UNIVERSITY OF CALIFORNIA,
IRVINE

Evaluation of the vitamin B12 analog cobinamide as a potential antidote in reversing hydrogen sulfide toxicity

THESIS

submitted in partial satisfaction of the requirements
for the degree of

MASTER OF SCIENCE

in Biomedical and Translational Science

by

Tina Saber

Thesis Committee:
Professor Matthew Brenner, Chair
Professor Sheldon Greenfield
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2019

DEDICATION

To

my father
and
all my loved ones
whom always supported and motivated me in this beautiful chaos of life

"Not in pursuit of pomp and of pageant, to this door we have come
For shelter from ill-fortune, here we have come.
Wayfarers of love's stage are we and from the limits of non-existence
Up to the climes of existence, all this way we have come."

Hafiz

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ACKNOWLEDGMENTS

Being determined and having defined academic and career goals is essential for future success, but this is not achievable without good mentoring, people who can provide us tools, guidance, support and the feedback we need to thrive in this journey. I wish to express my sincere gratitude to my mentors:

Professor Matthew Brenner, I am indebted to you for your invaluable guidance, support and your great mentorship. Thank you very much for giving me this opportunity, and encouraging me every day. Joining your lab and working with you and your team was a great experience in my medical research life.

Professor Sheldon Greenfield, thank you for your endless kindness, patience, and help during my graduate studies. I learned a lot from your insight and attitude, which gave me a great comprehensive view of the patient and healthcare system settings.

Professor Elliot Botvinick, thank you for your kindness and support. I always appreciate your positive energy which has been a source of motivation.

Professor Mohsen Davoudi, thank you for your never-ending guidance and support. Your positive and invincible attitude is admirable.

What I have learned most from you gentlemen is not just how to be a good scholar. You showed me how to simultaneously be a great clinician, scientist, teacher and mentor. In the hope that I could, one day, faithfully convey what I have learned from you to the next generation.

I also thank all the UCI MS-BATS, UCI Beckman Laser Institute and Brenner lab members; without their guidance and help this investigation and dissertation would have not been possible.

This work was financially supported by the NIH CounterACT program, 1U01NS087964.

ABSTRACT OF THE THESIS

Evaluation of the vitamin B12 analog cobinamide as a potential antidote in reversing hydrogen sulfide toxicity

By

Tina Saber

Master of Science in Biomedical and Translational Science

University of California, Irvine, 2019

Professor Matthew Brenner, Chair

Background: Hydrogen sulfide (H_2S) is a potent, toxic gas, frequently inhaled accidentally from natural or industrial (petroleum, metallurgy, agriculture) sources. Moreover, it can potentially be weaponized for malicious use, for civilian terrorism or against military personnel. Unfortunately, currently no effective antidote exists to neutralize the fatal effects of H_2S; this is a major public health concern. Preliminary studies have demonstrated that cobinamide (a precursor of cobalamin, vitamin B12) can inhibit H_2S toxicity. We developed a novel inhalation model, simulating realistic toxic exposure to investigate the efficacy of cobinamide.

Method: Twenty New Zealand White rabbits (10 in each, cobinamide and control groups) were anesthetized, intubated and placed in a respiratory inhalation circuit, delivering an anesthetic (isoflurane) and H_2S mixture, using a compressed air flow-by model. Rabbits' vital signs (heart rate, blood pressure, respiratory rate and oxygen saturation) and gas levels within the hood were continuously monitored. After any change in respiratory

pattern, 1cc cobinamide or normal saline was administered intramuscularly. The primary endpoint was mean survival time.

Result: Cobinamide-treated rabbits had an average 7-minute increase in survival time (12.6±3.37 min versus 19.5±7.26 min). Kaplan Meier survival curves showed significant difference. Log rank analysis of the two groups' survival curves was statistically different ($p<0.007$).

Conclusion: We have demonstrated the first utilization of cobinamide as an intramuscular antidote to H_2S in a rabbit inhalation model, successfully prolonging survival more than 50%. Further investigations are required to address the serious public health concern for hydrogen sulfide mass casualty.

INTRODUCTION

Hydrogen sulfide (H_2S) is a highly toxic and explosive gas with a smell of rotten eggs in low concentrations. Hydrogen sulfide is found in different sources like crude petroleum, natural gas, volcanos or hot springs. It is also produced from bacterial breakdown of human waste or organic decay. On the other hand, industrial activities that can produce the gas in huge amounts are gas drilling, refining, petroleum production, mining, etc. [1] Typical occupational hazard scenarios involve exposure to high concentrations of H_2S gas released from the stated sources.

Among fatal gas inhalation incidents which happen in the workplace or mass casualties, carbon monoxide with 36% is the first and hydrogen sulfide with 7.7% is the second most common. [2] Various governmental agencies like National Institute for Occupational Safety and Health (NIOSH) or Department of Health and Human Services (DHHS) have published documents and provided safety recommendations for high risk workers during emergency situations. [3] The medical literature does not include studies investigating fatal intentional exposures in the United States (U.S.); however, the incidence of suicide using H_2S in Japan has shown an exponential increase since 2007. [4] The wide availability of this gas, and the scattered studies published in the U.S., suggest that suicides are increasing in the U.S. as well [5], underestimated by public health officials and physicians. From 2008 to 2010, the reported cases of suicide by H_2S gas have increased from 2 to 18 cases. In the following years, from 2011 to 2013, data and reports show a total of 43 victim of hydrogen sulfide toxicity including 15 successful suicide victims and 21 victims who were first responders. [6] Officials should be aware of this trend in order to implement plans for

prevention of morbidity and mortality and, first responders must be educated about these trends in order to protect themselves when approaching a potential H_2S victim.

Recently, toxic chemicals, have attracted the attention of terrorists' as well, due to the devastating potential effects that weaponizing such chemicals could have on the general population. In 1995, the nerve gas sarin was used in an attack in Tokyo; more recently in Iraq, chlorine storage tanks were targeted. All these scenarios, demonstrate malicious interest toward chemical agents as a new way to cause disruption and devastation in a community. [7] Hydrogen sulfide is one significant, potential and attractive agent for this purpose, given its high toxicity, difficulty in diagnosing exposure to high concentrations, and the ease with which it can be produced. The first reports on this mode of utilization are from World War I, when H_2S was used by the British army as a chemical weapon. At the time, it was not considered to be an ideal war gas, but, while other gases were in short supply, it was used on two occasions in 1916. [8]

The primary objective and mission of the CounterACT program (Countermeasures Against Chemical Threats) is to foster and support translational research and development of new or improved therapeutics to mitigate the health effects of chemical threats, of which hydrogen sulfide is one. Unfortunately, there is no current approved antidote for H_2S, to neutralize its devastating effects in mass casualty accidents. Therefore, the development of antidotes that can be administered quickly in the field to treat civilian victims or military personnel in casualties are a priority. To be effective in mass casualty scenarios, the antidote must be rapidly acting and easily administered, preferably in small volumes by intramuscular injections.

The vitamin B12 analog, cobinamide, has been studied and is being developed in recent years as a novel therapy for mass casualty cyanide poisoning. [9] Cobinamide is highly water-soluble, can be absorbed rapidly across muscle, and has a high binding affinity for cyanide. Based on the similar mechanism of toxicity of cyanide and hydrogen sulfide on Cytochrome-C oxidase, in addition to cyanide, cobinamide also has a high affinity for hydrogen sulfide. Therefore, we hypothesized that cobinamide could have potential effectiveness in treating H_2S poisoning, and devised and initiated studies based on this hypothesis.

Goal of this study

The primary goal of this project is to show the effectiveness of intramuscular cobinamide for rescuing animals from H_2S poisoning. Due to the severe toxicity of hydrogen sulfide in humans, antidote candidates may not be tested for efficacy in humans. Thus, FDA regulations specify antidotes need to be approved through 'Animal Rule Pathways', requiring significant survival endpoint difference in animal models.

CHAPTER 1 – BACKGROUND

1.1. Exposure

Despite the adverse effects of hydrogen sulfide, it is important to know that at very low levels H_2S is a modulator in the body: it helps reduce inflammation, mediates cytoprotective signal transmission through NO signaling, controlling vascular tone, among others. [10, 11]

The remarkable characteristics which make hydrogen sulfide a dangerous gas are the low ignition rate and its heaviness. The latter causes H_2S to not rise and stay in low levels and travel along the ground below the air. [12] This could lead to people working in enclosed and poorly ventilated areas such as basements, manholes, sewer lines, etc. to be exposed to highly dangerous levels.

By far the most common primary route of exposure to hydrogen sulfide in mass casualties or workplace accidents is through inhalation, although eye or dermal contact, injection, and accidental ingestion are plausible routes as well. After inhalation, H_2S enters the circulation directly across the alveolar-capillary membrane, and dissociates in part into HS, and sulfide ion. Some also, remains as free H_2S in the blood and that fraction can interact with different enzymes. The infusion of sulfide ion alone into circulation mimics the systemic effects of H_2S inhalation but do not result in pulmonary edema, one of the mainstay complications of H_2S inhalation. [13]

The clinical effects of H_2S depend on its concentration, and the duration of exposure. [14] The exposure-response curve for lethality is extremely steep for hydrogen sulfide. [15] It is known that the primary determinant of toxicity in acute scenarios is the concentration rather than the duration of exposure. Thus, higher concentrations give little margin of safety.

Dose-related outcomes of hydrogen sulfide inhalation are summarized in Table 1. Most real-world toxicity studies have involved acute, uncontrolled incidents in which the exact concentration and pre-existing conditions are not known. On the other hand, controlled studies have invariably involved animals, whose results where later extrapolated to humans.

As a direct-acting metabolic toxin, hydrogen sulfide mostly affects organs that are critically dependent on oxidative metabolism, such as the nervous system, heart and respiratory system. In occupational or industrial exposures, workers often first notice hydrogen sulfide by the characteristic rotten egg odor. The olfactory detection limit of it is as low as 0.01-1.5 ppm. Unfortunately, the gas quickly paralyzes the olfactory receptors, hindering people from awareness of its existence, resulting in unexpected overexposure. [16] People exposed to low concentrations also report headaches, nausea and other symptoms. [17] People acutely exposed to around 100 ppm commonly experience lacrimation, photophobia, tachypnea, dyspnea, tracheobronchitis, nausea, vomiting, diarrhea, and cardiac arrhythmias. [18] Symptoms become more severe as concentrations increase. A variety of severe, acute central neurotoxicities (including dizziness, motor or memory dysfunction), pulmonary edema, arrhythmias, or coma are all effects which can occur with two to three breaths at high concentrations.

