UNIVERSITY OF CALIFORNIA SAN DIEGO

Development of Cobinamide as Potential Antidote for Azide Poisoning

A thesis submitted in partial satisfaction of the requirements for the degree Master of Science

in

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by

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The thesis of Cole D. Link is approved, and it is acceptable in quality and form for publication on microfilm and electronically:

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DEDICATION

I would like to dedicate this work to my friends and family for being there for me and pushing me to pursue my ambitions.

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Results, in part are currently being prepared for submission for publication of the material. Boss, Gerry; Tat, John; Link, Cole. The thesis author will be co-author of this material.

ABSTRACT OF THE THESIS

Development of Cobinamide as a Potential Antidote for Azide Poisoning

by

Cole D. Link

Master of Science in Biology

University of California San Diego, 2020

Professor Gerard R. Boss, Chair
Professor Matthew D. Daugherty, Co-Chair

Sodium azide, referred to as "NaN_3" or "azide" in this thesis, is a common pesticide, laboratory preservative, and propellent in vehicular airbags due to its explosive properties. Despite its practical utilities, azide is highly toxic. Ingestion of several grams can cause death in 1-2 hours. Azide's high toxicity in conjunction to its commercial availability are causes for concern and the chemical is listed as a potential terrorism threat (Holstege et al. 2007). There are two proposed mechanisms of azide toxicity: inhibition of cellular respiration via cytochrome C oxidase inhibition and generation of nitric oxide. Inhibition of cytochrome C by azide is well documented but generation of nitric oxide has only recently been found *in vivo*. Since there is no

FDA-approved antidote for azide poisoning, development of an antidote for azide poisoning is a public health priority. Members of the Boss Lab have shown that cobinamide (Cbi), a vitamin B_{12} analog, scavenges azide and nitric oxide. We hypothesized that cobinamide could antagonize azide poisoning based on azide's mechanism of toxicity and how cobinamide functions. We determined that azide inhibited cell growth and cobinamide offered protection. We saw that *D. melanogaster* (fruit flies) raised on food containing cobinamide were partially protected from azide toxicity, with survival as a readout. We found that azide induced nitric oxide generation in mice. Finally, we observed that cobinamide rescued mice poisoned with a lethal dose of azide. Altogether our results demonstrated azide toxicity *in vitro* and *in vivo* and that cobinamide could reverse these toxic effects.

Introduction

1.1 Sodium azide

Sodium azide (NaN_3) is a compound with many uses. It is used as an airbag propellant to allow for rapid inflation due to its extreme combustibility, leading to a peak in azide production at 10 to 12 million pounds per year (Chang et al. 2003). It is used in biomedical laboratories as a preservative since it inhibits microbial growth. It is found in explosives due to its combustibility (Dyer 2012). Finally, sodium azide is a common herbicide used in farms all over the world. The amount used can vary between 40 to 120 pounds per acre (Chang et al. 2003). Azide easily dissolves in water and does so with no noticeable odor or color. This, along with its accessibility—azide is very easy to obtain through online commercial retailers—and explosive qualities pose a threat to the public at-large (Leonard et al 2020). Azide has been used in terrorist attacks around the world (Okumura et al. 2003).

1.2 Azide poisoning

Azide's toxicity to humans and increased production along with its wide commercial use increase the potential for contact with humans. People are exposed to sodium azide in three mains ways: ingestion, transdermal, or transmucosal absorption. The common sequence of symptoms is headache, collapse, hypotension, tachypnea, and metabolic acidosis (Kurt et al. 2016). Symptoms appear within a few minutes of ingestion and systemic poisoning with just a few grams can occur within 1-2 hours (Dyer, 2012). Severity is dose dependent. In cases that cause health issues but did not result in death, patients were exposed to 0.01 mg/kg to 2 mg/kg. In cases that resulted in death, patients were exposed to >13 mg/kg (Chang et al. 2003).

The mechanisms by which azide kills is still under investigation. It is known that azide is a strong inhibitor of cytochrome C oxidase and therefore, cellular respiration (Yoshikawa 1974). Azide also causes hypotension, likely via its conversion to nitric oxide, a potent vasodilator (Puzserova, A, and I Bernatova, 2016). Azide can be oxidized by catalase and myeloperoxidase in the presence of H_20_2 to form nitric oxide (Vanuffelen, 1998). Cytochrome C oxidase inhibition and nitric oxide production potentially account for the metabolic acidosis and hypotension respectively seen in patients.

