UNIVERSITY OF CALIFORNIA SAN DIEGO

Development of Cobinamide as a Potential Antidote for Paraquat Poisoning

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

in

Biology

by

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Professor Immo E. Scheffler, Co-Chair
Professor Douglass Forbes

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The Thesis of Cassandra Tiffany Lai is approved, and it is acceptable in quality and form for publication on microfilm and electronically:

Co-Chair

Chair

University of California San Diego

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DEDICATION

I would like to dedicate this to my family for always supporting me and allowing me to follow my aspirations regardless of how far it took me from home.

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

ABSTRACT OF THE THESIS

Development of Cobinamide as a Potential Antidote for Paraquat Poisoning

by

Cassandra Tiffany Lai

Master of Science in Biology

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Professor Gerard R. Boss, Chair
Professor Immo E. Scheffler, Co-Chair

Paraquat is a commonly used herbicide that is highly toxic when ingested and has been increasingly used in suicide cases. No effective treatment for paraquat poisoning exists. There are two mechanisms of paraquat toxicity: the production of reactive oxygen species like superoxide and the inhibition of mitochondrial respiration. Cobinamide, the vitamin B_{12} precursor, has been shown to be an effective scavenger of reactive oxygen species (ROS). Due to cobinamide's ability to scavenge ROS, we hypothesized that

cobinamide could be used as a potential treatment for paraquat poisoning. We tested the hypothesis by assessing cobinamide's effectiveness in reversing paraquat-induced toxicity *in vitro* and *in vivo*. Experiments with mammalian cells and *Drosophila melanogaster* showed that cobinamide partially rescued these model organisms from paraquat toxicity. Further experiments were performed to determine the mechanism of this rescue. The production of ATP was assessed in COS7 cells showing a dose-dependent decrease in ATP levels in response to paraquat, but surprisingly, was not restored upon addition of cobinamide. Mechanistically, this is maybe due to the lack of binding between cobinamide and paraquat. Results suggest that the mechanism of antagonism occurs mainly through ROS scavenging. Overall, these findings suggest the potential use of cobinamide as an antidote for paraquat poisoning in the clinical setting.

INTRODUCTION

1.1 Paraquat

Acute pesticide poisoning is a major problem in developing countries especially due to the large usage of pesticides in agriculture and how easily accessible these highly toxic agents are (Jeyaratnam, 1990). In addition to accidental exposures, pesticides have become a widely used suicidal agent, contributing to almost 60% of deaths in Asia (Eddleston & Phillips, 2004, Jeyaratnam, 1990). For example, paraquat (PQ), one of the most commercially available pesticides, results in roughly 300,000 lethal ingestions each year (Gawarammana & Buckley, 2011). Paraquat is not only limited to developing countries, but has progressed to become a popular agent for self-harm in America, resulting in more deaths than any other pesticides in 2008 (Bronstein et al., 2009).

Paraquat is highly toxic upon ingestion, as even trace amounts can lead to damage or failure in target organs, such as the lungs, liver, and kidneys (CDC, 2013). In particular, paraquat accumulates in the lungs through active transport and remains in high concentrations even after the paraquat plasma concentration has depreciably decreased (Dinis-Oliveira et al., 2008). Paraquat also accumulates in the liver and kidneys as these organs detoxify toxic chemicals. In addition to oral ingestion, other routes of paraquat exposure include inhalation and skin contact, but toxicity only occurs at high concentrations and prolonged dermal exposure, respectively (CDC, 2013).

Currently, there is no antidote for paraquat poisoning. As such, treatment strategies vary greatly, depending on the training of the medical staff and available resources. However, most cases seem to involve treating the poisoning victims with activated charcoal, in addition to providing supportive care like fluid and ventilation (CDC, 2013). Survival

appears to be contingent on the ingestion dose and how quickly treatments were administered (Sabzghabaee et al., 2010). But in general, prognosis is poor since current treatment protocols do not focus on reversing paraquat toxicity at the mechanistic level, but rather by preventing further absorption of the toxic chemical. Thus, an important part of paraquat toxicology would be to develop antidotes that could antagonize paraquat at a molecular level.

In order to develop a potential antidote for paraquat, it is critical to understand the mechanisms of paraquat toxicity. Paraquat toxicity is proposed to occur via two routes: excess generation of reactive oxygen species and inhibition of mitochondrial oxidative phosphorylation (Bus & Gibson, 1984, Cochemé & Murphy, 2008, Castello et al., 2007). The first mechanism of toxicity occurs through a continuous redox cycle that results in the formation of ROS (Bus & Gibson, 1984). Mechanistically, paraquat is ingested in the +2 oxidation state, but can be reduced to the +1 oxidation state by electron carriers such as NADPH-cytochrome P450 reductase, xanthine oxidase, NADH-ubiquinone oxidoreductase and nitric oxide synthase (Bus & Gibson. 1984, Dinis-Oliveira et al., 2008). The paraquat radical ($PQ^{\cdot+}$) readily reacts with dimolecular oxygen (O_2) to form the reactive oxygen species superoxide ($O_2^{\cdot-}$) and return to its original +2 state where PQ^{2+} can proceed to oxidize more electron carriers. This cycle continuously occurs *in vivo,* specifically in the cytosol and mitochondrion of cells, generating large amounts of superoxide (Figure 1).

ROS such as superoxide, peroxide, and the hydroxyl radical are highly reactive molecules due to unpaired electrons present in their valence electron shell. As such, ROS readily reacts with biomolecules like proteins, lipids, polysaccharides and DNA to obtain a full octet. This process can induce oxidative stress, which damages biomolecules, leading to

diseases such as cardiovascular diseases, Parkinson's disease, and amyotrophic lateral sclerosis (Turrens, 2003).

