to metMbSH were observed. **D:** Spectrum of heart tissue following NaHS injection 2 min prior to $NaNO_2$. No metMbSH signals were detected.

Reactions with the Crucial Target of Sulfide Toxicity, Cytochrome *c* Oxidase. In many cases, changes in oxidation state and substitution of ligands at the oxygen-binding (active site) heme a_3 in cytochrome *c* oxidase can be conveniently followed by electronic absorption spectroscopy (*e.g.* Figure 22). As prepared, the fully oxidized ("resting") enzyme exhibits an absorption spectrum with two distinct features, a Soret band at 420-422 nm (Fig 22A, dot-dash trace) and a visible-region band at ~600 nm (Figure 22B, dot-dash trace). Upon the addition of sulfide, using a 5-fold excess of NaHS over enzyme, the spectrum changes to yield a more prominent Soret band at 428 nm (Fig 22A, dotted trace) and an increased intensity of the ~600 nm band (Figure 22B, dotted trace). It should be remembered that these absorption spectral envelopes (22A and 22B) result from two sets of overlapping signals arising from heme *a* and heme a_3 (any Cu_A and Cu_B contributions are minimal). This lack of resolution in the spectra means that while such measurements are very good indeed at revealing whether or not something has happened at either heme *a* or heme a_3 (oxidation-reduction and/or ligand substitution) they do not necessarily reveal exactly which heme was involved, or exactly what happened chemically – this usually has to be inferred from other information. It is quite clear from the absorption spectra that, following addition of sulfide, a new derivative of the enzyme has been formed, probably HS^- (rather than S^{2-} at pH 7.4) has bound to heme a_3 of the enzyme, but the oxidations states of the metal cofactors remain unspecified. Based on earlier studies,[174] it was most likely that HS^- became bound to ferric heme a_3 with reduction of Cu_B – and, indeed, our own EPR measurements with samples prepared in parallel proved to be in keeping with this assertion (not shown). Following exposure of the tentatively identified hydrosulfide adduct of the enzyme to excess NO gas, the 428 nm Soret band sharpened and increased in intensity (Figure 22A, dashed trace) while the visible-region band hardly changed (Figure 22B, dashed trace). The new spectrum obtained after the NO addition is

reminiscent of that of a partially reduced-NO adduct of the enzyme, where NO is bound to ferrous heme a_3 while heme *a* remains in the ferric form.[59] Interestingly, upon the addition of a strong reductant (sodium dithionite) to the partially-reduced sulfide-inhibited NO adduct, a more complicated envelope was obtained, with two maxima at 428 nm and 442 nm (Figure 22A, solid trace) together with a two-fold stronger band at 603 nm in the visible region (Figure 22B, solid trace). Absorption maxima at 442-444 nm and 603-605 nm are invariably associated with fully reduced derivatives of cytochrome *c* oxidase. The lack of any great shift in the 428 nm band of the NO adduct upon the addition of dithionite confirms that NO was bound to a ferrous heme in both cases. Also, the 428 nm and 442 nm features were slightly variable in relative intensity between samples (not shown) indicating that they represent two distinct chemical species (one associated with heme *a*, the other with heme a_3) rather than being two bands in the spectrum of a single chromophore. The present data do not definitively identify to which heme NO binds, but we assume this to be the oxygen binding site of heme a_3; which in turn suggests the 442 nm band to arise from reduced heme *a*. More importantly, the absorption spectra do unambiguously show that NO is able to displace the inhibitory ligand from the sulfide-inhibited enzyme. Moreover, when oxygen was admitted to the closed vessel containing the NO adduct, the absorption spectrum reverted to that of the starting, fully-oxidized enzyme (not shown) confirming any NO inhibition to be transient and providing a mechanistic basis for nitrite-derived NO antagonizing sulfide inhibition of cytochrome *c* oxidase.

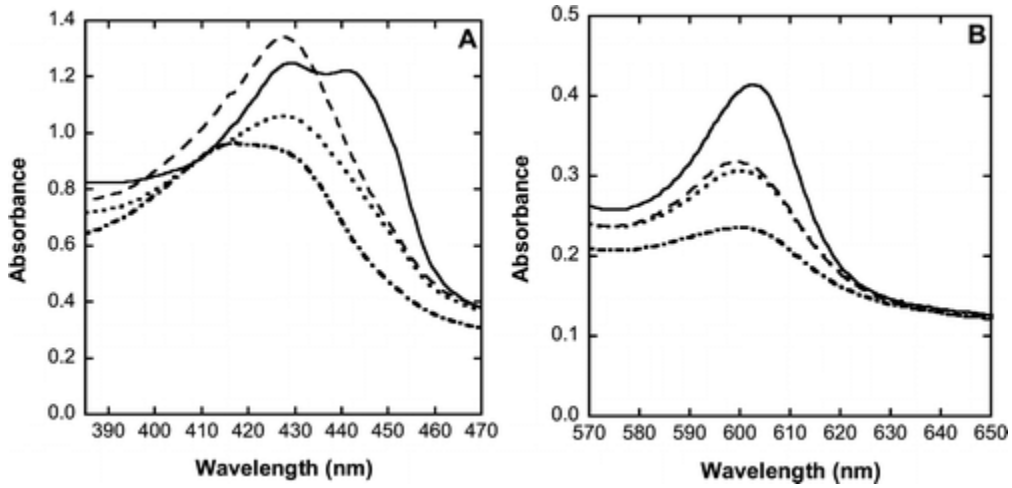

Figure 22. Electronic absorption spectra of cytochrome *c* oxidase derivatives showing displacement of HS^- by NO.

Samples were prepared in 100 mM aqueous potassium phosphate buffer, pH 7.4, 0.05 % lauryl maltoside, 25°C, 1.00 cm pathlengths**. Dash-dot trace:** Cytochrome *c* oxidase as isolated (oxidized, resting), 5 μM in enzyme; **dotted trace:** partially-reduced sulfide adduct 5 μM in enzyme, 0.2 mM in NaHS, **dashed trace:** partially-reduced sulfide adduct plus NO, 5 μM in enzyme, 0.2 mM in NaHS, 1.9 mM (1.0 atm) NO; **solid trace:** partially-reduced sulfide adduct plus NO, 5 mM in enzyme, 0.2 mM in NaHS, 1.9 mM (1.0 atm) NO, plus ~1 mM in $Na_2S_2O_4$. **A:** Soret region 380-470 nm. **B:** Q (or α) band region 570-650 nm.

Amelioration of Sulfide Toxicity by Nitrite in Cultured Cells. We have previously shown that proliferating (sub-confluent) bovine pulmonary artery endothelial cells (BPAEC) can be used to investigate changes in mitochondrial function within the cellular environment.[133] Furthermore, as BPAEC do not contain hemoglobin/myoglobin, it was possible to show with this cell line that NO antagonizes cell death due to cyanide in a manner that does not depend upon methemoglobin, or metmyoglobin, formation.[60] Leavesley *et al*[225] have reported a similar effect using NO donors in a neuronal line of cultured cells inhibited with KCN. In the previous studies with BPAEC, we successfully used the metabolic indicator dye alamarBlue to monitor cell proliferation. However, sulfide was found to interact directly with alamarBlue, reducing the dye and preventing its implementation in the current studies. Instead, we used propidium iodide staining as a marker of cell death, having first established that propidium iodide did not react with sulfide. Recent studies have shown that nitrite can be easily converted to NO in various

cells/tissues.[55, 137, 226] Plated cells were covered with Parafilm and inoculations of reagents made by injections through the cover to slow down the loss rates of gaseous H_2S and NO. Compared to controls, application of $NaNO_2$ did result in increased cell death after 1 hr (Figure23) which is not surprising, as the NO released will inhibit the mitochondrial electron-transport chain to some extent (*i.e.* transiently). Addition of NaHS (alone) to the media resulted in considerably more cell death 1 hr later (Figure 23) showing the sulfide dose to be considerably more inhibitory/toxic than the nitrite-derived NO dose. Most importantly, however, when the same doses of nitrite and sulfide were given together, their toxic effects did not combine additively, rather the level of cell death observed corresponded to that seen with nitrite alone (Figure 23). In other words, nitrite is observed to ameliorate the sulfide toxicity. These findings strongly support the proposition that methemoglobin formation by nitrite is not required for significant antidotal activity, but instead, NO generated from nitrite displaces bound sulfide from cytochrome *c* oxidase.

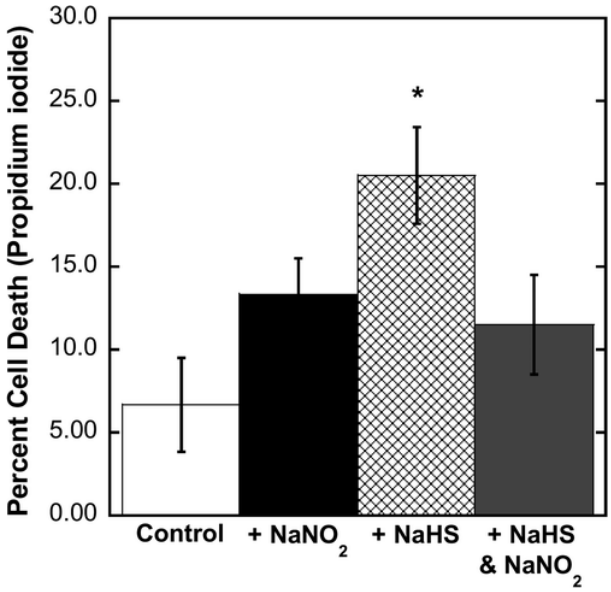

Figure 23. Resistance of bovine pulmonary endothelial cells (BPAEC) to sulfide toxicity is increased in the presence of sodium nitrite.

Effect of NaNO2 on BPAEC and BPAEC treated with NaHS. BPAEC were plated and then covered with Parafilm just prior to experiments. Aqueous solutions (in media) of both NaNO2 (1 mM) and NaHS (5 mM) were injected through the Parafilm into the cell media of wells while shaking the plates gently. Plates were incubated at 37°C for 1 hr and then treated with both SYBR-Green and propidium iodide dyes. Cell counts were taken using a Zeiss IM 35 fluorescent microscope and an Infinity 2 camera with the Infinity Analyze software.

Discussion

It is widely accepted that the lethal acute toxicities of HCN and H_2S are similar, cytochrome *c* oxidase within the central nervous system is the primary target for inhibition, and death results from respiratory paralysis.[96, 148] We have found nothing, to date, that would lead us to suspect that any of these assertions might be incorrect. It has also, however, previously been widely accepted that the antidotal action of sodium nitrite toward cyanide intoxication involves oxidation of hemoglobin to metHb, which then detoxifies cyanide through formation of cyanomethemoglobin (metHbCN). This in turn frequently led to the tautological opinion that sodium nitrite was an undesirable choice for a cyanide antidote because it would result in methemoglobinemia in patients with already challenged respiratory function.[227-229] Contrary to these hypotheses, our laboratory has shown in a series of studies that the nitrite anion probably functions as an NO donor able to reverse cyanide inhibition at cytochrome *c* oxidase and, at efficacious antidotal levels, metHb formation is minimal (< 2% total hemoglobin).[59-62] In support of this position, Lavon has very recently shown that the administration of isosorbide dinitrate in rabbits ameliorates cyanide toxicity without any metHb formation.[162] Now, in the present study of sulfide toxicity and its amelioration by nitrite, we have demonstrated an analogy. Sodium nitrite can clearly be protective against acute sulfide toxicity in mice (Figures 18 & 19) while metHb accumulation in the blood is negligible (Fig 20). Quantitation of the EPR data revealed that only 4% of the NaHS dose given to mice became trapped as metHbSH. This is less than the experimental variability of the toxicant delivery, since our dose error was about ±1 mg/kg (of 16 mg/kg total) or *ca.* ±6%, not enough to explain the observed level of protection. Using an approach suggested independently by Chen[230] and Way[231] who, before our group, were the most vocal critics of the nitrite-metHb hypothesis, we

attempted to further suppress nitrite-dependent metHb formation with methylene blue. Certainly, sodium nitrite was still fully protective against acute sulfide toxicity in mice also treated with methylene blue (data not shown) but we only found modest (< 20%) reduction in levels of metHb by EPR – in keeping with our position regarding the insignificance of metHb, but not adding much weight to the argument. The current rather ambiguous results of the methylene blue experiment could be due to the relatively low levels of metHb formation we now obtain. The present data obtained with isolated enzyme (Figure 22) and cultured cells (Figure 23) support the notion that nitrite-derived NO reversing the sulfide inhibition at cytochrome *c* oxidase is likely to be the principle antidotal mechanism.

Our findings are that experimental animals acutely poisoned with sulfide (LD_{40} given intraperitoneally as an aqueous NaHS solution) either succumb within minutes (typically < 5 min) or recover fully (by RotaRod assay) within about 15 min (Figure 2) irrespective of whether antidote was given or not. These observations seem to be in broad agreement with the recent report of Haouzi *et al*[102] who also found following administration directly into the vasculature of sheep and rats at sub-lethal levels, free sulfide species reacted and disappeared from the bloodstream within 1 minute. In short, effects that can truly be described as "acute" are very rapid. On the other hand, there are numerous accounts of suspected victims of H_2S gas inhalation reaching the clinic exhibiting "acute symptoms" such as unconsciousness and respiratory difficulty.[96, 106] Typically, these patients will have presented at the clinic approximately half an hour or more after collapse, many having received ventillatory support, and some succumb to the poisoning hours after the exposure. This chain of events is quite unlike observations made with laboratory animals, suggesting additional and slower mechanisms of toxicity are important in real-world exposures. While inhibition of cytochrome *c* oxidase may represent the key acute toxic action of sulfide, it

has been suggested that sulfide may engage a broad range of other biological targets such as carbonic anhydrase,[232] monoamine oxidase,[233] cholinesterase and Na+/K+-ATPase.[110] Alternately, sulfide inhibition of mitochondrial electron-transport chains in various parts of the brain, which is probably not relieved by ventillatory support, may lead to damagingly low ATP levels – sometimes this is misleadingly referred to as "brain/tissue *hypoxia*" in relation to cyanide/sulfide toxicity, whereas "bioenergetic hypoxia" might be more appropriate as there will actually be net *hyperoxic* conditions due to suppressed oxidative phosphorylation. Distinguishing between these possibilities will have to await further studies, but the current findings do reinforce the view from the experimental toxicologist's perspective that clinical presentations of sulfide poisoning can never be accurately described as "acute" (the patient would either be dead, or require no treatment) instead, they represent a range of more complicated "post-acute" conditions.

The current results (Figures 18 & 19) suggest that sodium nitrite may only be useful prophylactically and cannot be administered quickly enough to be of any antidotal value when given after toxicant dose. However, the lengthy survival time of sulfide-poisoned human victims compared to the laboratory animals indicates that this need not be so. There may be around half an hour or so, especially in the ambulance and before arrival at the clinic, during which time a beneficial intervention could be made with sodium nitrite, or other antidotes like decorporating agents. NO crosses the blood-brain barrier and, consequently, nitrite administration may be a practical way to address the potential brain "bioenergetic" hypoxia during ventillatory support. At this time, what is lacking is a good experimental paradigm for studying real-world exposures, which are almost all occupational accidents involving H_2S gas inhalation for a duration of at least several minutes. This is the first obvious limitation of the simple *ip* -injection approach we have adopted so far, which has such a short period of effect that meaningful testing of putative antidotes

post-toxicant delivery is impractical if at all possible. At some point inhalation exposures of animals will be required to address some particular pulmonary issues, such as the lung edema commonly found at autopsy in humans [234] and which, so far, has not been evident in our animals. Nevertheless, there is some value in pursuing non-inhalation methods as some of the pulmonary issues are likely due to H_2S acting as an irritant and it will be important to disentangle these from systemic toxic effects and their amelioration. An equally important limitation of the present study is the use of a mouse model. Humans and other larger mammals exposed to H_2S tend to exhibit coma and death without the transient and reversible torpor, associated with bradycardia and hypopnea, observed for small animals like mice. Perhaps this pattern of protective response helps the mice to avoid neurological sequelae in a manner not available to larger mammals.

Appendix B A Comparison of the Cyanide-Scavenging Capabilities of Some Cobalt-Containing Complexes in Mice

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Keywords: Cobalamin; cobinamide; complex IV; cyanide; cytochrome *c* oxidase; electron transport; mitochondria; macrocycle; porphyrin; respiratory poison; righting recovery; RotaRod

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Abstract

Four cobalt-containing macrocyclic compounds previously shown to ameliorate cyanide toxicity have been comparatively evaluated with an acute sub-lethal toxicity model in conscious (un-anesthetized) adult male Swiss-Webster mice. All of the compounds (the cobalt-corrins cobalamin and cobinamide, a cobalt-porphyrin, plus a cobalt-Schiff base macrocycle) given 5 min prior to the toxicant dose significantly decreased the righting-recovery time of cyanide-intoxicated mice, but the doses required for maximal antidotal effect varied. Additionally, all of the compounds tested significantly reduced the righting-recovery time when administered at either 1 or 2 min after cyanide intoxication, but none of the compounds tested significantly reduced the righting-recovery time when delivered 5 min after the toxicant dose. Using the lowest effective dose of each compound determined during the first (prophylactic) set of experiments, neuromuscular recovery following cyanide intoxication in the presence/absence of the cobalt-based antidotes was assessed by RotaRod® testing. All the compounds tested accelerated recovery of neuromuscular coordination and no persistent impairment in any group, including those animals that received toxicant and no antidote, was apparent up to 2 weeks post-exposures. The relative effectiveness of the cobalt compounds as cyanide antidotes are discussed and rationalized based upon the cyanide-binding stoichiometries and stability constants of the Co(III) cyano adducts, together with consideration of the rate constants for axial ligand substitutions by cyanide in the Co(II) forms.