Currently there is no known FDA approved antidote for azide poisoning. It is recommended by the CDC to primarily give supportive care to the patient/victim ("CDC" 2011). Since cyanide poisoning inhibits cytochrome oxidase similarly to that of azide, cyanide antidotes appear to be logical azide antidotes (Yoshikawa, 1974). However, the cyanide antidotes sodium thiosulfate and sodium nitrate have not proven effective at treating azide poisoning (Schwarz 2014). In particular, administration of sodium nitrate may exacerbate NO-induced hypotension. Hydroxocobalamin is another antidote approved for treating cyanide and may even work as an azide antidote, likely due its ability to scavenge nitric oxide (Bonnett 1963; Borron et al. 2007; Sharma 2003).

1.2 Cobinamide, a precursor to cobalamin

Cobalamin is commonly known as vitamin B_{12}. In the body, cobalamin exists in multiple forms (Ermens et al., 2003). Cobalamin analogs are coenzymes in several biological reactions. For instance, methylcobalamin is a coenzyme that works with methionine synthase in the production of pyrimidines and purines (Ermens et al., 2003).

Cobalamin's ability to detoxify cyanide rests with its structure. Cobalamin is a molecule with a cobalt center with six coordination sites (Figure 1A). Four of the coordination sites are

attached to nitrogens. The fifth coordination site is attached to a 5,6-dimethylbenzimidazole that is attached by a nucleotide tail. This leaves the final coordination site free to bind ligands such as cyanide (Sharma et al. 2003).

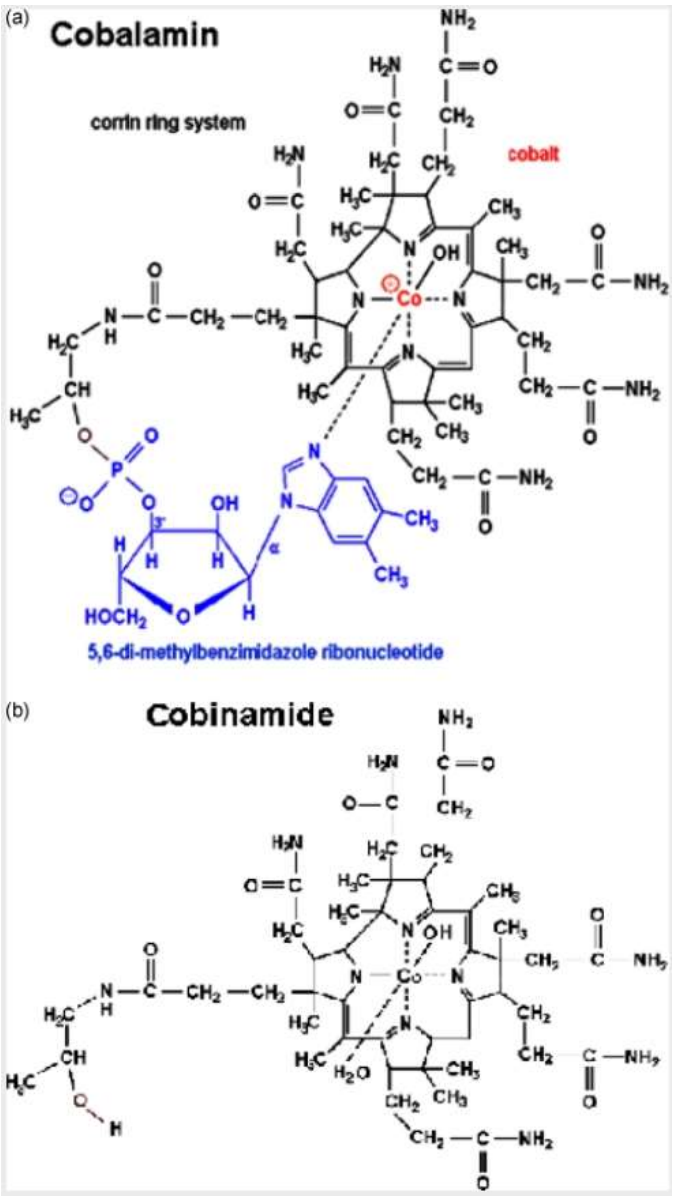


Figure 1: The molecules cobalamin (A) and cobinamide (B) (Brenner, 2010). Both cobalamin and cobinamide have a cobalt center with four nitrogen's attached. Cobalamin has a 5,6-dimethylbenzimidazole highlighted at the fifth coordinate and a free ligand at the other. The R group attached represents a hydroxyl group that is replaced when a ligand of a higher binding affinity attaches. The removal of the fifth coordinate indicates the structure of a cobinamide molecule. For this work, both the fifth and sixth coordinates were given a hydroxyl group allowing for cobinamide to have two free binding sites.