Cells have endogenous antioxidants like reduced glutathione, catalase, and superoxide dismutase to neutralize ROS (Simon et al., 2000). Ingestion of paraquat can lead to excessive superoxide formation that overwhelms the capacity of the body's endogenous antioxidants, resulting in severe toxicity (Bus & Gibson, 1984). Therefore, potential treatments should aim to neutralize the effect of the excess ROS produced in order to successfully reverse paraquat toxicity.

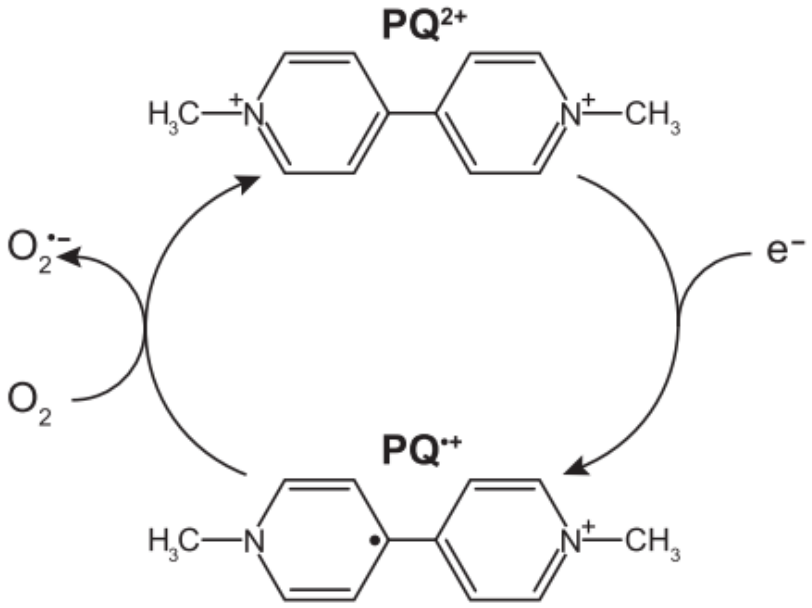


Figure 1. Paraquat Redox Cycle (Cochemé & Murphy, 2008). Paraquat (PQ^{2+}) is reduced by electron carriers to produce the paraquat radical ($PQ^{\cdot+}$). The radical can be oxidized by oxygen (O_2) to form superoxide ($O_2^{\cdot-}$) and PQ^{2+}.

The second proposed mechanism of paraquat is the inhibition of mitochondrial oxidative phosphorylation. Paraquat is actively taken up into the mitochondrion where it interacts with the electron transport chain (Cochemé & Murphy, 2008). The exact location of inhibition within the electron transport chain is still under debate. The current location of

paraquat binding is proposed as either complex I or complex III (Cochemé & Murphy, 2008, Castello et al., 2007). Regardless of the specific location of inhibition, this results in a decrease in ATP production.

1.2 Cobinamide, the Structural Precursor of Cobalamin

Cobalamin (OHCbl), or more commonly known as Vitamin B_{12}, is used as a treatment for cyanide poisoning as it directly binds to cyanide with high affinity (Bonnett, 1963). Cobalamin is composed of a cobalt atom within the center of its structure that has six coordination sites (Figure 2). The cobalt is coordinated to four nitrogen groups that produce the planar corrin ring. The fifth coordination site is occupied by a 5,6 dimethylbenzimidazole nucleotide tail, and the sixth coordination site is free to bind potential ligands such as cyanide or hydroxyl (Bonnett, 1963, Sharma et al., 2003). The free binding site can be altered to contain an additional R group that prevents cobalamin from binding to ligands that are not of interest.