Introduction

Other compounds, including dicobalt ethylenediaminetetraacetate (Kelocyanor®) [235] and 4-dimethylaminophenol [236], remain in use worldwide, but there are currently only two acceptable antidotes to cyanide poisoning available in the United States [237, 238]. The first, Nithiodote®, is a combination treatment of sodium nitrite and sodium thiosulfate [164]; in which (i) the nitrite anion probably acts as a nitric oxide (NO) donor leading to the removal of cyanide bound to cytochrome *c* oxidase [62, 157] rather than simply being a methemoglobin generator and (ii) the thiosulfate reacts with free cyanide in a reaction catalyzed by the enzyme rhodanese leading to formation of the considerably less toxic thiocyanate anion (SCN^-) [164]. Intravenous infusion of sodium nitrite in the pre-hospital setting must be undertaken with caution, due to the likelihood of induced hypotension and methemoglobinemia, the latter being of particular concern if there has been any concomitant carbon monoxide poisoning through smoke inhalation [238, 239]. The second antidote, Cyanokit®, contains hydroxocobalamin (Cb), a vitamin B_{12} derivative (Figure 24A) [240]. Cb binds a single cyanide anion to its central cobalt(III) cation, with high affinity thereby acting as a scavenger of the toxicant in the bloodstream. Unfortunately, while Cb appears to be efficacious and the safest available option for treating cyanide intoxication [241, 242], it is still a less than ideal antidote because it must first be dissolved (5 g solid in ~200 mL saline) before it can be intravenously infused (~15 mL per min for almost 15 min in adults) [240]. Particularly with regard to acute-poisonings/mass-casualty situations, this slow administration is a significant problem as cyanide is such a quick-acting toxicant.

Cobinamide (Cbi) lacks the dimethylbenzimidazole nucleotide tail of Cb (Figure 24B) allowing for the binding of two cyanide anions to the cobalt ion and has been shown to be a

potentially better cyanide scavenger [243, 244]. We have recently demonstrated the cyanide scavenging ability of cobalt(III)meso-tetra(4-N-methylpyridinyl)porphine (CoTMPyP) (Figure 24C) a water soluble metalloporphyrin complex [176, 245]. In addition, we have begun to investigate the cyanide binding and scavenging activities of Schiff-base macrocyclic compounds, including cobalt(III)2,12-dimethyl-3,7,11,17-tetraazabicyclo[11.3.1]heptadeca-1(7)2,11,13,15-pentaene (CoN_4[11.3.1]) (Figure 24D) [69]. These smaller cobalt cations, CoTMPyP and CoN4[11.3.1] or similar, which are also able to bind two cyanide anions per cobalt, may be soluble at higher concentrations in the bloodstream than Cb and Cbi, resulting in improved cyanide-scavenging capabilities. In this paper, to directly assess the relative merits of such cobalt-containing compounds (Table 12) as cyanide antidotes, we have undertaken a series of head-to-head comparative assays in mice, in which experiments with Cb were included as a benchmark.

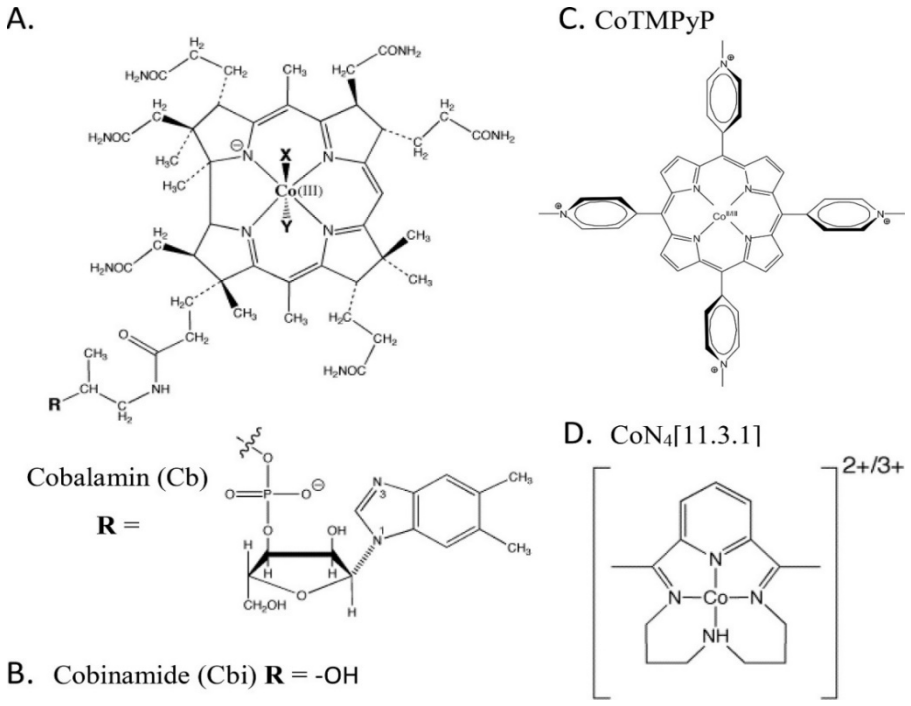

Figure 24. Structures of cobalt-containing compounds for comparison of cyanide scavenging abilities in mice. (A) Cobalamin (Vitamin B12),a FDA approved cyanide antidote, (B) cobinamide, the biological precursor to cobalamin, (C) CoTMPyP (Cobalt (II/III)meso-tetra(4-Nmethylpyridinyl)porphine), and (D) CoN4[11.3.1] (cobalt (II/III)(2,12-dimethyl-3,7,11,17-tetraazabicyclo-[11.3.1]heptadeca-1(7)2,11,13,15-pentaene).

Table 12. Selected Properties of the Cobalt-containing Trial Compounds.

Property	Hydroxocobalamin (Cb)	Cobinamide (Cbi)	CoTMPyP	CoN_4[11.3.1]
Molecular masses (of cations)	1329	990	678	317
Comparative (estimated) costs[a]	1[b]	63[c]	~0.8[d]	< 0.2[e]
Available sites for exogenous ligands	1	2	2	2
Stability constants[f]	10^5 M^{-1}	10^9-10^{10} M^{-2}	2 x 10^{11} M^{-2}	> 107 M^{-2} [g]
Rate constants[h]	> 10^2 $M^{-1}s^{-1}$ [i]	> 10^3 $M^{-1}s^{-1}$ [j]	10^2 - 10^3 $M^{-1}s^{-1}$ [k]	~10^5 $M^{-1}s^{-1}$ [l]

[a]Based on the Sigma-Aldrich catalog (accessed on line October 2017) molar comparisons. [b]Hydroxocobalamin hydrochloride $165/g (equating to $825 for a single adult dose). [c]Comparing prices of cyanocobalamin and dicyanocobinamide, the sole derivative of the latter available at the time. The Cbi was only available in mg quantities, so there is likely to be reduced cost associated with scaling up production; however, Cbi will remain many-fold more expensive than its precursor Cb as additional manufacturing steps (chemical modification, purification, recovery) are necessary. [d]CoTMPyP was not available, so this estimate is based on the mean of the pricing for cobalt(II)-5,10,15,20-tetraphenylporphine and cobalt(II)-5,10,15,20-tetrakis(4-methoxyphenyl)porphine, two similar metalloporphyrins. [e]CoN_4[11.3.1] is, so far as we are aware, not commercially available at this time (except by custom synthesis). This estimate is based on the catalog prices of the starting materials multiplied by 10. [f]Stability constants for cyano adducts in terms of total cyanide concentrations [HCN + CN^-] for Co(III) forms at pH 7.4 and 25°C. [g]The stability constant for the bis(cyano)Co(II) adduct was actually determined and found to be 3 x 10^5. There are remarkably few studies reporting the stability constants of cyano complexes of transition-metal ions in different oxidation states. However, based on available reliable data for (i) the hexacyanoferrate(II) and hexacyanoferrate(III) pair, plus (ii) the stepwise formation constants for some other polycyano complexes, we estimate the increase in the net stability constant for the Co(III) form of CoN_4[11.3.1] to be at least an order of magnitude per cyano ligand: 3 x 10^5 multiplied by 10^2 = 3 x 10^7, or greater. [h]Rate constants for cyanide binding to the Co(II) forms at pH 7.4 and 25 °C. Where dicyano complexes are formed, it is often the case that the rate for only one ligand association can be observed, the other presumably being too fast to measure. [i]The reported rate constant is for the Co(III) form (80 $M^{-1}s^{-1}$) hence that for the Co(II) form must be greater as Co(III) complexes are typically more substitution inert than their Co(II) counterparts. [j]The rate constant for the largest (~90%) of two [cyanide]-dependent phases observed for the Co(III) form is 3 x 10^3 $M^{-1}s^{-1}$, hence that for the Co(II) form must be greater. [k]Two [cyanide]-dependent phases of comparable extent were observed. [l]Praekunatham *et al*, manuscript in preparation.

For this investigation, we chose an acute-toxicity model using intraperitoneal (ip) injections of NaCN (saline solutions) in adult male Swiss-Webster mice. In this paradigm, which has previously been well-validated [22, 62, 156, 157] the mice are conscious and freely moving at the time of toxicant injection, avoiding the confounding influence of anesthesia present in some other animal protocols [62, 130]. The ameliorative capabilities of the compounds in question given both prophylactically and therapeutically have been compared. Antidotal effects in the short term (< 30 min) have been quantitatively assessed by observing shortening of recovery times following

cyanide-induced unconsciousness ("righting recovery"). Antidotal effects in the longer term (24 hr – 2 weeks) have been quantitatively assessed by measuring the duration that the animals were able to remain in position on a rotating cylinder (RotaRod®) a test of neuromuscular coordination). The results suggest that the trial compounds all work by similar mechanisms and identify potential strengths/weaknesses of each in the pursuit of cobalt-containing compounds as antidotes to cyanide poisoning.

Experimental Section

Chemicals. All non-gaseous reagents, obtained from Fisher or Sigma-Aldrich, were ACS grade or better and used without further purification. Argon and nitrogen gases were purchased from Matheson Incorporated. Previously described procedures were employed to prepare Cbi [246], CoTMPyP [245] and CoN_4[11.3.1] [69]. Solutions of sodium cyanide in saline were prepared immediately prior to use in septum-sealed vials with minimized headspaces and volumetric transfers made with gastight syringes.

Animal Exposures. The University of Pittsburgh Institutional Animal Care and Use Committee (Protocol Number 13092637) approved all animal produces used in these experiments. The Division of Laboratory Animal Research of the University of Pittsburgh provided all veterinary care during this study. With the exception of most animals exposed to sodium nitrite (see below), male Swiss-Webster mice weighing 35-40 g (6-7 weeks old) were purchased from Taconic, Hudson, NY, housed four per cage and allowed access to food and water *ad libitum*. Animals were allowed to adapt to their new environment for one week prior to carrying out experiments. All animals were randomly assigned to experimental groups of predetermined size.

All solutions administered to mice were prepared by dilutions into sterilized saline in septum-sealed vials using gastight syringes and were given by ~0.1 mL intraperitoneal (ip) injections.

Similar procedures (Protocol Numbers 0808101 and 1008725) approved by the University of Pittsburgh Institutional Animal Care and Use Committee were employed in the case of the majority of mice treated with sodium nitrite. Veterinary care was provided by the Division of Laboratory Animal Research of the University of Pittsburgh. Male Swiss-Webster mice 16-20 weeks old, weighing 40-45 g were purchased from Charles River Laboratories, Wilmington, MA. All solutions were prepared by dilutions into sterilized saline and administered through ~0.1 mL intraperitoneal (ip) injections. In general, a group of at least 4 mice were tested for each experimental point. Efficacy was tested through the recovery of righting ability after $NaNO_2$ (12 mg/kg) was injected 2, 4, 8, 12, 16 or 20 min following the administration of NaCN (100 μmol/kg).

Righting Recovery. Following cyanide administration, the duration of time required for the recovery of righting ability in mice was measured following a simplification [157] of the procedure originally used by Crankshaw *et al* [156]. The toxicant (5 mg/kg NaCN) was administered to mice (ip) and they were then placed in a transparent but dark green-colored plastic tube (Kaytee CritterTrail, available from pet stores) in a supine position. The time it took from the initial administration of the toxicant until the mouse flipped from the supine to a prone position in the plastic tube was taken as the endpoint (righting-recovery time).

Prophylactic Dose-Response. The established antidote (Cb) and potential prophylactic antidotes (Cbi, CoTMPyP or CoN_4[11.3.1]) were injected (ip) into mice (n = 6-8 per dose) at levels of 30, 40, 45, 50 or 70 μmol/kg, 5 minutes before the administration (ip) of 100 μmol/kg NaCN. Control animals received cyanide alone. The righting-recovery times were recorded for mice that survived the cyanide intoxication. A single injection of cyanide administered at this sub-lethal

dose typically results in a persistent state of unconsciousness within 1-2 min that can last for more than 25 min [62, 157] allowing for multiple measures of trial-compound efficacy given both prophylactically and therapeutically. The lowest dose of each putative antidote (Cbi, CoTMPyP or CoN_4[11.3.1]) having the maximal ameliorative response following prophylactic administration was determined. Based on these results we then selected a single dose ("lowest dose having maximal antidotal effect") for subsequently testing the therapeutic powers of the trial compounds. For comparison, Cb was given at 70 μmol/kg in these subsequent experiments (see below) as there was no maximal antidotal effect apparent for this compound in the experimental range.

Therapeutic Time Response. After determining the lowest doses having maximal antidotal effect for each putative antidote, mice (n = 6-8 per dose) were given 100 μmol/kg NaCN and subsequently (1, 2, or 5 min later) injected with either 50 μmol/kg CoN_4[11.3.1] or, alternately, 70 μmol/kg Cb, Cbi, or CoTMPyP. Righting-recovery times were recorded for mice that survived the toxicant injections and, later, the same animals were used in the RotaRod assessments described below.

RotaRod Testing. The accelerating RotaRod® (Coulbourn Instruments, Whitehall, PA), a rotating cylindrical apparatus, was used to assess motor skill, learning and recovery subsequent to cyanide intoxication. Well-established experimental protocols were followed [247, 248]. An individual trial was started by placing a mouse on the RotaRod device turning at 4 rpm. Subsequently, acceleration was varied linearly from 4 to 22 rpm over the course of 60 s. Trials ended when the mouse either fell off, or had remained on the rotating cylinder for 60 s. Latency to fall, and highest speed reached were recorded for each trial. The animals were evaluated over a period of three consecutive days. On the first day, each mouse was trained in a series of 8 sequential trials on the RotaRod device. The baseline motor performance was established on the

second day by determining the max speed (rpm) reached before each animal fell off the RotaRod apparatus, averaged over three trials. On the second day, animals were tested in sets of 3 trials at 15 min intervals (with one set of trials made prior to any injections) over a period of 2.5 hrs. On the third day, 24 hr after the previous experiments, mice were tested again for a single set of 3 trials to determine whether any latent longer-term differences between experimental groups had emerged. A subset of the animals were further tested on the RotaRod apparatus up to 2 weeks following their injections. Data were analyzed using 2-way ANOVA to determine the main effect of treatment and time with Tukey's multiple-comparison test to determine the differences between groups.

Results

Comparison of the Prophylactic Effects of Cb, Cbi, CoTMPyP and CoN_4[11.3.1] on Acute (Sub-Lethal) Cyanide Toxicity. All of the cobalt containing compounds selected for comparison in this paper have been reported to be protective against acute cyanide poisoning [69, 176, 237, 240, 245], but they have not previously all been tested in a head-to-head fashion in the same model system. We chose to use the prophylactic administration of the cyanide scavenging compounds in mice as a starting point for comparing the efficacy of the four chosen compounds (Cb, Cbi, CoTMPyP and CoN_4[11.3.1]) against cyanide toxicity. In order to determine the lowest dose having maximal antidotal effect in each case, Swiss-Webster mice (age 7-8 weeks) were injected intraperitoneally (ip) with either 30, 40, 45, 50, or 70 μmol/kg of each cobalt compound to be tested, followed 5 min later by injection (ip) of NaCN (100 μmol /kg). Control animals that received NaCN alone were "knocked down" (i.e. became unconscious) within 1-2 minutes, 20%

(16 of 82) died within 4 min of the toxicant delivery, and those that survived exhibited a mean righting recovery time of 24 ± 7 min (n = 66).

All of the compounds employed in this prophylactic paradigm significantly decreased the righting recovery time of the cyanide-intoxicated mice, but the dose of each test compound required for the maximal effect (*i.e.* minimal recovery time) varied (Figure 25). While the righting recovery time seemed to decrease as the Cb administered was increased from 30 to 50 μmol/kg, the only significantly effective dose was 70 μmol/kg (Figure 25A). The righting recovery time was 9 ± 5 min (compared to 24 min for controls) and, in addition, 100% of the mice receiving this Cb dose (15 of 15) survived. Interestingly, Cb was the only antidotal compound of the four for which a majority of the mice (14 of 15) still experienced knock-down, even at the highest dose tested. Lower doses of Cbi (45, 50 and 70 μmol/kg) compared to Cb significantly reduced the righting-recovery times of cyanide-intoxicated mice to 8 ± 8 min, 12 ± 10 min, and 5 ± 6 min, respectively (Figure 25B) – all the animals survived (16 of 16) and half experienced knock-down (8 of 16). However, there was noticeably more variation within the Cbi-treated groups of mice compared to all the other groups, particularly in mice administered 45 or 50 μmol/kg Cbi, and the response was not strictly linear. The most effective dose of Cb (70 μmol/kg), against cyanide intoxication, was ~1.6 times a similarly effective dose of Cbi (45 μmol/kg). The lower effective dose of Cbi can be rationalized by consideration of its structure (Figure 24B) in which the loss of the dimethylbenzimidazole ribonucleotide tail of the Cb structure (Figure 24A) allows Cbi to bind two exogenous cyanide anions rather than only one.