Cobinamide is structurally similar to cobalamin, but the fifth coordinate's 5,6-dimethylbenzimidazole functional group is replaced with a hydroxyl group that is readily displaced by ligands that can bind to cobinamide (Brenner 2010). So, instead of having one upper ligand binding site, cobinamide has upper and lower ligand binding sites. Also, the 5,6-dimethylbenzimidazole group has a negative trans effect on the upper binding site which in turn further reduces its binding affinity (Brenner 2010). Since cobinamide has two free ligand binding sites, cobinamide binds cyanide at a higher binding affinity than hydroxocobalmin (Brenner, 2010). Besides scavenging cyanide, cobinamide scavenges nitric oxide as well (Broderick et al., 2005). Furthermore, a former master's student in the Boss Lab has shown that cobinamide can bind azide directly (Lin 2012).

1.3 Central Goal

To determine azide's mechanism of toxicity and evaluate whether cobinamide can reverse the toxic effects.

Materials and Methods

2.1 Vitamin B_{12} analogs

Cobinamide (Cbi) was synthesized from hydroxocobalamin by base hydrolysis. It was then converted to histidylcobinamide by adding histidine (Sigma-Aldrich). Sodium azide, referred to throughout this text as azide was obtained from Research Products International (Sodium Azide, S24080, Research Products International, Mt. Prospect, IL, USA).

2.3 Cell culture

A549 and COS-7 cells were grown in Gibco DMEM GlutaMax (Dulbecco's Modified Eagle Medium) that contained 4.5g/L D-glucose and 10% fetal bovine serum (Sigma-Aldrich). COS-7 cells were derived from kidney cells of the African green monkey and acquired from ATCC (Manassas, VA, USA). A549 cells are human cancer cells taken from pulmonary tissue and were obtained from ATCC (Manassas, VA, USA). Cells were cultured at 37ºC at 5% CO_2 atmosphere.

2.5 Cell imaging

A549 and COS-7 cells were seeded at 300,000 cells per well in a six-well plate and given 24 hours to settle. Next, the cells were treated with azide with or without cobinamide for 24 hrs. Etoposide treatment served as a positive control. We used phenol-free Gibco DMEM 1X in this experiment to allow for clearer imaging. The EVOS M5000 Imaging System (Thermo Fisher) was used to capture the images.

2.4 Clonogenic survival assay

A549 Cells were plated on six-well plates at 125 cells per well and given 24 hours to settle. The cells were then treated with azide, with and without cobinamide for 24 hours. Cells were then released in drug-free medium for two weeks to allow colonies to form. Medium was then aspirated off and cells were washed with PBS. Cell colonies were stained with 0.5% crystal violet overnight and then counted by hand. Image was scanned using Epson Perfection 4990 Photo. One-way ANOVA with Bonferroni correction was used to determine statistical significance.

2.2 Fruit fly experiments

Drosophila melanogaster (fruit flies) were raised on standard food or food containing 400 μM cobalamin (OHCbl) or 400 μM dihistidyl-cobinamide (DHCbi or Cbi in this thesis). For experimentation, flies were anesthetized with ice and placed in a vial with a gauze containing either sodium phosphate (Na_3PO_4) pH 7.4 or 100 mM azide made in sodium phosphate pH 7.4. Intoxication and death were caused via azide ingestion. Life/death observation was recorded every hour for eight hours. Two-way ANOVA with Bonferroni correction was used to determine statistical significance

2.7 Cardiac puncture and blood draw

C57/black 6 mice were injected intraperitoneally with either 20 mg/kg of azide or water. Mice were then euthanized by carbon dioxide asphyxiation at 15 minutes, 30 minutes, 60 minutes, 120 minutes, 180 minutes, and 240 minutes post azide exposure. An incision was made in the mouse abdomen to access the heart, where a syringe and needle were used to draw blood directly from the heart.

2.8 Nitrite measurement

Nitrite levels were measured using a modified Griess assay. To this end, blood harvested from cardiac punctures was first spun down in microcentrifuge tubes (Thomas Scientific) to separate serum from plasma. The serum was taken out and spun down again in Vivaspin 500 centrifugal concentrator to reduce proteins in the sample (VS0112, Vivaproducts, Littleton, MA, USA). Then, 50 μL of each serum sample or 1X assay buffer for the control were put in a corresponding well of a 96-well plate. Since there was likely still azide in the serum, and azide inhibits nitrate reductase, an enzyme in the Griess assay, 5 μL of 0.5 M sulfuric acid was added and the samples were incubated at 37ºC at 5% CO_2 atmosphere for two hours to convert residual azide into hydrazoic acid, which was liberated. Any lost liquid was determined and replaced with 1X Assay Buffer. 5.1 μL of 1M NaOH and 2.5 μL 1 M Tris pH 7.4 was added to neutralize the pH in each sample. 10 μL of nitrate reductase and 20 μL of cofactors (Active motif, Lot# 13920037) were added to each well to convert nitrate to nitrite. The plate was then placed on the shaker for one minute and incubated at room temperature in the dark for 30 minutes. Finally, 100 μL of Griess reagent was added and allowed to react for 20 minutes. The absorbance was measured by BioTek™ Synergy™ 2 Multi-Mode Microplate Readers at 540 nm wavelength.