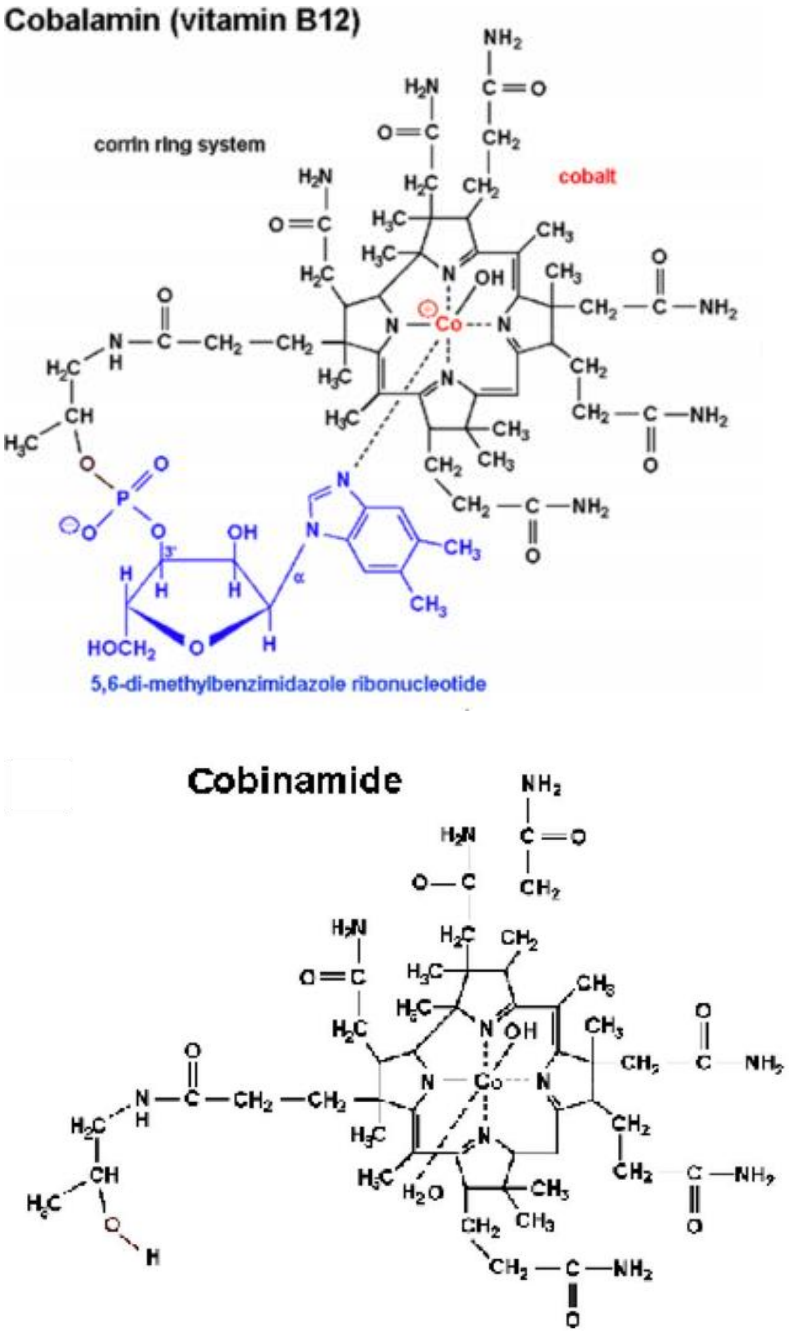


Figure 2. The structure of cobalamin and cobinamide (Brenner et al., 2010). The cobalt atom highlighted in red indicates the center of the corrin ring structure. The group highlighted in blue represents the 5,6 dimethylbenzimidazole nucleotide tail (fifth coordination site). Removal of this group represents the structure of cobinamide. The R group depicted is a hydroxyl group that is replaced when a ligand with higher affinity is encountered. For the following experiments, cobinamide has two hydroxyl groups coordinated to the cobalt atom.

Cobinamide (Cbi), the immediate biological precursor of cobalamin, lacks the 5,6 dimethylbenzimidazole nucleotide tail bound to the cobalt atom. The absence of the dimethylbenzimidazole group allows cobinamide to have two potential ligand binding sites, in comparison to just one ligand binding site found in cobalamin. This absence also removes the negative trans-effect normally observed on cobalamin's free ligand binding site and allows cobinamide to have greater affinity for its potential ligands (Sharma et al., 2003). We have shown that cobinamide binds cyanide and hydrogen sulfide with higher affinity than cobalamin (Broderick et al., 2006, Jiang et al., 2016).

In addition to cyanide and hydrogen sulfide, workers of the Boss lab hypothesized that cobinamide binds to superoxide. Superoxide can actively reduce cytochrome c. This effect would be neutralized if cobinamide binds to superoxide. To test this hypothesis, a spectrophotometric analysis was conducted on samples containing cytochrome C, superoxide, and cobinamide. Cytochrome c absorbance was used as an indicator for superoxide concentrations. Increased cytochrome c absorbance correlates with increased reduction by superoxide. When cobinamide was added to samples, there was a dose-dependent reduction of cytochrome c absorbance (Figure 3). It was calculated that cobinamide actively competes against cytochrome c to bind to superoxide with an equilibrium constant of 2×10^8 $M^{-1}s^{-1}$. Therefore, it was concluded that another potential ligand of cobinamide was superoxide, leading to a working model that cobinamide could be used as a ROS scavenger. Thus, it was hypothesized that cobinamide could reverse paraquat toxicity by neutralizing the excessive amount of superoxide.

