

OXIDATIVE STRESS AS A TARGET FOR ALZHEIMER'S DISEASE THERAPEUTICS

by

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## OXIDATIVE STRESS AS A TARGET FOR ALZHEIMER'S DISEASE THERAPEUTICS

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## ABSTRACT

Alzheimer's Disease (AD) is a progressive neurodegenerative disease that is currently ranked as the sixth leading cause of death in the United States. Those affected by the disease experience symptoms such as cognitive decline, memory loss, confusion, and difficulty with language. Two of the main hallmarks of AD are neuroinflammation and oxidative stress. Inflammation is normally a natural response that aids in fighting off pathogens. However, constant inflammation can be toxic to brain tissue. Oxidative stress is characterized by the accumulation of free radicals and reactive oxygen species (ROS) and can be caused by many mechanisms such as mitochondrial destruction and dysfunction of the antioxidant system. Neuroinflammation and oxidative stress are linked processes that exacerbate one another. For example, the presence of ROS can activate microglia to produce increased levels of pro-inflammatory cytokines. Additionally, both of these processes interact with AD pathology, further contributing to the presence of neurodegeneration. The current study is aimed at investigating whether L2, a powerful antioxidant created in Dr. Kayla Green's lab at TCU, could produce a rebound effect on the viability of microglial cells undergoing oxidative stress and inflammation. BV2 microglial cells were used in conducting the MTT assays performed to measure percent cell survival. First, the oxidative stressor, H<sub>2</sub>O<sub>2</sub>, was administered to the cells at increasing concentrations in order to establish a negative, dose dependent relationship between oxidative stress and cell viability. The second portion of the procedure involved adding L2 compound at increasing concentrations to the microglial cells with a constant concentration of 3.0 μM H<sub>2</sub>O<sub>2</sub> administered to all wells one hour later. This set-up allows us to measure the potential rescue capacity of L2. Finally, LPS was administered to the cells at increasing concentrations in order to substantiate an inflammatory state in the microglia. H<sub>2</sub>O<sub>2</sub> was found to be a powerful oxidative stressor, causing substantial cell

death. However, LPS did not produce as drastic results, only causing a limited decline in microglial cell viability. Finally, L2 showed a noticeable rescue capacity prior to H<sub>2</sub>O<sub>2</sub> administration, suggesting a possible future for L2 as a therapeutic method for AD and other neurodegenerative diseases.

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## INTRODUCTION

Alzheimer's Disease (AD) is a progressive neurodegenerative disorder that is currently ranked as the sixth leading cause of death in the United States. With an increasing average life-expectancy among the world's population, AD has solidified itself as a major health-care problem. Those affected by the disease experience symptoms including, but not limited to, mental decline, memory loss, confusion, difficulty with language, and behavioral and mood changes. The basic biological markers of AD include increased CSF levels of total Tau, an abundant neuronal protein that stabilizes microtubules, hyperphosphorylated Tau (P-Tau), and increased levels of beta-amyloid peptide (Souza et. al., 2014).

One of the hallmarks of AD is inflammation that occurs both centrally in the brain and in the periphery. While inflammation is the natural response to activation of our immune system and is critical for our survival, neuroinflammation is heavily associated with AD pathology. Two main cellular mediators involved with the inflammatory response are members of the glial cell family, microglial cells and astrocytes. Microglial cells play a role in remodeling of the brain, acting as macrophages of the central nervous system. Astrocytes are the most numerous glial cells resident in the brain, regulating electrical synapses, providing metabolic support, and removing toxins. (Wyss-Coray and Rogers, 2012). When chronic inflammation is uncontrolled, it can lead to harmful cell and tissue damage. The continuous presence of damaged neurons results in the constant activation of microglia and astrocytes. This generates a neuroinflammatory environment which is thought to promote neurodegeneration (Roman and Olaf, 2015). Linked to inflammation, another process commonly upregulated in the brains of aging individuals is that of oxidative stress, which plays a major role in age-related neurodegeneration and cognitive decline. There are many contributing factors that play a role in oxidative stress. The process can

occur due to dysfunction of the antioxidant system, causing the accumulation of reactive oxygen species (ROS) in the brain (Huang et. al., 2016). Microglial activation, due to neuronal damage, can also generate superoxide radicals. It is also thought that the presence of ROS can activate macrophages, in specific microglia, which can then produce major pro-inflammatory cytokines such as IL-1 $\beta$  and TNF- $\alpha$  (McGarry et. al., 2018). This illustrates the connection between oxidation and inflammation in the brain. Other contributing factors include the high metabolic demand of glial cells and the low rate of brain regeneration (Manoharan et al., 2016). Oxidative damage of mitochondrial proteins and DNA occurs in early stages of the disease, suggesting a role of chronic oxidative stress in disease progression (Roman and Olaf, 2016).

