Hydroxylpyclen as a Potential Therapeutic for Alzheimer's Disease

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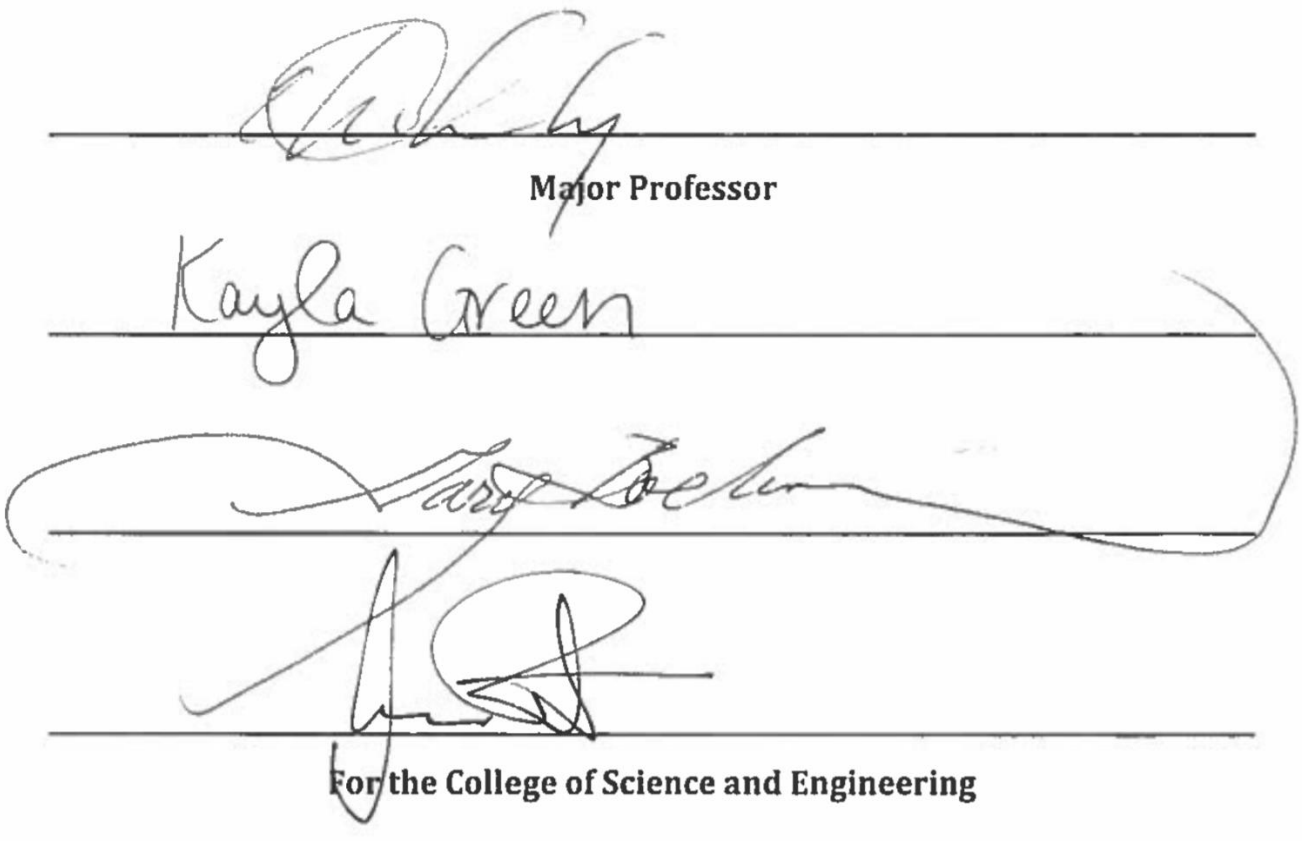

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Hydroxylpyclen as a Potential Therapeutic for Alzheimer's Disease

1. Introduction

Alzheimer's disease (AD) is the sixth leading cause of death in America, and is the only one of the top ten leading causes of death that cannot currently be prevented, cured, or slowed. AD presents with debilitating cognitive deficits evolving into the inability to accomplish daily tasks and progressing until the disease becomes lethal. One in every nine Americans age 65 and older is faced with AD. This fact becomes more important with the realization that the oldest members of the large baby boomer generation turned 70 in 2016. Sixty one percent of those over 70 with AD are predicted to fall victim to the disease before reaching the age of 80. At the current time, someone in the United States develops AD every 66 seconds, but by 2050, the incidence of AD will double, leaving someone to develop AD every 33 seconds. (Alzheimer's Disease Facts and Figures, 2016). This means that we are in the beginning of a rapid increase in AD prevalence with no therapeutics to slow or stop the progression of the disease.

AD symptomatically presents with a range of cognitive deficits depending on the specific stage of the disease. Early pathology attacks the limbic regions of the brain effecting a patient's ability to recall newly formed memories, but as the disease progresses more regions of the brain are affected and a wider array of the symptoms of dementia become apparent (Weintraub et al., 2012). The most common symptoms of full scale dementia are problems with recalling recent conversations, names and events, depression, difficulties with communication, confusion, behavioral changes, and difficulties with speaking, swallowing and

walking (Alzheimer's Disease Facts and Figures, 2016). The inability to perform basic bodily functions such as breathing and swallowing are often what make the disease lethal. The specific initiator of these symptoms is not known, however one of the most well understood pathologies consistent in AD is amyloid beta plaques. In fact, AD begins approximately two decades before symptoms arise, and it is understood that amyloid beta is one of the early agents in the neurodegenerative cascade (Lesné et al., 2013). Amyloid plaque burden has been shown to precede cognitive decline, and while studies do not show a direct link between plaque burden and the severity of cognitive decline, they do show this correlation with soluble amyloid beta levels which would suggest an association between amyloid beta and cognitive symptoms (Serrano-Pozo et al., 2011). This suggests that efforts made to diminish amyloid beta burden, especially early in the disease progression, could significantly decrease the cognitive challenges in an AD patient.