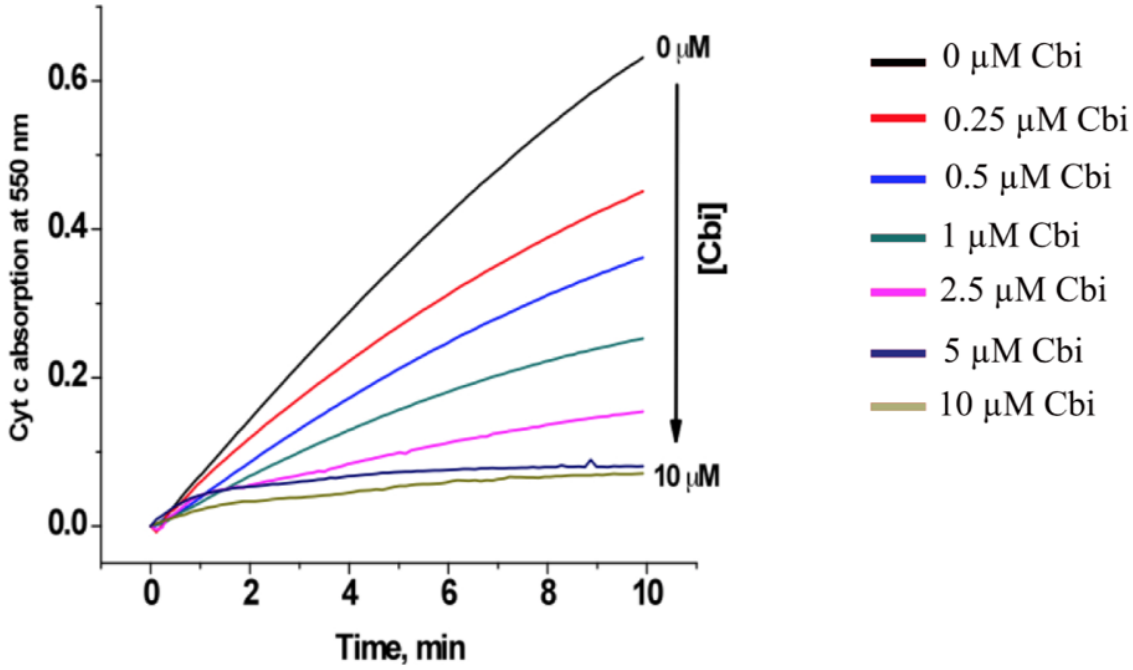


Figure 3. Cobinamide neutralizes $O_2^{\cdot-}$. Cytochrome c absorbance was measured for 10 minutes at 550 nm. Different colors correlate to the concentration of Cbi used in the sample.

MATERIALS AND METHODS

2.1 Materials

Aquohydroxocobinamide, referred to as cobinamide (Cbi) throughout the text, was synthesized from hydroxocobalamin acetate by cerium (III) hydroxide through base hydrolysis (Blackledge et al., 2010). Paraquat was obtained from Sigma-Aldrich (paraquat dichloride hydrate, 75365-73-0, Sigma-Aldrich, St. Louis, MO, USA). CellTiter-Glo reagent was obtained from Promega (CellTiter-Glo© Luminescent Cell Viability Assay, G7572, Promega, Madison, WI, USA).

2.2 Cell Culture

COS7 and HeLa cells were grown in Gibco DMEM 1X (Dulbecco's Modified Eagle Medium), which contained 4.5g/L D-glucose and GlutaMAX. Medium was supplemented with 10% fetal bovine serum (FBS) obtained from Sigma-Aldrich, 1% antimycotic obtained from Gibco, and 1% penicillin streptomycin glutamine obtained from Gibco. Cells were kept at 37°C at 5% CO_2 atmosphere. COS7 cells were derived from the kidney of African green monkeys and obtained from ATCC (Manassas, VA, USA). HeLa cells were derived from a human cervical cancer tumor and obtained from ATCC.

2.3 Cell counting studies

COS7 or HeLa cells were plated in 6-well plates at 50,000 cells/well 24 hours prior to treatment. Four conditions were used in experiments: untreated, paraquat alone, Cbi alone, or paraquat combined with Cbi. Experiments were performed in duplicates. Cells were incubated with respective conditions for three hours before drugs were removed, followed by phosphate buffer saline (PBS) wash and a 48 hour release in fresh medium. After 48 hours, cell number was assessed using a hemocytometer.

2.5 ATP Concentration Assay

COS7 cells were plated in 12-well plates at 25,000 cells/well for 24 hours in regular growth medium. Cells were then switched to glucose-free medium for 24 hours. Glucose-free medium was obtained from Gibco and supplemented with 10% FBS, 1% antimycotic, 1% penicillin streptomycin glutamine, and 4.5g/L D-galactose. Five conditions were used for assaying: untreated, 0.25 mM paraquat, 0.5 mM paraquat, 0.025 mM Cbi, and 0.25 mM paraquat with 0.025 mM Cbi. Each condition was plated in triplicates, but ATP readout was an average from technical duplicates. The additional third replicate for each condition was used for assessing cell number so that ATP could be normalized to 100,000 cells. Cells were treated for 18 hours and then harvested. At harvest, cells were washed with cold PBS and then the CellTiter-Glo reagent was added. Cells were lysed on an orbital shaker and transferred into microcentrifuge tubes. Tubes were spun down and lysates were transferred into 96-well plates. ATP concentrations of cells were determined using CellTiter-Glo© Luminescent Cell Viability Assay. The assay uses an irreversible luciferase reaction to generate luminescence that is proportional to the ATP concentration (Figure 3). Mechanistically, luciferase first catalyzes the reaction between luciferin and ATP in the presence of magnesium. The intermediate product then reacts with dimolecular oxygen to produce oxyluciferin and luminescence. Luminescences of samples were read using Tecan Infinite™ 200 Microplate Reader.