Table 1. Dose-related effects of acute hydrogen sulfide inhalation exposure

Concentrations (ppm)	*Effects*
0.01- 1.5	Odor threshold; when "rotten egg" smell is first noticeable to some
3–5	Odor becomes more pronounced. Prolonged exposure may cause nausea, tearing, headache or loss of sleep
10–20	Eye irritation, fatigue, loss of appetite, headache, irritability, poor memory, dizziness
50–100	Conjunctivitis, severe eye injury
100–150	Olfactory fatigue or paralysis; can no longer smell hydrogen sulfide odor
150–250	Irritation of nose and lungs, nausea, headache, vomiting, dizziness
250–500	Severe respiratory irritation, pulmonary edema
500–1000	Staggering, CNS stimulation, knockdown, seizures, myocardial infarction, apnea, coma, death
1000 and above	Unconsciousness at once, with early cessation of respiration and death in a few minutes (death from respiratory paralysis)

Source: OSHA, Guidotti T.L. Occup. Med 1996

1.2. Molecular pathology and mechanism

As stated in the beginning of this chapter, the sulfide ion can be an endogenous signal transmitter; at low concentrations it donates electrons to complex II of the electron transport chain (ETC) in mitochondria, hence stimulating ATP production. [19]

In the blood, it dissociates to hydrosulfide (HS^-), is delivered to tissues by circulation, and raises sulfide levels in tissue. [19] The half-life of hydrogen sulfide in tissues is very short and hydrosulfide quickly is cleared, thus the effects of exogenous hydrogen sulfide in the form of blood hydrosulfide are brief. On the other hand, it is known that the endogenous level of sulfide in the human brain is relatively high, so the flux may not be detectable. And it is likely that the addition of a sulfide ion load can pass the threshold level of narcosis or anesthesia. [2]

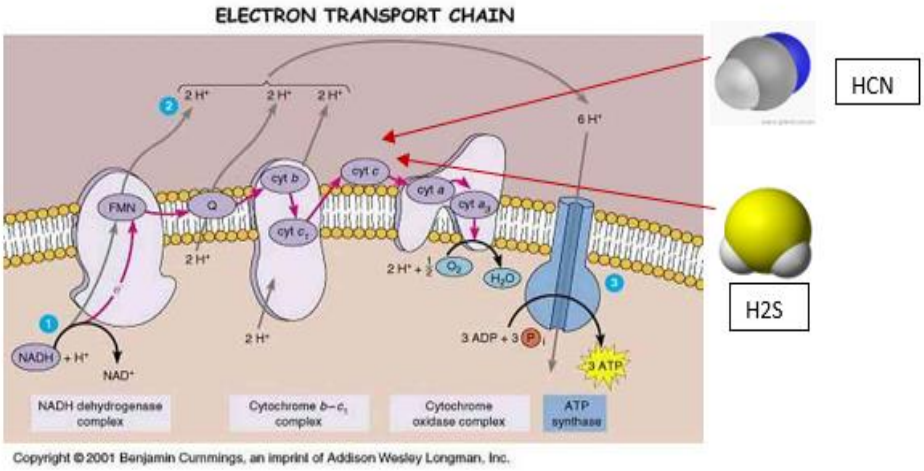
In acute and especially high-dose exposure levels, the toxicity of hydrogen sulfide is through binding to and inhibiting cytochrome-C oxidase in complex IV of the electron transport chain in mitochondria, the fundamental step for oxidative respiration and aerobic metabolism in cells. [20] This effect is the same as oxygen deprivation or asphyxiation except that it acts far more rapidly. That is why hydrogen sulfide along with cyanide, carbon monoxide and azide are classified as cellular asphyxiants. (Fig. 1)

Yet, this is not the only mechanism of toxicity. It has been suggested H_2S toxicity can also be due to mechanisms other than aerobic respiration inhibition which are likely to be at least as important but, still not well-studied or understood. "Knockdown process" is one of those proposed complications: a H_2S- induced acute central nervous toxicity leading to reversible unconsciousness. At high and prolonged concentrations (above 500 ppm) it can easily be fatal, but in transient exposures, such as in oil fields where there are air

movements, it may be rapidly reversible. In the knockdown process, cytochrome oxidase poisoning is likely not the cause, because the cellular anoxia would be devastating to the brain, immediately irreversible, and even if the victim did recover, there would be anoxic brain injury. [2, 15]

Also, sulfide has been reported to both reduce and increase oxidative stress in cells and in whole animals. Jiang et al. described protein kinases JNK and Erk are activated when cells are stressed by different agents, including reactive oxygen species. NaSH increases the generation of hydroxyl radical, one of the most damaging reactive oxygen species. [19]

Figure 1. The ETC (Electron Transport Chain) in mitochondria, showing the points of action of hydrogen sulfide (H_2S) and hydrogen cyanide (HCN). [Cummings B]



1.3. Available approaches in cyanide poisoning; a potential option for hydrogen sulfide as well

Cyanide, another cellular asphyxiant, also exerts its toxic effects by binding to cytochrome-C oxidase. For years, Cyanide Antidote Kit (CAK; Lilly) has been an available antidote approved by the U.S. Food and Drug Administration. CAK contains amyl nitrite pearls, sodium nitrite solution, and thiosulfate. Sodium nitrite induces the formation of methemoglobin in RBCs. Cyanide rapidly complexes with methemoglobin, due to its higher affinity for methemoglobin instead of cytochrome-C oxidase. This releases cytochrome-C oxidase to resume normal cellular respiration. [21] This approach had its own toxicity; the main concerning one is the production of methemoglobin. The CAK is expensive and not suited for out-of-hospital use. These defects prevent indiscriminate use in all but confirmed cyanide intoxications, and can thus result in delays of life-saving interventions. In 2006, FDA approved intravenous hydroxocobalamin for the treatment of known or suspected cyanide poisoning. [22] It has been recognized as an antidote for cyanide toxicity for more than 40 years, [21, 23] and is in active use in France as a cyanide antidote.

1.4. Vitamin B_{12} analogs; hydroxocobalamin and cobinamide chemistry, and their comparison

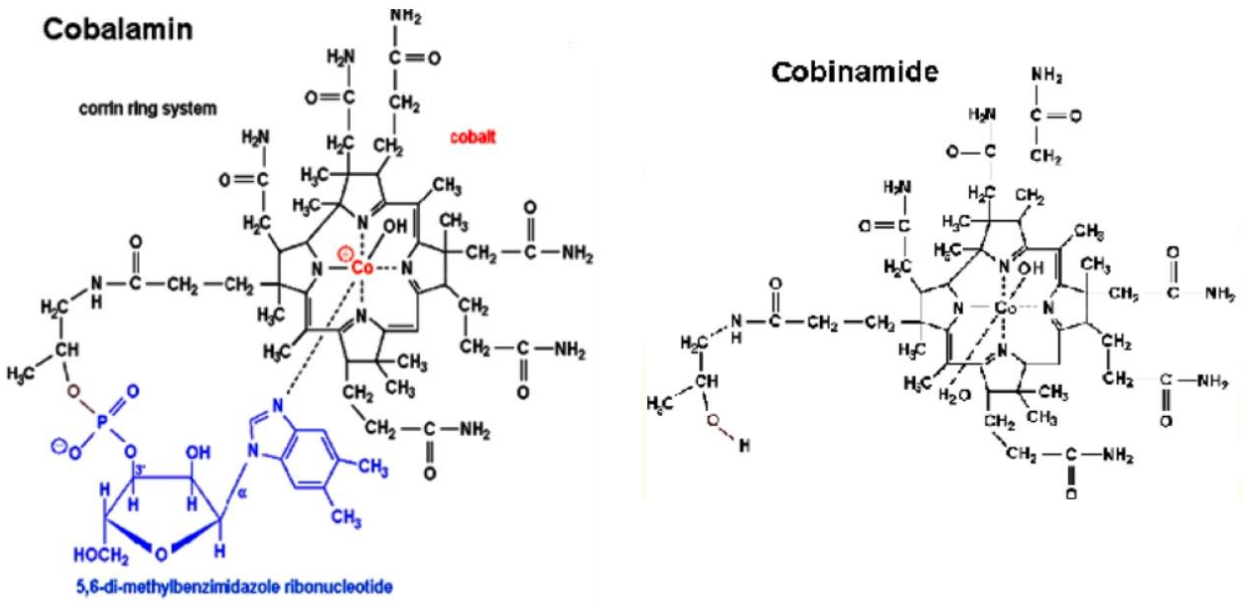
Hydroxocobalamin (OHCBI), is a form of vitamin $B_{12,}$ an approved treatment for cyanide poisoning. It is a precursor of cobalamin (vitamin B12) and has been used for 10 years in France as the treatment of choice for cyanide poisoning. Hydroxocobalamin binds cyanide in both intracellular and intravascular spaces to form cyanocobalamin, which is then

excreted in the urine. The core of hydroxocobalamin consists of a cobalt atom in the center of a corrin ring system. From six coordination sites in cobalt, four are used to bind the corrin ring. The fifth site binds the dimethyl-benzimidazole group and the sixth site is free and capable of binding to various ligands. [24]

Cobinamide is a novel agent for treating cyanide. It is more water soluble and is the penultimate precursor in the biosynthesis of cobalamin which lacks the dimethyl-benzimidazole tail (Fig. 2). Removal of the dimethyl-benzimidazole group allows cobinamide an additional free site for binding ligands. In addition, the dimethyl-benzimidazole group exerts a negative trans-effect on the upper binding site, thereby reduces hydroxocobalamin's affinity for ligands as well. [25] This means cobinamide is more effective than hydroxocobalamin in cyanide poisoning. [26]

The mentioned characteristics, i.e. high binding affinity, two binding sites for ligands, and water solubility, all suggest cobinamide's potential to be formulated for administration in concentrated solutions for intramuscular injections, and eventual development of an antidote for mass casualty exposures.

Figure 2. Molecular structures of cobalamin and cobinamide. Note the absence of the dimethyl-benzimidazole-nucleotide in cobinamide, thus increasing its affinity for ligands. [Brenner M. et al.]



1.5. Sulfide mitochondrial respiration inhibition: reversal by cobinamide

Currently there are several treatment options for hydrogen sulfide, most of which are palliative and not effective therapies; these include sodium nitrite, hydroxocobalamin, oxygen supplementation or hyperbaric oxygen. Of these, the two agents with low activity are hydroxocobalamin and sodium nitrite. Nitrite treatment is based on the same theory that was discussed for cyanide yet with less efficacy, because the complex between hydrogen sulfide and methemoglobin does not last long enough to make a difference.