The other two compounds forming bicyano adducts, CoTMPyP and CoN_4[11.3.1], were then tested. CoTMPyP significantly reduced the righting recovery time at all doses tested (Figure 25C) with righting recovery times of 15 (± 10) min, 9 (± 7) min, 9 (± 5) min, 5 (± 4) min, and 1 (±

3) min for doses of 30, 40, 45, 50, and 70 μmol/kg, respectively. The dose of Cb (70 μmol/kg) most effective against cyanide intoxication, was ~1.8 times a similarly effective dose of CoTMPyP (40 μmol/kg). No deaths (100% survival) were observed when mice were administered either 45, 50 or 70 μmol/kg CoTMPyP before cyanide intoxication. Out of 13 mice that were administered 70 μmol/kg of CoTMPyP, only two knocked-down.

Finally, dose-response testing demonstrated that CoN_4[11.3.1] administered at 45, 50, or 70 μmol/kg significantly reduced the righting recovery time versus control mice to 5 (± 7), 3 (± 4), 3 (± 3) min, respectively (Figure 25D) – all the animals survived (15 of 15) and about half experienced knock-down (7 of 15). Since, we found that the average time of righting recovery is unchanged when the dose administered was increased from 50 to 70 μmol/kg, the lower dose (50 μmol/kg) was chosen for the therapeutic experiments (see below). The dose of Cb (70 μmol/kg) most effective against cyanide intoxication, was at least 1.6 times a similarly effective dose of CoN_4[11.3.1] (45 μmol/kg) – this value could be up to 1.8 times (70 μmol/kg Cb:40 μmol/kg CoN_4[11.3.1]) but for a single outlying point in the data (Figure 25D).

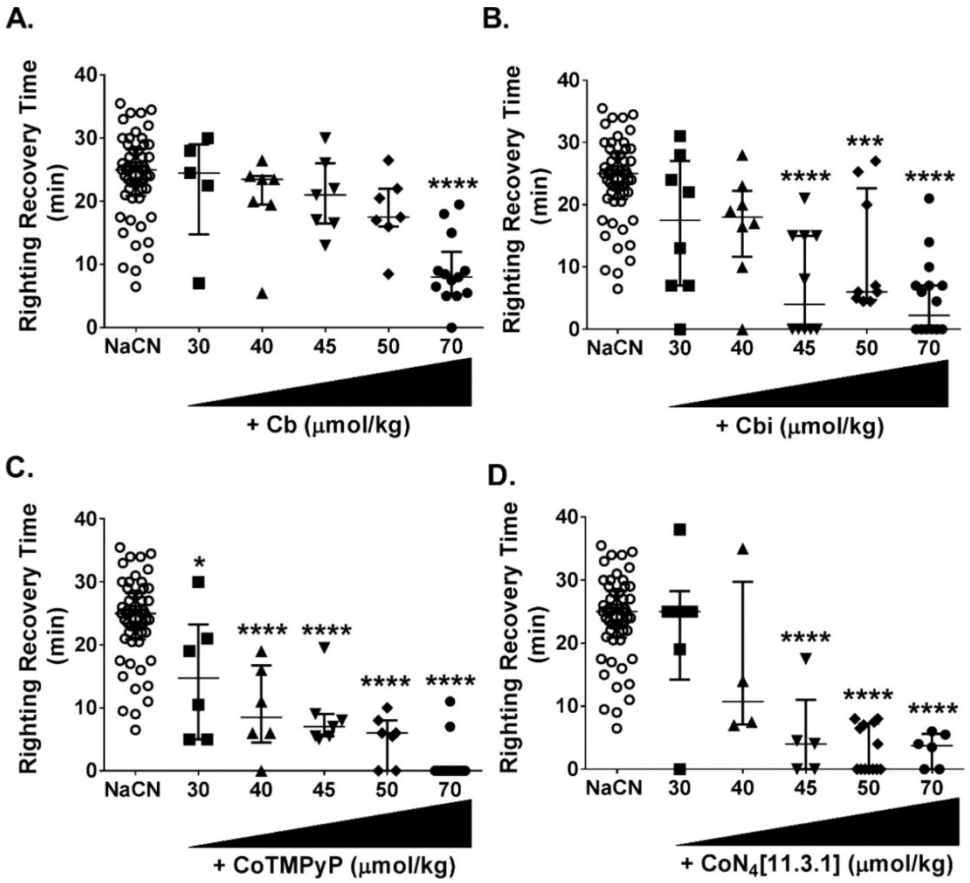

Figure 25. Dose-response profiles for prophylactically administered Cb, Cbi, CoTMPyP and CoN_4[11.3.1] in cyanide-intoxicated male mice as determined by righting-recovery times.
Swiss-Webster male mice (7-8 weeks of age) were injected (ip) with either Cb (A), Cbi (B), CoTMPyP (C) or CoN_4[11.3.1] (D) 30, 40, 45, 50, and 70 μmol/kg in saline, 5 min before the administration of 100 μmol/kg NaCN (in saline). Control animals received only 100 μmol/kg NaCN injections (open circles). Righting recovery times were recorded and the medians and interquartile ranges are shown. One-way ANOVA with Tukey's multiple comparisons post-test was performed for each compound tested to determine the significance of the righting-recovery time as compared to controls. ****$p \leq 0.0001$, ***$p \leq 0.001$, **$p \leq 0.01$ and *$p \leq 0.05$.

Comparison of the Therapeutic Effects of Cb, Cbi, CoTMPyP and CoN_4[11.3.1] on Acute (Sub-Lethal) Cyanide Toxicity. To examine the ability of the cobalt-containing compounds to ameliorate the effects of cyanide intoxication when given after the toxicant, Cb, Cbi, CoTMPyP, or CoN_4[11.3.1] were administered at the maximally effective doses (as described above) to male Swiss-Webster mice (7-8 weeks of age) at either 1, 2, or 5 min after injection of NaCN (100 μmol/kg). That is, all the cobalt compounds were administered at a dose of 70

μmol/kg, except for CoN_4[11.3.1], which was given at a dose of 50 μmol/kg. The righting-recovery times following the cyanide injections were recorded and compared with the controls given NaCN only (Figure 26).

The mean recovery times for animals given 70 μmol/kg Cb at 1 or 2 min after cyanide injections were found to be 11 ± 8 min and 14 ± 3 min, respectively (Figure 26A), longer than that observed when the same dose was prophylactically administered (9 ± 5 min, Figure 25A). Mice receiving 70 μmol/kg Cbi at 1 or 2 min post-cyanide intoxication had righting-recovery times of, respectively, 7 ± 4 min and 11 ± 2 min, again longer than for the prophylactically administered dose (5 ± 6 min, Figure 25B). CoTMPyP performed no better than Cb or Cbi in this therapeutic test (Figure 25C) exhibiting recovery times of 10 ± 7 min and 11 ± 6 min, for delivery of 70 μmol/kg CoTMPyP at 1 and 2 min, respectively, after cyanide intoxication compared to prophylaxis (1 ± 3 min, Figure 25C). Additionally, the majority (6/7) of mice tested knocked-down when CoTMPyP was administered at 1 min after cyanide injections, comparable to the effect seen with both Cb and Cbi. However, CoN_4[11.3.1] performed better than the other compounds when given 1 min after cyanide intoxication (Figure 26D) with a righting recovery time of 4 ± 3 min, comparable to its prophylaxis (3 ± 3 min, Figure 25D) even though a lower dose of the antidote (50 μmol/kg) was used. When given 2 min after the toxicant, the mean righting-recovery time for CoN_4[11.3.1] was 13 ± 6 min, no better than the other cobalt compounds at the same time point. Additionally, when the CoN_4[11.3.1] had been administered at 1 min after the cyanide injections, 4 of the 12 mice tested did not knock-down, a noteworthy improvement compared to the effects seen with the other compounds.

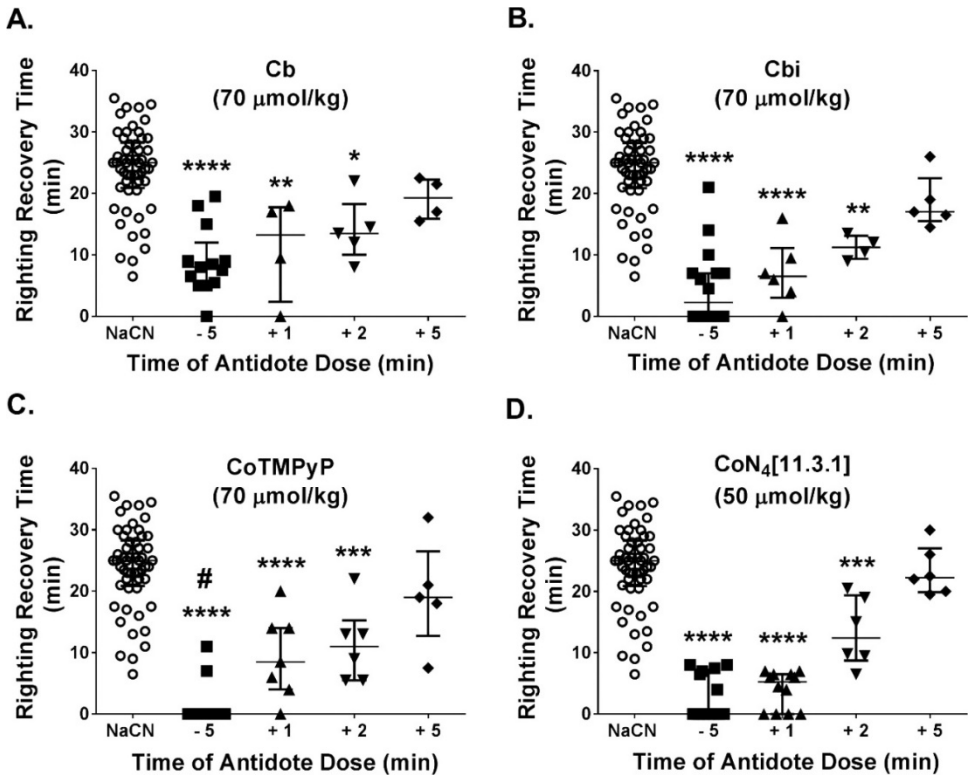

Figure 26. Therapeutic effects of cobalt-containing compounds in male mice after cyanide intoxication. Swiss-Webster mice were injected with either 70 μmol/kg of Cb (A), Cbi (B), CoTMPyP (C), or with 50 μmol/kg of CoN_4[11.3.1] (D) at 1, 2, or 5 min post cyanide intoxication. NaCN given alone (100 μmol/kg) and with the test compounds (at the doses above) injected prophylactically at -5 min are included in each plot for comparison purposes. Righting-recovery times were recorded for each set of injections; the median and interquartile range are shown. One-way ANOVA with Tukey's multiple comparisons post-test was performed for each compound tested to determine the significance of the righting-recovery time as compared to controls. ****$p \leq 0.0001$, ***$p \leq 0.001$, **$p \leq 0.01$ and *$p \leq 0.05$. #median and interquartile range are both equal to 0.

In summary, all of the cobalt compounds tested significantly reduced the righting-recovery time when administered at either 1 or 2 min after cyanide intoxications, but none of the compounds tested had any measurable impact on the righting-recovery time when delivered 5 min after the cyanide administration (Figure 26). Using exactly the same righting-recovery approach in sub-lethally intoxicated mice (16-20 weeks of age) we have previously shown that sodium nitrite clearly is an effective cyanide antidote when given therapeutically more than 5 min after the toxicant dose [157]. Such is the significance of this earlier result that we present data collected from

the younger mice (7-8 weeks, closed diamonds) in this study along with the data previously published for the older mice here (Figure 27, solid circles) both for emphasis and as a positive control. In terms of preventing knock-down and the shortest righting recovery observed, prophylactically administered CoTMPyP appeared to be the most effective of the cobalt compounds (Table 25). Otherwise, CoN_4[11.3.1] was the most efficacious of the cyanide scavengers tested by several criteria, not the least of which was that it performed comparably, or slightly better than the others, at lower relative dose.

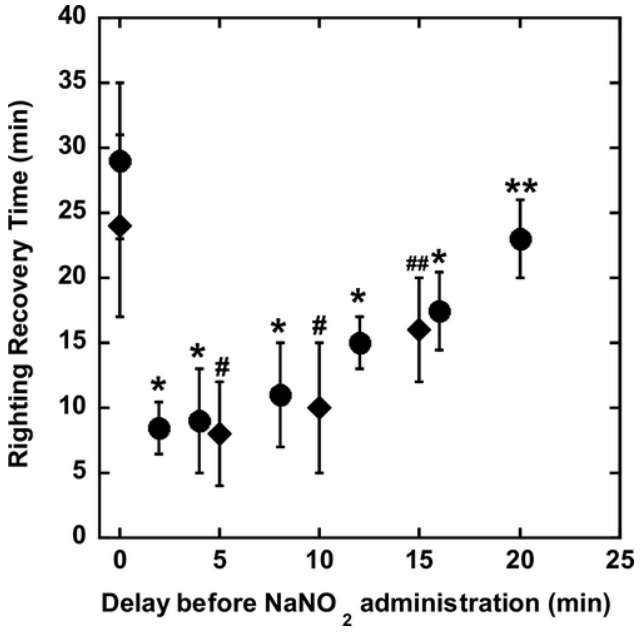

Figure 27. The ameliorative effect of $NaNO_2$ on cyanide intoxication.
Swiss-Webster mice (males, 16-20 weeks of age, solid circles and males 7-8 weeks of age, solid diamonds) injected with NaCN (100 μmol/kg, ip) and then administered $NaNO_2$ (12 mg/kg, ip) 2 to 20 min after cyanide. Control animals received NaCN only. Values represent means and standard deviations. In general, at least 4 animals per point were used, except for control (n = 17 for 16-20 weeks of age and n = 66 for mice of 7-8 weeks of age). One-way ANOVA with Tukey's multiple comparisons post-test was performed to determine the significance of the righting-recovery time as compared to controls.*p ≤0.0001, **p ≤ 0.05 (16-20 week old mice) #p ≤0 .0001. ##p ≤ 0.01 (mice of 7-8 weeks of age). Solid circles (mice of 16-20 weeks of age) are reformatted data from Cambal *et al.*

Table 13. Distinguishing Animal Data for the Cobalt-containing Trial Compounds.

Assessment	Controls (100 µmol/kg NaCN)	Hydroxocobalamin (NaCN + Cb, 70 µmol/kg)	Cobinamide (NaCN + Cbi, 70 µmol/kg)	CoTMPyP (NaCN + 70 µmol/kg)	CoN_4[11.3.1] (NaCN + 50 µmol/kg)
Prophylaxis[a]: Death/group	16/66	0/15	0/16	0/13	0/15
Prophylaxis: knock-downs/group	66/66 (100%)	14/15 (93%)	8/16 (50%)	2/13 (15%)	7/15 (47%)
Prophylaxis: (5 min pre-NaCN): righting recovery time (min)	24	9	5	1	3
Prophylaxis: effectiveness ratio[b]	N/A	1.0	1.6	1.8	1.7
Therapeutic[c]: knock-downs/group[d]	N/A	4/4 (100%)	5/6 (83%)	6/7 (86%)	8/12 (67%)
Therapeutic: righting recovery time (min)[d]	N/A	11	7	10	4

[a]Putative antidotes given 5 min before NaCN.
[b] Relative to Cb (see text for explanation).
[c]Putative andidotes given 1 min post NaCN administration
[d]For antidotal doses delivered at 1 min time points.

Comparison of Neuromuscular Recovery in Mice Administered Acute (Sub-Lethal) Cyanide Doses (Controls) or Saline (Shams). The RotaRod testing paradigm employed (Figure 28A, see Experimental Methods for further details) involves measuring the duration that individual animals can remain in position walking on a rotating cylinder; any shortening of the observed duration following some experimental insult being taken as evidence of impairment. While primarily a measurement of neuromuscular coordination [247, 248] the technique also routinely provides some assessment of learning capability and memory. During the training periods (day 1, 1 – 8 min; Figure 28B, 28C and 28D) the performance of all the animal groups increased steadily, indicating the mice were adapting to (*i.e.* learning) the test. The trained performance was essentially maintained by 24 hr later with a very slight loss in the previous day's level (pre-ip data sets, Figure 28B, 28C and 28D) and sham mice receiving saline solution without toxicant continued to adapt until reaching a plateau in their performance (open circles, day 2, 15 – 150 min;

Figure 28B). Control mice receiving only 100 μmol/kg NaCN were still completely incapacitated 15 min after the toxicant dose, unable to remain on the rotating cylinder for any time at all, but then steadily improved over the next 2 hr (filled squares, day 2, 15 – 150 min; Figure 28B). The performances of the sham and control animals remained indistinguishable 24 hr later (day 3, 24 hr points; Figure 28B). A subset of these animals were evaluated at longer times post injections and, in fact, the performances of the sham and control animals subjected to training regimens remained indistinguishable at 48 hr, 1 week and 2 weeks after the intoxications (see Supporting Information).

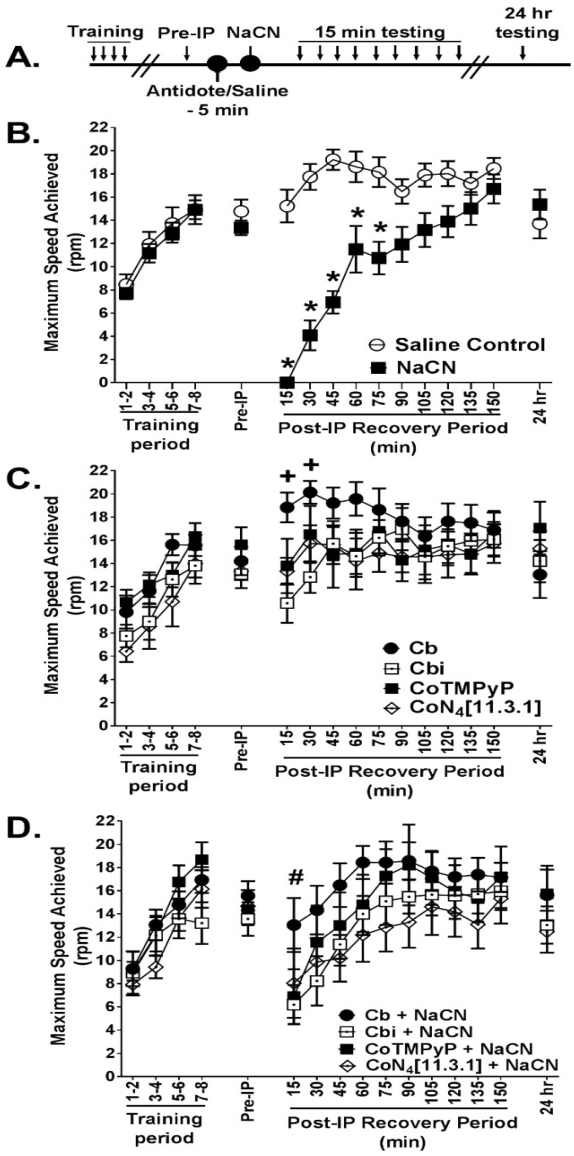

Figure 28. Neuromuscular coordination comparison, using RotaRod testing, of Cb, Cbi, CoTMPyP and CoN_4[11.3.1] amelioration following NaCN administration in male Swiss-Webster mice.