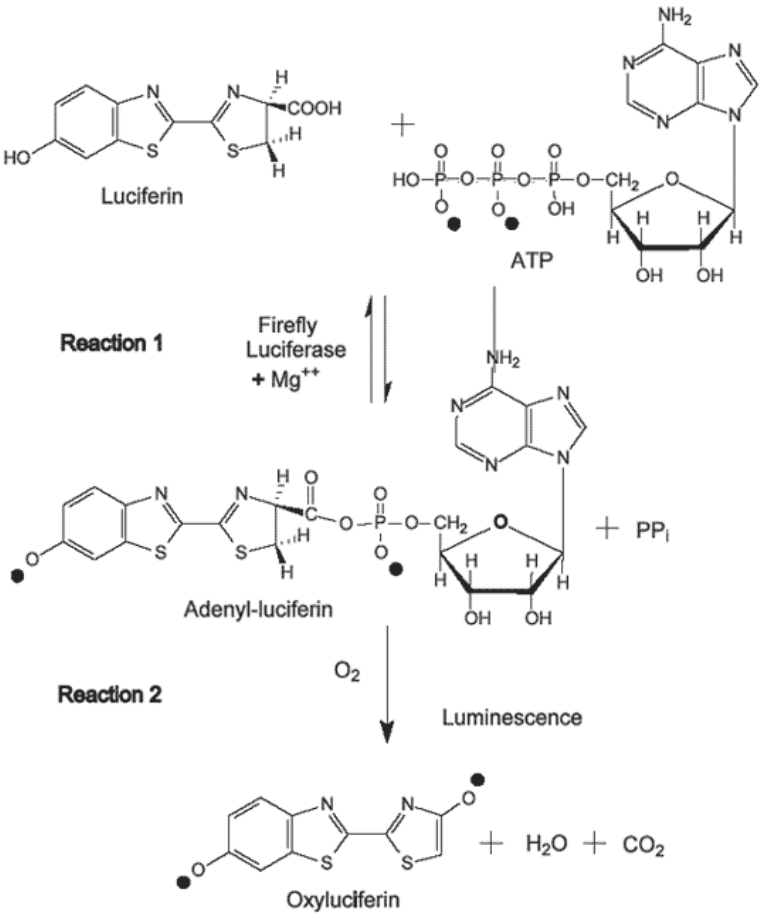


Figure 4. The luciferase reaction in the CellTiter-Glo© Luminescent Cell Viability Assay (Sigma-Aldrich, 2019). The amount of luminescence is proportional to the amount of ATP present in wells.

2.6 UV-Vis Spectrophotometry

The UV-Vis spectrum of paraquat was measured using a Kontron 860 Spectrophotometer. Paraquat binding curves were generated at a constant amount of 25 µM cobinamide. Four concentrations of paraquat were used: 25 µM paraquat, 50 µM paraquat, 125 µM paraquat, and 250 µM paraquat.

2.7 *Drosophila melanogaster* Survival Experiments

D. melanogaster were grown in vials containing standard fly food. To perform survival experiments, *D. melanogaster* were anesthetized on ice prior to being transferred

into vials of food that had been mixed with drugs. Four conditions were used: untreated, 20 mM paraquat, 800 µM Cbi, and 20 mM paraquat with 800 µM Cbi. Surviving number of *D. melanogaster* was counted each day for seven days following transfer into drug treated vials.

2.8 Statistical Analyses

One-way ANOVA with Bonferroni correction was used to analyze statistical significance in selected conditions for cell culture experiments. Two-way ANOVA was used to analyze statistical significance in *D. melanogaster* survival experiments. Conditions considered significant had a p value < 0.05. Analyses were performed on Prism 5.0.

RESULTS

3.1 Cobinamide Partially Rescues Paraquat Treated Mammalian Cells

Initial experiments asked if cobinamide could rescue mammalian cells treated with paraquat. COS7 and HeLa cells were used for these experiments as both cell lines are mammalian in lineage, easily manipulated, and grow rapidly. After seeding for 24 hours, cells were treated for three hours with paraquat in the presence or absence of cobinamide to simulate acute exposure. Cells were then released for 48 hours before cell counts were used as a readout for the effect of drug treatments. Paraquat toxicity was observed in both COS7 and HeLa cells. Paraquat treatment resulted in a 49% and 48% decrease in cell counts for COS7 and HeLa cells, respectively (Figure 5). Combined treatment of 0.1 mM Cbi with 1 mM paraquat for COS7 cells resulted in roughly a 30% increase in cell counts when compared to 1 mM paraquat alone. Similarly, combined treatment for HeLa cells also resulted in a 25% increase in cell count. It was concluded that cobinamide partially reverses paraquat toxicity *in vitro*.