As previously stated, one of the primary biological markers of AD is the aggregation of Amyloid beta (A $\beta$ ). The plaques that are formed consist of aggregates of A $\beta$ , a peptide around 40 AA in length, generated by proteolytic cleavage of the amyloid precursor protein (APP) (Finder et. al., 2007). Furthermore, it is believed that the aggregation of these peptides contributes to the cause of AD rather than acting as a product of the disease. A $\beta$  itself has been shown to act as a pro-inflammatory agent, promoting the activation of many inflammatory components. A $\beta$  can activate NADPH oxidase in primary cultures of cortical neurons, causing the generation of ROS within the cells (Roman and Olaf, 2016). The accumulation of A $\beta$  aggregates is thought to be exacerbated by essential biometal ions such as zinc and copper. Metal ions are critical for life. They often bind to metalloproteins and allow for essential functions such as the maintenance of cell structure, the regulation of gene expression, the mediation of cell signaling, and the catalyzation of enzymatic activity (Kim et. al., 2018). However, it has been found that an accumulation of these metal ions in the brain can contribute to A $\beta$  pathology in AD. For



instance, copper that is unbound to protein and found free in the cytoplasm has been shown to increase APP cleavage activity.

Dr. Kayla Green's lab in the TCU Chemistry Department has successfully created compounds that can simultaneously chelate metal ions and act as powerful antioxidants. They have developed a family of compounds all consisting of N-heterocyclic amines that, in turn, have the capacity to perform radical scavenging and metal ion capture. For this project, the compound L2, (3,6,9,15-tetraazabicyclo[9.3.1]penta-deca-1(15),11,13-trien-13-ol), was used. Their lab has shown that L2 acts as a promising therapeutic for neurodegenerative diseases involving oxidative stress and metal ion dysregulation.

For this project, BV2 microglial cells were plated into 96 well plates and run through MTT assays using increasing concentrations of both H<sub>2</sub>O<sub>2</sub> and LPS as treatments to test for decreased cell viability. Once decreased cell viability was established, the compound L2 was introduced to the microglial cells in addition to the treatments (H<sub>2</sub>O<sub>2</sub> and LPS) in order to test for a rebound of viability. The overall objective of the study is to measure the antioxidant and metal ion chelating abilities of L2 in a microglial cell line. This relationship could then be extended to neuroinflammatory diseases in general, with the possibility of this compound acting as a possible therapeutic for AD and other diseases that present with increased inflammation and oxidation.

## MATERIALS AND METHODS

### **BV2 Cell Maintenance**

BV2 microglial cells were maintained in a cell incubator at 37 degrees Celsius at 5% CO<sub>2</sub>. Cells were grown in 10 cm tissue culture dishes in complete cell medium containing 10 mL of DMEM, 5% Pen/Strep, 5% L-Glutamine, and 15% Fetal Bovine Serum. When the cells became around 80-90% confluent, they were passaged. Cells were passaged by first aspirating

off the medium and washing with 5 ml of PBS. After washing the cells, 5 ml of complete medium were added and a cell scraper was used to remove the cells from the bottom of the dish. The cells were then spun down in a centrifuge at 1.2 rcf for 10 minutes in a 15 ml conical tube. Once a pellet was formed, the supernatant was aspirated, leaving a 2ml solution in the tube. The cells were counted using a hemocytometer, adding 10 microliters of the resuspended cells to each side and manually counting using a microscope.

### **Experimental Treatment of Cells**

Cells were treated with either H<sub>2</sub>O<sub>2</sub> or LPS. H<sub>2</sub>O<sub>2</sub> was added at concentrations of 12 μM, 6 μM, 3 μM, 1.5 μM, and a control of 0 μM. LPS was added at concentrations of 20 μg/mL, 10 μg/mL, 5μg/mL, 2.5μg/mL and a control of 0 μg/mL. All cells received 100 microliters of the designated treatment concentration. All treatments lasted 16 hours prior to measurement.