(A) RotaRod testing paradigm: arrows indicate RotaRod testing times; lines with circles indicate injection times (all ip). Mice were trained on the RotaRod 24 h before injection, and a baseline performance was obtained 1 h before injection (Pre-ip). Mice were tested every 15 min after injections for 2.5 h (up to 150 min) and again at 24 h to assess recovery. One-way ANOVA with Tukey's multiple comparisons post-tests were performed to determine the significance between controls and a particular compound tested or between compounds tested as noted. (B) Comparison of performance (maximum speed achieved) for injections of 100 µmol/kg NaCN (closed square) and saline control (open circle). *$p \leq 0.001$ vs saline control. (C) Comparison of performance (maximum speed achieved) for mice injected (ip) with 70 µmol/kg of Cb (closed circle), Cbi (open square), CoTMPyP (closed square) or 50 µmol/kg CoN_4[11.3.1] (open diamond). +$p \leq 0.01$ vs Cbi. (D) Comparison of performance of mice (maximum speed achieved) injected with either 70 µmol/kg of Cb (closed circle), Cbi (open square), CoTMPyP (closed square) or 50 µmol/kg CoN_4[11.3.1] (open diamond) and 100 µmol/kg cyanide. #$p \leq 0.05$ for Cbi & NaCN vs Cbl & NaCN. Numbers of animals (in parentheses) used in each set of experiments are as follows: NaCN (16), saline (13), Cb (8), Cbi (8), CoTMPyP (6), CoN_4[11.3.1] (7), Cb & NaCN (7), Cbi & NaCN (7), CoTMPyP & NaCN (6) and CoN_4[11.3.1] & NaCN (7).

Comparison of Neuromuscular Recovery in Mice Administered Cb, Cbi, CoTMPyP and CoN_4[11.3.1] Prior to Acute (Sub-Lethal) Cyanide Exposures. Before testing any ameliorative capabilities, an initial set of experiments was performed in which the cobalt compounds were administered in the absence of cyanide to investigate the presence of possible undesirable side effects (Figure 28C). Interestingly, the performance of mice on the RotaRod device was enhanced at 15 and 30 min after the administration of Cb (filled circles, Figure 28C, $p < 0.01$ compared to saline shams in Figure 28B); the effect being quite small, but reproducible over multiple days of testing. In contrast, Cbi-treated mice had a significantly decreased rate of performance on the RotaRod device at 15 and 30 min post-cyanide administration (open squares, Figure 28C, $p < 0.01$ compared to saline shams in Figure 28B). From about 90 min onwards, the data obtained for the animals treated with Cb, Cbi and the saline shams were indistinguishable (Figure 28C). There were no significant differences in the entire experimental range from 15 min – 24 hr between the performances of either CoTMPyP- or CoN_4[11.3.1]-treated animals (filled squares and open circles, Figure 28C) when compared to the performance of the sham mice injected with saline (Figure 28B).

In a second set of experiments, animals were injected with the cobalt cyanide-scavenging compounds 5 min prior to the toxicant dose, then tested every 15 min for 2.5 hr and again at 24 hr (Figure 28D). Mice administered 70 μmol/kg Cb (5 min before the toxicant dose) had significantly improved performance up to 105 min after the cyanide injection (filled circles, Figure 28D) when compared to mice receiving cyanide alone (filled squares, Figure 28B). Interestingly, when comparing the performance of cyanide-challenged mice given the different antidotal compounds, the performance of the mice administered Cb was significantly improved compared to the others at the earliest time (15 min) after the cyanide intoxication (filled circles, Figure 28D). Otherwise,

the performances of the cyanide-challenged mice administered Cb, Cbi, CoTMPyP, or CoN_4[11.3.1] were not significantly different (Figure 28D). Compared to the results for control animals given cyanide only (filled squares, Figure 28B) all the test compounds appeared to be significantly antidotal in mice in the 15 min to ~90 min window. Consistent with a previous report [69] there appears to be no persistent impairment of neuromuscular coordination detectable as judged by RotaRod testing at 24 hr in any group that received cyanide, irrespective of whether antidote was also given, or not (Figure 28B,C and D). In fact, no impairment was observed at 2 weeks time post toxicant and/or antidote for any mice using the RotaRod testing (see Supporting Information).

Importance of the Essentially Irreversible Kinetics of Antidotal Cyanide-Scavenging Compounds. We have now repeatedly argued [69, 176] that the cobalt-based compounds Cb, Cbi, CoTMPyP and CoN_4[11.3.1], even if administered in their Co(III) forms, are all quickly converted to Co(II) forms in circulating blood due to the presence of endogenous reductants such as ascorbate. Following cyanide binding, however, the reduction potentials of the central cobalt ions become lowered to the extent that the cyanide adducts revert to oxidized forms. This is crucially important because Co(III) complexes are typically more substitution inert than their Co(II) counterparts [118, 249, 250] and the cyanide forms are thus stabilized, so that they may be excreted rather than assist in systemic redistribution of the toxicant. This point of view is, seemingly, reinforced by the observation that assimilation of the cobalamin cofactor in B_{12}-dependent enzymes requires reductive decyanation of cyanocobalamin catalyzed by its chaperone [251]. As further demonstration of this principal, we undertook a set of experiments employing gallium nitrate as a trial antidote to both cyanide and sulfide (*i.e.* H_2S/HS^-) intoxication in mice. Ga(III) is known to be a relatively safe ion when introduced into mammals, including humans, for other purposes [252, 253]. It is also well-known to form a stable complex with cyanide, but this is

substitution labile rather than inert [254, 255]. Accordingly, when attempts were made to investigate Ga(III) as a cyanide antidote in mice using the righting-recovery procedure, no effect was observed, beneficial or otherwise (Table 14). On the other hand, the similarly acting mitochondrial poison sulfide [22, 256] forms a precipitate with Ga(III) [257]. This is not a readily reversible process and, consequently, Ga(III) was clearly efficacious in ameliorating sulfide intoxication (Table 14) in keeping with the suggestion that a degree of irreversibility associated with the final adduct is a highly desirable characteristic for effective scavenging.

Table 14. Effects of $Ga(NO_3)_3$ on Cyanide and Sulfide Toxicity in Swiss-Webster Mice.

	Survivors/group (%)	Time until deaths of non-survivors (min)
5 mg/kg NaCN only (control)	50/66 (76%)	~3
50 mg/kg $Ga(NO_3)_3$ given 1 min after NaCN	6/8 (75%)	~3
18 mg/kg NaHS only (control)	16/24 (67%)	< 4
50 mg/kg $Ga(NO_3)_3$ given 1 min after NaHS	8/8 (100%)	———

Discussion

When given prophylactically, Cb, Cbi, CoTMPyP and CoN_4[11.3.1] all clearly ameliorate the toxic effects of cyanide as assessed by righting recovery (Figure 25) and restoration of neuromuscular coordination (Figure 28D compared to filled squares in Figure 28B). In all cases, including mice given only the toxicant, or only one of the cobalt compounds, recovery of normal neuromuscular function at 24 hr appeared complete (Figure 28B, 28C, 28D). That is, there was no sign of any long-term impairment (up to 2 weeks), providing encouragement for the continued

development of the latter three compounds as potential cyanide antidotes. The slightly toxic effect detected in the case of Cbi at 15 and 30 min (open squares, Figure 28C) is, however, of some minor concern. The value of these behavioral assessments of toxicity and antidote-dependent recovery with conscious mice should be fully appreciated – in addition to providing information not necessarily accessible with unconscious animals, such experiments avoid the well-known confounding complications of anesthesia [62, 130].

In experiments where the putative antidotes were administered after the toxicant, Cbi, CoTMPyP and CoN_4[11.3.1] (Figures 26B, 26C and 26D) all performed better than Cb (Figure 26A). Particularly if the lower dose is considered, CoN_4[11.3.1] (Figure 26D, 50 μmol/kg) was measurably better than Cbi and CoTMPyP (Figures 26B and 26C, each 70 μmol/kg) as a therapeutic. This lower effective dose of CoN_4[11.3.1] could, of course, be of importance for treating higher levels of cyanide intoxication than would be possible with the other compounds. In relation to management of public health emergencies, there is currently some interest in stockpiling cyanide antidotes and, consequently, other factors may become important for ranking these cobalt compounds. The relatively low cost of CoN_4[11.3.1] (Table 12) should not be overlooked in this regard. Also, however, the storage requirements for Cb and Cbi (biological materials) are that they be refrigerated (-20 °C) whereas we have stored CoTMPyP, CoN_4[11.3.1] and similar complexes in darkened, screw-top vials at room temperature for months (years in some cases) without noticeable decomposition (assessed by mass spectrometry and spectral analysis) beyond some slow oxidation of Co(II) to Co(III).

The stability constants (K) of all four cyano adducts (Table12) are large enough to ensure that the compounds are efficient cyanide scavengers. For example, if Cb and cyanide are present in approximately equal quantities, $K = 105$ implies that > 99.99% of the cyanide will be bound to

the Co(III) center; alternately, if CoN_4[11.3.1] and cyanide are present in approximately equal amounts, K = 3 x 105 (the lower end of the possible range – see footnote g to Table 12) implies that > 99.9% of the cyanide will be bound to the Co(II) center; in the other two cases, the stability constants are larger and even more of the cyanide will be scavenged. Furthermore, since the rate constants for cyanide binding by the other three compounds are all similar or larger than that for Cb, we may argue that equilibrium is attainable in each case at fast enough rates that the relative efficacies of these compounds as antidotes should simply be given by the number of exogenous cyanide anions they can bind. In other words, the antidotal capabilities of Cbi, CoTMPyP and CoN_4[11.3.1] (all binding 2 CN^- per Co(III)) should be comparable on a molar basis and they should be better than Cb (binding 1 CN^- per Co(III)) by no more than a factor of approximately 2. In the present investigation, this is exactly what we find, the "effectiveness ratios" of Cbi, CoTMPyP and CoN_4[11.3.1] compared to Cb are all approaching 2 (Table 13). It requires comment that prophylactically administered Cbi has previously been reported to be 3-fold to 11-fold more efficient than Cb as an *in vivo* cyanide scavenger in mice [244]. Certainly, the anesthesia used in this previous study may have influenced the results, but when the toxicant was given as NaCN solutions (ip injections) the 3-fold increased effectiveness of Cbi compared to Cb observed is not very different from the 2-fold increase we suggest here to be limiting. Much more surprising is the finding that Cbi was 11-fold more effective than Cb when the toxicant dose was delivered as inhaled HCN. This simply does not appear possible if the only relevant activity of Cbi is straightforward complexation of 2 CN^- per Co(III); strongly suggesting that there must be some presently unrecognized aspect to the antidotal action of Cbi particularly associated with toxicant inhalation. In the earlier study [244] the animals were given the antidotes 15 min before starting the

HCN dose which was then continued for a period of 30 min, so there was plenty of time for other processes to become involved.

In the therapeutic experiments (Figure 26) where antidotes were given after the toxicant, the CoN_4[11.3.1] performed best of the four when given 1 min after the cyanide (Table 13) perhaps reflecting its significantly faster reaction rate than the others (Table 12). All the compounds were comparably effective when given up to 2 min after the cyanide, but if a delay of 5 min was allowed before giving antidote, none were effective (Figure 26). We interpret this observation to indicate that within 5 min of receiving a cyanide dose the toxicant has bound to its molecular target, namely, cytochrome *c* oxidase in the mitochondria, and the cobalt-based scavengers are not able to reverse the inhibition of the enzyme as we have unambiguously demonstrated for at least Cb and Cbi [246]. To the contrary, sodium nitrite clearly does work therapeutically if given as an antidote at times longer than 5 min after the cyanide dose (Figure 27). It should be noted that the majority of these nitrite data were obtained employing older mice and from a different supplier compared to the other animal experiments. The older control animals given no antidote exhibited a mean righting recovery time of 29 min, enabling effectiveness to be demonstrated if the nitrite was given up to 20 min following the cyanide dose. On the other hand, younger control animals given no antidote exhibited a mean righting recovery time of 24 min, enabling effectiveness to be demonstrated if the nitrite was given up to only about 15 min following the cyanide dose. It is to be understood that the 15-20 window of effectiveness represents a limitation of the method, it does not necessarily mean that the nitrite would be ineffective if the therapeutic doses were to be delayed for more than 20 min following a toxicant dose in some other poisoning scenario. We have previously shown that nitric oxide (NO) is able to reverse cyanide inhibition of cytochrome *c* oxidase [59, 65] and used this observation to infer a plausible mechanism by which nitrite anion (acting as an NO donor)

may be antidotal toward cyanide intoxication independent of methemoglobin formation [62, 157]. Therefore, unlike the cobalt-based scavengers, nitrite anion can reverse the toxic effect of cyanide at its principal molecular target by reversing the inhibition of cytochrome *c* oxidase; hence its broader window of action. It follows that there are numerous experimental protocols that could inadvertently be undertaken that might misleadingly suggest cobalt-based scavengers (or any other cyanide-complexing compounds) can be of therapeutic benefit 5 min or more after the toxicant dose has ceased. For instance, if a putative cyanide scavenger is given as a nitrite salt, or NO complex, then this really represents a combination therapy, not an unambiguous assessment of the scavenger. Less obviously, any animal model in which cyanide scavengers are to be tested, but where there may be inflammation (with accompanying upregulation of inducible nitric oxide synthase), or where analgesics/anesthetics are employed (at least some of which are known [62] to behave like stimulators of endogenous NO production), a combination therapy is probably, if unintentionally, being investigated.

We have previously shown [69, 176, 246] that the cobalt-based compounds Cb, Cbi, CoTMPyP and CoN_4[11.3.1] are all quickly converted to Co(II) forms by reductants at the levels they are present in circulating blood, facilitating suitably rapid cyanide binding. Upon binding cyanide, however, the reduction potentials of the central cobalt ions become lowered to the extent that the cyanide adducts become oxidized to stable forms that may be excreted. Here, we have described a set of experiments (Table 11) employing gallium nitrate as a trial antidote to both cyanide and sulfide (i.e. H_2S/HS^-) providing further confirmation of the importance that the final adduct is essentially substitution inert – otherwise, the intended scavenger will only assist in the systemic redistribution of the toxicant. The overall similarity in the relevant physicochemical properties (Table 12) of the cobalt compounds able to bind two cyanide anions per metal ion, namely Cbi,

CoTMPyP and CoN_4[11.3.1] and the comparability of their performances in the antidotal trials (Table10) suggests that many cobalt complexes with four approximately equatorial nitrogen donors should display the necessary oxidation-reduction chemistry and ligand substitution characteristics suitable for application as cyanide scavengers. Such compounds may, however, differ considerably the type of macromolecular biomolecules with which they interact. That is, their relative toxicities will likely depend to some extent on the peripheral structures of their chelating ligands.

Supplemental Materials and Figures

Raw data for the righting recovery of cyanide intoxicated mice establishing prophylactic dose response, therapeutic time responses using Cbi, Cb, CoTMPyP and CoN_4[11.3.1]. Raw data of RotaRod assessments for cyanide intoxicated mice and those treated with Cbi, Cbl, CoTMPyP.

Mouse experiments were carried out as previously presented in the Experimental section. In addition to all individual data points used to make Figures 2, 3 and 5, we have included means and standard deviations of each data set.

Table 15. Raw data of the dose-response profiles for prophylactically administered Cb, Cbi, CoTMPyP and CoN_4[11.3.1] in cyanide-intoxicated male mice as determined by righting-recovery times (as shown in Figure 25).