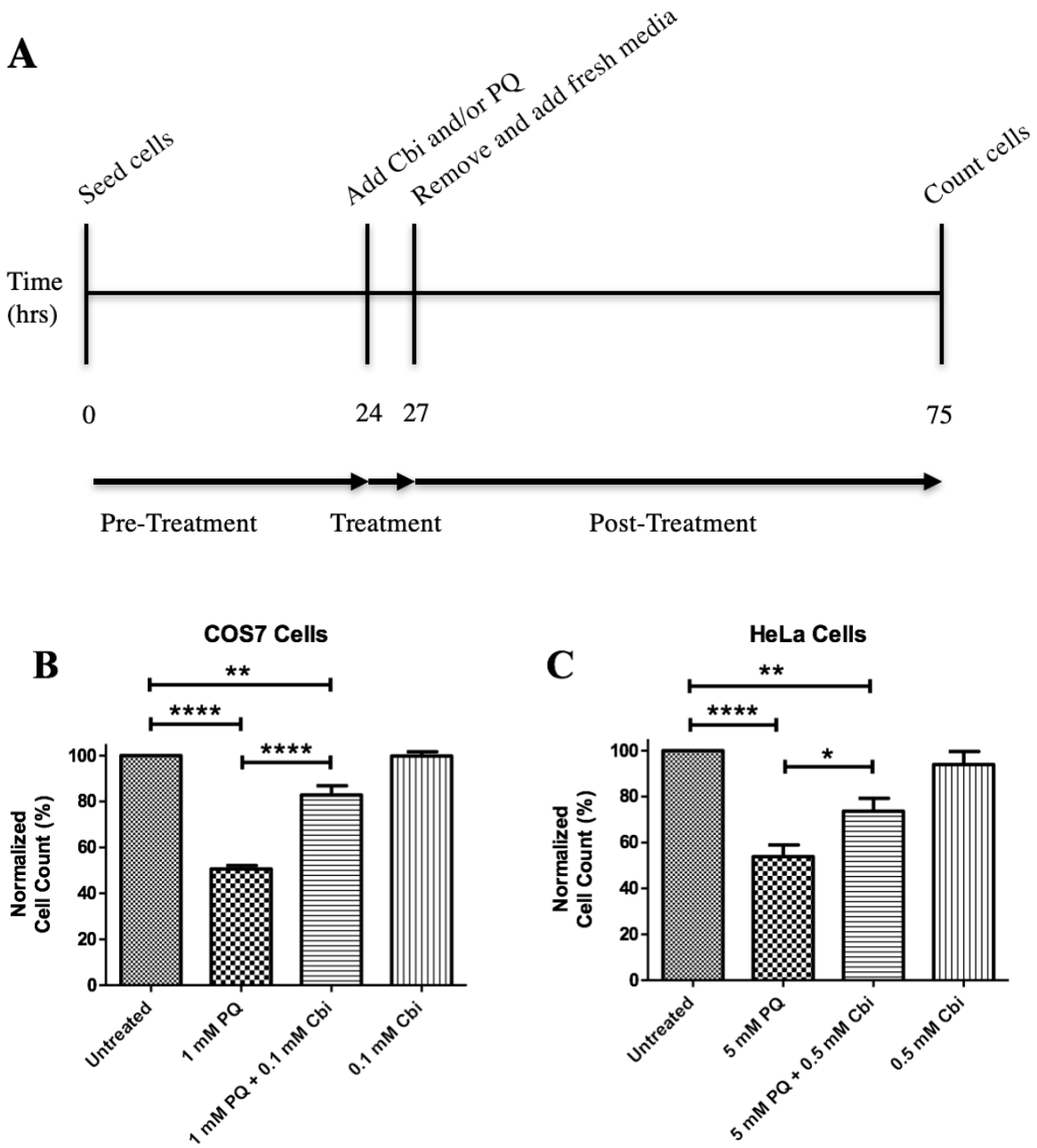


Figure 5. Cobinamide partially rescues paraquat treated cells. (A) A timeline of cell counting experiments. Cobinamide (Cbi) rescues (B) COS7 cells and (C) HeLa cells after three hour treatment with paraquat (PQ). Both graphs are composed of three to five independent experiments, performed as technical duplicates. Data are presented as normalized means ± standard error of the mean (SEM). One-way ANOVA with Bonferroni correction was used to determine statistical significance; one asterisk (*) represents $p<0.05$, two asterisks (**) represents $p<0.01$, and four asterisks (****) represents $p<0.0001$.

3.2 Cobinamide Partially Rescues Paraquat Treated *D. melanogaster*

We used *D. melanogaster* to study if cobinamide is also effective at rescuing whole organisms from paraquat toxicity. Flies were used because they have a short life cycle and share many genes with humans. Flies were transferred into vials containing food treated with paraquat in the presence or absence of cobinamide to simulate chronic exposure. Survival was observed for seven days and used to determine the effect of drug treatment. There were negligible deaths among *D. melanogaster* fed with normal food and food containing 800 µM cobinamide. *D. melanogaster* fed food containing 20 mM paraquat progressively died over a span of seven days (Figure 6). However, there was significant rescue seen when 800 µM cobinamide was combined with 20 mM paraquat. Similarly to *in vitro* experiments, cobinamide treatment did not fully reverse paraquat toxicity.