2.9 Mouse survival

Male C57/black 6 mice were anesthetized with isoflurane and then injected with azide intraperitoneally (IP). Immediately after azide exposure, mice were administered cobinamide intramuscularly. Survival was observed over a three-hour period. The statistical test was log-rank.

Results

Azide Treatment Inhibits Cell Growth; Cobinamide Rescues Cells

To determine the effect of azide on A549 and COS-7 cell growth, cells were treated in indicated conditions for 24 hours and then images were taken to determine morphological changes. Cobinamide treated cells showed no change in appearance, relative to untreated controls. Etoposide, an anti-cancer agent, led to appearance of shriveled cells, suggesting apoptosis. Azide-treated cells did not have the same apoptotic characteristics that the etoposide-treated cells had. Instead, they showed what seemed like reduced growth relative to untreated controls. The combined azide and cobinamide treatments both did not show apoptotic characteristics and seemed to have more growth than the azide alone.

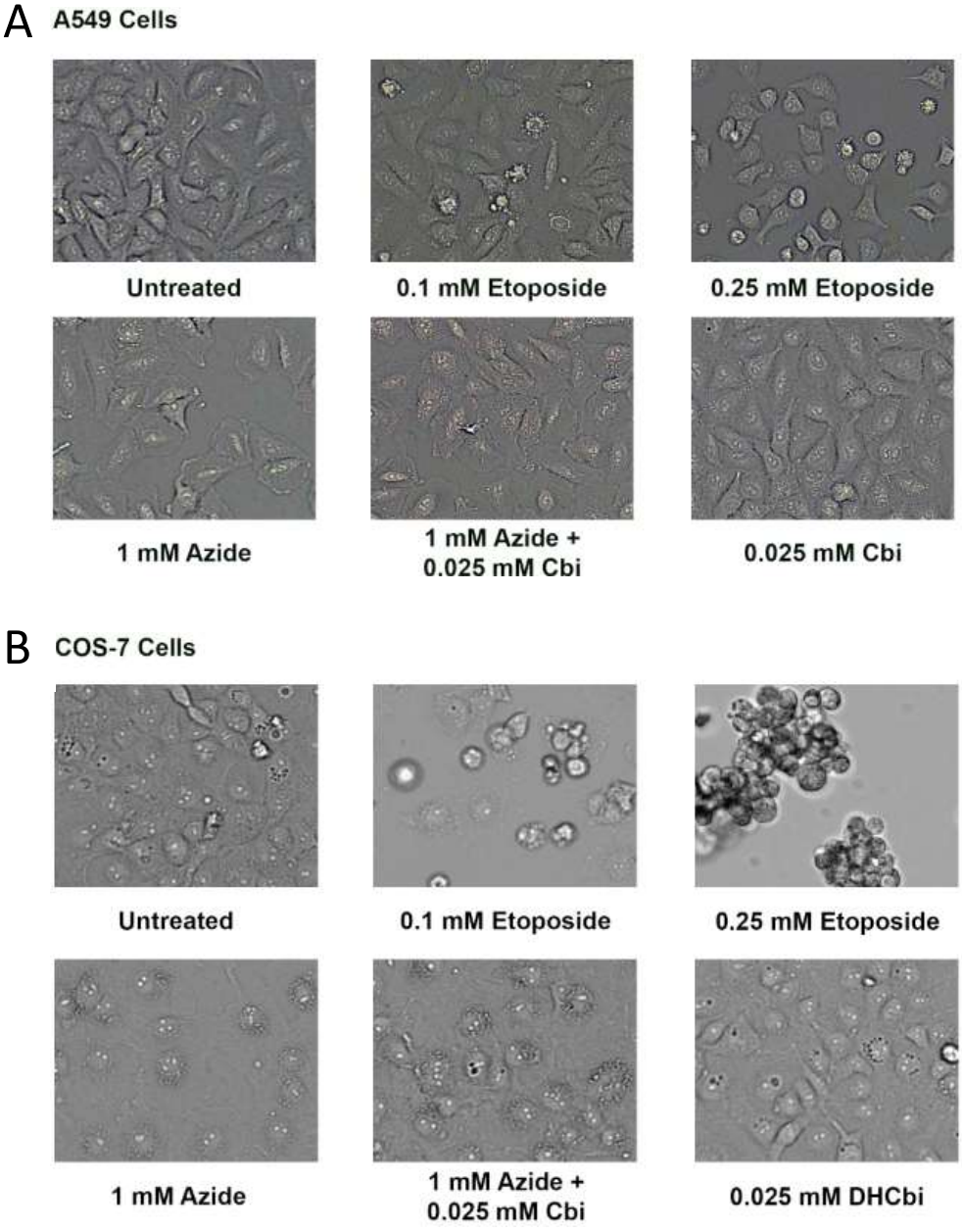


Figure 2: A549 (A) and COS-7 (B) treated cells do not show apoptotic characteristics.

