REDUCING ROS AND CHELATING METAL IONS IN NEURONAL CELLS USING NOVEL COMPOUNDS

by

Olivia Kinsinger

Submitted in partial fulfillment of the

requirements for Departmental Honors in

the Department of Biology

Texas Christian University

Fort Worth, Texas

May 4, 2020

REDUCING ROS AND CHELATING METAL IONS IN NEURONAL CELLS USING NOVEL COMPOUNDS

Project Approved:

Supervising Professor: Giridhar Akkaraju, PhD

Department of Biology

Kayla Green, PhD

Department of Chemistry

Laura Luque, PhD

Department of Biology

ABSTRACT

It is estimated that 45% of people over the age of 85 in the U.S. suffer from Alzheimer's disease. Patients with Alzheimer's disease, which is characterized by cognitive deficiencies and memory loss, have higher concentrations of amyloid plaques in brain tissue than patients without the disease. Abnormal levels of transition metal ions Fe, Zn, and Cu in brain tissue are associated with amyloid beta plaques and also have been shown to catalyze the generation of excess reactive oxygen species (ROS) and cause oxidative stress. The combination of the ROS generation and the amyloid plaque formation results in neuronal cell death and subsequent neurodegeneration, which ultimately causes the memory loss and death associated with Alzheimer's. We have synthesized the compounds **L2** and **L4** which are designed to chelate metal ions and scavenge ROS, thus reducing the damage they cause. We hypothesize that due to their chelating properties and pyridol groups, **L2** and **L4** should reduce oxidative damage in neuronal cells by chelating metal ions and scavenging free radicals. The cytotoxicity of the compounds was first tested on HT-22 neuronal cells. Next, neuronal cells were treated with,compounds that induce formation of ROS, in the presence and absence of **L2** and **L4.** If our hypothesis is correct, our compounds should reduce the oxidative damage induced by these compounds.

Introduction

Alzheimer's Disease (AD) is a neurodegenerative disease characterized by dementia, language problems, impaired cognition and is ultimately fatal. The disease impairs normal functioning of everyday activities and impedes overall quality of life. Patients afflicted with AD usually survive with the disease for years, but symptoms worsen over time. As the disease progresses, the symptoms evolve beyond cognitive deficits as parts of the brain involved in basic physiological functions become impaired. This leads to a sharp decline in health as patients become unable to carry out basic activities such as walking and swallowing. In the final stages of the disease, patients require extensive attention from care-givers and the disease ultimately results in death (Alzheimer's and Dementia 322).

According to data reported by the 2010 U.S. Census and the Chicago Health Aging Project, it is estimated that there were 5.8 million Americans afflicted with Alzheimer's in 2019. Of the 5.8 million living with the disease, 81 percent are seventy-five years old or older. Another statistic notes 1 in 10 adults age 65 and older in America is living with Alzheimer's disease. Data from the study Established Populations for Epidemiologic Study of the Elderly estimated the incidence of Alzheimer's disease in America. The data approximated 487,000 adults over the age of 65 will develop the disease in the year 2019 (Alzheimer's and Dementia 331).

To diagnose a patient with Alzheimer's disease, physicians look at beta-amyloid levels obtained from a PET scan. Beta-amyloid serves as a biomarker for Alzheimer's disease. To diagnose patients with dementia, a symptom of Alzheimer's, physicians have to take a cognitive approach. Physicians will look at patients' psychiatric records, perform cognitive tests on patients, and speak with their care takers to learn more about the patients' cognitive and behavioral changes (Alzheimer's and Dementia 335).

Much of the pathology describing the mechanism of Alzheimer's remains unknown. However, throughout the years researchers have discovered changes in the brains of those afflicted with Alzheimer's at both the structural level as well as at the cellular level. Cognitively, Alzheimer's leads to a loss of episodic memory. On a structural level, there has been a correlation with medial temporal lobe shrinkage, reduction in gray matter, and atrophy of the entorhinal cortex. MRI was used to measure volume reduction. The atrophy of brain regions results from loss of axons and dendrites, myelin reduction, and neuronal cell death (Roetne & Jacobsen 3032). Previous research has also shown that neurons in the hippocampus, a brain structure that plays an imperative role in memory formation, have decreased by 80% in patients in the late stages of Alzheimer's disease (Roetne & Jacobsen 3032).

One of the major hallmarks of Alzheimer's disease are amyloid-β plaques. Structurally, they first occur in the neocortical area of the brain. The plaques then spread to areas in the midbrain and finally to the brainstem (Roetne & Jacobsen 3033). Aggregation of amyloid-β plaques has been hypothesized as the causative agent of Alzheimer's Disease. The amyloid hypothesis posits that in the aging brain accumulation of amyloid-β peptide aggregates leads to the cognitive dysfunctions associated with Alzheimer's disease. Amyloid-β (Aβ) is generated from Amyloid Precursor Protein (APP). APP is cleaved by secretases α,β, and γ. The cleavage of APP by these secretases results in the release of amyloid-β which aggregates in plaques (Strooper 467). Amyloid-β plaques are a major biomarker of Alzheimer's Disease.