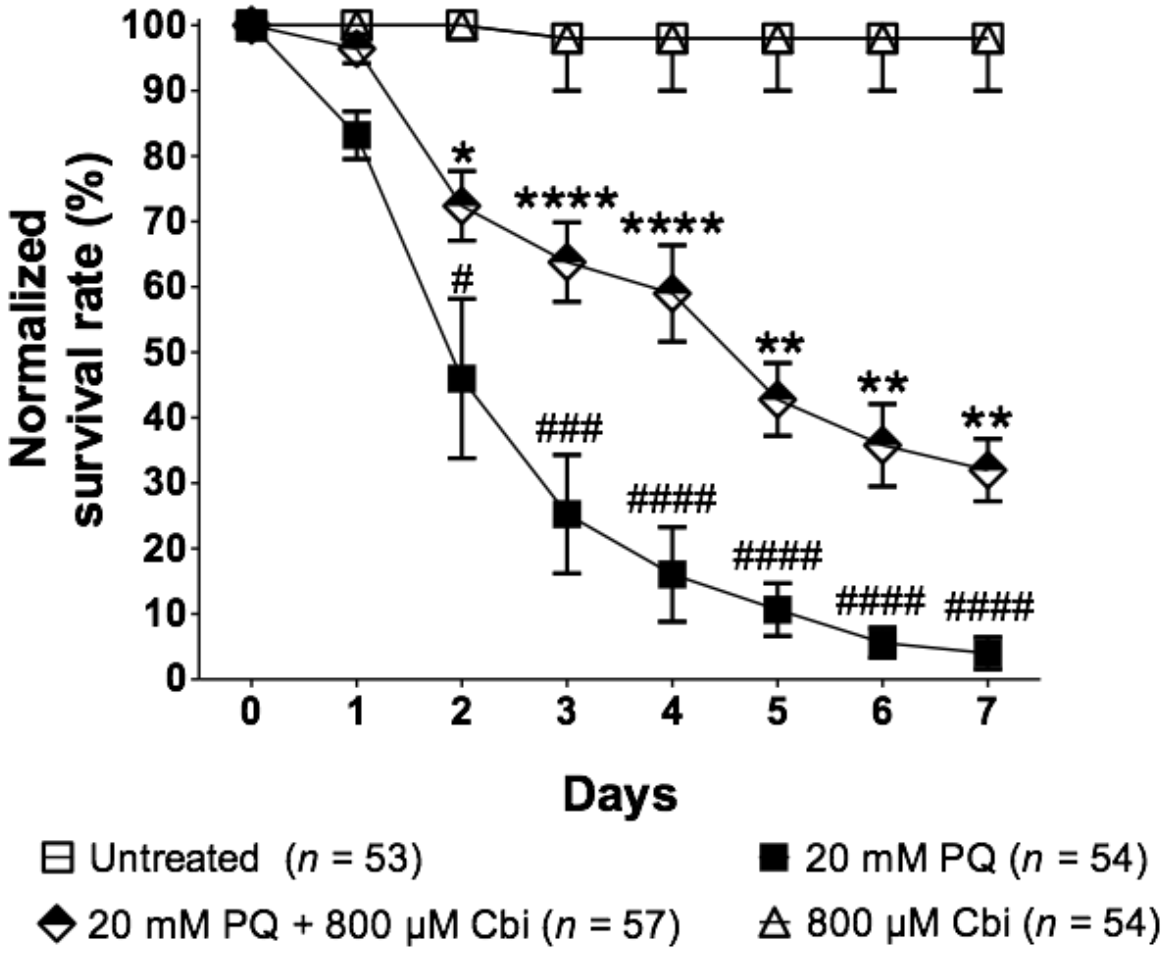


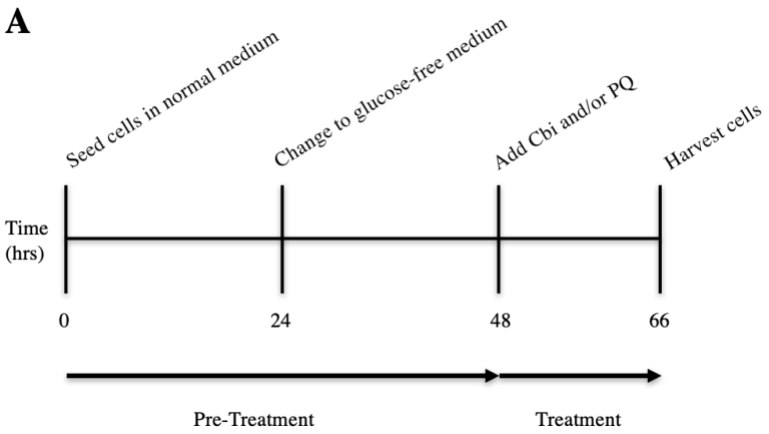
Figure 6. Cobinamide partially rescues *D. melanogaster* treated with paraquat. The graphs are composed of five independent experiments over the span of seven days. Data are presented as normalized means ± SEM. Statistical analysis marked with asterisks (*) compared *D. melanogaster* fed 20 mM paraquat (PQ) with *D. melanogaster* fed 20 mM PQ + 800 µM cobinamide (Cbi). Statistical analysis marked with hashtags (#) compared *D. melanogaster* fed normal food with *D. melanogaster* fed 20 mM PQ. Two-way ANOVA was used to determine statistical significance; */# represents $p<0.05$, **/## represents $p<0.01$, ***/### represents $p<0.001$, and ****/#### represents $p<0.0001$. No asterisk or hashtag represents not significant.

3.3 Cobinamide Does Not Restore ATP Concentrations in Paraquat Treated Cells and Does Not Bind to Paraquat

We wanted to delineate how cobinamide reverses paraquat toxicity and possibly explain why full reversal was not obtained. The next experiments focused on assessing the effect of paraquat on ATP production and whether the addition of cobinamide could reverse the effect. Initially, cells were seeded for 24 hours in regular growth medium and then switched to glucose-free medium for an additional 24 hours (Figure 7A). This medium switch ensured all ATP production would occur through oxidative phosphorylation and not glycolysis. Cells were then treated for 18 hours to allow ample time for ATP depletion. COS7 cells exposed to paraquat resulted in a dose-dependent decrease in ATP concentrations when compared to untreated cells (Figure 7B). Cbi at 0.025 mM had a small positive effect on ATP concentration when compared to untreated cells. However, addition of 0.025 mM Cbi with 0.25 mM paraquat did not increase ATP concentration relative to 0.25 mM paraquat alone. Paraquat negatively affects cellular ATP concentration, but the addition of cobinamide was not able to reverse the effect.

Cobinamide's lack of restoration of ATP after paraquat treatment led to a subsequent experiment focused on explaining these results. A UV-Vis spectrum was performed to determine if cobinamide directly binds to paraquat. This experiment was performed by Sameh Ali. Molecules that react directly with the cobalt atom in cobinamide change the UV-Vis spectrum of cobinamide, thus, providing a useful method to verify cobinamide's binding partners. It was found that increasing concentrations of paraquat did not alter the UV-Vis spectrum of cobinamide (Figure 7C).