Cobinamide Partially Rescues Colony Formation in Cells Treated with Azide

To further evaluate the effect of azide on cell growth, we carried out a clonogenic survival assay. The *in vitro* study was done with A549 cells only, due to their propensity to form colonies, whereas COS-7 cells were not included because they do not form discernable colonies as A549 cells do. After cells were allowed to settle for 24 hours, they were treated with azide, cobinamide, or a combination of azide and cobinamide for 24 hours. The media was then changed, and cells released in regular medium for two weeks to form colonies. Colonies were then stained and counted (Figure 3A). After treatment and subsequent detection, azide treated cells formed 42% fewer colonies than the untreated cells (Figure 3B). The cobinamide alone had no effect on the colony number. The wells with both cobinamide and azide had 17% more colonies formed than azide alone.

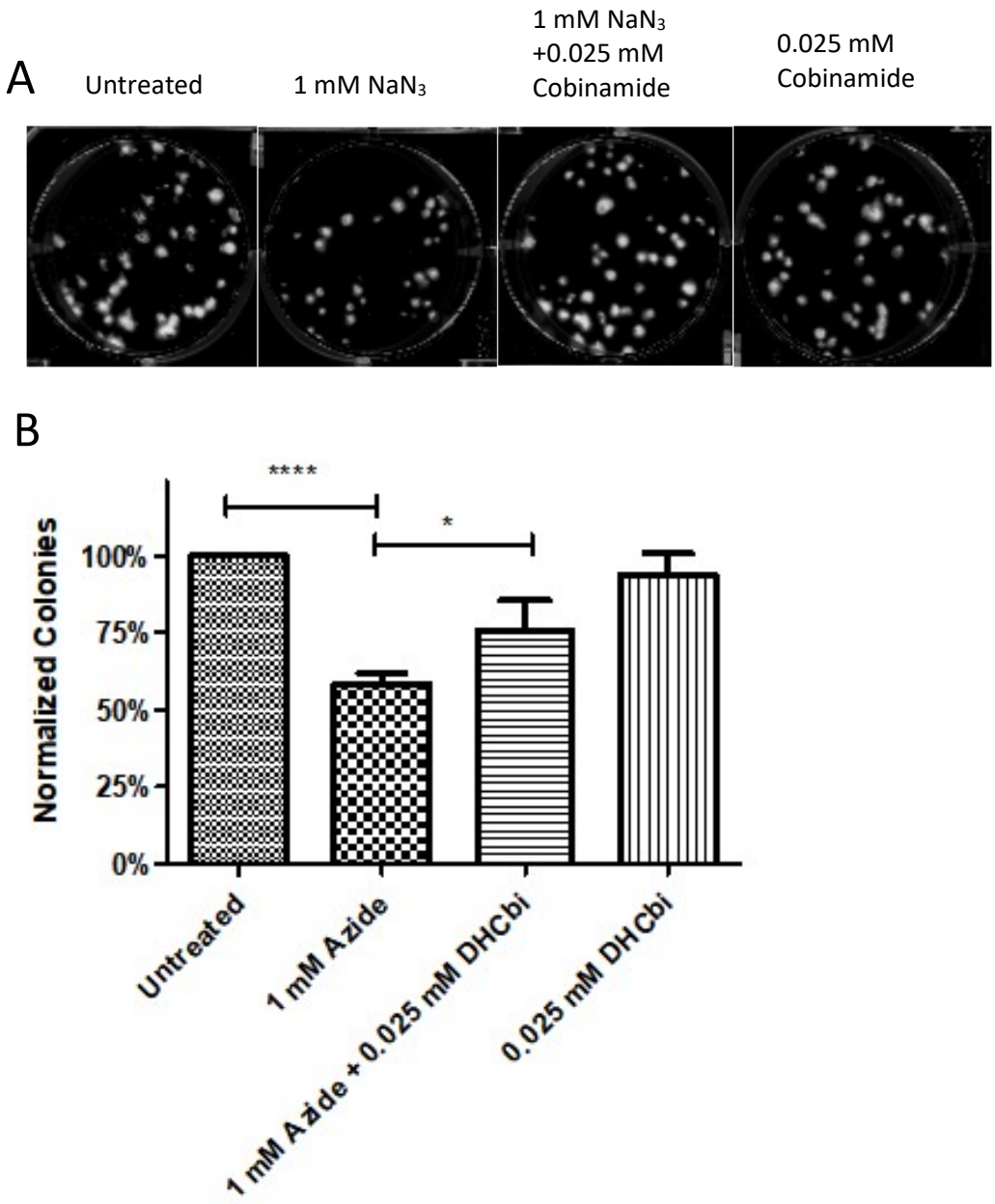


Figure 3: Azide treated cells lose their ability to form colonies; cobinamide rescued. Images of listed conditions in assay (A). Cobinamide rescued colony (B). Graph is composed of four independent experiments. Data are presented as normalized means to untreated ±standard error of the mean (SEM). One-way ANOVA with Bonferroni correction was used to determine statistical significance; one asterisk (*) represents $p<0.05$ and four asterisks (****) represents $p<0.0001$.

Drosophila melanogaster Pretreated with Cobinamide Have Increased Survivability

Given that cobinamide rescued cells from azide toxicity, we hypothesized that cobinamide could also rescue whole animals from azide toxicity. Cobinamide has two free ligand binding sites compared to cobalamins one and can bind cyanide, a toxin similar to azide, with a much higher affinity than cobalamin (Brenner, 2010). Moreover, we hypothesize the protection conferred by cobinamide would be better than cobalamin in the presence of azide *in vivo*. To show this, we chose *Drosophila melanogaster* as a model organism because they are well-studied and have a short life cycle. Flies were pretreated with normal food, or food containing 400 μM cobalamin or 400 μM cobinamide for a month. This ensured the treated flies had cobinamide or cobalamin in their system. For experimentation, flies were transferred to vials containing a gauze doused with 100 mM azide made in sodium phosphate pH 7.4 or sodium phosphate pH 7.4 alone for eight hours. Sodium azide has a pKa of 4.6. Sodium phosphate pH 7.4 was used to ensure the pH was high enough so that azide stayed in solution and did not convert to hydrazoic acid. The toxic effects of the sodium phosphate on any of the flies were negligible, whereas flies that were fed normal food and ingested azide died rapidly over time. However, flies pretreated with 400 μM cobalamin or 400 μM cobinamide food showed significantly greater survival than flies pretreated with normal food. The flies pretreated with cobinamide food showed significantly greater survivability than that of cobalamin. Neither treatments fully protected the flies from azide poisoning.