Furthermore, hydrogen sulfide disappears rapidly from the circulation under conditions of good oxygenation. Also, evidence suggests that nitrite can only be effective within the first few minutes following exposure and may actually slow hydrogen sulfide removal thereafter.

Regarding hydroxocobalamin, because of its poor water solubility, it must be administered in relatively large volumes intravenously, making it impractical for treating mass casualties or in accident fields by non-health professionals. Thus, development of a drug which can be given rapidly in a small volume by intramuscular injection would be highly desirable. With similarities in the mechanism of toxicity in cyanide and hydrogen sulfide, cobinamide has attracted researchers' attention as a potential antidote for hydrogen sulfide as well. Recent years, many studies and research labs are running various animal models to investigate its efficacy.

<u>In vitro studies:</u>

Dr. Gerry Boss's group in UCSD (University of California, San Diego) have shown ultraviolet-visible (UV-vis) spectrum changes of cobinamide and how the sulfide reaction changes this spectrum. In a paper reporting from this study group, Jiang et al. graphed the relation of intracellular oxygen consumption during sulfide toxicity and after treatment with cobinamide (Fig. 3). [19] Jiang, et al. found that 1mM NaSH rapidly reduced oxygen consumption in neurons; cell oxygen consumption gives us a good measure of mitochondrial function. They also demonstrated that concomitantly with the fall in oxygen consumption, there is an increase in cellular acid production. This is due to a switch to anaerobic metabolism and lactate generation (Fig. 4).

Figure 3. Oxygen consumption measured in cortical neurons, as a surrogate measure of mitochondrial function. The effect of NaSH with and without cobinamide can be seen in exposed and control subjects. [Jiang et al.]

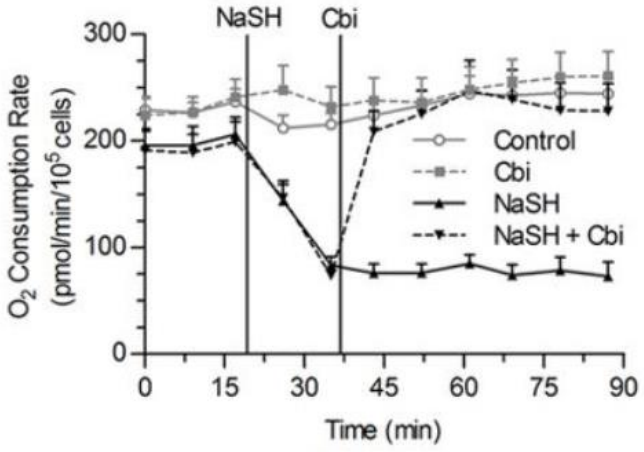
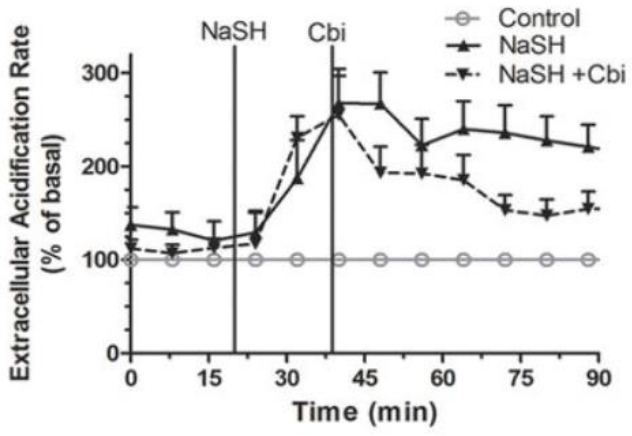


Figure 4. Concomitant with the fall in O_2 consumption, sulfide increases cellular acid production (presumed to be due to anaerobic metabolism and lactate generation). The effect of NaSH with and without cobinamide can be seen in exposed and control subjects. [Jiang et al.]



In vivo studies:

In several animal models it has been shown that cobinamide is the most effective therapy in treating hydrogen sulfide, compared to others previously published. In 2014, Brenner et al. reported the comparative effects of three cobinamide derivatives (different ligand forms) versus hydroxocobalamin and saline for control, in rabbits poisoned intravenously with NaHS. Their results showed groups treated with all cobinamide forms tolerated higher doses of hydrogen sulfide, and had increased survival times. [25] In 2017, Bebarta et al. reported the three-way comparison of intravenous cobinamide, hydroxocobalamin or saline in a large animal model study. He used swine for this purpose and demonstrated that all cobinamide-treated animals survived while none of the controls or hydroxocobalamin-treated animals survived. [6] In another study in 2018, Patrick Ng et al. evaluated the intramuscular administration of cobinamide for hydrogen sulfide poisoning. Here also, swine were poisoned with H_2S through NaHS intravenous infusion. Treated animals with intramuscular cobinamide were compared with saline controls. Cobinamide group had 100% survival, compared with 0% in the control group. [27]

CHAPTER 2 – METHODS

2.1. Materials

Based on the initial results of previous studies, in vitro and in vivo, as well as the information about the therapeutic agent, for the next phase in development, we proposed to study an in vivo model to investigate the efficacy of intramuscular cobinamide in an inhalation hydrogen sulfide gas exposure scenario.

Due to the highly complex nature of the interactions of metabolic poisons with multiple organ systems, it would be unethical to deliberately expose healthy human volunteers to a lethal toxic metabolic agent. In these circumstances, FDA has specific regulations and relies on evidence from animal studies. The FDA Animal Rule requires a survival endpoint difference in animal groups for efficacy approval. These FDA regulations specify: "1) there is a well-understood mechanism of the toxicity of the substance and its prevention or substantial reduction by the product, 2) the effect is demonstrated in more than one animal species expected to react with a response predictive for humans, unless the effect is demonstrated in a single animal species that represents a sufficiently well-characterized animal model for predicting the response in humans, and 3) the animal study endpoint is clearly related to the desired benefit in humans, generally the enhancement of survival or prevention of major morbidity." [28]

To come up with an effective treatment, having a carefully controlled model, which can simulate a realistic scenario of toxic inhalation exposure, as would likely be experienced by human victims, is essential.

2.2. Animal subjects

New Zealand White Rabbit

After initial screening in zebrafish and mice by groups we collaborate with, moderate and/or large animal size studies and models are needed to enable progression of development of promising candidate antidote. The rabbit model has been chosen because: 1) they are the smallest and least sentient species able to readily, simultaneously accommodate the monitoring devices (hemodynamic monitoring devices and CWNIRS), and 2) the physiologic mechanism of toxicity is more analogous to toxicity in humans than rodents.

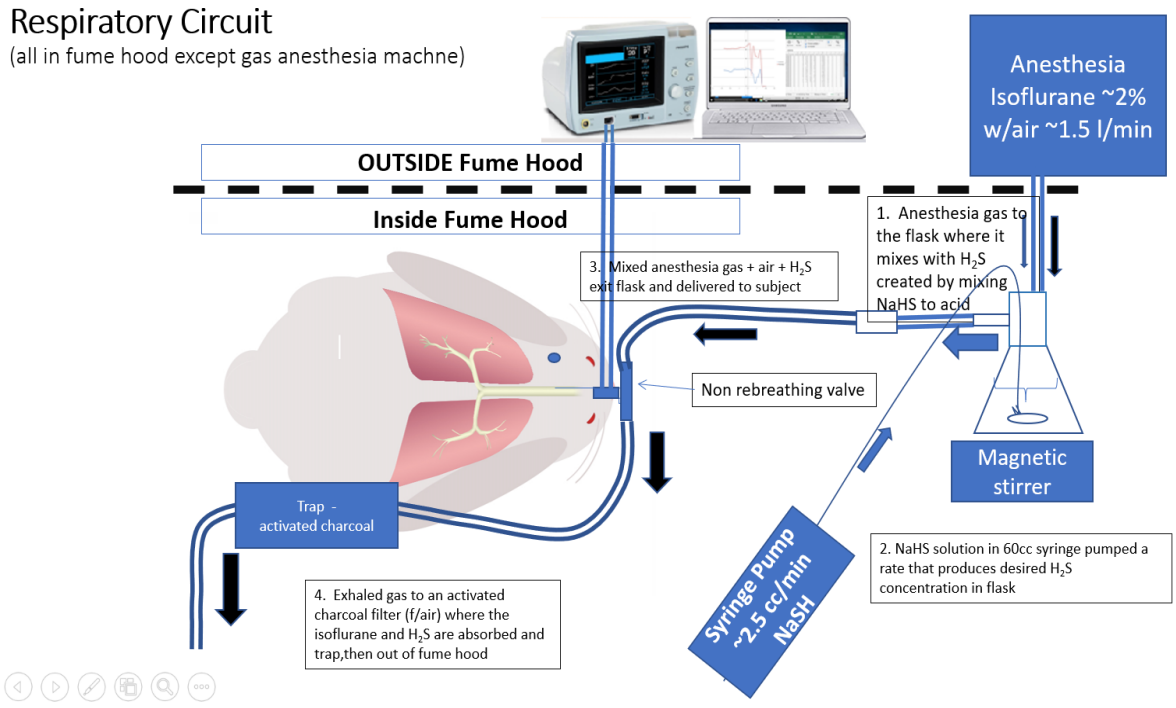
For these reasons, pathogen-free adult male New Zealand White rabbits weighing 3.5-5 kg were used in our studies. All studies and procedures were approved by the University of California, Irvine, Institutional Animal Care and Use Committee.

2.3. Study design and setting

To simulate likely real-time hydrogen sulfide inhalation exposure scenarios as closely as possible, a custom gas inhalation circuit was designed to deliver a mixture of hydrogen sulfide and isoflurane anesthesia in a compressed airflow to intubated rabbits. Anesthesia was required by our ARC in order to minimize discomfort/distress to the animals. Compressed air was supplied by Ohmeda VMC anesthesia machine, which vaporized isoflurane. It was set at 2.25 L/min to provide a flow-by rate of gas, and the rate was dropped to 1.8 L/min when the animal developed apnea with H_2S exposure. Sodium hydrosulfide, dissolved in saline at a concentration of 18mg/mL, was prepared minutes before the experiments. Then, the H_2S gas was produced by dripping the 320 mM NaHS

solution at a 2.5 mL/min rate into a reaction chamber containing 150 ml 3N-HCl. A disposable respiratory non-rebreathing valve was used to deliver the H_2S, air, and isoflurane mixture from the reaction vessel to the animal subject. This entire set-up, including the: [1] reaction chamber, [2] animal and [3] respiratory circuit, were all located inside a fume hood for safety. The exhaled gas was then sent to a container of activated charcoal where the isoflurane and H_2S were absorbed. During the experiment, H2S gas levels were monitored within the hood and circuit. This in situ generation system enables high dose exposure without requiring a large tank of dangerous lethal gas present.