Figure 7. Cobinamide does not reverse the decrease in ATP concentration in paraquat treated COS7 cells and does not bind to paraquat. (A) A timeline of ATP concentration assays. (B) ATP concentration does not change when cobinamide (Cbi) is added to paraquat (PQ) treated cells. The graph is composed of four independent experiments, performed in technical duplicates. Data are presented as normalized mean ± SEM. One-way ANOVA with Bonferroni correction was used to determine statistical significance; four asterisks (****) represents $p<0.0001$ and n/s represents not significant. (C) The UV-Vis Spectrum of cobinamide with increasing concentrations of paraquat. Different lines correlate to the concentration of PQ used in the sample.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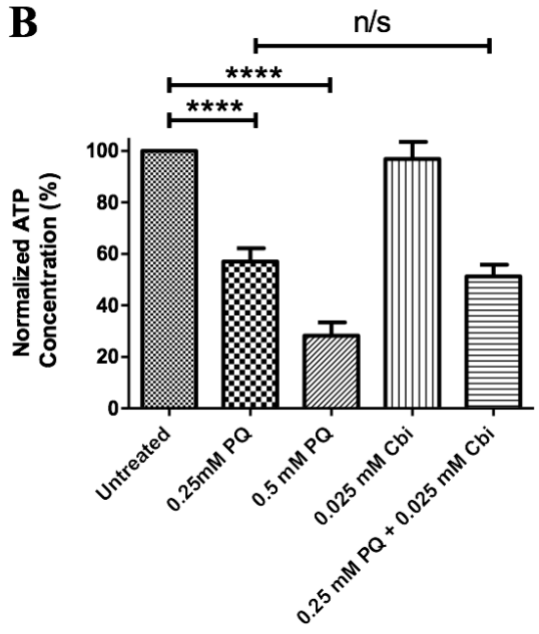
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Normalized ATP Concentration (%)
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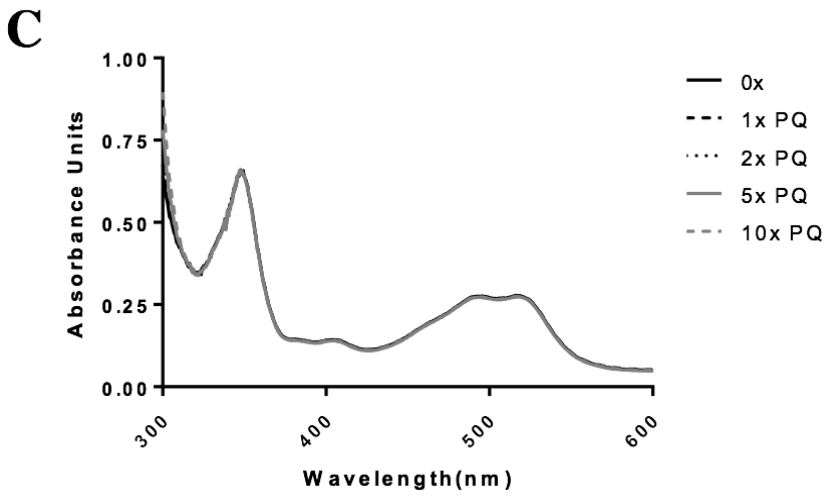
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10x PQ

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

DISCUSSION

The incidence of paraquat poisoning has drastically increased due to its use in suicide cases in developing countries, contributing to roughly 300,000 deaths each year in Asia (Jeyaratnam, 1990, Gawarammana & Buckley, 2011). There is no antidote for paraquat poisoning and current treatments focus on preventing further absorption of paraquat rather than reversing toxicity (CDC, 2013). Thus, there is a need to develop a paraquat antidote that reverses toxicity at a molecular level.

The Boss lab is actively developing cobinamide as an antidote for other toxic chemicals. Cobinamide has been shown to be an effective antidote for cyanide and hydrogen sulfide poisoning, reversing toxicity in many *in vivo* models such as mice and pigs (Anantharam et al., 2017, Ng et al., 2018). Additional types of cobinamide like nitrocobinamide and dihistidylcobinamide are currently being developed that have an altered free ligand binding site which reduce cobinamide's toxicity in animals (Chan et al., 2015, unpublished work).

Thus, experiments explored whether cobinamide could be used as a potential treatment for paraquat toxicity. Paraquat was highly toxic to mammalian cells, decreasing cell growth by 50% in both COS7 and HeLa cells (Figure 5). Addition of cobinamide partially rescued paraquat treated cells.

Additionally, next experiments were deployed to investigate the central hypothesis *in vivo*. Paraquat was highly toxic to *D. melanogaster* causing over 70% of samples to die as early as three days after exposure commenced (Figure 6). However, *D. melanogaster* fed food that was treated with paraquat and cobinamide had less than a 40% decrease in survival rates. Flies fed combined treatment had a significantly higher survival rate which started two

days after exposure when compared to samples fed only paraquat. Similar to *in vitro* experiments, only partial reversal was seen in *D. melanogaster*. In general, cobinamide was successful in partially rescuing both mammalian cells and *D. melanogaster* from paraquat toxicity.

Since cobinamide was unsuccessful in reversing the decrease in paraquat-induced ATP production (Figure 7B), a binding curve was performed to assess the interaction between cobinamide and paraquat (Figure 7C). There were no alterations in the UV-Vis spectrum even when high concentrations of paraquat were used to overcome the potential low binding affinity, indicating that cobinamide does not directly bind to paraquat. This lack of binding could potentially explain the lack of restoration found in the ATP concentration assay. More importantly, it could potentially explain why full reversal of paraquat toxicity was not observed *in vitro* and *in vivo*. Briefly, partial reversal may have occurred since cobinamide is only reversing one mechanism of paraquat toxicity: the excessive generation of superoxide.

In conclusion, the widely-accessible herbicide paraquat poses serious health threats to humans. At present, no specific antidote for paraquat poisoning exists. This study explored the potential use of cobinamide as a paraquat treatment with encouraging results. Future experiments should test whether paraquat induces ROS and if cobinamide rescues cells and animals by squelching the ROS. Potential *in vitro* experiments include lipid peroxidation assays, OxyBlotting, and 8-hydroxydeoxyguanosine assays to test for oxidative stress in lipids, proteins and DNA, respectively. Moreover, mouse survival studies will be useful in determining the effects cobinamide has on paraquat toxicity in a complex animal. Success in future experiments could demonstrate cobinamide's potential to be a specific antidote for

paraquat poisoning and may eventually be used as a treatment for paraquat ingestion in clinical settings.

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