Another hypothesis for the generation of Alzheimer's Disease is the tau hypothesis. In normally functioning neurons axonal transport is essential. In this type of intracellular transport, motor proteins carry cargo through microtubules. Microtubules composed of tubulin bind microtubule-associated proteins (MAPs). Tau is the most important MAP in axonal

transport. That Tau protein has a variety of phosphorylation sites. Some of these phosphorylation sites have been associated with Alzheimer's Disease. Patients with Alzheimer's Disease have abnormally high levels of hyper-phosphorylated tau. Abnormal levels and phosphorylation of tau can lead to the depolymerization of microtubules, which subsequently leads to damage to the intracellular transport system and diminished neural transmission. These changes ultimately lead to decreased cognitive functioning, a major symptom of Alzheimer's Disease (Roetne & Jacobsen 3034).

Another factor that leads to Alzheimer's Disease is oxidative damage. The brain accounts for roughly 2% of the weight of the human body but disproportionately uses about 20% of the body's total oxygen. Therefore, it is especially susceptible to oxidative damage (Chen & Zhong 271). In neurons, the oxidation of lipids, proteins and nucleic acids has been cited as pathological markers of Alzheimer's Disease. The literature has frequently cited oxidative damage and the production of Reactive Oxygen Species (ROS) as a contributor to the pathogenesis of Alzheimer's, however the mechanism of its formation is not entirely known. Research has indicated it could be the result of metal accumulation, hyperphosphorylation of tau, inflammation and amyloid beta accumulation (Chen & Zhong 271). To further complicate this mechanism, oxidative damage / ROS can exacerbate Aβ accumulation and hyperphosphorylation of tau, thereby contributing to the pathogenesis of Alzheimer's Disease.

ROS arises primarily from mitochondrial dysfunction at the electron transport chain. Mitochondrial dysfunction and the subsequent impaired metabolism have been observed in Alzheimer's patients. In the literature, Aβ has been shown to interfere with the electron transport chain and consequently lead to increased ROS and oxidative damage (Chen & Zhong 273). Furthermore, metal ions can bind to Aβ to produce ROS by redox activity. Specifically,

copper, zinc and iron can react via Fenton reactions to generate H_2O_2 and other hydroxyl radicals. This contributes to the oxidative damage characteristic of Alzheimer's.

Another biomarker of Alzheimer's that plays a role in pathogenesis is abnormally elevated levels of transition metal ions, most notably copper, zinc and iron ions. Under normal biological conditions, transition metal-ions are important components of the brain that are essential for detoxification of free-radicals, electron and oxygen transport processes, neurotransmitter biosynthesis, and neuronal signaling (Johnston et al. 2019). However, if these processes become unregulated these metal ions can become dysfunctional. Unregulated metal ions further contribute to the pathogenesis of AD by reacting with Aβ to increase ROS generation. Additionally, research has shown iron metal ions may upregulate APP leading to increased Aβ deposition which contributes to the development of Alzheimer's according to the Amyloid Hypothesis (Bonda et al. 2011). Therefore, due to the pathogenic effects unregulated metal ions can have in the brain, the literature has proposed that the development of chelating compounds that bind to and remove transition metal ions could be a potential therapy in treating Alzheimer's Disease (Bonda et al. 2011).

A new potential therapy for Alzheimer's Disease proposes the use of small tetra-azamacrocyclic molecules with both antioxidant and radical scavenging features. These compounds were designed with the intent to reduce oxidative damage/ ROS and chelate and remove metal ions associated with Aβ plaque aggregation. Two of these molecules specifically are denoted as **L2** and **L4** and are the focus of this study**. L2** has previously been shown to have metal binding preference for Cu^{2+} and an IC50 of 298.0 μM in HT-22 cells (Green et al 2019). **L4** was synthesized to enhance the antioxidant capabilities of **L2** with the addition of an extra pyridol group. Data from DPPH assays affirmed **L4**'s enhanced ability to reduce ROS

compared to **L2.** These compounds were further investigated for their abilities to reduce oxidative damage in cell culture. The potential for these antioxidant, metal chelating compounds would be for therapeutic use in Alzheimer's patients.