Figure 5. Schematic experiment setup.



2.4. Animal preparation

Animals were initially anesthetized with an intramuscular injection of a 2:1 ratio of ketamine HCl (100mg/ml): Xylazine (20 mg/ml) at a dose of 0.75 cc/kg. The animals were then intubated with endotracheal tubes and were connected to the anesthesia machine with isoflurane 2%. A pulse oximeter, placed across the cheek, was used to measure SpO2 and heart rate. Blunt dissection was performed to isolate the femoral artery and vein on the left thigh (using a femoral sheath) for central venous and arterial catheter placement, used for systemic blood pressure monitoring and blood sampling.

2.5. Continuous wave near infrared spectroscopy (CWNIRS) and other vital signs measurements

CWNIRS was delivered over the rabbits' brain using a fiber optic probe to investigate the effects of sulfide on tissue oxyhemoglobin and deoxyhemoglobin concentrations.

Technical background: Visible light is absorbed and scattered by several tissue components, so it penetrates only short distances. On the other hand, near infrared (NIR) photons (700~900 nm) are capable of deeper penetration. They can penetrate bones, which make them practical for trans-cranial oximetry. Briefly, a NIR Spectroscopy (NIRS) setup consists of a light source, a spectrometer and customized optical fiber guides. The beam is directed to the target site using a fiber optic probe. On its way down, the beam interacts with different components, being partly reflected and scattered at certain tissue components. The NIR light will be partially absorbed by optical pigments, such as hemoglobin; thus depending on the oxygenation status of hemoglobin, the resultant spectrum will change. The fraction of light returning to the source light fiber will be

collected by detectors. [29] In this manner, the CWNIRS system provides rapid, real-time measurement of tissue oxygenation state, and is able to show continuous real-time changes in tissue circulating hemoglobin oxygenation status, during H_2S inhalation. [30]

2.6. Monitoring, and timing of antidote administration, and euthanasia

For all rabbits, vital signs including SPO2, heart rate, blood pressure and minute ventilation were monitored and recorded continuously during the entire experiment.

Antidote administration:

The time of administration of treatment was defined as observation of the first signs of any agonal breathing or apnea; as soon as this was recorded, a 1 cc injection was administered into the animal's left triceps. For the antidote treatment group this was 1 cc 150mM/ml of 5-acetyl tetrazole cobinamide; for the control group this was 1 cc normal saline.

Euthanasia:

The rabbit subjects' death had been defined as systolic blood pressure less than 20 mmHg on the continuous intra-arterial monitor. When this was recorded, in order to attempt a humane death, the animal was euthanized using an intravascular injection of 1cc Euthasol (pentobarbital sodium and phenytoin sodium).

2.7. Data analysis

a. Power analysis

Power analysis showed a requirement of minimum of 9 animals per group, assuming a survival rate of 10% in the control and 80% in the treatment group, with power for two tail $p<0.05$ and 80% chance of avoiding beta error.

So, by having 10 animals per group we would cover the range of potential control and treatment arm results expected, for reasonable efficacy of treatment in lethal chemical exposure. In our study, power analysis indicated that a total sample of 20 (10 rabbits each in the control and treatment groups) would be sufficient to detect a 4.5 minutes difference (E size=4.5) in the time-to-death with 80% power (α= 0.05) and standard deviation of 3.37.

b. Descriptive statistics

Baseline statistics across groups were compared. Specific two-group comparison testing was accomplished with a two-sample t-test. A two-tailed p value <0.05 was considered significant. All data were analyzed using IBM SPSS Statistics version 22.

c. Primary outcome

The primary outcome was to investigate efficacy of intramuscular cobinamide by answering this question: whether there is a significant difference in survival time/time to death among the treated group versus control groups. Our independent dichotomous variable is treatment group; control (saline) versus treated (Cob), and dependent continuous variable is time to death. For pursuing this aim, survival times were analyzed by developing Kaplan-Meier curves and the survival distributions were compared using log-rank test.

Also, continuous outcome was analyzed by using cox regression model after adjusting for potential confounder, like total minute ventilation volume.

CHAPTER 3 – RESULTS

3.1. Characteristics of study subjects

The NaHS dose produced in our system was precise among all animals and was calculated to achieve 100% fatality. In our lethal model, animals were exposed to concentrations of approximately 2000 ppm of H2S, although the amount of gas inhaled depends on other factors like, respiratory rate and minute ventilation. A total of 20 animals were studied, 10 received an intramuscular (IM) injection of saline, and 10 received IM injection of 1cc 150mM 5-acetyl tetrazole cobinamide.

One lethal endpoint of hydrogen sulfide exposure (the usual experimental endpoint in rats) is respiratory paralysis, a dose-dependent reduction in ventilatory drive resulting in apnea, which follows an initial reflex hyperpnea. [2] We gave the Injections when any of these changes in the respiratory pattern were seen, such as apnea or agonal breathing.

At baseline, the groups had similar vital signs and biological variables. There was no significant difference in weight between the two groups. Comparing the amount of gas inhaled by animals in liter, showed a p value < 0.05.

.

Table 2. Study subject characteristics

	Control (saline) group n=10	**Cobinamide group n=10**	**Mean Difference between groups (95% CI)**	**P value**
Weight (kg)	4.3 ± 0.4	4.05 ± 0.4	0.25 (-0.12,0.6)	0.17
Time to death	12.6 ± 3.37	19.5 ± 7.26	-6.9 (-12.3, -1.4)	0.018
Total ventilation volume (lit)	8.67 ± 2.21	11.66 ± 5.29	-2.9 (-6.8, 0.8)	0.11

Data presented as mean ± standard deviation

3.2. Primary outcome

The mean survival time for the control group was 12.6 ± 3.37 versus 19.5 ± 7.26 for the cobinamide group. Log Rank analysis indicated a significant difference in survival time between the cobinamide and control (saline) group (p .007). The Kaplan Meier survival curve (Fig 6) shows the increased survival of animals receiving antidote compared with the survival of controls. It is important to emphasize the H_2S inhalation exposure continued throughout the study, so animal groups with the highest survival curves inhaled more total toxic gas.

Figure 6. Kaplan Meier survival curve, comparing time to death in control group (0 = blue) to the cobinamide treatment group (1 = green).

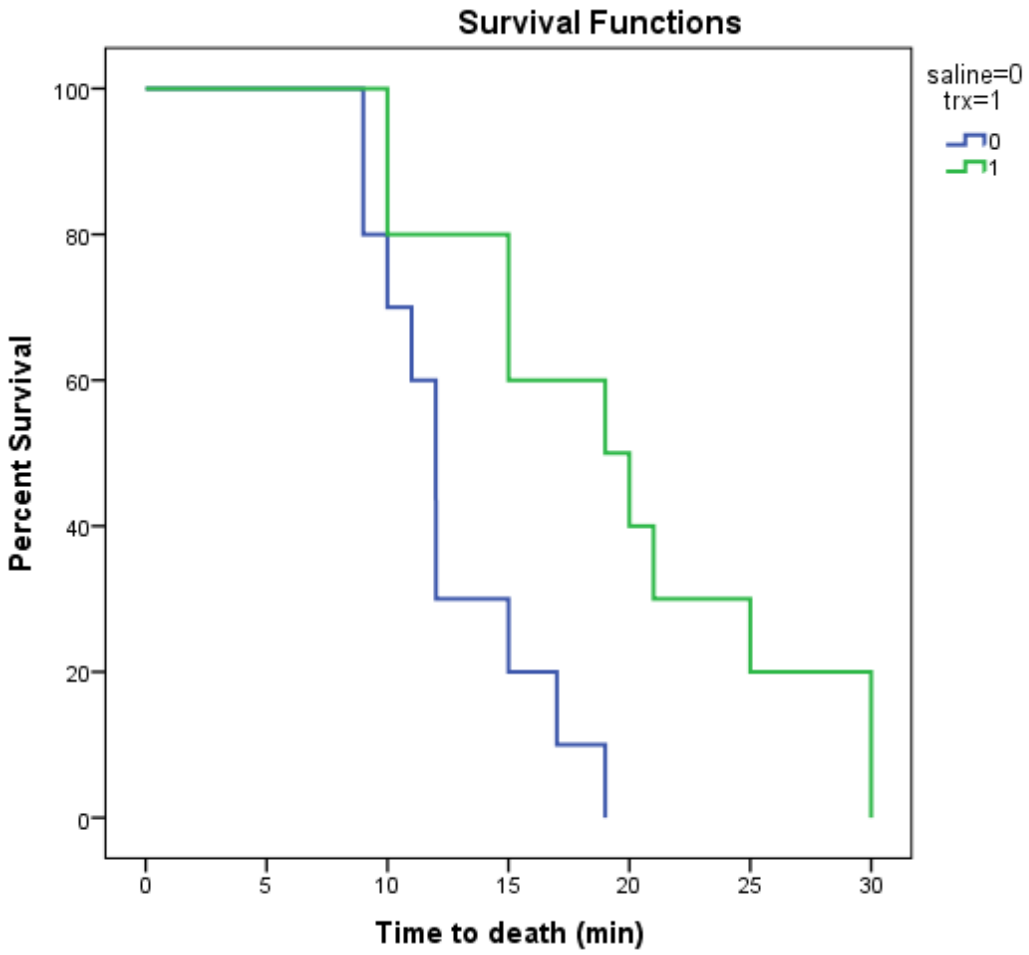


Table 3. Log rank analysis

Overall Comparisons

	Chi-Square	df	Sig.
Log Rank (Mantel-Cox)	7.258	1	.007

Test of equality of survival distributions for the different levels of saline=0 trx=1.

Table 4. cox regression analysis

Variables in the Equation

	B	SE	Wald	df	Sig.	Exp(B)	95.0% CI for Exp(B)	
							Lower	Upper
treatment	-1.264	.598	4.463	1	.035	.283	.088	.913
TotalVent	-.243	.088	7.619	1	.006	.784	.660	.932

Since the primary outcome was significant, on the second step, we modeled our analysis with cox regression, after adjusting for total ventilation volume. Since our treated group with cobinamide survived longer, they inhaled larger amounts of hydrogen sulfide and were exposed to toxic levels of approximately 2000 ppm for an extra 6 minutes each. This increased the chances of developing pulmonary edema, anoxia, systemic circulatory failure, and other fatal outcomes. After adjusting, the p value is still less than 0.05 and significant.