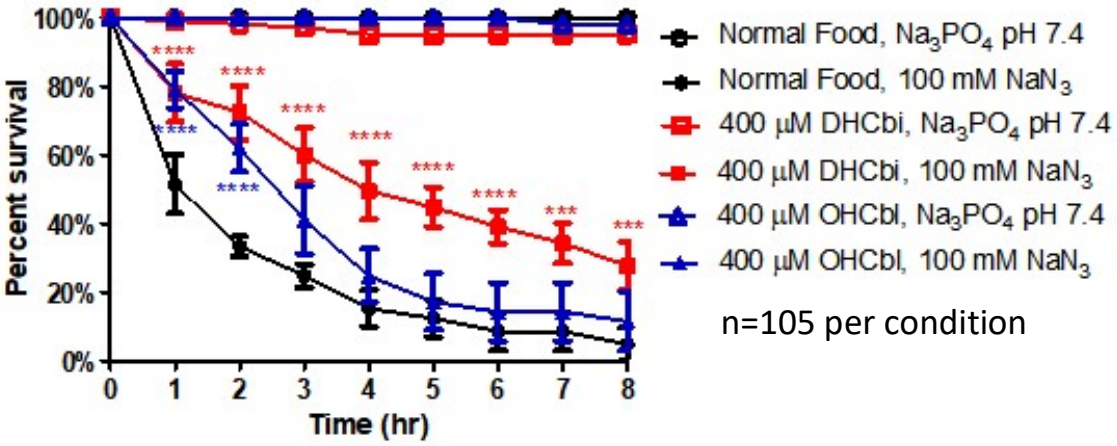


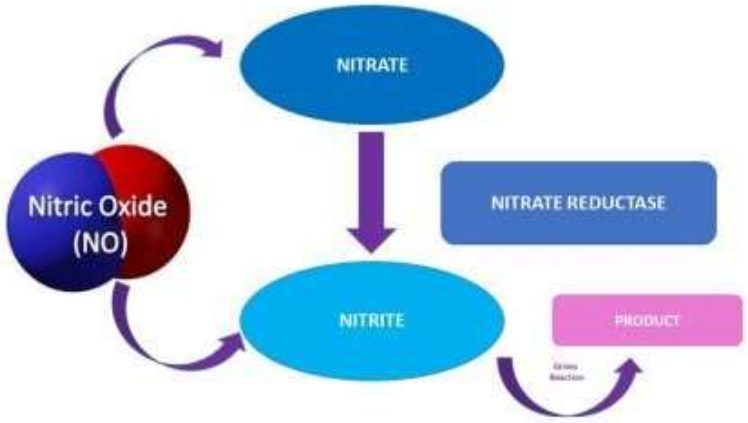
Figure 4: Cobinamide and cobalamin partially protected *Drosophila melanogaster* from azide toxicity. Flies that were exposed to azide are represented by filled symbols (filled black circles are azide alone; open black circles are control flies). Cobinamide (red open squares and filled squares) and cobalamin (shown as blue open triangles and filled triangles) provided partial protection over an eight-hour period. The data are the mean ±standard error of the mean (SEM) of seven independent experiments with 15 flies in each condition per experiment. Two-way ANOVA with Bonferroni correction was used to determine statistical significance; one asterisk (*) represents $p<0.05$, two asterisks (**) represents $p<0.01$, three asterisks (***) represent $p<0.001$, and four asterisks (****) represents $p<0.0001$.

Mice injected with azide have elevated levels of Nitric Oxide in Serum

Hypotension is a clinical symptom of azide toxicity. Nitric oxide is a well-known vasodilator and increased serum nitric oxide after azide exposure may account for hypotension. To determine whether azide poisoning would affect nitric oxide levels, C57 black 6 male mice were injected with 20 mg/kg of azide or water and euthanized at various time points post injection. This dose was sublethal. Sera were harvested and assessed for nitrite levels as a proxy for nitric oxide (Figure 5A).