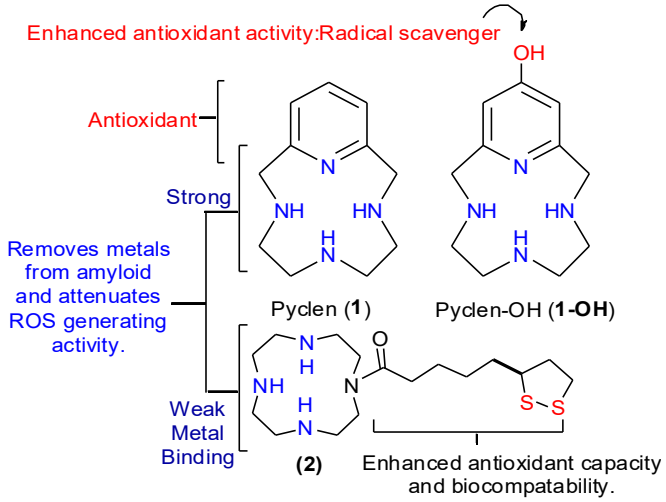


Figure 1- Structure of L2

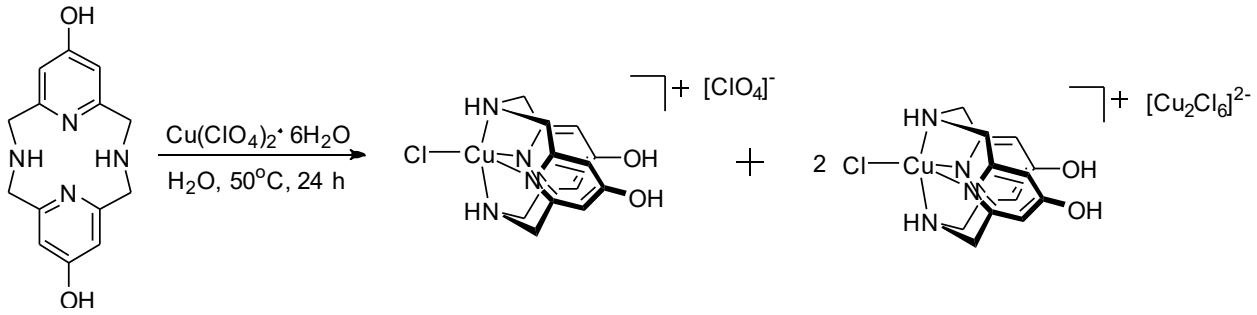


Figure 2- Structure of L4

This study aims to test these compounds in cell culture. The cytotoxicities of these compounds in neuronal cells will be investigated as well as their ability to scavenge radicals in cell culture. This study also aims to determine the different effects of L2 versus L4 in cell culture.

Methods

Cell Culture

HT-22 and FRDA cells were grown in complete Dulbecco's Modified Eagle Medium (DMEM) (10% fetal bovine serum, 1% nonessential amino acids, 2mM L-glutamine, 100 units of penicillin, and 1.7mM streptomycin) separately in T75 tissue culture flasks at 37°C, 5% CO_2 95% air. Upon reaching confluency, the cells were aspirated in the tissue culture hood and washed with 5mL 1x PBS. The PBS was then aspirated. Following, 3mL of 0.05% Trypsin was added to the flask to remove the cells from the bottom of the flask. Next, 7mL of complete DMEM was added to the trypsin and cells to quench them. The resulting solution was broken up via trituration with a pipette and the cells were counted by hand using a hemocytometer. Lastly, the cells were plated for DCFH-DA and MTT experiments in black-bottomed 96 well plates. Cells for the MTT assay were plated at a concentration of 5,000 cells/well and cells for the DCFH-DA were plated at a concentration of 25,000 cells/well. 1 mL of the remaining cells were added to a new T75 flask filled with 12mL DMEM. The medium and trypsin were obtained from Sigma-Aldrich.

MTT Cell Viability Assay

Mouse hippocampal (HT-22) cells were plated at a concentration of 5,000 cells/well in a 96 well plate and incubated in a 37°C incubator overnight. The next day, the cells were treated with varying concentrations of compound dissolved in serum-free medium obtained through serial dilution. The cells treated with compound were incubated overnight for 16 hours. On the third day, the medium was removed and replaced with 100uL/well of 1mg/mL solution of thiazolyl blue tetrazolium bromide (MTT) dissolved in serum-free medium. The cells treated with MTT were incubated for 4 hours at 37°C. Next, the medium was again removed and replaced with 100uL of DMSO/well and placed on a shaker for 5 minutes at Room

Temperature (RT). Lastly, absorbance was measured in a spectrophotometer at 540nm and the data was analyzed via the Omega Data Analysis program (Basau et al. 1990)