3.3. Other characteristics compared between the two groups

During the experiment, the animals' four major vital signs (respiratory rate [RR], oxygen saturation rate [SpO2], heart rate [HR] and blood pressure [BP]) were measured every minute, starting from the baseline (t = -1, one minute before exposure to the gas) to the end of the study (euthanasia administration time). This data was recorded and is shown below. For each group (cobinamide vs. control), the animals' vital signs plots have been graphed separately. Subsequently the mean of the vital sign variable at each one-minute time point for all 10 animals in the cobinamide versus control groups are compared together in one chart. To calculate the mean for each minute after the animals start dying, a value of 0 (zero) is entered for each dead animal, and the denominator is kept as 10.

Figures 7A, 7B, 7C. Recorded respiratory rates (RR), measured at one minute intervals, starting from one minute before H_2S inhalation was initiated (t= -1) to time of death of last rabbit, shown for (A) Control subjects, Rabbits 1-10, (B) Cobinamide subjects, Rabbits 11-20, and (C) Graph comparing mean RR for all 10 Control vs. 10 Cobinamide subjects. To calculate the mean for each minute after the animals start dying, a RR value of 0 (zero) is entered for each dead animal, and the denominator is kept as 10.

Figures 7A.

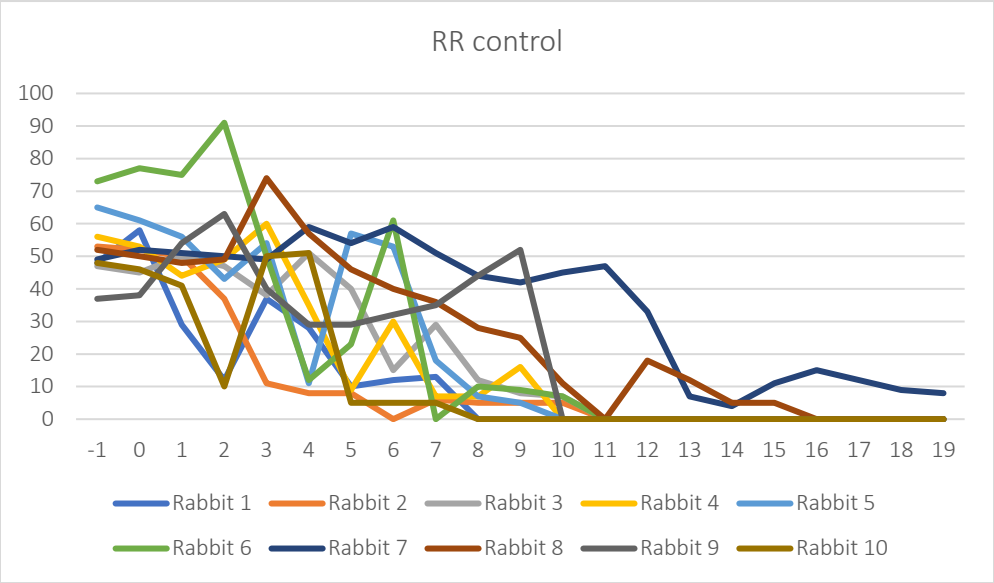


Figure 7B.

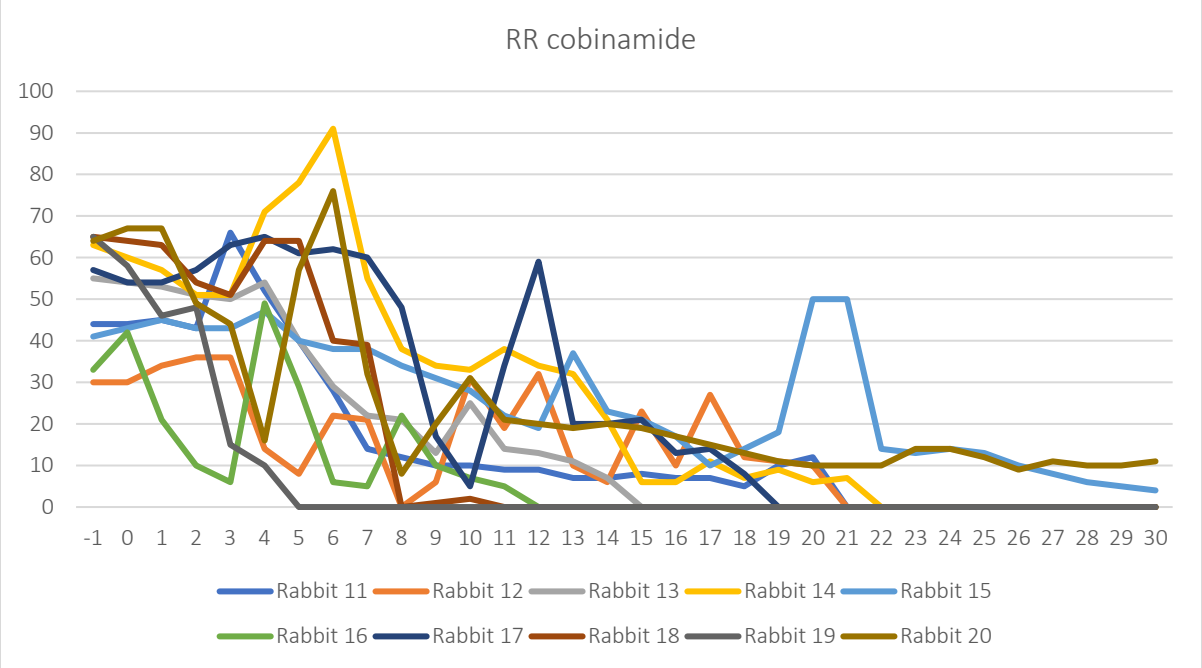
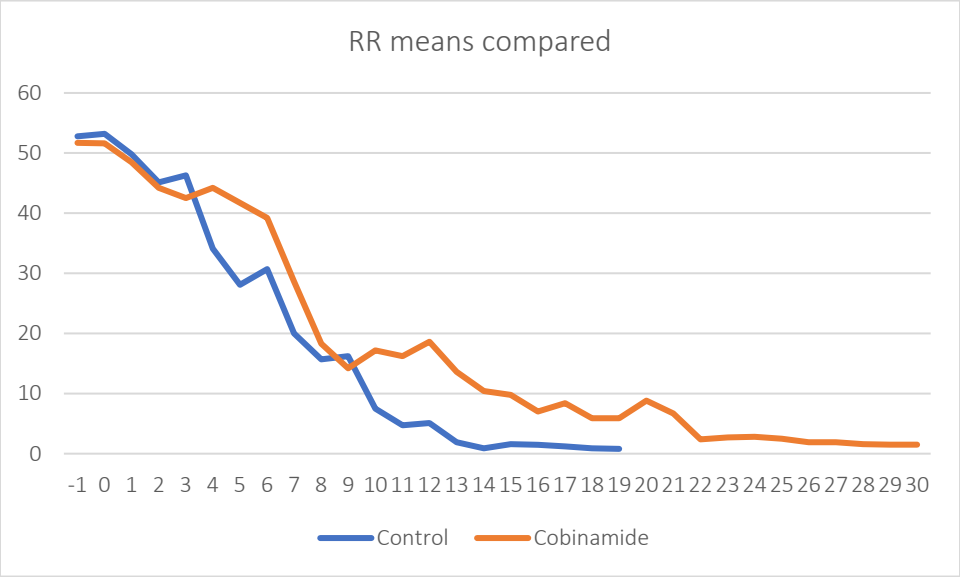
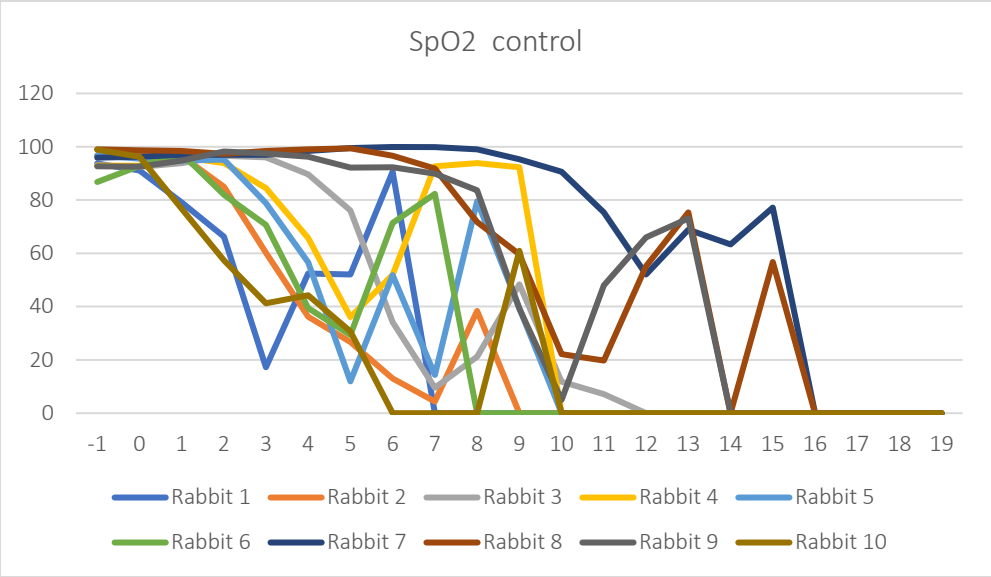


Figure 7C.

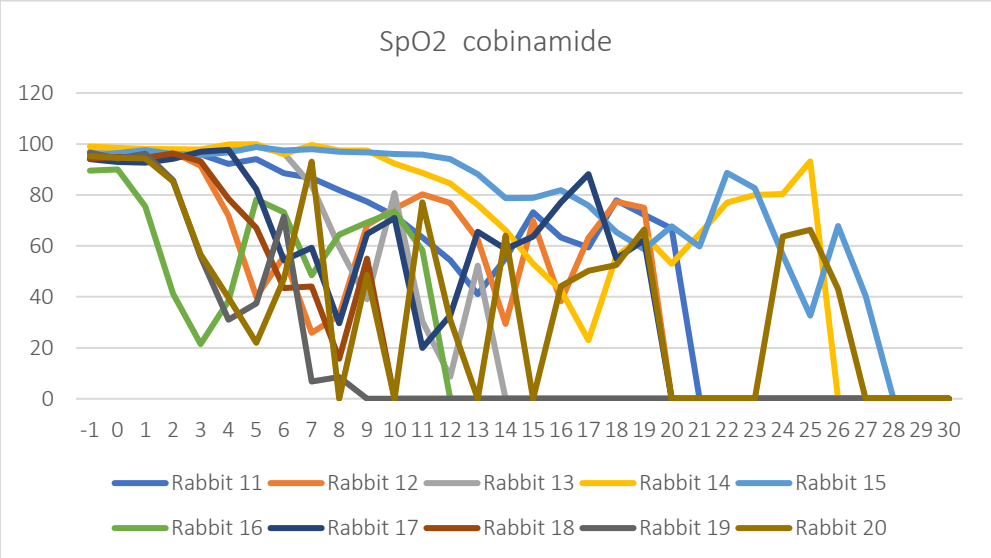


Figures 8A, 8B, 8C. Recorded oxygenation saturation rates (SpO2), measured at one minute intervals, starting from one minute before H_2S inhalation was initiated (t= -1) to time of death of last rabbit, shown for (A) Control subjects, Rabbits 1-10, (B) Cobinamide subjects, Rabbits 11-20, and (C) Graph comparing mean SpO2 for all 10 Control vs. 10 Cobinamide subjects. To calculate the mean for each minute after the animals start dying, a SpO2 value of 0 (zero) is entered for each dead animal, and the denominator is kept as 10.