### **L2 Compound**

Cells were administered L2 one hour prior to treatment with H<sub>2</sub>O<sub>2</sub>. L2 was added at concentrations of 12.5 μM, 1.25 μM, 0.125 μM, 12.5 nM, 1.25 nM, 0.125 nM, 12.5 pM, 1.25 pM, 0.125 pM, and a control of 0 pM at 100 microliters per well. All cells were then administered 100 microliters of H<sub>2</sub>O<sub>2</sub> (3.0 μM). L2 compound was provided by Dr. Kayla Green.

### **MTT Assay**

To measure cell viability of the BV2 cells, MTT (3-(4,5-Dimethylthiazonal-2-yl)-2,5-Diphenyltetrazolium Bromide) assays was performed. BV2 cells were plated in 96 well plates at 5000 cells per well. Cells were then treated with the appropriate treatment (H<sub>2</sub>O<sub>2</sub> or BSO) at the previously listed concentrations. After a 16-hour treatment time, the plate was dumped and 100 μL of MTT reagent (1mg/1ml) was added to each well, aside from the well designated as the blank. The plate was then incubated for four hours. After incubation, the plate was dumped again

and 100  $\mu\text{L}$  of DMSO was added to each well, followed by 5 minutes of gentle shaking. The cells were read on a plate reader (BMG LabTech FLUOstar Omega, Cary, NC) at an absorbance of 540 nm.

## RESULTS

### **$\text{H}_2\text{O}_2$ acts as a powerful oxidative stressor in microglial cells**

In order to induce oxidative stress in the microglial cells,  $\text{H}_2\text{O}_2$  was added to the cells at increasing concentrations. Cell viability was then measured using an MTT assay. Our results illustrated a negative, dose-dependent relationships between  $\text{H}_2\text{O}_2$  and cell survival rate (see Figure 1). Therefore, our data supports the idea that  $\text{H}_2\text{O}_2$  acts as a powerful oxidative stressor.

### **LPS had a weaker effect on overall microglial cell viability**

In order to induce inflammation in the microglial cells, LPS was administered to the cells at increasing concentrations. Cell viability was then measured using an MTT assay. The decrease in BV2 cell survival was not as drastic as seen with the  $\text{H}_2\text{O}_2$  treatment (see Figure 2). This showed that LPS had a weaker effect on overall microglial cell viability.

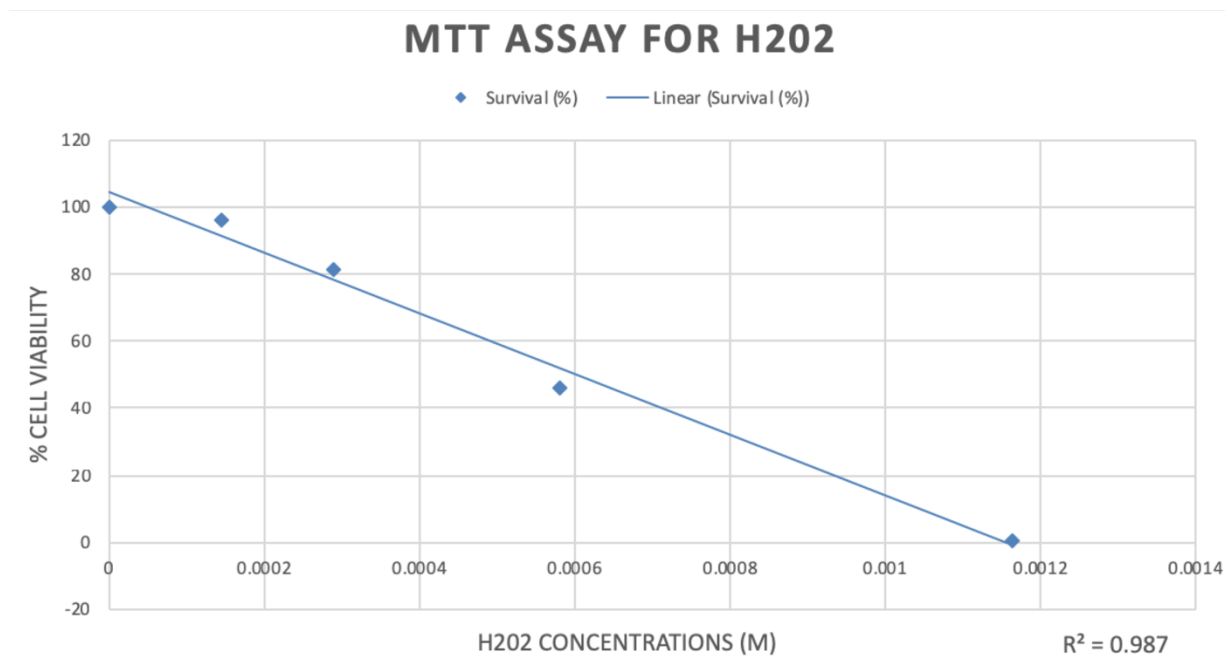
### **L2 administration had a rescue capacity in BV2 cells**