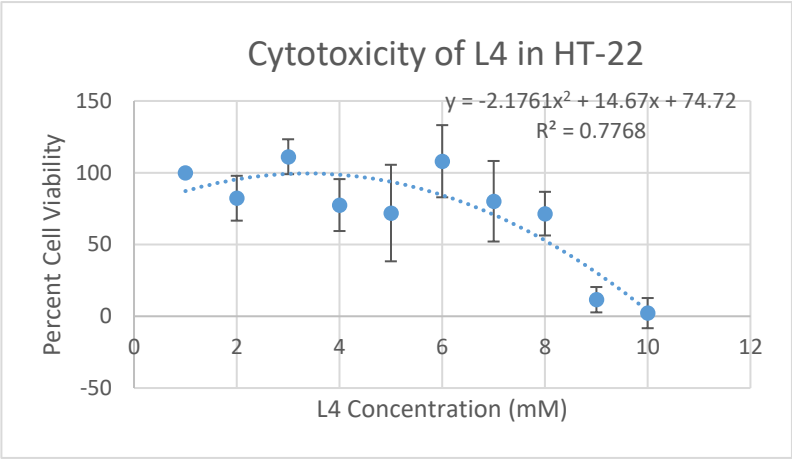
DCFH-DA ROS Measuring Assay

Mouse hippocampal (HT-22) cells and FRDA cells were separately plated at a concentration of 25,000 cells/well in a 96 well plate and incubated in a 37°C incubator overnight. The next day, the medium was removed and replaced with 100uL/well of a solution of 25uM 2'7'-Dichlorodihydrofluorescein diacetate (DCFH-DA) dissolved in serum-free medium, and incubated at 37°C for 45 minutes. Following this step, the medium was again removed and replaced with 100uL/well of complete DMEM. Next, the cells were treated with varying concentrations of compound dissolved in serum-free medium obtained through serial dilution. The plate was then incubated at 37°C overnight for 16 hours. The following day, the fluorescence was measured in a spectrophotometer with an excitation filter of 475 nm and emission filter of 520 nm, and the data was analyzed via the Omega Data Analysis program (Eruslanov 2010)

Results

To be able to use these compounds in neuronal cells, it was first important to determine their cytotoxicity. This allows for proper selection of dosage in future experiments. First, we determined the cytotoxicity of the compounds L4 (Figure 1a) and L2 (Figure 1b) by performing MTT cytotoxicity assays. The cytotoxicity was measured first to gain information about the concentration of compounds that should be used in subsequent experiments. HT-22 mouse hippocampal cells were treated with increasing concentrations of L2 and L4 (both water soluble) in separate plates for 16 hours. Next, cell viability was measured using an MTT assay. The

results indicate both L4 and L2 exhibit dose-dependent cytotoxicity in HT-22 cells. The EC-50



of L4 was found to be 7.83mM The EC-50 of L2 was found to be 18.92mM.

Figure 1a- *Cytotoxicity of L4 in HT-22*

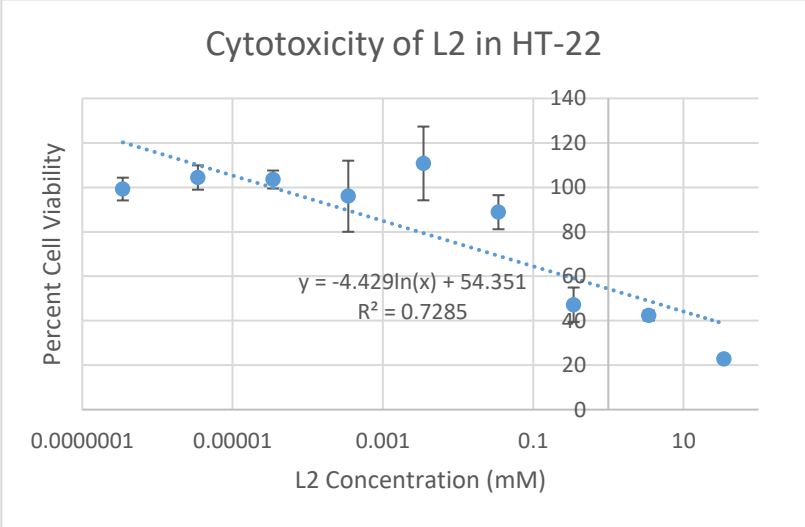


Figure 1b- *Cytotoxicity of L2 in HT-22*

Next, a DCFH-DA assay was performed to determine the amount of ROS generated in FRDA cells with H_2O_2 alone in increasing concentrations. The DCFH-DA assay is capable of measuring intracellular ROS levels. This experiment was conducted to determine a baseline concentration of H_2O_2 to be used in future experiments in order to induce oxidative damage to

mimic Alzheimer's conditions. Fluorescence in the DCFH-DA assay corresponded with fold ROS compared to the untreated control. The results (Figure 2) indicated a dose-dependent increase in ROS with increasing concentration of H_2O_2. The highest increase in ROS was found to be a 34.5 fold increase in comparison to the control not treated with H_2O_2.