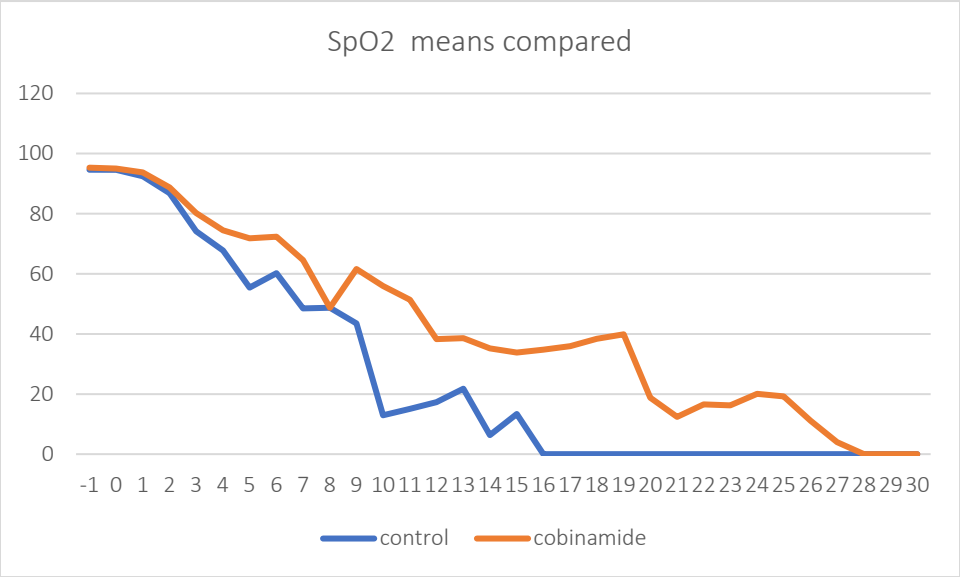
Figures 8A.



Figures 8B.

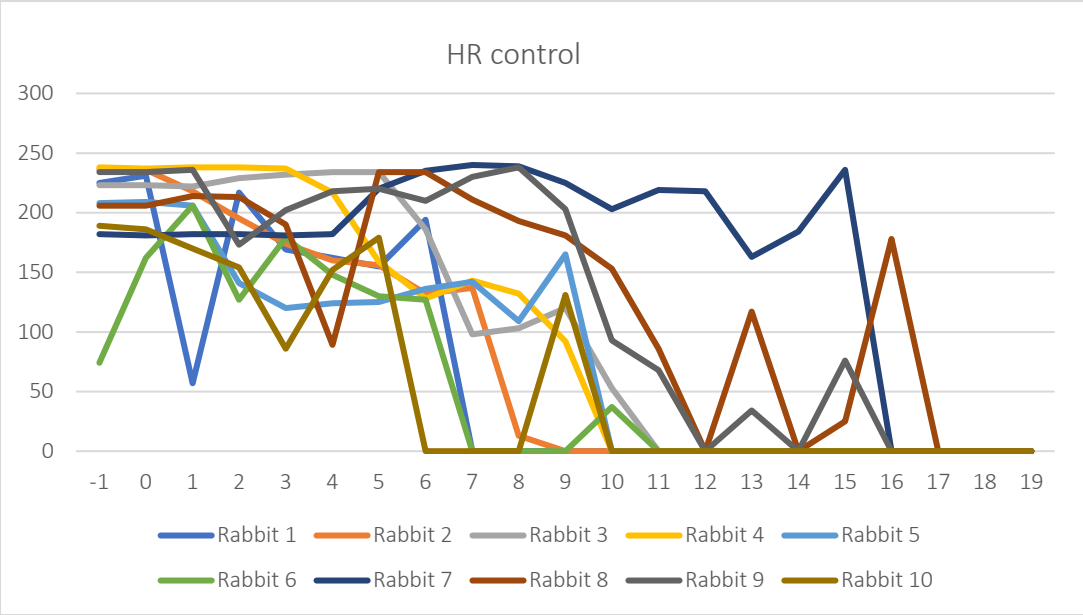


Figures 8C.

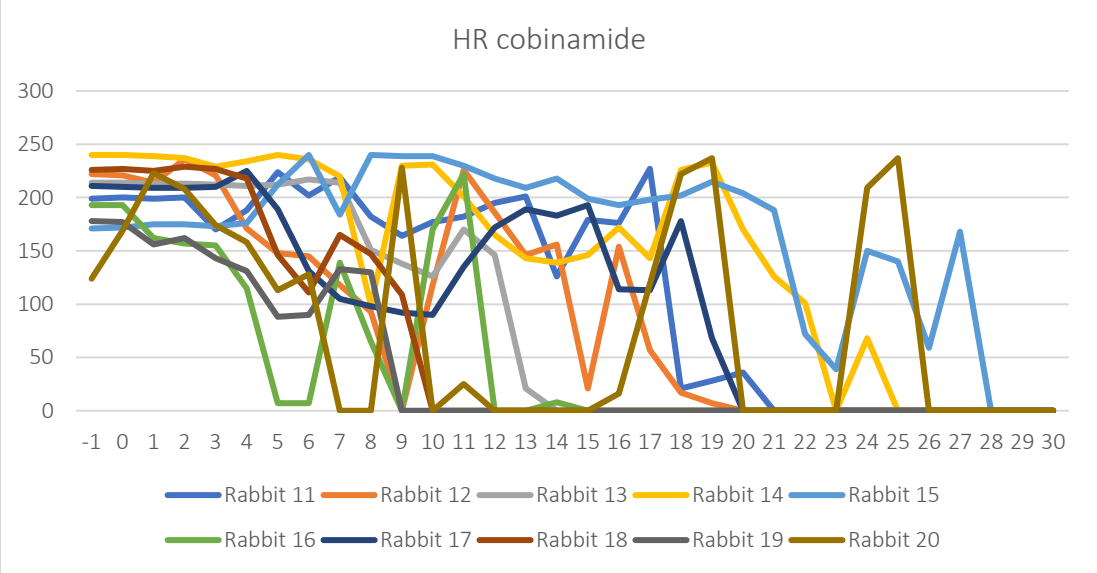


Figures 9A, 9B, 9C. Recorded heart rates (HR), measured at one minute intervals, starting from one minute before H_2S inhalation was initiated (t= -1) to time of death of last rabbit, shown for (A) Control subjects, Rabbits 1-10, (B) Cobinamide subjects, Rabbits 11-20, and (C) Graph comparing mean HR for all 10 Control vs. 10 Cobinamide subjects. To calculate the mean for each minute after the animals start dying, a HR value of 0 (zero) is entered for each dead animal, and the denominator is kept as 10.

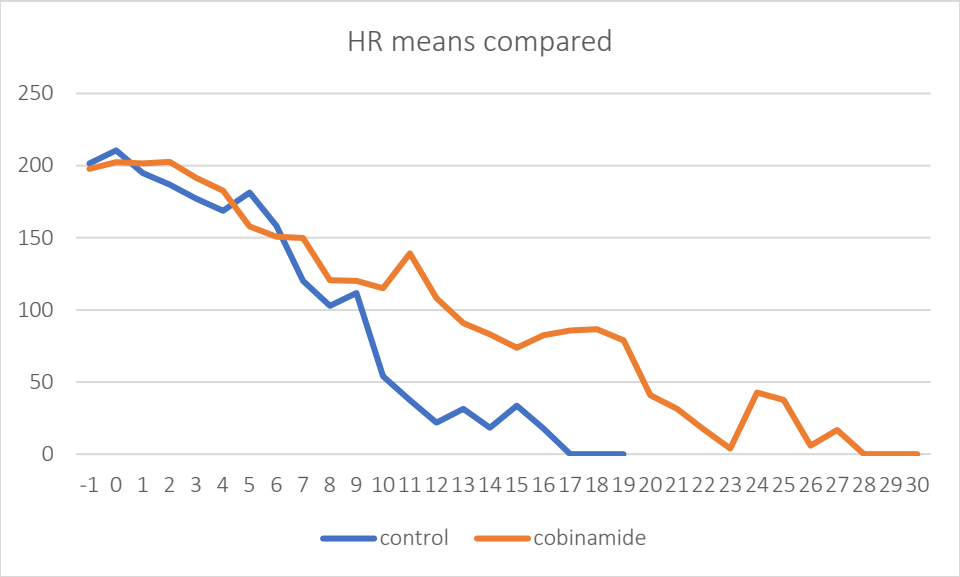
Figures 9A.



Figures 9B.

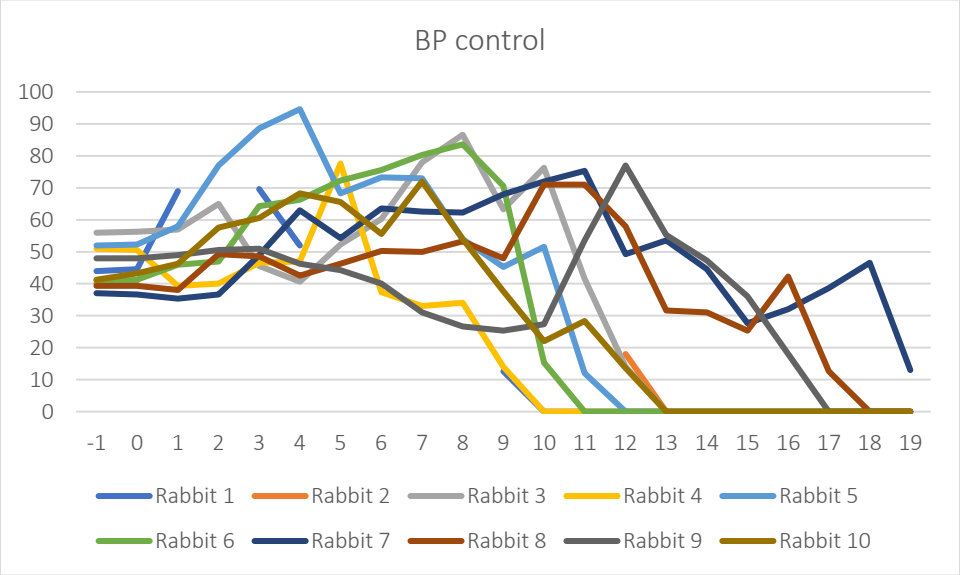


Figures 9C.