Untreated mice had no change in serum nitrite concentration upon water injection. Azide exposure led to a striking increase in nitrite concentration, as compared to the control mice (Figure 5B). The increased nitrite did not return to baseline until about 240 minutes after injection.

A



B

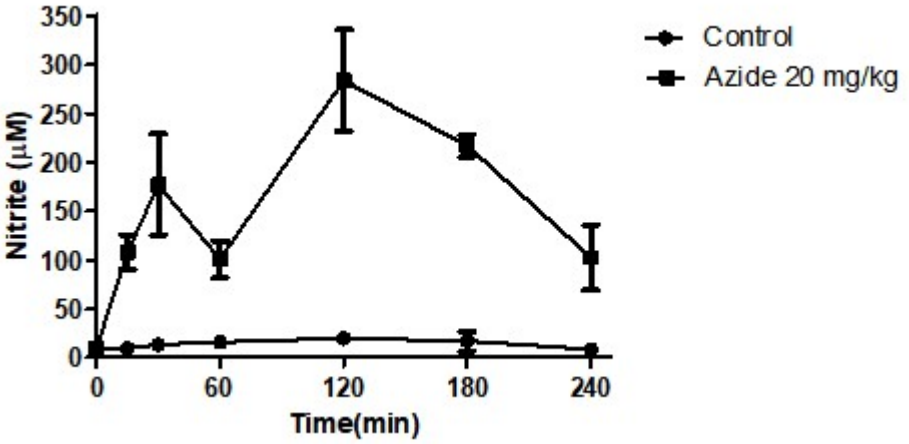


Figure 5: Azide induces nitric oxide in male mice. Nitrate/nitrite formation for detection via Griess reagent (A) (Bioquochem.com). Mice that were injected with azide produce more nitrate than those injected with water (B). Mice injected with 20 mg/kg of sodium azide (NaN_3) or water and euthanized at 15 minutes, 30 minutes, 60 minutes, 120 minutes, 180 minutes, and 240 minutes post injection. Control mice are indicated by solid black circles and azide treated mice are indicated by solid black squares. Mice were between 4 and 19 months old. Three mice were euthanized at each time point. The data are the mean ±standard error of the mean (SEM).

Cobinamide Partially Rescues azide Poisoned Mice

Since we verified that azide induces nitric oxide formation in mice, we then hypothesized that cobinamide, a nitric oxide and an azide scavenger, would rescue mice after lethal azide exposure. To keep our data consistent, male C57 black 6 mice were used again. Mice were injected with 27.5 mg/kg of azide with or without 350 mg/kg of cobinamide and survival was tracked for 180 minutes. Azide injected mice had only 20% survival, with death occurring within 30 minutes of azide injection. Mice injected with azide and cobinamide had 100% survival (Figure 6).