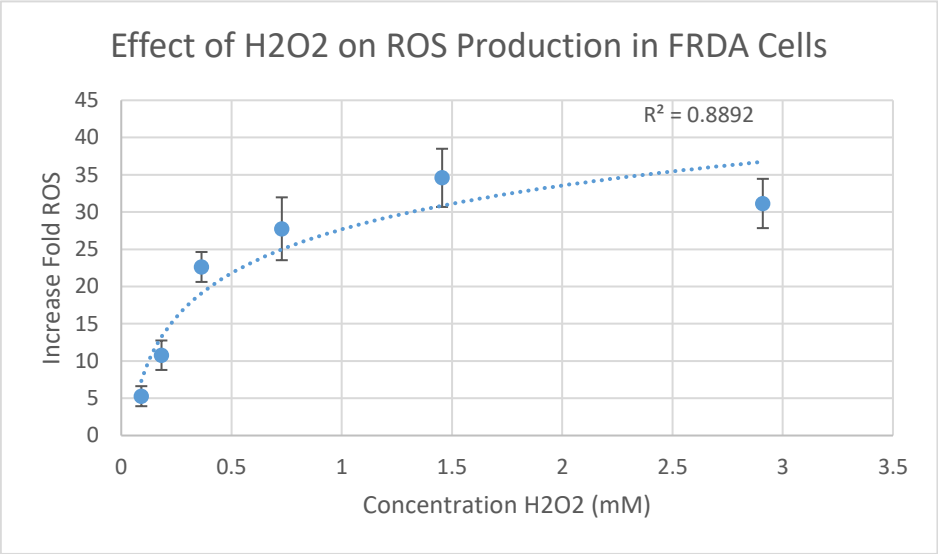


***Figure 2**- ROS generated by H_2O_2*

A DCFH-DA assay was also performed to test the ability of Buthionine sulfoximine (BSO) to induce ROS in FRDA cells as shown in Figure 3. BSO is an agent that knocks down the generation of glutathione, a natural antioxidant in cells. BSO does so by inhibiting gamma-glutamylcysteine synthetase, the enzyme in the first step of glutathione synthesis. This experiment was conducted to determine a baseline concentration of BSO to be used in future experiments. BSO alone was added to the cells in increasing concentrations. ROS was measured as a function of the untreated control. The highest increase in ROS was found to be 1.6 fold higher than the control not treated with BSO. BSO was not used as an inducer of ROS in subsequent experiments because it was determined to be less effective at inducing ROS than H_2O_2.

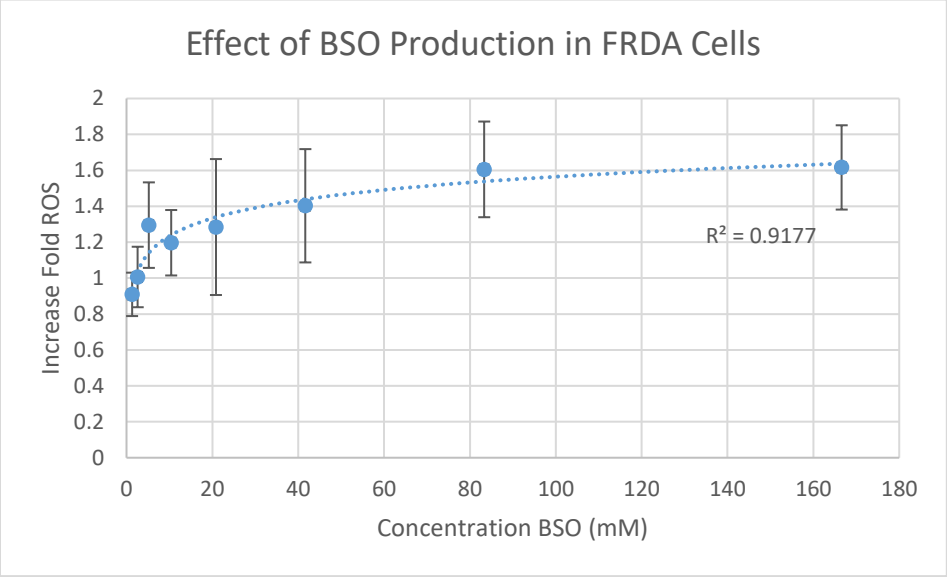


***Figure 3**- ROS generated by BSO*

Using the data from the H_2O_2 DCFH-DA assay, a baseline concentration was used to induce ROS in FRDA cells. Butylated Hydroxytoluene (BHT) was used as a control antioxidant to show a decrease in ROS compared to a control not treated with BHT. The results as shown in Figure 4 indicate a slight dose-dependent reduction in ROS in cells treated with the antioxidant BHT.