Mouse #	Righting Recovery Time (min)																				
	NaCN	Cb (µmol/kg)					Cbi (µmol/kg)					CoTMPyP (µmol/kg)					CoN_4[11.3.1] (µmol/kg)				
		30	40	45	50	70	30	40	45	50	70	30	40	45	50	70	30	40	45	50	70
1	20.5	24.5	23.5	16.5	16.0	5.0	7.0	0.0	15.0	25.3	14.0	10.5	6.0	5.0	0.0	0.0	25.0	7.0	0.0	0.0	5.5
2	34.5	30.0	26.5	17.0	20.5	5.0	28.0	28.0	0.0	27.0	0.0	5.0	16.0	8.0	10.0	0.0	38.0	14.0	4.0	0.0	0.0
3	22.0	7.0	24.0	30.0	17.0	8.5	0.0	16.5	15.0	4.5	10.0	19.0	0.0	5.5	5.5	0.0	25.0	7.5	17.5	0.0	3.5
4	30.0	22.5	23.5	26.0	22.0	6.5	24.0	19.0	0.0	6.0	0.0	5.0	6.0	7.0	6.0	11.0	25.0	35.0	0.0	0.0	0.0
5	24.0	28.0	5.5	22.0	8.5	18.0	7.0	23.0	0.0	5.0	0.0	30.0	11.0	19.5	0.0	0.0	0.0		4.5	0.0	6.0
6	23.0		19.5	13.0	17.5	19.5	31.0	17.0	15.0	20.0	0.0	21.0	19.0	5.5	6.0	0.0	19.0			4.0	4.0
7	24.0		20.0	21.0	26.5	9.0	13.0	10.0	21.0	6.0	0.0			9.0	8.0	0.0				7.0	
8	25.5					0.0	22.0	20.0	0.0	7.0	21.0					0.0				8.0	
9	20.5					5.5			0.0	4.5	7.0					0.0				0.0	
10	22.0					9.0			8.0		0.0					0.0				8.0	
11	23.5					7.5					0.0					0.0				0.0	
12	9.5					8.0					4.5					0.0				7.5	
13	17.0					15.0					6.0					7.0				6.5	
14	25.0										7.0					0.0					
15	35.5										7.0										
16	25.5										0.0										
17	17.5																				
18	22.0																				
19	24.0																				
20	32.0																				
21	25.5																				
22	17.5																				
23	11.0																				
24	25.0																				
25	23.0																				
26	6.5																				
27	29.0																				
28	26.0																				
29	25.0																				
30	27.0																				
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34	15.0																				
35	13.5																				
36	30.0																				
37	16.0																				
38	30.0																				
39	25.0																				
40	28.0																				
41	34.0																				
42	29.0																				
43	27.0																				
44	9.0																				
45	13.0																				
46	23.0																				
47	31.0																				
48	33.0																				
49	21.0																				
50	25.0																				
51	23.0																				
52	27.0																				
53	23.5																				
54	34.0																				
Mean	23.8	22.4	20.4	20.8	18.3	9.0	16.5	16.7	7.4	11.7	4.8	15.1	9.7	8.5	5.1	1.3	22.0	15.9	5.2	3.2	3.2
Std. Dev.	6.7	9.1	7.0	5.9	5.6	5.5	11.3	8.5	8.4	9.5	6.2	10.0	7.1	5.1	3.8	3.4	12.5	13.1	7.2	3.7	2.6
Std. Error	0.9	4.1	2.6	2.2	2.1	1.5	4.0	3.0	2.7	3.2	1.5	4.1	2.9	1.9	1.4	0.9	5.1	6.6	3.2	1.0	1.1

Swiss-Webster male mice (7-8 weeks of age) were injected (ip) with either Cb, Cbi, CoTMPyP or CoN_4[11.3.1] 30, 40, 45, 50, and 70 µmol/kg in saline, 5 min before the administration of 100 µmol/kg NaCN (in saline). Cyanide intoxicated animals received 100 µmol/kg NaCN injections.

Table 16. Raw data of righting recovery times for therapeutic effects of cobalt-containing compounds in male mice after cyanide intoxication (as shown in Figure 26).

Mouse #	Righting Recovery Time (min)																
	NaCN	Cb				Cbi				CoTMPyP				CoN_4[11.3.1]			
		-5min	+1min	+2min	+5min	-5min	+1min	+2min	+5min	-5min	+1min	+2min	+5min	-5min	+1min	+2min	+5min
1	20.5	5.0	17.0	8.0	15.5	14.0	16.0	9.0	14.5	0.0	0.0	22.0	7.5	0.0	7.0	15.0	20.0
2	34.5	5.0	0.0	22.0	22.5	0.0	0.0	10.5	16.5	0.0	20.0	5.5	32.0	0.0	0.0	9.8	22.5
3	22.0	8.5	9.5	12.0	21.5	10.0	7.0	12.0	17.0	0.0	14.0	13.0	19.0	0.0	6.0	9.5	30.0
4	30.0	6.5	18.0	14.5	17.0	0.0	9.5	13.5	19.0	11.0	14.0	13.0	21.0	0.0	4.5	19.0	19.5
5	24.0	18.0		13.5		0.0	6.0		26.0	0.0	8.5	5.5	18.0	0.0	6.5	6.5	22.0
6	23.0	19.5				0.0	4.0			0.0	4.0	9.0		7.0	6.0	20.5	26.0
7	24.0	9.0				0.0				0.0	6.0			8.0	0.0		
8	25.5	0.0				21.0				0.0				0.0	0.0		
9	20.5	5.5				7.0				0.0				8.0	6.5		
10	22.0	9.0				0.0				0.0				0.0	4.0		
11	23.5	7.5				0.0				0.0				7.5	7.0		
12	9.5	8.0				4.5				0.0				6.5	0.0		
13	17.0	15.0				6.0				7.0				4.0			
14	25.0					7.0				0.0							
15	35.5					7.0											
16	25.5					0.0											
17	17.5																
18	22.0																
19	24.0																
20	32.0																
21	25.5																
22	17.5																
23	11.0																
24	25.0																
25	23.0																
26	6.5																
27	29.0																
28	26.0																
29	25.0																
30	27.0																
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32	29.0																
33	28.0																
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36	30.0																
37	16.0																
38	30.0																
39	25.0																
40	28.0																
41	34.0																
42	29.0																
43	27.0																
44	9.0																
45	13.0																
46	23.0																
47	31.0																
48	33.0																
49	21.0																
50	25.0																
51	23.0																
52	27.0																
53	23.5																
54	34.0																
Mean	23.8	9.0	11.1	14.0	19.1	4.8	7.1	11.3	18.6	1.3	9.5	11.3	19.5	3.2	4.0	13.4	23.3
Std. Dev.	6.7	5.5	8.3	5.1	3.4	6.2	5.4	1.9	4.4	3.4	6.9	6.2	8.7	3.7	3.1	5.7	4.0
Std. Error	0.9	1.5	4.2	2.3	1.7	1.5	2.2	1.0	2.0	0.9	2.6	2.5	3.9	1.0	0.9	2.3	1.6

Swiss-Webster mice were injected with either 70 µmol/kg of Cb, Cbi, CoTMPyP or with 50 µmol/kg of CoN_4[11.3.1] at 1, 2, or 5 min post cyanide intoxication. NaCN given alone (100 µmol/kg) and with the test compounds (at the doses above) injected prophylactically at -5 min are included for comparison purposes.

Table 17. Raw data for the neuromuscular coordination comparison, using RotaRod testing, of Cb, Cbi, CoTMPyP and CoN_4[11.3.1] and of their amelioration following NaCN administration in male Swiss-Webster mice (as shown in Figure 28 B,C and D).

	Maximum Speed Achieved (rpm)																														
	Saline Control															NaCN															
Training Period															Mean / SD																Mean / SD
1-2	10.5	9.5	7.5	11.5	8.0	5.0	7.0	8.0	8.5	4.0	7.5	6.5	16.5		8±3	9.0	5.5	5.5	6.5	6.5	8.5	11.0	5.5	6.0	7.0	8.5	8.0	6.0	8.5	10.5	10.5	8±2
3-4	17.0	10.5	13.5	14.0	13.5	8.0	7.5	11.5	13.5	6.0	9.5	10.5	20.0		12±4	13.5	13.0	6.5	5.5	11.0	13.5	10.5	5.5	10.5	8.0	15.0	13.5	15.0	12.5	11.0	14.0	11±3
5-6	17.5	10.5	19.0	18.0	14.0	8.0	9.0	18.0	14.0	4.5	13.0	11.0	22.0		14±5	15.5	12.0	7.5	8.5	15.5	16.5	15.0	8.0	14.0	9.5	12.5	15.0	10.5	13.0	14.0	20.0	13±3
7-8	17.0	10.5	17.0	18.0	16.0	16.0	8.5	21.0	13.0	6.5	14.5	14.0	22.0		15±5	15.5	12.0	12.5	14.0	14.0	12.5	17.0	11.0	21.5	10.0	12.5	17.0	15.5	16.0	16.5	21.0	15±3
Pre-IP	14.0	10.7	17.7	11.7	13.0	20.3	14.7	16.0	15.0	8.3	13.3	15.7	21.7		15±4	19.0	11.0	11.7	10.7	11.0	13.0	13.7	11.7	14.7	9.3	12.7	15.7	16.3	13.7	16.0	13.7	13±3
Post-IP Recovery Period																															
15	14.7	21.0	12.7	13.7	21.0	5.0	15.3	14.3	16.7	6.7	17.7	17.3	22.0		15±5	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0±0
30	16.7	20.7	14.0	15.3	22.0	22.0	19.0	14.3	14.7	9.3	19.3	21.3	22.0		18±4	0.0	16.3	4.3	0.0	12.7	0.0	0.0	5.0	6.7	0.0	0.0	0.0	7.3	8.7	0.0	4.3	4±5
45	21.3	17.0	11.0	19.0	22.0	19.3	21.7	19.0	19.0	15.7	21.3	21.7	22.0		19±3	8.7	10.0	8.0	6.7	15.3	6.7	4.3	4.7	5.3	13.3	0.0	3.0	10.0	6.3	3.3	5.3	7±4
60	21.0	28.7	12.7	12.0	22.0	17.7	19.7	13.7	13.3	18.0	21.0	20.3	22.0		19±5	29.7	16.3	19.0	9.0	12.3	4.0	5.3	2.7	5.0	9.7	0.0	5.0	16.0	16.7	11.3	22.0	12±8
75	16.7	22.0	7.7	20.7	21.3	18.0	22.0	14.7	10.7	18.3	20.3	21.7	22.0		18±5	19.3	9.7	13.0	11.0	14.0	4.3	5.0	4.3	9.7	10.3	4.0	5.7	18.0	13.7	8.0	22.0	11±6
90	16.3	19.7	10.7	13.7	19.0	16.0	19.3	11.0	12.0	15.3	21.0	18.3	22.0		16±4	20.0	10.3	14.3	11.3	19.0	4.0	4.3	9.0	11.7	10.7	0.0	8.7	19.0	16.0	11.7	20.7	12±6
105	22.0	18.7	19.3	13.0	20.0	20.0	14.7	12.3	13.0	16.3	22.0	19.3	22.0		18±4	19.3	13.3	19.0	13.0	19.3	4.0	4.0	10.0	13.3	9.0	4.7	12.0	20.0	15.3	12.3	22.0	13±6
120	22.0	19.3	13.7	9.0	16.3	16.3	21.0	15.3	17.3	20.7	19.7	21.7	22.0		18±4	22.0	11.3	12.0	14.3	15.0	12.7	5.0	10.3	15.0	7.3	5.3	14.7	22.0	17.0	16.3	22.0	14±5
135	22.0	18.0	14.0	16.7	12.0	16.0	17.7	11.0	15.7	18.7	17.7	22.0	22.0		17±4	22.0	9.7	11.3	13.0	22.0	18.7	5.0	11.7	17.0	11.7	6.0	14.3	22.0	19.3	14.7	22.0	15±6
150	22.0	22.0	14.7	14.3	16.0	18.0	20.0	17.0	13.7	16.7	21.7	22.0	22.0		18±3	22.0	13.3	16.0	12.7	22.0	18.3	10.3	15.7	19.7	12.7	5.0	17.3	22.0	22.0	16.3	22.0	17±5
24 hr	18.0	16.3	11.7	17.3	6.3	18.0	10.3	7.0	10.3	12.7	13.3	15.0	22.0		14±5	18.0	13.3	12.7	9.0	21.7	16.0	13.7	9.7	17.0	10.7	4.3	20.0	20.3	20.0	22.0	17.3	15±5

Mice were trained on the RotaRod 24 h before injection, and a baseline performance was obtained 1 h before injection (Pre-ip). Mice were tested every 15 min after injections for 2.5 h to and again at 24 h to assess recovery

A. Raw RotaRod data of mice injected (ip) with 70 µmol/kg of cobalamin (Cbl) cobinamide (Cbi), CoTMPyP or 50 µmol/kg CoN_4[11.3.1].

	Maximum Speed Achieved (rpm)																																										
	Cb									Cbi										CoTMPyP										CoN_4[11.3.1]													
Training Period									Mean / SD										Mean / SD										Mean / SD										Mean / SD							
1-2	8.0	11.0	6.5	12.5	6.5	5.5	17.5	11.0	10±4	6.5	6.5	13.0	9.0	6.0	4.0	7.5	9.5	8±3	8.0	13.5	11.0	14.0	9.5	8.0	11±3	6.0	5.5	8.5	11.0	5.0	4.0	5.0	6±2										
3-4	11.0	15.0	12.5	15.0	6.0	6.0	11.5	16.0	12±4	5.0	5.0	16.5	10.5	7.0	5.5	8.0	14.5	9±4	12.5	13.0	9.5	16.5	9.5	12.0	12±3	5.5	7.0	13.5	18.0	4.5	6.0	5.5	9±5										
5-6	19.0	16.5	15.5	15.5	11.0	13.0	16.5	18.0	16±3	11.0	11.0	21.0	13.0	7.5	12.0	9.5	16.0	13±4	12.0	13.5	11.5	18.0	11.0	12.0	13±3	9.0	11.0	14.5	21.0	4.5	5.5	9.5	11±6										
7-8	18.5	16.5	14.0	16.0	11.0	15.5	14.0	19.0	16±3	14.5	15.5	22.0	15.0	8.5	11.5	8.5	15.0	14±4	13.0	16.5	13.5	20.5	16.5	18.0	16±3	12.0	10.5	15.5	19.5	13.5	11.0	16.0	14±3										
Pre-IP	17.7	11.0	15.7	9.7	14.0	11.3	14.7	19.7	14±3	14.3	13.3	19.0	12.3	8.0	14.0	9.0	14.7	13±3	9.3	18.3	13.0	18.3	18.3	16.3	16±4	14.0	12.7	13.0	15.7	16.0	10.7	11.3	13±2										
Post-IP Recovery Period																																											
15	22.0	22.0	21.0	17.0	14.0	13.3	19.3	22.0	19±4	11.0	8.7	21.0	5.3	6.7	10.7	12.3	9.0	11±5	5.3	14.0	9.7	20.3	19.7	13.7	14±6	15.0	15.3	8.7	17.3	13.0	14.0	10.0	13±3										
30	22.0	20.3	21.7	22.0	14.0	18.3	20.7	22.0	20±3	13.7	18.0	13.3	4.7	12.3	14.3	11.3	15.0	13±4	10.0	21.7	10.0	22.0	22.0	13.3	17±6	21.0	20.3	12.0	18.0	10.7	15.7	12.3	16±4										
45	21.7	17.3	22.0	21.3	13.0	14.3	22.0	22.0	19±4	13.7	16.0	21.7	8.7	14.0	14.3	17.3	20.0	16±4	7.7	19.3	9.7	22.0	22.0	8.0	15±7	21.3	17.0	16.3	17.0	16.0	15.0	6.7	16±4										
60	20.5	22.0	22.0	22.0	12.0	14.0	22.0	22.0	20±4	9.7	16.0	22.0	13.0	8.7	13.7	17.0	17.0	15±4	10.3	20.7	11.7	21.3	14.7	10.7	15±5	21.7	22.0	16.0	13.0	8.7	13.7	4.3	14±6										
75	21.7	19.3	22.0	22.0	9.3	11.3	21.3	22.0	19±5	11.0	17.0	22.0	14.7	10.7	14.0	19.3	21.0	16±4	12.0	21.3	10.7	22.0	17.3	17.3	17±5	21.3	19.7	13.0	13.7	13.3	8.7	14.7	15±4										
90	19.0	20.7	22.0	18.3	8.3	15.0	18.7	19.0	18±4	8.3	22.0	21.0	14.7	13.3	13.7	20.7	22.0	17±5	10.7	17.7	9.3	21.0	14.7	12.3	14±4	19.7	18.7	11.0	15.0	11.7	14.7	11.3	15±4										
105	16.3	20.3	21.3	20.7	7.3	14.7	17.0	13.0	16±5	7.3	13.0	21.3	14.3	9.0	11.3	18.3	22.0	15±5	8.7	12.7	11.0	22.0	17.0	19.7	15±5	18.3	19.7	16.3	22.0	9.3	9.7	6.7	15±6										
120	20.0	21.3	20.3	21.3	9.3	13.0	16.3	19.3	18±4	10.3	16.0	21.0	12.0	12.3	14.3	16.3	21.7	15±4	11.0	21.0	11.3	19.0	13.0	18.3	16±4	19.3	17.0	16.3	22.0	9.0	8.7	11.0	15±5										
135	20.0	20.3	22.0	21.7	9.0	15.3	14.3	17.3	17±4	11.0	16.3	22.0	13.7	11.3	18.0	13.3	22.0	16±4	11.7	22.0	13.0	17.0	11.7	13.7	15±4	15.7	19.3	15.0	22.0	10.3	9.3	12.0	15±5										
150	14.7	19.3	22.0	20.3	8.0	16.3	17.3	17.0	17±4	13.0	15.3	22.0	13.7	11.0	18.3	13.3	22.0	16±4	11.7	22.0	13.7	19.3	12.0	21.0	17±5	20.0	16.7	15.3	22.0	13.7	8.7	13.7	16±4										
24 hr	17.7	18.0	22.0	7.0	7.3	12.0	13.0	7.3	13±6	14.7	10.3	21.3	10.3	8.0	11.7	15.3	22.0	14±5	13.7	19.7	7.7	22.0	22.0	17.3	17±6	11.7	10.3	19.3	20.0	17.3	11.7	16.7	15±4										

B. Raw RotaRod data of mice injected (ip) with 70 µmol/kg of cobalamin (Cbl) cobinamide (Cbi), CoTMPyP or 50 µmol/kg CoN_4[11.3.1].