To measure whether L2, a powerful antioxidant, could have a potential rebound effect on microglial cell survival rates, L2 was administered one hour prior to  $\text{H}_2\text{O}_2$  treatment (3.0  $\mu\text{M}$ ). Our results illustrate an increase in percent cell survival from 0.12- 12.0  $\mu\text{M}$  (see Figure 3). This illustrates that L2 offers a protective effect against oxidative stress in the BV2 cell line.

## DISCUSSION

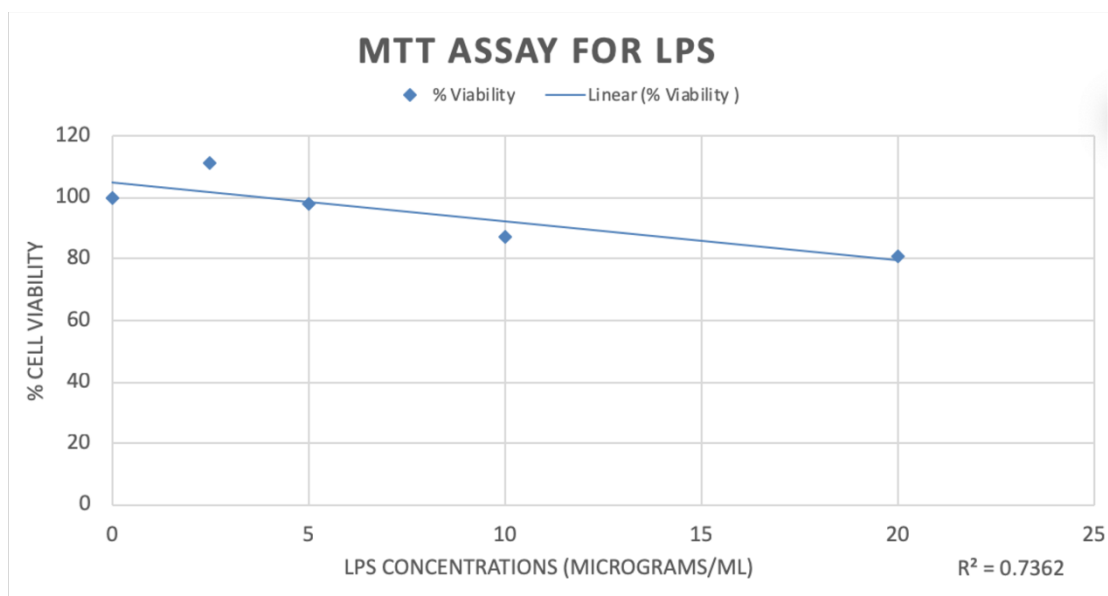
In the current study, we wanted to test whether L2 could procure a rebound effect in microglial cells undergoing oxidative stress and inflammation. To test this, we first had to create a state of oxidative stress in the cells. This was accomplished by administering H<sub>2</sub>O<sub>2</sub> at increasing concentrations to the microglial cells. Our results from the MTT assay showed that H<sub>2</sub>O<sub>2</sub> acts as a powerful oxidative stressor, causing a negative dose dependent effect in regard to overall percent cell viability. Additionally, in order to create an increased inflammatory state in the cells, we administered increasing concentrations of LPS. However, as previously stated, there was a less drastic drop in cell survival with increased LPS assault. We believe that the LPS may be increasing levels of pro-inflammatory cytokines in the microglial cells, but perhaps this is not leading to cell death. In future studies, we would want to administer L2 and LPS to the cells in a similar fashion, but instead of using an MTT assay, we would utilize a TNF-alpha ELISA to measure specific pro-inflammatory cytokine levels. This would allow us to see whether L2 offers a protective mechanism by reducing levels of pro-inflammatory cytokines such as TNF- $\alpha$  and IL-6. The last part of our study set out to measure the protective effect of L2 compound on microglial cells undergoing oxidative stress. When treating with L2 one hour prior to H<sub>2</sub>O<sub>2</sub>, the results from the MTT assay showed a rebound in cell viability at the 0.12–12.0  $\mu$ M region. In future studies, we hope to expand the range of L2 concentrations as well as perform a toxicity study to find the level of L2 that is detrimental to cell viability.