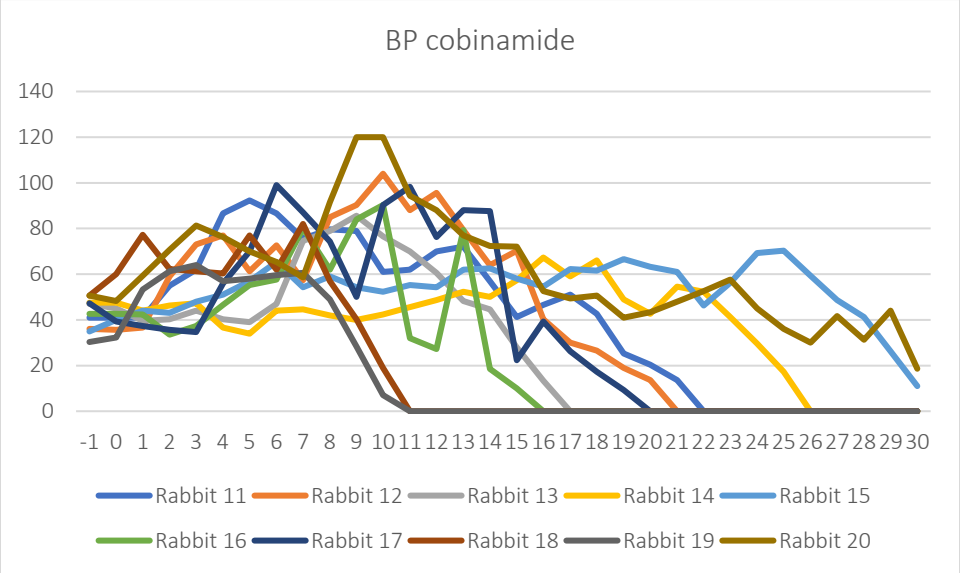


Figures 10A, 10B, 10C. Recorded mean blood pressures (BP), measured at one minute intervals, starting from one minute before H_2S inhalation was initiated (t= -1) to time of death of last rabbit, shown for (A) Control subjects, Rabbits 1-10, (B) Cobinamide subjects, Rabbits 11-20, and (C) Graph comparing mean BP for all 10 Control vs. 10 Cobinamide subjects. To calculate the mean for each minute after the animals start dying, a BP value of 0 (zero) is entered for each dead animal, and the denominator is kept as 10.

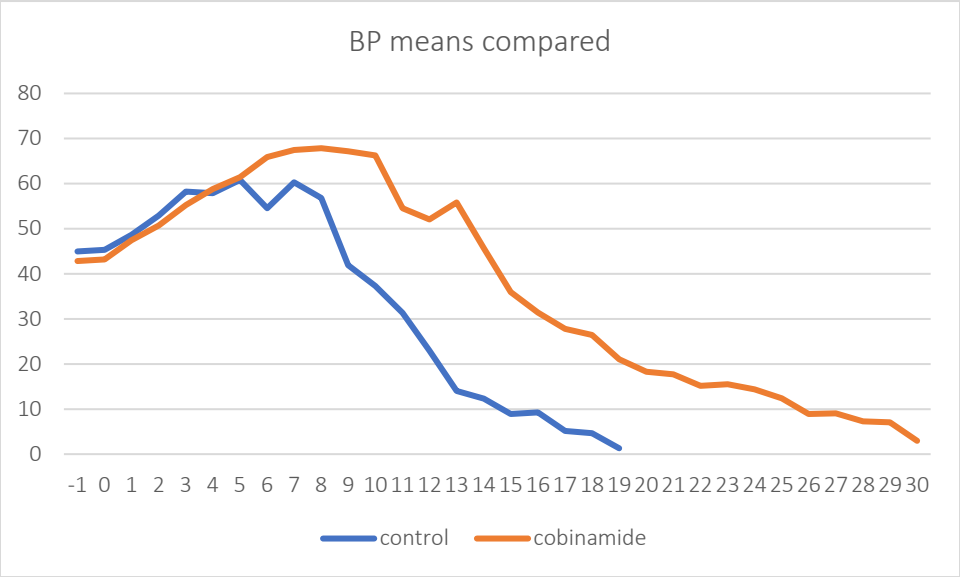
Figures 10A.



Figures 10B.



Figures 10C.


BP means compared
80
70
60
50
40
30
20
10
0
-1 0 1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21 22 23 24 25 26 27 28 29 30
control
cobinamide

CHAPTER 4 – DISCUSSION

To our knowledge this is the first inhalation study of H_2S in a non-rodent animal. [31] In our study, intramuscular administration of cobinamide caused hydrogen sulfide toxic animals to survive an average seven minutes longer.

Apnea or diminished respiratory effort occur quickly after H2S exposure. A diminished respiratory effort, in the absence of full apnea, by virtue of lowering the air flow rate actually increases the inhaled H_2S concentrations the animal is exposed to, making the injury even worse. This is because H_2S is generated in the flask at a constant rate, with the air flowing by the flask surface picking up the volatile H_2S. When the animal breathes normally, since air flows by the surface faster, there is a lower concentration of H_2S in the flow-by gas. As the animal's respiratory effort diminishes, with a decrease in the flow-by rate, the H_2S concentration rises. (This is done to make sure a highly lethal level of H_2S gas is administered.) After full apnea occurs, this situation can likely be similar to the clinical scenario where a victim is removed from the hydrogen sulfide-contaminated area. At the point where apnea has occurred, there is no more H_2S being inhaled, while the rabbit has received the cobinamide antidote; this is much like a patient who has been moved to fresh air and received cobinamide.

We modeled our study based on pilot experiments, and thus created a precise, reasonably consistent lethal inhalation model whereby all animals were exposed to 1600-2000 ppm hydrogen sulfide. We hypothesized that if we could detect a statistically meaningful survival time difference in this lethal model, the antidote would be more promising to be

efficacious in real-life exposures, where victims are hopefully evacuated in the first few minutes. So, in the next phase of antidote development studies, we could confirm this in a lower concentration inhalation model, in which treatment animals will have their exposure stopped at some point and become full survivors; this would be analogous to being removed from the exposure site in a timely safe period.

One acute, fatal complication of H_2S toxicity, other than central neurotoxicity and apnea, which occurs frequently in inhalation exposures is pulmonary edema. [30] We observed that clinical evidence of pulmonary edema developed in some of our animals in the cobinamide treatment group, who had longer survival times; this was manifested by audible rale and frothy pinkish respiratory secretions.

Our current study has several limitations. First, anesthetized and intubated animals do not fully reproduce real-life exposures, and for some metabolic agents, anesthesia may have some partial protective effects. However, since awake animal studies would be inhumane, current animal care guidelines discourage and limit the use of awake animals in toxic studies such as hydrogen sulfide. Yet this limitation could have been partially mitigated by the fact that both hydrogen sulfide exposed groups of control and cobinamide antidote treatment animals were similarly anesthetized and intubated.

Second, anesthesia might bias our data by the fact that inducing hypotension could increase the susceptibility of the animals to the cardio-respiratory depressant effect of hydrogen sulfide. Although, conversely, in the inhaled model, the effect of general anesthetics could protect the animals through decreased minute ventilation, or by preventing hyperventilation in response to gas exposure. [32]

Third, our study was not conducted in a randomized model. However, all animals were from the same supplier and the same inbred strain.

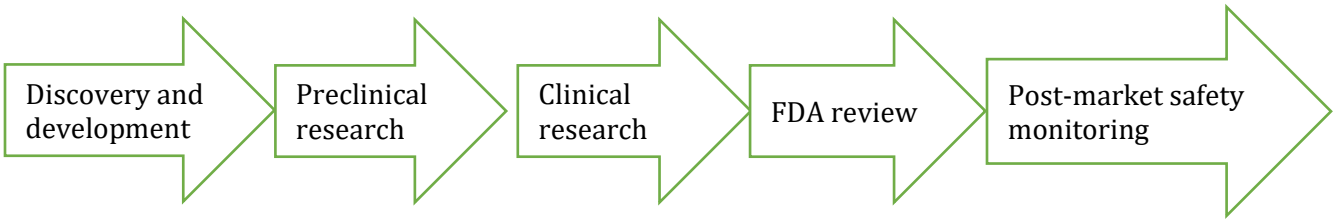
Despite the limitations, this study demonstrates that cobinamide shows considerable promise as an antidote for H_2S poisoning. Cobinamide has a long pharmacokinetic half-life of 32 minutes [32], suggesting it could potentially be used for prophylaxis in high-risk scenarios such as first responders, and for stabilization until victims are removed from the exposed site.

CHAPTER 5 – FUTURE WORK DESIGN

Future prospective

The development of a new drug requires a strategic plan, consisting of different phases of evaluation: these encompass a chain of events which may overlap or transcend the non-clinical and clinical investigation steps.

Figure 11. Essential steps of the drug development process



The evaluation of non-clinical activity and efficacy of a new drug candidate includes *in vitro* and *in vivo* assays that can be carried out throughout all stages of drug development. These tests are essential in order to provide the basic pharmacodynamics knowledge about the drug, which will then be required to allow the drug to enter clinical studies. [33]

Before moving to clinical trials and testing the promising drug on humans, the important step is to determine the safety threshold of the agent. The FDA requires scholars to use Good Laboratory Practices (GLP) for this aim.