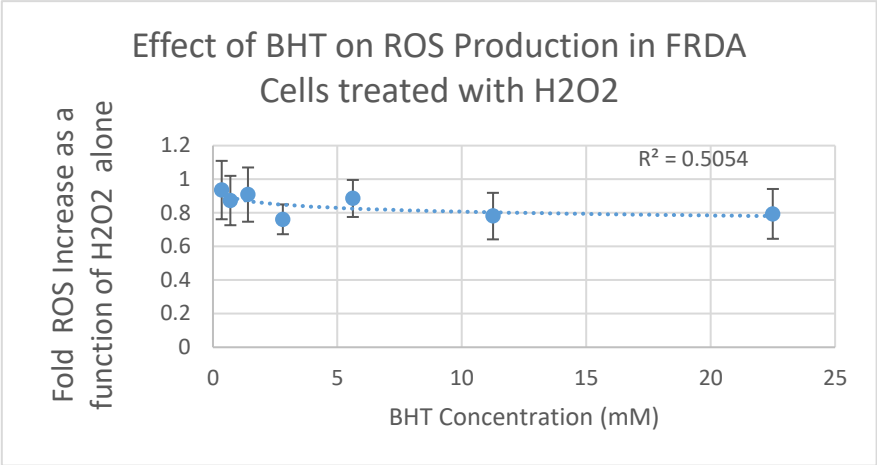


Figure 4- *Effect of BHT on ROS Production in FRDA Cells treated with H_2O_2*

A DCFH-DA assay was performed to test the protective effects of L2 against ROS produced by a baseline concentration of H_2O_2 in FRDA cells. The results (Figure 5) indicate a slight dose-dependent reduction in ROS in cells treated with L2. The reduction was compared against a control not treated with L2. However, the p value was calculated to determine if L2 had a significant effect on reduction in ROS compared to the control. The p value was 0.242 which is greater than α=0.05. Therefore, the null hypothesis was accepted that there was no significant difference in ROS production between cells treated with L2 and untreated.

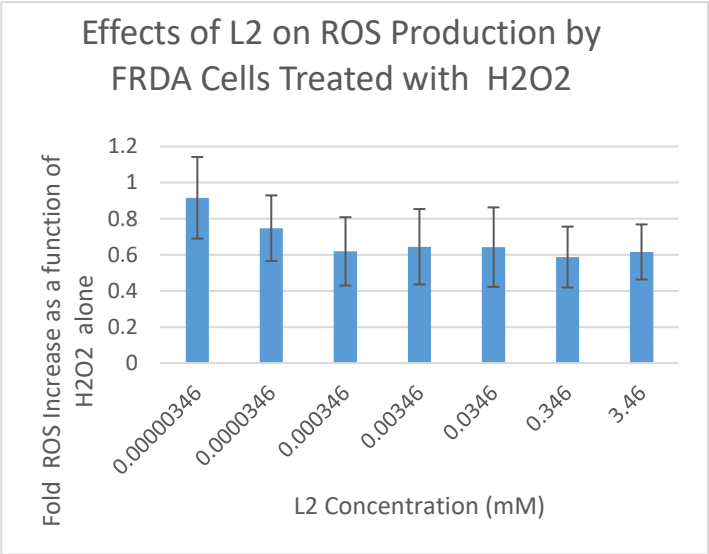


Figure 5- *Effect of L2 on ROS Production in FRDA Cells treated with H_2O_2*

Another DCFH-DA assay was performed to determine the amount of ROS generated in HT-22 cells with H_2O_2 alone in increasing concentrations. The previous experiments were conducted in FRDA cells so this experiment provided information about how a different cell line responded to H_2O_2. This experiment was conducted to determine a baseline concentration of H_2O_2 to be used in future experiments. Fluorescence in the DCFH-DA assay corresponded with fold ROS compared to the untreated control. The results (Figure 6) indicated a dose-dependent increase in ROS with increasing concentration of H_2O_2 until the highest concentration in which ROS was reduced The highest increase in ROS was found to be a 15.9 fold increase in comparison to the control not treated with H_2O_2.