Figure 1



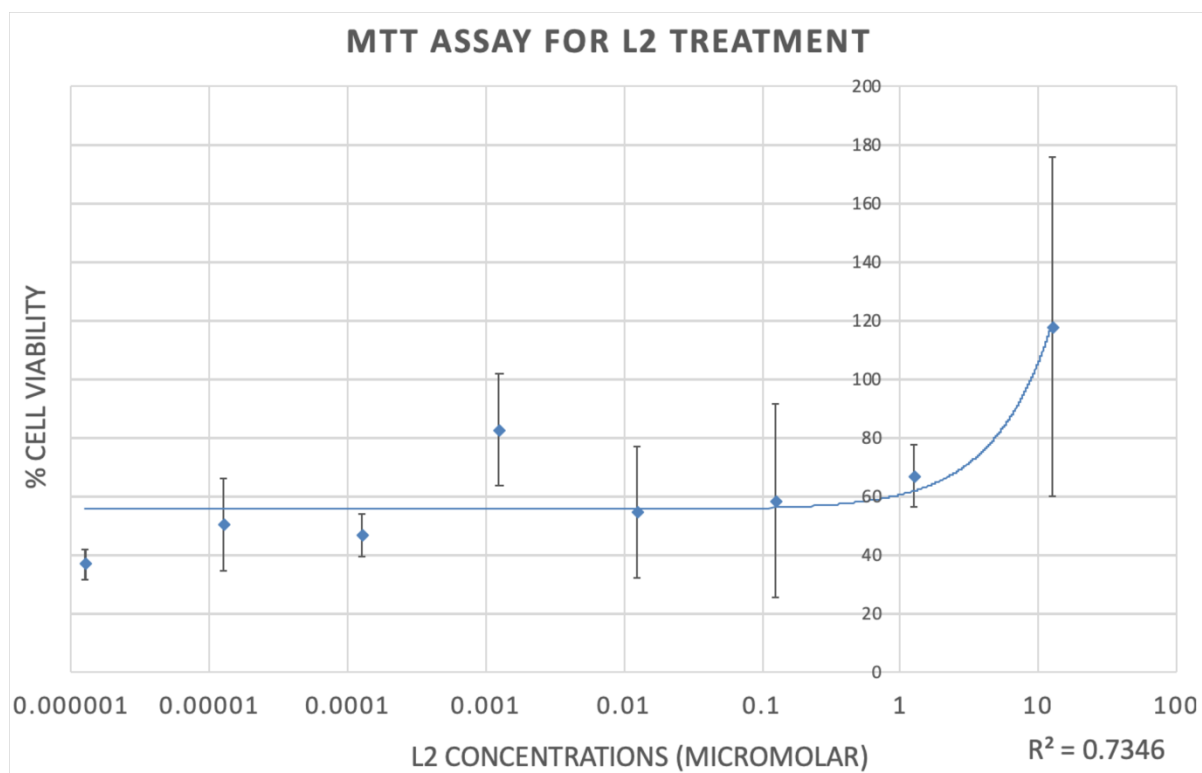
**Figure 1.** BV2 cell survival decreased with increased H<sub>2</sub>O<sub>2</sub> concentration. All cells received 3.0 μM H<sub>2</sub>O<sub>2</sub>.

Figure 2



**Figure 2.** LPS had a weak effect on overall BV2 cell viability.

Figure 3



**Figure 3.** L2 treatment enhanced BV2 cells survival following H<sub>2</sub>O<sub>2</sub>, oxidative stress inducer. This demonstrates that L2 could play a possible therapeutic role in protecting microglial cells from oxidative stress.

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