Table 17 Continued

	Maximum Speed Achieved (rpm)																													
	Cb + NaCN								Cbi + NaCN								CoTMPyP + NaCN								CoN_4[11.3.1] + NaCN							
Training Period								Mean / SD								Mean / SD							Mean / SD								Mean / SD
1-2	12.5	17.0	7.0	7.5	7.5	7.5	6.0	9±4	18.5	10.0	8.5	4.5	5.5	5.5	10.0	9±5	10.0	10.5	8.5	5.5	11.0	9.0	9±2	7.5	6.0	5.0	12.0	6.5	10.5	8.0	8±3
3-4	14.5	19.0	9.5	15.0	13.0	11.5	9.0	13±3	16.5	18.5	12.0	6.0	10.0	8.0	14.0	12±5	17.0	13.5	8.0	8.0	14.5	12.5	12±4	9.0	9.0	7.0	11.5	6.0	10.0	13.5	9±3
5-6	10.0	19.0	12.5	16.5	16.0	16.5	13.0	15±3	17.0	22.0	11.0	10.0	10.5	12.0	12.5	14±4	22.0	20.0	13.0	16.0	14.0	15.5	17±4	9.0	13.0	11.5	15.5	15.0	13.5	18.0	14±3
7-8	14.5	21.5	14.0	20.5	15.5	17.0	15.5	17±3	16.5	20.0	14.0	5.0	10.0	13.5	13.5	13±5	22.0	22.0	18.5	21.0	13.5	15.0	19±4	10.5	15.5	11.0	22.0	18.5	16.0	19.5	16±4
Pre-IP	9.3	19.7	14.3	18.0	15.3	17.3	15.0	16±3	16.0	16.3	16.7	6.3	11.0	13.0	15.7	14±4	16.7	16.7	13.3	17.0	8.3	14.3	14±3	9.7	13.7	11.0	18.3	16.3	14.0	19.7	15±4
Post-IP Recovery Period																															
15	6.3	17.7	14.0	20.7	16.0	13.3	3.3	13±6	0.0	0.0	9.3	7.0	6.7	11.0	9.3	6±4	7.3	0.0	0.0	11.0	9.0	14.0	7±6	2.0	7.0	3.0	20.0	6.3	0.0	18.0	8±8
30	6.3	20.0	16.7	22.0	12.7	12.3	10.3	14±6	0.0	15.3	15.7	7.0	5.7	5.3	8.7	8±6	3.3	15.7	2.7	16.3	9.3	22.0	12±8	7.0	5.3	5.0	17.0	5.0	14.7	15.0	10±5
45	10.0	21.3	21.3	22.0	12.7	15.0	13.0	16±5	7.7	13.3	21.3	10.0	10.0	7.0	10.3	11±5	6.0	20.3	6.0	16.7	8.3	20.7	13±7	5.0	5.3	5.0	15.0	9.0	15.7	16.3	10±5
60	13.5	19.0	21.5	22.0	17.5	22.0	13.5	18±4	12.0	15.7	22.0	10.0	11.7	15.3	11.3	14±4	22.0	19.3	6.3	10.7	10.7	19.7	15±6	4.3	11.0	6.0	19.7	9.3	17.7	17.3	12±6
75	11.7	22.0	22.0	21.3	19.3	21.3	11.3	18±5	9.7	19.7	22.0	7.0	13.3	16.7	17.3	15±5	22.0	22.0	10.3	16.7	10.7	22.0	17±6	5.0	11.7	6.3	14.7	15.0	19.7	17.7	13±6
90	13.0	21.7	21.3	20.3	19.7	22.0	12.0	19±4	13.0	19.3	22.0	12.3	11.7	15.0	15.0	15±4	20.0	22.0	11.0	16.0	8.3	32.0	18±9	6.0	10.0	7.3	13.7	15.3	20.0	20.7	13±6
105	13.7	20.7	21.7	20.7	13.7	21.7	11.7	18±4	15.0	21.7	21.3	12.0	10.3	11.3	18.0	16±5	18.3	22.0	13.0	19.7	7.7	22.0	17±6	5.3	15.7	6.0	16.0	18.7	21.0	19.7	15±6
120	13.3	22.0	17.3	20.0	12.7	22.0	13.0	17±4	13.0	18.3	21.3	12.7	9.7	16.7	17.3	16±4	15.0	20.0	12.7	19.0	6.7	22.0	16±6	6.0	14.0	9.3	11.3	18.0	18.7	22.0	14±6
135	16.0	20.0	16.0	21.7	13.3	22.0	12.7	17±4	14.0	21.3	22.0	14.0	10.0	12.7	16.0	16±4	17.3	16.0	12.7	16.7	7.3	22.0	15±5	5.3	10.3	11.0	11.3	20.3	13.0	20.3	13±5
150	15.0	20.7	15.0	19.0	14.7	22.0	13.7	17±3	19.3	19.0	22.0	13.7	10.0	12.3	15.3	16±4	20.0	21.0	12.0	21.7	6.3	22.0	17±6	4.7	15.3	10.7	20.0	19.3	18.0	19.3	15±6
24 hr	10.3	22.0	18.3	20.7	9.7	19.7	8.7	16±6	14.0	13.0	21.3	10.0	7.7	12.0	13.3	13±4	18.7	21.3	10.3	19.7	6.3	18.0	16±6	8.0	11.0	7.3	20.0	12.0	18.0	11.0	12±5

C. Comparison of performance of mice injected with either 70 µmol/kg of cobalamin cobinamide, CoTMPyP or 50 µmol/kg CoN_4[11.3.1] (5 min before cyanide dose) and 100 µmol/kg cyanide

Mouse/injection and rpm obtained	NaCN	Cb	Cbi	CoTMPyP	CoN4[11.3.1]	Cb&NaCN	Cbi&NaCN	CoTMPyP&NaCN	CoN4[11.3.1]&NaCN
#1	14	12.5	11	16	19	14	16	18	17
#2	13	16	13	20	16	14	18	22	16
#3	20	17	15	12	22	16	13	12	13
#4	17	20	16	15	13	17	15	15	16

D. Comparison of RotaRod performance of mice injected with either 70 µmol/kg of cobalamin cobinamide, CoTMPyP or 50 µmol/kg CoN_4[11.3.1] (given alone or 5 min before cyanide dose) and/or 100 µmol/kg cyanide at 48 hours post injection.

The kinetics of the reaction of cyanide with Co(II)N_4[11.3.1] (cobalt(II) 2,12-dimethyl-3,7,11,17-tetraazabicyclo-[11.3.1]-heptadeca-1(7)2,11,13,15-pentaenyl cation) were determined using an Applied Photophysics laser-flash/stopped-flow spectrometer (LKS.60-SX.18MV-R system) to measure the rapid reaction kinetics and the resultant data was fit with the PC Pro-K software (!SX.18MV) provided by the manufacturer. All reactions were run under pseudo-first-

order conditions (4-16 mM potassium cyanide) following a decrease in absorbance at 462 nm (a band found in the absorption spectrum of Co(II)N_4[11.3.1]) in phosphate buffer and at a thermostatically-controlled temperature. Both Co(II)N_4[11.3.1] and potassium cyanide solutions were prepared anaerobically (using a Schlenk line), prior to loading into the stopped-flow spectrophotometer. Co(II)N_4[11.3.1] was prepared in 100 mM potassium phosphate buffer and KCN was prepared in basic solution (1 mM NaOH) just prior to the reaction in order to prevent any loss of HCN. The final buffer (50 mM phosphate) pH was found to be pH 7.6, measured after the reaction occurred. The binding kinetics proved to be biphasic and very fast at 25°C, thus the reaction temperature was lowered to 10°C in order to better follow the absorbance changes. The first phase was still too rapid to be observed but the second phase was found to be linear in cyanide. This rate constant for the binding of cyanide to Co(II)N_4[11.3.1] was obtained from linear fit of the individual observed rates to the cyanide concentrations (using Kaleidegraph software) and found to be 8.0 (±0.5) x 10^4 M^{-1} s^{-1} (pH 7.6, 50 mM potassium phosphate buffer, final pH and buffer concentrations) at 10°C.

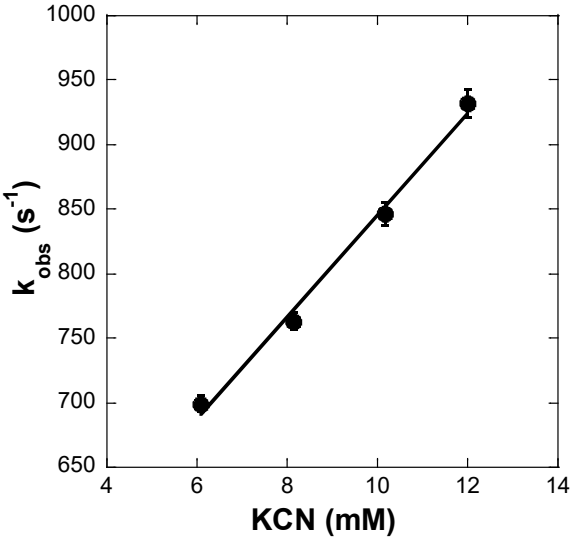

Figure 29. Kinetics of cyanide binding to Co(II)N_4[11.3.1] under anaerobic conditions.
Linear dependence of the reaction of Co(II)N_4[11.3.1]Br_2 (0.6 mM) in sodium phosphate buffer (0.1 M, pH 7.4) with KCN (prepared in 1 mM NaOH, pH 11.6) at 10°C, anaerobic conditions (final pH = 7.6, 50 mM potassium phosphate).

Appendix C The Antidotal Action of Some Gold(I) Complexes Toward Phosphine Toxicity

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Abstract

Phosphine (PH_3) poisoning continues to be a serious problem worldwide for which there is no antidote currently available. An invertebrate model for examining potential toxicants and their putative antidotes has been used to determine if a strategy of using Au(I) complexes as phosphine scavenging compounds may be antidotally beneficial. When *Galleria mellonella* larvae (or wax worms) were subjected to phosphine exposures of 4300 (±700) ppm•min over a 20 min time span, they become immobile (paralyzed) for ~35 min. The administration of Au(I) complexes auro-sodium bisthiosulfate (AuTS), aurothioglucose (AuTG) and sodium aurothiomalate (AuTM) 5 min prior to phosphine exposure resulted in a drastic reduction in the recovery time (0-4 min). When the putative antidotes were given 10 min after the phosphine exposure, all the antidotes were therapeutic, resulting in mean recovery times of 14, 17 and 19 min for AuTS, AuTG, AuTM, respectively. Since AuTS proved to be the best therapeutic agent in the *G. mellonella* model, it was subsequently tested in mice using a behavioral assessment (pole-climbing test). Mice given AuTS (50 mg/kg) 5 min prior to a 3200 (±500) ppm•min phosphine exposure exhibited behavior comparable to mice not exposed to phosphine. However, when mice were given a therapeutic dose of AuTS (50 mg/kg) 1 min after a similar phosphine exposure, only a very modest improvement in performance was observed.

Introduction

Worldwide, ingestion of pesticides is seemingly the most common method of suicide, accounting for approximately one third of all such deaths.[258, 259] Since the early 1980s, particularly in parts of Asia, phosphine (PH_3) released from pelleted phosphides has become increasingly used as the poison within this genre,[260-265] yet there appears to be no antidote currently available. Throughout North America, phosphides (particularly of aluminum and zinc) are legally obtainable from many commercial outlets in pelleted form for use as rodenticides. There are dozens of sub-lethal occupational exposures annually in the U.S.[266, 267] and occasional domestic accidents leading to fatalities in Canada and the U.S.;[268, 269] but of greater public health concern is the possibility that phosphine may be deliberately put to malicious purposes, since the phosphide pellets release the toxic gas simply upon contact with mildly acidic water. A key target for the acute toxic action of phosphine is believed to be the mitochondrion, seemingly by inhibition of cytochrome *c* oxidase (complex IV).[270-274] Unfortunately, rigorous verification of this mechanism of action at the biochemical and cellular levels is lacking, representing a barrier to the rational development of possible antidotes. Phosphine, however, is slow acting, relatively stable *in vivo* and a ligand much used in synthetic chemistry; it follows, therefore, that a scavenging approach employing metal ion complexes designed to bind phosphine ought to significantly ameliorate its toxicity.

In this investigation, we studied a number of compounds that are commercially available and have previously been evaluated for their pharmacological activity and safety, although not as decorporating agents. The essential desired activity we sought was the ability to rapidly bind phosphine with reasonably high affinity and, based on general inorganic principles,[207, 275] we proposed that some gold(I) complexes should prove to be good candidate phosphine antidotes.

Phosphine is a "soft" ligand with a marked preference for binding in σ-donor/π-acceptor fashion to "soft" metal ions, typically 2nd and 3rd row transition metals in low oxidation states. Gold in its univalent state, Au(I), is the softest metal ion and, given that Au(I) compounds have been widely used to treat rheumatoid arthritis for about a century, [276, 277]we gave Au(I) complexes a high priority for investigation as potential phosphine-scavenging agents. Non-life threatening side effects develop in about one third of patients given repeated high doses of Au(I) anti-arthritics, but these are usually minor and manageable/reversible.[278, 279] Gold salts are about an order of magnitude more expensive than salts of most first-row transition metals, but this cost is still small in in comparison to that of the overall purified product.

In addition, we have previously shown that *Galleria mellonella* larvae (caterpillars) can usefully be applied to the screening of antidotes for mitochondrial toxicants; namely, azide, cyanide and sulfide.[280] Accordingly, we have employed *G. mellonella* larvae to find an exposure level to phosphine gas useful for testing both the prophylactic and therapeutic effects towards phosphine of three Au(I) compounds (each in use for decades as anti-rheumatoid arthritis drugs[278, 279]) namely, auro-bisthiosulfate (AuTS), sodium aurothiomalate (AuTM) and aurothioglucose (AuTG). All of these compounds contain sulfide donors, keeping the gold in its reduced univalent state, lowering toxicity and promoting affinity for phosphine. The outcomes of these experiments with the larvae were then used to guide the development of a protocol for testing the potential antidotes to phosphine in mice.

Experimental Section

Reagents. All chemicals, ACS grade or otherwise stated, were purchased from either Sigma-Aldrich or Fisher and used without additional purification. Aluminum phosphide (AlP) and argon gas purchased from American Elements (Los Angeles CA) and Matheson, respectively, were also used as supplied. Gold compound solutions were prepared using phosphate-buffered saline (pH = 7.4). Phosphine gas was generated through the reaction of either calcium phosphide (Ca_3P_2) or aluminum phosphide (AlP) with sulfuric acid. In the exposure of mice or *G. mellonella* larvae, 20 mL of 1.2 M sulfuric acid was added to ~0.5 g of Ca_3P_2 to slowly release phosphine in a closed container. The time-dependent variation in phosphine production was observed by infrared spectroscopy at 2,325 cm^{-1} (Thermo-Nicholet 6700 FT-IR) using Beer's law to quantitate the measurements. Calibration, was by a standard additions method using pure phosphine gas generated by the action of sulfuric acid on AlP, a more rapid reaction than that employed in the inhalation chamber. The exposures to phosphine gas are reported as integrated concentration (ppm) × time (min) of phosphine exposure.

Animal Studies – *G. mellonella* Larvae. Larvae of the Greater Wax Moth, *G. mellonella,* were purchased from Vanderhorst Wholesale, Inc. (Saint Mary's, Ohio) and were acclimated at 25°C for six days. Larvae were randomly selected for groups of 10 organisms each. Groups were exposed to phosphine gas generated from calcium phosphide pellets using sulfuric acid in a sealed container (4.8 L volume) as above. In addition, a group of larvae were exposed to sulfuric acid only as an added control. Phosphine exposure lasted for 20 minutes and the atmosphere was pumped with a 30 mL syringe every 5 minutes in order to circulate the phosphine. After exposure, the wax worms were removed from the chamber and monitored for recovery from paralysis

("knockdown"). Recovery was determined by the repeated performance of righting behavior as described by Frawley *et al*.[280] Gold compounds and control solutions were administered at determined time points either before or after phosphine exposure. All injections were a maximum volume of 10 μL and into the most distal left abdominal proleg. Solutions were prepared with filtered PBS, which also served as the injection control. Organisms were monitored for immune activity signaled by a melanin-mediated color change.[78] *G. mellonella* mitochondrial particles (mainly broken mitochondria) were prepared by cooling about 20 larvae to 4°C for 12 minutes. Larvae were then minced in 1 mL EDTA/KCL solution (154 mM KCl, 1 mM EDTA; pH adjusted to 6.8).[159] The minced tissue was then gently homogenized using a glass homogenizer in 5 mL EDTA/KCl solution, filtered through cheesecloth and subsequently centrifuged at 1500 g, 4°C for 8 min to collect mitochondrial particles. The pellet obtained was then washed with 200 μL EDTA/KCl solution, suspended in 200 mL of the same solution and placed on ice subsequent to use in respirometric experiments.

Animal Studies – Mouse Model. The University of Pittsburgh Institutional Animal Care and Use Committee (Protocol Number 17091400) approved all animal protocols used in this study. The Division of Laboratory Animal Research of the University of Pittsburgh provided all veterinary care during these experiments. Male Swiss-Webster (CFW) mice weighing 35-40 g (6-7 weeks old) were purchased from Taconic, Hudson, NY, housed four per cage and allowed access to food and water *ad lib*. Animals were allowed to adapt to their new environment for one week prior to carrying out experiments. All animals were randomly assigned to experimental groups of predetermined size. Animals, two at a time (one subject given test antidote, plus one control), were exposed to phosphine gas in a procedure that was otherwise identical to that used for the *G. mellonella* larvae (see above) for 15 min.

Following phosphine exposure, the duration of time required for the recovery of pole climbing in mice was measured following a procedure originated by Frawley *et al.*[280] This test evaluates the ability of the mouse to climb a lightly roughened, twenty four inch pole (3/8" diameter) before and after exposure to a toxicant, as well as an evaluation of the recovery, post exposure when receiving a treatment. This test is based on the observed natural curiosity of the mouse to climb to the top of the pole, it is relatively simple, and requires minimal equipment. The pole test was started at 20 minutes post toxicant exposure, or as soon the mouse righted itself, and continues every 10 minutes until the mouse was fully recovered. Full recovery was assessed by the mouse scoring the highest rating possible (3), three times in a row. Briefly, the pole was placed in the horizontal position (45° angle) and the mouse placed onto the end. The pole was thengradually raised to the vertical position (through a 90° angle). Once the mouse climbed to the top, or not, it was removed from the pole, scored and replaced in its bucket until the next trial. The mice were scored by performance, receiving scores (see Supplementary information) from zero (fall off/ can't grasp pole) to three (climbs to the top readily with no issues).