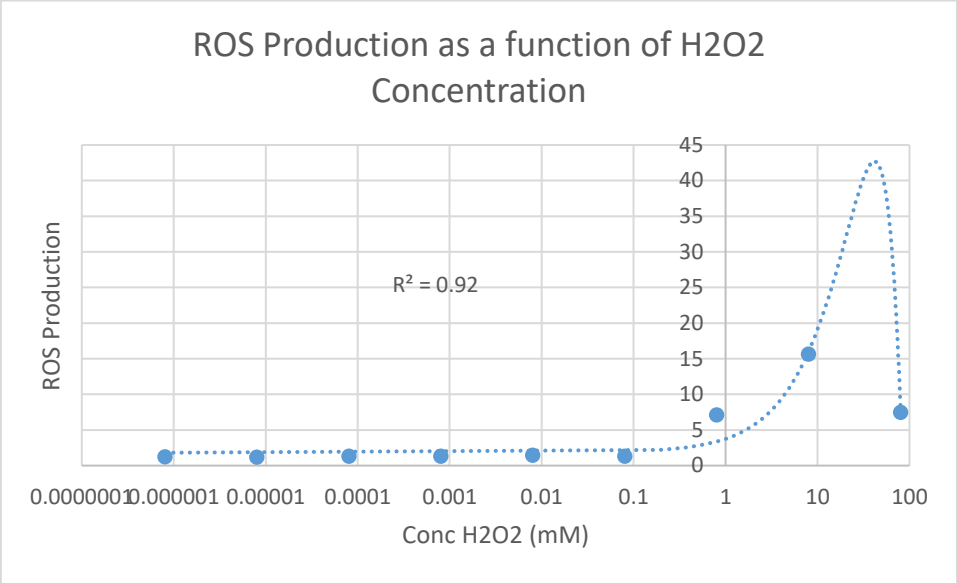


***Figure 6**- ROS production from H_2O_2 in HT-22 Cells*

A DCFH-DA assay was performed to test the protective effects of lipoic against ROS produced by a baseline concentration of H_2O_2 in HT-22 cells. Lipoic acid is a known antioxidant. Future research plans on linking lipoic acid to L2 to increase the radical-scavenging abilities of the compounds. Therefore, the effects the two compounds have individually on the production of ROS were tested. The results (Figure 7) indicate a slight dose- dependent increase in ROS in cells treated with lipoic acid. The reduction was compared against a control not treated with lipoic acid.

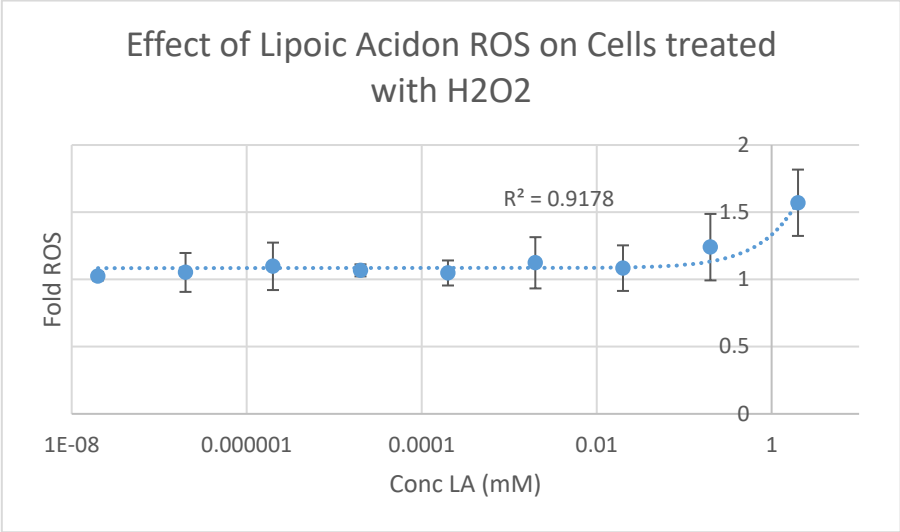


Figure 7- *Effect of Lipoic Acid on ROS Production in HT-22 Cells treated with H_2O_2*

Discussion

The goal of our research project was to examine the abilities of the compounds L2 and L4 to reduce oxidative damage in cell culture. Ultimately, the goal of this project would be to evaluate the utility of these compounds to reduce oxidative damage in patients afflicted with Alzheimer's Disease. It is known from the literature that Alzheimer's Disease is characterized by amyloid beta plaques, increased ROS and unregulated metal ions. The compounds L2 and L4 were therefore synthesized with the goal of reducing oxidative stress and chelating metal ions. Both had previously been shown to reduce ROS in solution and their chemical properties had previously been studied. The structures of the compounds were described as having radical scavenging pyridol groups and metal chelating pockets to bind to dysregulated metal ions. Due to its extra pyridol group, L4 was hypothesized to be a better radical scavenger than L2. We were interested in understanding how these compounds would function in cell

culture. We first sought to ascertain the cytotoxicity of these compounds in cell culture. The next goal was to test L2 and L4 for protective effects against ROS.

First, the cytotoxicity of L2 and L4 was tested on HT-22 neuronal cells. Both exhibited dose-dependent increases in cytotoxicity. It was determined that L2 was less cytotoxic than L4. The result needs to be repeated because for therapeutic purposes, if L4 is a better radical scavenger and thus a more effective drug option clinically, then being less cytotoxic would be beneficial. Gaining information regarding the cytotoxicities of these compounds was necessary in order to determine the appropriate baseline concentrations for use in future ROS reduction assays.