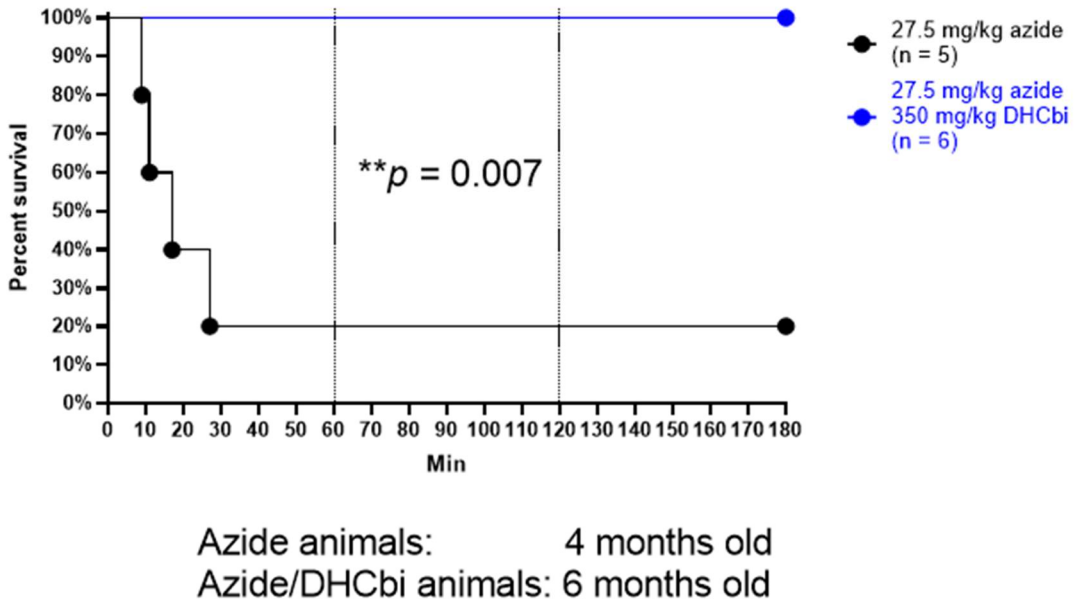


Figure 6: Cobinamide rescues mice from lethal azide poisoning. Male mice were injected with 27.5 mg/kg of azide with or without 350 mg/kg of cobinamide. Survival was recorded for 180 minutes. Mice were 4-6 months old. Azide control mice are indicated with solid black circles. Azide and cobinamide treated mice are indicated by blue circles. Log-rank was used to determine statistical significance.

Discussion

Sodium azide is highly toxic to mammalian cells. In humans, azide is rapidly absorbed on ingestion. Hypotension occurs within minutes and ingestion of several grams can cause death in 1-2 hours. Its effects on humans have been reported as early as 1927 (Chang 2003). The common onset of symptoms is headache, collapse, hypotension, tachypnea, and metabolic acidosis (Kurt et al. 2016). Azide inhibits cytochrome C oxidase similar to cyanide, though most cyanide antidotes are not effective in treating azide poisoning (Schwarz 2014). Commercial availability makes azide especially dangerous since it would be accessible to those with nefarious intentions. Indeed, azide has been used in executed and planned terrorist plots around the world (Okumura et al. 2003). No antidote exists for azide poisoning and treatment is supportive.

Cobinamide, a precursor of cobalamin (vitamin B_{12}), is in late-stage pre-clinical development as an antidote for multiple mitochondrial poisons. Due to its structure (Figure 1) cobinamide has potential to be a more effective antidote than cobalamin.

In our initial experiments, we sought to determine the effects of azide on A549 and COS-7 cells and whether cobinamide could reverse these effects (Figure 2). Both A549 and COS-7 cells, when treated with azide, did not show apoptotic characteristics but did have less cell density. The results suggest that the effect of azide on mammalian cells is growth inhibition as opposed to inducing apoptosis.

To further investigate this theory, a clonogenic survival assay was preformed (Figure 3). Cells treated with azide alone had significantly fewer colonies formed than untreated controls, suggesting azide inhibited cells' ability to form colonies. Cells treated with azide

and cobinamide had significantly more colonies formed than azide alone demonstrating cobinamide can rescue cells from azide toxicity.

After showing cobinamide can rescue mammalian cells from azide toxicity, we showed that cobinamide protected fruit flies from azide toxicity, and this protection was better than that with cobalamin (Figure 4).

We showed mice that were injected with azide had a dramatic rise in serum nitric oxide levels, as compared to controls (Figure 5B). This verifies work done previously showing azide exposure induced nitrosylhemoglobin in mice (Frawley et al. 2020). NO generation may account for hypotension found in azide poisoned patients.

To further investigate cobinamide's efficacy as an antidote to azide poisoning, mice were subjected to a lethal dose of azide then given cobinamide. Cobinamide rescued mice from azide poisoning (Figure 6). Since cobinamide is a nitric oxide scavenger in blood, nitric oxide scavenging could be a mechanism by which cobinamide antagonizes azide toxicity. Co-workers at the Boss lab are currently working to determine if cobinamide and azide injected mice show reduced nitric oxide in blood.

Together, our results suggest cobinamide, a nitric oxide scavenger, is a suitable antidote to azide poisoning and verifies azide as a nitric oxide inducer.

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