The potential prophylactic antidote, AuTS, was injected (ip) into mice (3-6 animals) at 50 mg/kg, 5 minutes before the exposure to phosphine. Control animals received phosphine alone. In addition, the putative antidote (AuTS, 50 mg/kg) was given 1 min after the exposure of the mice to phosphine.

Respirometric Experiments. An Oxygraph O2k Polarographic instrument (Oroboros Instruments, Innsbruck, Austria), equipped with a Clark-type electrode for high-resolution respirometry was used to measure oxygen flux. Mitochondrial buffer, MiR05,[35] (2.1 mL) was added to both chambers of the respirometer and allowed to equilibrate for 20 minutes at 25°C before adding ~100 µL of the mitochondrial particles (prepared as above). Mitochondrial

respiration was then observed with the addition of cytochrome *c* (final concentration 10 μM), succinate (final concentration 10 mM) and rotenone (to prevent back reaction through complex I, final concentration 0.5 μM). Phosphine was added in 25-100 μL increments to the respiring mitochondria particles from a saturated phosphine solution prepared by adding deoxygenated phosphate buffer to AlP in a septum-capped vial with minimal headspace.[281]

Protein Isolations and Enzyme Assay. Cytochrome *c* oxidase was prepared as previously described[59] from intact bovine heart mitochondria using a modified Harzell-Beinert procedure (without the preparation of Keilin-Hartree particles). The enzyme was determined to be spectroscopically pure if the 444 nm to 424 nm ratio for the reduced enzyme was 2.2 or higher.[282] Enzyme concentrations were determined as total heme *a* using the differential (absorption) extinction coefficient of $\Delta\varepsilon_{604}$ = 12 $mM^{-1}cm^{-1}$ for the reduced minus oxidized spectra of the mammalian and bacterial enzymes, respectively.[283] Concentrations throughout are given on a per enzyme concentration basis (*i.e.* [heme *a*]/2).

Steady-state kinetics were performed with the isolated enzyme as described by Nicholls *et al*.[284] The concentration changes of the electron donor, bovine ferrocytochrome *c*, were monitored through its absorbance at 550 nm (minus 540 nm, an isosbestic point in the spectrum of cytochrome *c*) in the presence of excess sodium ascorbate (14.5 mM) in normoxic solution, 0.1 M potassium phosphate, pH 7.44, 0.02% laural maltoside (Anatrace). The fractional oxidase activity, [E], was determined by the following equation:

$$[E] = [c^{2+}]_0 \times [c^{3+}]_t/[c^{3+}]_0 \times [c^{2+}]_t.$$

where $[c^{2+}]_0$ = fraction at time 0, $[c^{3+}]_t$ = fraction at time t, $[c^{3+}]_0$ = fraction at time 0 and $[c^{2+}]$ = fraction at time 0.

Statistical Analysis. Data was analyzed using Kaleidagraph software. A p-value ≤0.05 was considered significant.

Results

Using *G. mellonella* as a Model for Testing Antidotes to Phosphine.

We have previously shown that *G. mellonella* caterpillars provide a reasonable model for screening antidotes to cytochrome *c* oxidase toxicants, such as cyanide.[280] Oxygen flux was initiated by adding cytochrome *c*, to replace any lost when the mitochondria were lysed, the electron donor succinate and rotenone (complex I inhibitor) to prevent backflow. The respirometric inhibition (decreased oxygen flux, JO_2) of *G. mellonella* mitochondrial particles[280] observed was quite linear (R^2=0.95) with respect to micromolar phosphine additions as shown in Figure 30.

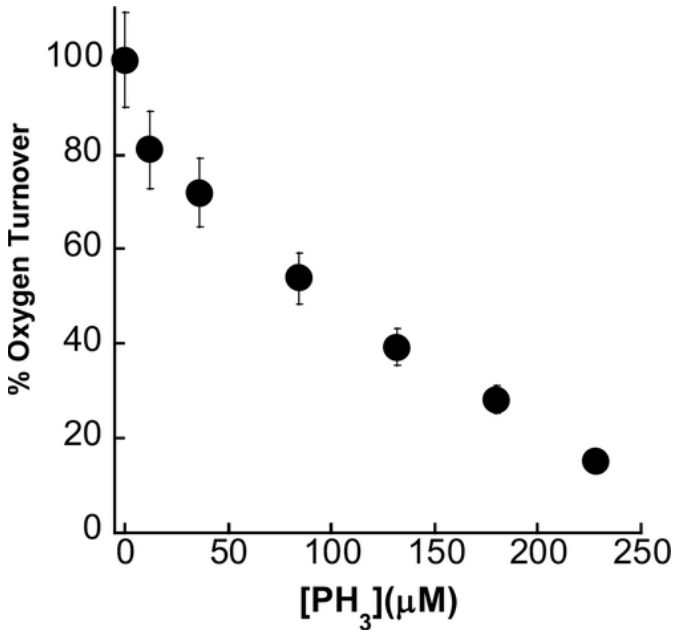

Figure 30. Respirometric response of *G. mellonella* mitochondrial particles titrated with PH_3.
Oxygen consumption was assessed in *G. mellonella* mitochondrial particles diluted in MiR05 respirometric solution (see Experimental Section for details). The tissue suspension (2.1 mL) was allowed to equilibrate in chamber for ~10 minutes prior to measuring oxygen consumption. Default respirometric settings of block temperature, 25°C; stir bar speed, 400 rpm and data recording, 2 s were used. All reagents/substrates quanitations are given as final concentrations. Cytochrome *c* (10 μM), succinate (0.5 mM) and rotenone (0.5 μM) were added to the 2.1 mL of respirometric solution containing 100 μL of mitochondrial particles and the oxygen flux recorded over 5 min (JO_2 of ~140 pmol/s ● mL). Subsequently, PH_3 was added, from a saturated solution, resulting in PH_3 concentrations of 12-230 μM in the respirometer and the oxygen flux was followed until it was constant (~5 min).

Exposure of *G. mellonella* larvae to phosphine in a closed container for 20 min caused ~ 50% of the larvae to become immobile (paralyzed) and the time (known as the recovery time) until the paralyzed larvae regained their ability to move was then recorded. Any larvae that were not immobilized were recorded as having a recovery time of zero. A dose-response of this adjusted recovery time of the *G. mellonella* larvae exposed to phosphine (12-10,000 ppm•min) was subsequently determined (see Figure 31). An exposure of 4300 (±700) ppm•min phosphine (over a 20 min time span) induced a state of paralysis that lasted ~35 min, a conveniently repeatable response.

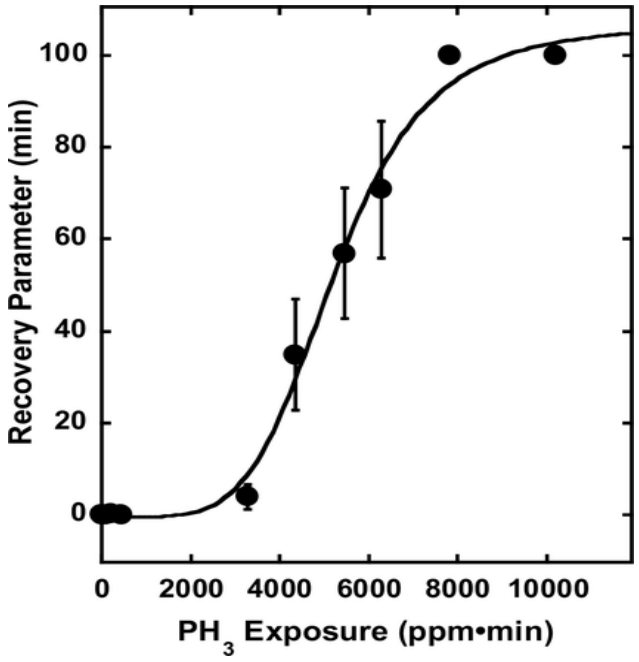

Figure 31. Dose-response data for G. mellonella larvae exposed to varying amounts of PH3.
The larvae were exposed to PH_3 gas generated by the reaction of Ca_3P_2 with 20 mL of 1.2 M H_2SO_4 in a closed vessel (see Experimental section). In sham controls, the addition of the same amount of acid to a reaction vessel (separated from the larvae) caused no change in the larval behavior. The time from which each larva ceased movement until movement began again (recovery time) was recorded. Each group of larvae (~10) were exposed to PH_3 at 12 -10,000 ppm●min. Roughly 60% of control larvae were incapacitated and any larvae that did not knockdown were assigned recovery times of 0 min. The few larvae that had recovery times over 120 min were not scored.

Once a reproducible recovery time for the larvae was obtained, putative antidotes at levels that showed no visible toxicity to the *G. mellonella* larvae (AuTS, 25 mg/kg; AuTM, 1g/kg; AuTG, 1g/kg) were administered by injection into the most distal left abdominal proleg 10 min prior to exposure to phosphine gas. All the gold complexes tested significantly decreased the mean time until the larvae recovered (AuTM 3(±3) min, AuTG 0 min, AuTS 2.1(±0.7) min; Figure 32A) and decreased the median time to be zero for all the antidotes when used prophylactically. More impressively, when the antidotes were given to the larvae at 10 minutes after exposure to phosphine, all proved to also be effective when given therapeutically (mean times until recovery: AuTM, 19(±6) min, AuTG, 17(±6) min, AuTS, 15(±4) min; median times until recovery: AuTM, 3 min, AuTG, 0 min, AuTS, 0 min; Figure 32B).

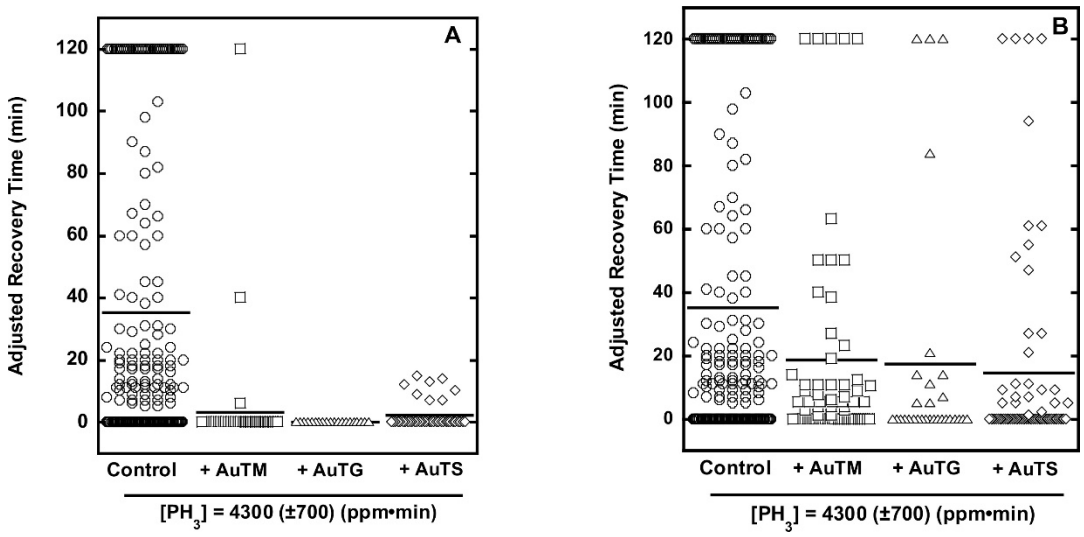

Figure 32. Prophylactic and therapeutic use of sodium aurothiomalate (AuTM), aurothioglucose (AuTG) and auro sodium bisthiosulfate hydrate (AuTS) against phosphine toxicity in *G. mellonella* larvae.
Larvae were injected with each of the three putative antidotes (AuTM, 1g/kg; AuTG, 1 g/kg; AuTS, 25mg/kg) either 5 min prior (A) or 10 min after (B) a PH_3 exposure of 4300 (±700) ppm●min. The mean time until recovery measured for each putative antidote vs control. Roughly 60% of control larvae were incapacitated after PH_3 exposure. Any larvae that did not knockdown were assigned recovery times of 0 min. The few larvae that had recovery times over 2 hours were scored as 120 min. In panel A (prophylactic testing) for each gold compound tested, $p<0.005$ when compared to control; in panel B (therapeutic testing, for each gold compound tested, $p<0.01$ when compared to controls. The means for each group is shown as a bar. The median recovery times were 0 in all cases but the therapeutic administration of AuTM which resulted in a median recovery times of 3 min.

Since it is difficult to isolate cytochrome *c* oxidase from *G. mellonella* larvae in large enough amounts in order to do steady-state turnover experiments and it has been shown that minimal differences between enzymes isolated from different species exist, the turnover experiments were performed with enzyme isolated from bovine hearts. The steady-state turnover of oxygen by bovine cytochrome *c* oxidase was monitored by following the absorbance changes in cytochrome *c*, the oxidase electron donor, after providing a source of electrons for cytochrome *c*, sodium ascorbate (Figure 33A, open circles). Once the steady-state was established, phosphine (to 100 μM) was added (at ~ 100 s) resulting in inhibition of the enzyme. The resulting time course of the inhibition of cytochrome *c* oxidase was fit by a single exponential: $[E]_{active} = 0.87e^{-0.033t} + 0.13$ (Figure 33B). The inactivation rate was proportional to the phosphine concentration with a

k_{on} calculated to be 3.3 x 10^3 $M^{-1}s^{-1}$ with 13% of the enzyme still active at the phosphine concentration of 100 μM. This residual activity is proportional to the apparent K_i, which was determined to be 13 μM, similar to those previously reported.[285] To test if the observed amelioration of phosphine toxicity by gold(I) complexes (Figure 32) could indeed be attributable to antagonism of cytochrome *c* oxidase activity, AuTS (300 μM) was added to the enzyme solution prior to the initiation of steady state conditions by addition of ascorbate (Figure 33A, closed diamonds). When phosphine (100 μM) was subsequently added, the steady-state turnover was roughly 70% of that observed for the normally functioning enzyme.

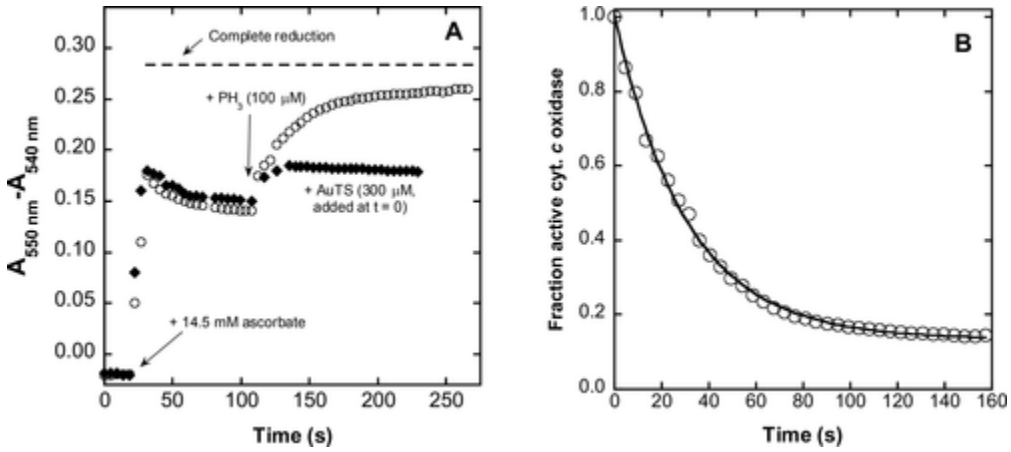

Figure 33. Cytochrome *c* oxidase steady state turnover: inhibition by phosphine and rescue by auro sodium bisthiosulfate hydrate (AuTS).

Cytochrome *c* oxidase (0.194 μM) turnover was followed by observing the oxidation of reduced cytochrome *c* (14 μM) monitored spectroscopically at A550 nm-A540 nm (T=25°C, 0.1 M sodium phosphate, pH 7.44, 0.02% lauryl maltoside) over time. Turnover was initiated by the addition of 1.45 mM sodium ascorbate. (A) Representative plots for the addition of phosphine (100 μM, open circles) to the active enzyme. Addition of 300 mM AuTS prior to ascorbate initiation of turnover (closed diamonds, symbols have been slightly offset so as to view both data sets) and subsequent addition of 100 μM phosphine. (B) A single exponential fit (solid line) to the fraction of active enzyme vs. time (open circles) calculated according to Eq. 1 (see Experimental Section). All reagents/quantities are given as final concentrations.

After screening the potential antidotes in *G. mellonella* larvae, a preliminary set of similar experiments were then carried out in mice. Swiss-Webster mice were exposed to phosphine gas, produced by the same method as used with the *G. mellonella* larvae, in a closed container for 15 min. This dose of phosphine, 3200 (±500) ppm•min) did not cause the mice to "knockdown" but did induce a severely lethargic state (motionless in open field). The mice were then examined by a pole-climbing behavioral assessment (see Supplemental Material section for details). Mice were examined 5 days prior to the phosphine experiments in order to obtain a baseline and then subjected to the pole test starting immediately following their exposure to phosphine. Mice given the AuTS antidote (50 mg/kg, previously determined to cause no change in behavior by pole testing and chosen based on the mean recovery time in *G. mellonella*) 5 min prior to the phosphine exposure performed as well as control mice that had never been exposed to phosphine (Figure 34A). However, when mice were exposed to phosphine and given the AuTS antidote 1 min after the toxicant exposure, the results of the pole test were less impressive with only a very modest improvement in performance (Figure 34B).