GLP was instituted in the USA in the 1970s due to concerns about validity of non-clinical safety data submitted to FDA for New Drug Applications. There were different reasons behind the need for GLP's being established, such as inadequate planning and incompetent

execution of studies, insufficient documentation of results, and even cases of outright fraud. [34] The GLP regulations provide the basis for assurance that outcomes of studies submitted to FDA are faithful and completely comply and document the experimental work conducted. Thus, the concept given the term "GLP" is defined as "a quality program related to organizational processes and conditions where non-clinical health and environmental safety are planned, performed, monitored, recorded, reported and archived". [34] The basic requirements in GLP regulations are found in the Code of Federal Regulations (CFR), title 21 [35], setting the minimum basic requirements, which are:

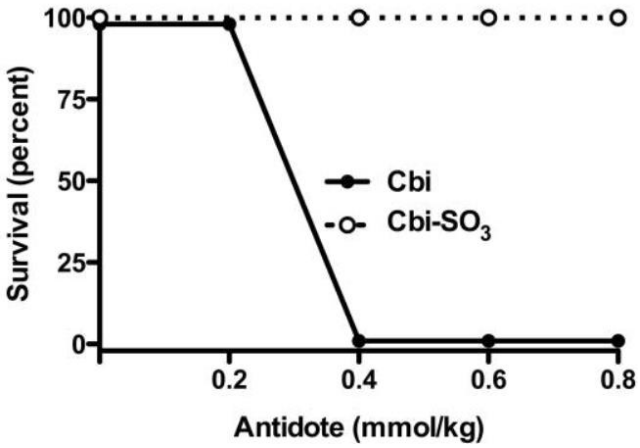
- Study conduct
- Personnel
- Facilities
- Equipment
- Written protocols
- Operating procedures
- Study reports
- A system of quality assurance oversight for each study, to help assure the safety of FDA-regulated product

Another subpart of CFR Title 21 (part 314: Application for FDA approval to market a new drug; Sec. 314.500, scope) applies to specific new drug products which have been studied for their efficacy and safety to ameliorate or prevent serious or life-threatening conditions which are caused by exposure to lethal toxic substances. These are studies that would be unethical to deliberately expose healthy human volunteers to a lethal or permanently disabling toxic agent, in order to investigate the product's safeness and effectiveness. In these circumstances FDA approval is based on adequate and well-controlled animal studies

(preferably more than one species), if the results of those animal studies establish that the drug is reasonably likely to produce clinical benefit in humans.

In our study the promising effectiveness of cobinamide has been demonstrated, and currently more studies are going on in other translational research laboratories. The first in vivo safety/toxicity study for a new drug is a dose range finding (DRF), in rodents. It is more practical to explore adverse effects in rodent species prior to non-rodent species. This step will also increase the available information for the design of non-rodent studies. Adriano Chan et.al, performed the general range-finding toxicity studies in mice. [32] He reported animals injected with 0.2 mmol/kg (200 mg/kg) tolerated this dose with no adverse effect. At 0.4 mmol/kg (400 mg/kg) they showed reduced activity followed by respiratory distress, bent posture, piloerection and finally death after 36 hours. LD-50 for cobinamide was calculated as 0.32 mmol/kg. Chan postulated that this adverse effect could be from cobinamide binding to endogenous NO, which may lead to systemic hypertension. Therefore, they next tested a ligand form of cobinamide; cobinamide sulfite. At doses up to 2 mmol/kg (2000 mg/kg), cobinamide sulfite induced no clinical signs of toxicity and animals survived at least for seven days. [32]

Figure 12. Range finding toxicity studies in mice. [Adriano Chan et. al]



The next step to support cobinamide safety, would be to imply the previous design to non-rodent GLP DRF studies, initially in rabbits, and later as a confirmatory in swine. In this testing, we will administer cobinamide intramuscularly, as this is the route it will be administered in humans, in the final product delivery mode. We will use a standard up-and-down procedure to find LD-50 and maximum tolerated dose (MTD) for rabbits. In this method, our test animals will be dosed one at a time. Each animal will be observed for 1 or 2 days. If it survives, the dose for the next animals is increased. If it dies, we will decrease the dose. [36]

Although animal models can generate the relevant information on the non-clinical safety and efficacy of cobinamide, they may be far from reproducing all the potential signs and symptoms in humans. Thus, the definitive proof can only be confirmed in completing phase I and II clinical trials. Nonetheless, animal studies are the pre-requisite and essential initial steps in guiding the early stages of drug development, especially in order to make the

determination whether to continue the project or not. Although efficacy studies cannot be performed in humans, safety studies can be performed for the candidate antidote. Therefore, after completion of animal GLP toxicity studies, safety studies in humans can be performed based on the animal studies of toxicity and therapeutic dose ranges.

My proposed clinical safety trial protocol for Cobinamide:

1- Title: Phase I dose-escalation, safety study of cobinamide in healthy volunteers

2- Objectives: primary objective is to define safety and tolerability of cobinamide in healthy humans by determining dose-limiting toxicity (DLT)

3- Study population:

This is going to be the first pilot study examining the proposed antidote on humans. Up to 20 healthy volunteers will be enrolled.

3-1- Inclusion Criteria

Patients eligible for inclusion in this trial must fulfill all the following criteria:

- Provide signed and dated informed consent prior to initiation of the study
- Male or Female patients aged ≥18 years
- Normal renal function [creatinine 0.9-1.3 mg/dl for adult males and 0.6- 1.1 mg/dl for adult females] or GFR of ≥ 90 mL/min
- Normal hepatic function [albumin, total bilirubin, AST, ALT value in the normal range of the laboratory]

- Normal cardiac function; left ventricular ejection fraction (LVEF) of ≥ 60% as assessed by echocardiography.
- For woman of child-bearing age: negative pregnancy test

3-2- Exclusion criteria

Patients who fulfill 1 or more of the following criteria will not be eligible to participate in this trial:

- Concomitant malignancies
- Uncontrolled or severe intercurrent medical condition
- Any known controlled or uncontrolled central nervous system involvement
- Any concurrent or prior use of cytotoxic regimens with delayed toxicity within the last four weeks
- Major surgery within the last four weeks
- Any major gastrointestinal or intracranial bleeding within the last four weeks
- Pregnant or breast-feeding women
- Any medical illness or abnormal laboratory finding that would increase the risk of participating in this study (based on the investigator's judgment)

4- Methodology/study design:

4-1- Dose conversion from animals to humans

We extrapolate the initial starting dose from previous animal species. Allometric scaling is an empirical approach whereby the exchange of drug dose is based on normalization of dose to body surface area (BSA). This approach assumes that there are some unique

characteristic physiological and biological processes in each species that account for the possible differences in pharmacokinetics. [37] Figure13 shows the conversion of animal doses to human equivalent doses (HED), based on BSA.

Figure 13. Conversion of animal doses to human equivalent doses (HED) based on body surface area (BSA). [Nair et al.]

Species	Reference body weight (kg)	To convert dose in mg/kg to dose in mg/m^2, divide by K_m	To convert human dose in mg/kg to AED in mg/kg, either	
			Multiply human dose by	Divide human dose by
Human	60	37		
Mouse	0.02	3	12.3	0.081
Hamster	0.08	5	7.4	0.135
Rat	0.15	6	6.2	0.162
Ferret	0.30	7	5.3	0.189
Guinea pig	0.40	8	4.6	0.216
Rabbit	1.8	12	3.1	0.324
Dog	10	20	1.8	0.541
Monkeys (rhesus)	3	12	3.1	0.324
Marmoset	0.35	6	6.2	0.162
Squirrel monkey	0.60	7	5.3	0.189
Baboon	12	20	1.8	0.541
Micro pig	12	27	1.4	0.730
Mini pig	40	35	1.1	0.946

*Data adapted and modified from FDA draft guidelines.[7] FDA: Food and Drug Administration, AED: Animal equivalent dose

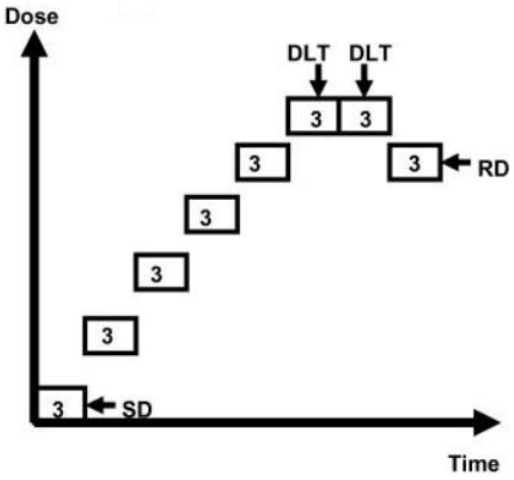
4-2- Dose-escalation phase:

We will propose to use the traditional 3+3 design. This method is the prevailing approach for conducting phase I cancer clinical trials. [38] The advantage of this method is that it requires no modeling of the typical dose-toxicity curves beyond the classic assumption of cytotoxic drugs that toxicity increases with increased dose.

The rationales for choosing this approach are as follows. First, this is going to be the first-in-human phase I clinical trial for cobinamide with no previous data about clinical complications or outcomes. Second, our information is only from animal-based studies and it would be unethical to expose healthy volunteers to a promising antidote with no in-human information. Third, in these circumstances the smoothest approach would be assuming the antidote like an anticancer drug, while later, based on further clinical information, we could propose other dose-escalation methods as well.

The 3+3 design will be conducted as follows. Initially, 3 volunteers will be enrolled and receive the first starting dose. If none of the three volunteers experiences a dose-limiting toxicity (DLT), another three volunteers will be treated at the next higher dose level. However, if one of the first three volunteers experiences a DLT, three more will be treated at the same dose level. This dose escalation will continue until at least two volunteers among three to six volunteers experience DLT (≥33% of volunteers with a DLT at that dose level). [38]

Figure 14. Graphical illustration of dose escalation 3+3 method [Le Tourneau et al.]



5-Statistical method:

The sample size for phase I is determined by clinical rather than statistical considerations, with approximately 20 volunteers usually recruited.

Continued vigilance for safety is critical as more data and experience is gathered from a broader population, once the product is on the market. This path that a drug travels from a lab to the medical field is usually long, and every drug may have a unique route, based on a wide variety of factors.

CHAPTER 6 – CONCLUSION

Many countries are under the constant threat of a mass casualty hydrogen sulfide disaster. This can be the result of industrial accidents, terrorist attacks, or hazardous material transportation incidents. The currently known incidents leading to mortality and morbidity are mostly industrial and work-related accidents and attempted suicides, while it is unfortunately acknowledged that the extent and burden of H_2S incidents appear to be generally underreported.

Absence of an FDA approved antidote or a reliable approach to treat acute hydrogen sulfide poisoning has raised considerable public health concern in recent years. The nature of moderate-to-severe H_2S intoxication demands rapid and definite diagnosis and intervention by medical personnel out of hospital to reduce morbidity and mortality. This would mean the ability and competency of first-responder personnel to ventilate patients and provide an antidote at the disaster site. H_2S suicides are on the rise, and industrial exposures continue to be problematic occupationally. The investigations conducted to-date are promising, but more is needed to be done to precisely uncover any unknown potential mechanisms of hydrogen sulfide toxicity and find effective and safe treatments for each.

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