Next, DCFH-DA assays were performed to measure intracellular ROS levels in the presence of L2 and L4 when cells were exposed to H2O2. The initial assays were performed to optimize the DCFH-DA assay. The first assay measured the ROS levels generated by H_2O_2, a known inducer of oxidative damage, to ascertain the concentration of H_2O_2 needed to generate an appropriate level of ROS to test compounds in future experiments. As expected H_2O_2 is a potent inducer of ROS and the results of our experiment were used to calculate a baseline concentration of H_2O_2 to be used in future experiments. FRDA cells were used in this experiment due to their ability to grow well in cell culture and known susceptibility to oxidative damage. The same experiment was repeated using BSO as a ROS inducer. The levels of ROS generated by BSO were not significant. There H_2O_2 was determined to be the more effective inducer of oxidative damage than BSO because H_2O_2 was capable of producing a maximum increase of 34.4 fold increase in ROS compared to the maximum 1.6 fold increase observed in the BSO experiment. Therefore, H_2O_2 was used in future experiments to induce ROS in future experiments.

The next assay used Butylated hydroxytoluene (BHT) as a control antioxidant to reduce ROS in cells assaulted with H_2O_2. This control experiment was performed as a positive control in order to ensure a reduction in ROS from the antioxidant could be measured with a DCFH-DA assay. At the highest concentration of BHT a 20.7% reduction increase in ROS was observed when compared to cells not treated with the antioxidant.

To test the ability of the compounds to protect against ROS, FRDA cells were pre-treated with L2 and then assaulted with H_2O_2 at a concentration determined from previous experiments in order to generate intracellular ROS. L2 reduced ROS by a maximum of 41.2% compared to control (cells treated without L2).

Future iterations of L2 will involve linking lipoic acid to L2 to enhance the compound's antioxidant abilities. First the compounds were tested individually. The same experiment was performed with lipoic acid in HT-22 cells. Lipoic acid did not show a protective effect against oxidative damage like L2 did. Instead, cells treated with lipoic acid exhibited a 1.55 fold increase in ROS compared to cells untreated with lipoic acid. As shown above, L2 exhibited a protective effect against ROS in FRDA cells. Future studies should test the ability of L2 and L4 to reduce ROS in HT-22 cells as well. Also, cytotoxicity experiments should be performed for cells pre-treated with L2 and L4 and assaulted with H_2O_2 to determine if the compounds have cytoprotective effects in addition to a protective effect against ROS.

The results of our research suggest L2 is able to reduce ROS levels in cell culture. This information confirms the hypothesis that the structure of the compound confers a protective effect against oxidative damage. Lipoic acid did not exhibit this antioxidant effect in cell culture and further research should be conducted to confirm this finding before linking the L2 or L4 and Lipoid acid together with the intent of increasing the ROS-reducing ability. Due to

its demonstrated ability to reduce ROS, L2 may be a potential therapeutic tool for the treatment of Alzheimer's disease. This conclusion is supported by the reduction in ROS observed in cells assaulted with H_2O_2, which mimics the increased ROS environment of the brains of patients with Alzheimer's Disease. The cytotoxicity of L2 and L4 found in this experiment can be used in future cell culture as well as *in vivo* experiments with the compounds.

Future experiments should be performed to test the metal-chelating properties of these compounds in cells. Overall, L2 shows promise as an antioxidant with potential to be used to reduce the oxidative damage observed in Alzheimer's Disease.

References

"2019 Alzheimer's Disease Facts and Figures." *Alzheimer's & Dementia*, vol. 12, no. 4, 2019, pp. 459–509., doi:10.1016/j.jalz.2016.03.001

Bart De Strooper. (2010). Proteases and proteolysis in alzheimer disease: A multifactorial view on the disease process. *Physiological Reviews, 90*(2), 465-494. doi:10.1152/physrev.00023.2009

MTT assay: Basu, A., Teicher, B. A., and Lazo, J. S. (1990) J. Biol. Chem. 265, 8451-8457

Bonda et al. (2011). Role of metal dyshomeostasis in Alzheimer's disease. *Metallomics 3(*2), 267-270. DOI: 10.1039/c0mt00074d

Chen & Zhong. Oxidative stress in Alzheimer's Disease (2014). *Neuroscience Bulletin, 30,* 271-281. oi: 10.1007/s12264-013-1423-y

DCF Assay: Eruslanov E., Kusmartsev S. (2010) Identification of ROS Using Oxidized DCFDA and Flow-Cytometry. In: Armstrong D. (eds) Advanced Protocols in Oxidative Stress II. Methods in Molecular Biology (Methods and Protocols), vol 594. Humana Press, \ Totowa, NJ

DPPH assay: Blois MS. Antioxidant determinations by the use of a stable free radical. Nature. 1958;181:1199–1200. doi: 10.1038/1811199a0.

Jakob-Roetne, R., & Jacobsen, H. (2009). Alzheimer's disease: From pathology to therapeutic approaches. *Angewandte Chemie International Edition, 48*(17), 3030-3059. doi:10.1002/anie.200802808. doi: 10.1007/s12264-013-1423-y

Hannah M. Johnston et al. (2019). *Inorganic Chemistry* **2019** *58* (24), 16771-16784

DOI: 10.1021/acs.inorgchem.9b02932