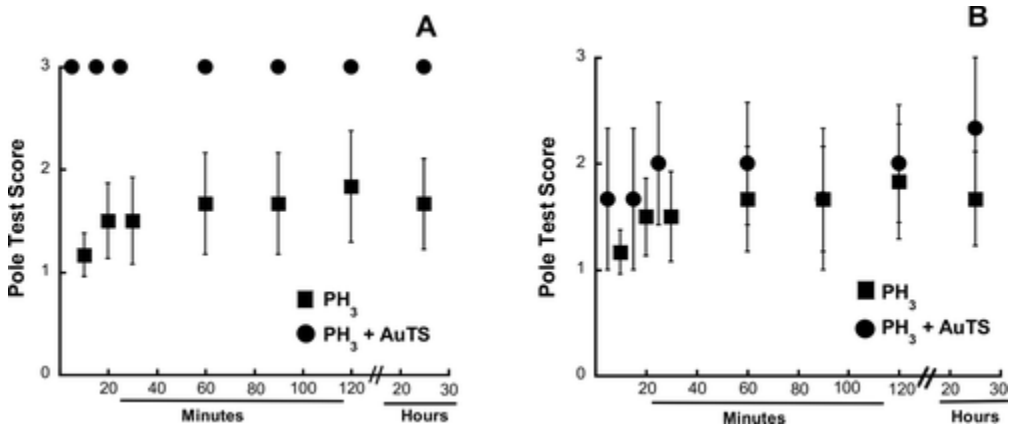

Figure 34. Prophylactic and therapeutic use of aurobisthiosulfate (AuTS) in PH_3 exposed mice.
Mice were given 50 mg/kg AuTS intraperitoneally (closed circles) either 5 min before (A) or 1 min after (B) a PH_3 (closed squares) exposure of 3200 (±500) ppm●min (over a 15 min time period), The mice were examined using a behavioral assessment (pole climbing test, see Experimental section) to evaluate their response to the toxicant and putative antidote (AuTS).

Discussion

It is intrinsically clear from the data (Figures 32) that, in the caterpillars, all three of the Au(I) compounds are highly effective antidotes for phosphine. That is, the original concept has been at least circumstantially validated in a biological system; Au(I) complexes are indeed able to detoxify phosphine, presumably through scavenging (coordination) and decorporation. Regarding the trials with mice, the compounds are certainly prophylactically effective (Figure 34A) but when administered after the toxicant, any beneficial effects were much more modest in these assays (Figure 34B). This result should not, however, be taken to indicate that that the approach will ultimately prove to be of no therapeutic application for two main reasons. First, it is presently unclear exactly why Au(I) complexes seem to be significantly therapeutic in the caterpillars (Figure 32B) but not the mice (Figure 34B). The compounds may have significantly different pharmacodynamic/kinetic characteristics in the two organisms; in which case, there may be related structures exhibiting such properties more suitable for therapeutic use in mammals. At this juncture, especially given that there is no antidote for phosphine currently available, any detectable ameliorative effect is encouraging. Second, most human victims reach the clinic having ingested a phosphide salt and the exposure is ongoing as the phosphide continues to release phosphine gas through hydrolysis in the stomach. Additionally, while phosphides are employed as fumigants in western countries, for indoor control of insects and outdoor control of rodents, it is not clear that their use is so effectively regulated worldwide. The recent increase in the application of drones to crop-dusting operations, particularly in Asia, could conceivably lead to future exposures of larger human populations, either through accident or with malicious intent, not to mention the possibility of release by deliberate detonations. Any individuals thus exposed to particulate phosphides

dispersed in air, will likely have infiltration of phosphide particles into the esophagus, airways and adhered to clothing. In all such cases where slow and continuing release of phosphine gas is to be expected, the availability of effective prophylactics to prevent any further toxic dose exacerbating the condition of the victims could have life-saving consequences.

The mechanism(s) through which phosphine exerts its toxicity is (are) seemingly complicated[270, 286, 287] and remain incompletely delineated.[288-290] We think it pertinent to consider if the present findings shed any light on these matters. For almost half a century, mitochondria have been [274] and continue to be [291] identified as key targets for disruption by phosphine through inhibition of cytochrome *c* oxidase.[285, 292-295] In response to sub-lethal phosphine exposure, the *G. mellonella* larvae used in the current study exhibit dose-dependent (Figure 31) temporary paralysis (knockdown) from which they appear to fully recover. This behavior is analogous to that obtained employing the *bona fide* cytochrome *c* oxidase inhibitors azide, cyanide and sulfide.[280] Consequently, while we have not set out to examine this particular question rigorously, our observations concerning the caterpillars do appear to be at least consistent with a mechanism of acute phosphine toxicity primarily involving inhibition of cytochrome *c* oxidase. If this is so, then it follows that the different response to the antidotes of phosphine-challenged mice (Figure 34) compared to the caterpillars (Figure 32) is plausibly due to there being another toxic mechanism operating in the mice, that might not involve reversible inhibition of cytochrome *c* oxidase.

In mammals, acute phosphine/phosphide poisoning is reported to lead to death by cardiopulmonary failure, with microscopically visible injury to myocardial tissue.[287, 296] This shares some similarity with acute cyanide and sulfide toxicity in mammals, where death is also the result of cardiopulmonary collapse, but cyanide and sulfide act more rapidly[38, 61] and principally on the central nervous system stimulating cardiopulmonary function.[96, 148] The measured

inhibition constant (K_i = 13 μM) and on-rate (k_{on} = 3.3 × 10^3 $M^{-1}s^{-1}$) for phosphine reacting with isolated cytochrome *c* oxidase (Figure 33) are, respectively, two orders of magnitude greater and three orders of magnitude slower than the corresponding reaction of the enzyme with cyanide,[297] in keeping with the less toxic nature of phosphine compared to cyanide. It follows that lethal doses of inhaled phosphine may require prolonged exposure as recently reported,[298-300] but there remains an observable difference between the behavior of phosphine and the better characterized mitochondrial toxicants in mammals, again suggesting that there could be at least one other toxic mechanism in play, possibly non-mitochondrial. Rahimi *et al.*[301] have recently shown that phosphine poisoning in rats can be ameliorated through blood transfusion, clearly implicating some component of the blood/vasculature as a target for the toxicant. This finding seems to be in keeping with earlier observations[302, 303] that hemolysis and methemoglobinemia may correlate with severity of outcome in aluminum phosphide-poisoned human patients. There is a paucity of information regarding the reaction of phosphine with hemoglobin and red blood cells, the available literature now being more than twenty-five years old.[304, 305] Further effort in this area now appears to be warranted.

Supplemental Material and Figures

Raw data for the pole climbing (see Experimental Section for details) assessment of phosphine intoxicated mice establishing prophylactic and therapeutic responses using auro-bisthiosulfate (AuTS, 50 mg/kg) (see Table 18). Raw data for the recovery assessment of *G. mellonella* larvae after exposures of phosphine along with the prophylactic and therapeutic administration of putative antidotes Au(I) complexes auro-bisthiosulfate (AuTS), sodium aurothiomalate (AuTM) and

aurothioglucose (AuTG). When *Galleria mellonella* larvae (or wax worms) were subjected to PH_3 exposures of 4300 (±700) ppm●min over a 20 min time span, they become immobile (paralyzed) for ~35 min. The administration of Au(I) complexes auro-sodium bisthiosulfate hydrate (AuTS), sodium aurothiomalate (AuTM) and aurothioglucose (AuTG) 5 min prior to PH_3 exposure resulted in a drastic reduction in the recovery time (0-4 min). When the putative antidotes were given 10 min after the PH_3 exposure, all the antidotes were therapeutic, resulting in recovery times of 19, 17 and 14 min for AuTM, AuTG and AuTS, respectively.

Raw data for the pole climbing (see Materials and Methods for details) assessment of phosphine intoxicated mice establishing prophylactic and therapeutic responses using auro-bisthiosulfate (AuTS, 50 mg/kg) (see Table 18)

Table 18. Raw data of the responses of phosphine-intoxicated male mice and the putative antidote AuTS as determined by a pole-climbing test (as shown in Figure 33).

		Pole Test Score at Time Point					
	Time (min)	**10**	**20**	**30**	**60**	**90**	**120**
- 5 min	mouse 1	3	3	3	3	3	3
	mouse 2	3	3	3	3	3	3
	mouse 3	3	3	3	3	3	3
+ 1 min	mouse 4	3	3	3	3	3	3
	mouse 5	1	1	2	2	1	2
	mouse 6	1	1	1	1	1	1
controls	mouse 7	2	2	3	3	3	3
	mouse 8	1	1	1	1	1	1
	mouse 9	1	3	3	3	3	3
	moue 10	1	1	1	1	1	1
	mouse 11	2	2	1	2	2	3
	mouse 12	0	0	0	0	0	0

Swiss-Webster male mice (7-8 weeks of age) were exposed phosphine gas, 3200 (±500) ppm min for 15 min (see controls). Mice were administered 50 mg/kg AuTS either 5 min before

phosphine gas or 1 min after the mice were exposed to phosphine. Mice were exposed in pairs: 1 control and 1 given the antidote.

Table 19. Dose-response recovery times of *G. mellonella* larvae in response to increasing Phosphine exposures as shown in Figure 31.

i	[PH_3] ppm•min	Recovery T (min)	i	[PH_3] ppm•min	Recovery T (min)
1	12.2	0	51	4362.5	0
2	12.2	0	52	4362.5	0
3	12.2	0	53	4362.5	7
4	12.2	0	54	4362.5	14
5	12.2	0	55	4362.5	17
6	12.2	0	56	4362.5	22
7	12.2	0	57	4362.5	29
8	12.2	0	58	4362.5	60
9	12.2	0	59	4362.5	100
10	12.2	0	60	4362.5	100
11	73.1	0	61	5447	0
12	73.1	0	62	5447	0
13	73.1	0	63	5447	16
14	73.1	0	64	5447	22
15	73.1	0	65	5447	41
16	73.1	0	66	5447	90
17	73.1	0	67	5447	100
18	73.1	0	68	5447	100
19	73.1	0	69	5447	100
20	73.1	0	70	5447	100
21	219.3	0	71	6287.8	0
22	219.3	0	72	6287.8	0
23	219.3	0	73	6287.8	8
24	219.3	0	74	6287.8	100
25	219.3	0	75	6287.8	100
26	219.3	0	76	6287.8	100
27	219.3	0	77	6287.8	100
28	219.3	0	78	6287.8	100
29	219.3	0	79	6287.8	100
30	219.3	4	80	6287.8	100
31	438.7	0	81	7835.4	100
32	438.7	0	82	7835.4	100
33	438.7	0	83	7835.4	100
34	438.7	0	84	7835.4	100
35	438.7	0	85	7835.4	100
36	438.7	0	86	7835.4	100
37	438.7	0	87	7835.4	100
38	438.7	0	88	7835.4	100
39	438.7	0	89	7835.4	100
40	438.7	0	90	7835.4	100
41	3265.8	0	91	10187.3	100
42	3265.8	0	92	10187.3	100
43	3265.8	0	93	10187.3	100
44	3265.8	0	94	10187.3	100
45	3265.8	0	95	10187.3	100
46	3265.8	0	96	10187.3	100
47	3265.8	0	97	10187.3	100
48	3265.8	0	98	10187.3	100
49	3265.8	20	99	10187.3	100
50	3265.8	20	100	10187.3	100

Table 20. Dose-response recovery times of *G. mellonella* larvae in response to prophylactic and therapeutic treatment with Au(I) complexes auro-bisthiosulfate (AuTS, 25 mg/kg), sodium aurothiomalate (AuTM, 1g/kg)

No Treatment Control	PBS Injection Control	AuTM Injection Control (1000ppm)	AuTG Injection Control (1000ppm)	AuTS Injection Control (25ppm)	PH_3 4300 (±700) ppm•min	Prophylactic PBS	Post Exposure PBS	Prophylactic AuTM (1000ppm)	Prophylactic AuTG (1000ppm)	Prophylactic AuTS (25ppm)	Post Exposure AuTM (1000ppm)	Post Exposure AuTG (1000ppm)	Post Exposure AuTS (25ppm)	H_2SO_4 Control
0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
0	0	0	0	0	0	0	0	0	0	0	0	0	0	
0	0	0	0	0	0	0	0	0	0	0	0	0	0	
0	0	0	0	0	0	0	0	0	0	0	0	0	0	
0	0	0	0	0	0	0	0	0	0	0	0	0	0	
0	0	0	0	0	0	0	0	0	0	0	0	0	0	
0	0	0	0	0	0	0	0	0	0	0	0	0	0	
0	0	0	0	0	0	0	0	0	0	0	0	0	0	
0	0	0	0	0	0	0	0	0	0	0	0	0	0	
0	0	0	0	0	0	0	0	0	0	0	0	0	0	
0	0	0	0	0	0	0	0	0	0	0	0	5	0	
0	0	0	0	0	0	0	0	0		0	0	5	0	
0	0	0	0	0	0	0	0	0		0	0	7	0	
0	0	0	0	0	0	0	0	0		0	0	11	0	
0	0	0	0	0	0	6	0	0		0	0	14	0	
0	0	0	0	0	0	8	0	0		0	0	14	0	
0	0	0	0	0	0	9	0	0		0	0	21	0	
0	0	0	0	0	0	9	0	0		0	0	84	0	
0	0	0	0	0	0	10	0	0		0	0	120	0	
0	0	0	0	0	0	10	0	0		0	0	120	0	
0	0	0	0	0	0	10	0	0		0	0	120	0	
0	0	0	0	0	0	11	0	0		0	0		0	
0	0	0	0	0	0	12	0	0		0	0		0	
0	0	0	0	0	0	12	0	0		7	0		0	
0	0	0	0	0	0	13	0	0		7	0		0	
0	0	0	0	0	0	13	0	0		9	0		0	
0	0	0	0	0	0	16	0	0		10	2		0	
0	0	0	0	0	0	17	0	0		12	2		0	
0	0	0	0	0	0	24	0	0		13	5		0	
0	0	0	0	0	0	35	6	0		14	5		0	
0	0	0	0	0	0	40	6	0		15	7		0	
0	0	0		0	0	51	8	0			7		0	
0	0	0		0	0	79	8	0			7		0	
0	0	0		0	0	120	8	0			10		0	
0	0	0		0	0	120	8	0			10		0	
0	0	0		0	0	120	8	0			10		1	
0	0	0		0	0	120	8	0			10		2	
0	0	0		0	0	120	10	0			10		5	
0	0	0		0	0	120	0	6			10		5	
0	0	0		0	0	120	0	40			10		5	
0	0	0		0	0	120	10	120			11		5	
0	0	0		0	0		11				13		7	
0	0	0		0	0		11				14		7	
0	0	0		0	0		13				16		9	
0	0	0		0	0		14				16		9	
0	0	0		0	0		19				19		9	
0	0	0			0		20				20		11	
0	0	0			0		23				20		11	
0	0	0			0		23				20		21	
0	0	0			0		31				20		27	
0	0	0			0		34				23		27	
0	0	0			0		86				26		47	
0	0	0			5		115				35		51	
0	0	0			5		120				43		55	
0	0	0			6		120				50		61	
0	0	0			6		120				71		61	
0	0	0			7		120				74		94	
0	0	0			7		120				93		120	
0	0	0			7		120				93		120	
0	0	0			7		120				93		120	
0	0	0			8		120				117		120	
0	0	0			8		120				120			
0	0	0			9		120				120			
0	0	0			9		120				120			
0	0	0			9		120				120			

Table 20 continued

No Treatment Control	PBS Injection Control	AuTM Injection Control (1000ppm)	AuTG Injection Control (1000ppm)	AuTS Injection Control (25ppm)	PH_3 4300 (±700) ppm●min	Prophylactic PBS	Post Exposure PBS	Prophylactic AuTM (1000ppm)	Prophylactic AuTG (1000ppm)	Prophylactic AuTS (25ppm)	Post Exposure AuTM (1000ppm)	Post Exposure AuTG (1000ppm)	Post Exposure AuTS (25ppm)	H_2SO_4 Control
0	0	0			9		120				120			
0	0	0			10		120				120			
0	0	0			10		120				120			
0	0	0			11		120				120			
0	0	0			11		120				120			
0	0	0			11		120				120			
0	0	0			11		120				120			
0	0				11		120				120			
0	0				11		120				120			
0	0				11		120				120			
0	0				12		120				120			
0	0				12		120				120			
0	0				12		120				120			
0	0				12		120				120			
0	0				13		120				120			
0	0				14		120				120			
0	0				14		120				120			
0	0				16		3							
0	0				17		5							
0	0				17		7							
0	0				17		19							
0	0				18		0							
0	0				19		0							
0	0				20		0							
0	0				20		0							
0	0				20		0							
0	0				22		120							
0	0				22									
0	0				22									
0	0				24									
0	0				25									
0	0				29									
0	0				30									
0	0				30									
0	0				31									
0	0				31									
0	0				38									
0	0				40									
0	0				40									
0	0				41									
0	0				45									
0	0				45									
0	0				57									
0	0				60									
0	0				60									
0	0				60									
0	0				64									
0	0				66									
0	0				67									
0	0				70									
0	0				80									
0	0				82									
0	0				87									
0	0				90									
0	0				98									
0	0				103									
0	0				120									
0	0				120									
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0	0				120									
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Table 20 continued

No Treatment Control	PBS Injection Control	AuTM Injection Control (1000ppm)	AuTG Injection Control (1000ppm)	AuTS Injection Control (25ppm)	PH_3 4300 (±700) ppm•min	Prophylactic PBS	Post Exposure PBS	Prophylactic AuTM (1000ppm)	Prophylactic AuTG (1000ppm)	Prophylactic AuTS (25ppm)	Post Exposure AuTM (1000ppm)	Post Exposure AuTG (1000ppm)	Post Exposure AuTS (25ppm)	H_2SO_4 Control
0	0				120									
0	0				120									
0	0				120									
0	0				120									
0	0				120									
0	0				120									
0	0				120									
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0	0				120									
0	0				120									
0	0				0									
0	0				0									
0	0				0									
0	0				18									
0	0				18									
0	0				18									
0	0				20									
0	0				22									
0	0				28									
0	10				120									
0	0				0									
0	0				0									
0	0				0									
0	0				0									
0	0				11									
0	0				20									
0	0				20									
0	0				24									
0	0				120									
0	0				120									
0	0				12									
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