Amyloid beta arises from the cleavage of amyloid precursor protein (APP), whose biological function is not fully understood. APP has been shown to aid in neuronal maintenance processes including neurite growth, synaptogenesis, and neuronal protein transport (Zheng et. al, 2011). Despite these positive roles shown for APP, its cleavage can yield two different proteins, one of which is the amyloid beta implicated in AD. APP in a non-AD brain is consistently cleaved by the enzyme alpha secretase yielding a non-amyloidogenic product, but in an AD brain this cleavage competes with cleavage by beta and gamma secretases yielding an amyloidogenic form of amyloid beta (Tyler et al., 2002). In an AD brain, the activity of alpha secretase is 80% less than normal, while the cleavage by beta and gamma

secretases increases 185% (Tyler et al., 2002). Amyloid beta peptides generated through this cleavage will begin as soluble alpha-helical structures, and can become insoluble as the secondary structure changes into beta sheets capable of interdigitation via electrostatic interactions (Sinha et al., 2012). In addition to the change in secondary structure, research has also shown that there is a high affinity binding sight on the amyloid beta peptide for metal ions (Singh et al., 2013). This suggests that metal ions may be directly involved in drawing the soluble peptides into an insoluble plaque form. Amyloid beta begins as a monomer, and then aggregates into soluble dimers, trimers, and oligomers, and insoluble fibrils and plaques that are well documented in AD.

Both the insoluble and soluble forms of this pathogenic amyloid beta have been shown to have toxic effects on neuronal cells. Soluble amyloid beta oligomers can interact with a variety of different receptors on the surface of neuronal cells which can cause several toxic downstream effects including synapse loss and apoptosis (Sakono and Zako, 2010). Specifically, soluble amyloid beta interacts with postsynaptic N-methyl-D-aspartate (NMDA) receptors causing a dysregulation of calcium which ultimately leads to impairment in synaptic function (Mota et al., 2014). In fact, research shows that the soluble amyloid beta oligomers are the most neurotoxic because their levels correlate with the level of cognitive decline seen in AD patients (Yankner & Lu, 2009). Research has shown that the direct addition of soluble amyloid beta oligomers to hippocampal neuron cultures causes loss of long term potentiation, increases in long term depression, and overall decreases in dendritic spine density (Shankar et al., 2007, and Shankar et al., 2008). While not

directly correlated with cognitive decline, insoluble amyloid beta plaques can exacerbate the toxic environment surrounding important brain cells like neurons (Butterfield and Lauderback, 2002). This toxic environment leads to additional neurodegeneration typical of AD. Therefore, both soluble and insoluble amyloid beta are linked to detrimental neuronal changes which are directly associated with synaptic plasticity and the cognitive challenges typical of AD.

Copper and zinc are all metal ions that are necessary for normal brain function, but are also implicated in the pathology of AD when their levels are no longer in homeostasis. Zinc and copper are utilized by normal neurons for appropriate synaptic signaling. In an AD brain, zinc is maintained in the synapse causing excessive stimulation of the neuron through the NMDA receptor which leads to increased release of copper into the synapse. Reuptake of this copper is also not efficient in the AD brain, leading to an excess of metal ions sitting in the synapse which can aid in amyloid beta aggregation between the synapses (Kepp, 2012, Duce & Bush, 2010 Metal review). Sequestering these metal ions in amyloid beta fibrils and plaques leaves them deficient in other places where their role is crucial. By limiting the access to free zinc ions, proteases responsible for degrading amyloid beta such as neprilysin and insulin degrading enzyme are not properly metalated and cannot function as they were designed to (Duckworth et al., 1994, El-Amouri et al., 2008). This deficiency causes an absence of one of the known amyloid beta clearance mechanisms in the brain. Additionally, low copper levels have been shown to reduce the prevalence of the amyloid beta transporter, low-density lipoprotein receptor-related protein 1 (LRP1), in brain capillaries which leads to higher levels of

amyloid beta in the brain as it is not being efficiently transported out (Singh et al., 2013). This body of research on metal ions suggests that their dyshomeostasis plays a large role in the increase in plaque burden and decrease in the mechanisms to clear the pathogenic protein.

One of the main consequences resulting from amyloid beta is a toxic oxidative stress environment. Oxidative stress results from an imbalance between pro-oxidants and antioxidants leading to the formation of reactive oxygen species (ROS) (Bernhard and Wang, 2007). Examples of ROS include superoxide anion (O_2-), hydrogen peroxide (H_2O_2), and hydroxyl radical (OH·), but there are also reactive nitrogen species (RNS) such as nitric oxide (NO) that can lead to more reactive species production and increased damage (Fridovich, 1978). While reactive species are normal by-products of cellular metabolism, specifically mitochondrial respiration, their imbalance has been shown to have a multitude of negative downstream effects (Poyton et al., 2009). Reactive oxygen species (ROS) can be directly generated by the metal ion core of the amyloid beta plaques. Research has shown that copper ion cores in amyloid beta plaques can partake in Fenton-like chemical reactions in which the copper ions are reduced in the presence of oxygen to produce hydrogen peroxide which is a known pro oxidant (Lynch et al., 2000). Additionally, APP has a copper binding sight which causes additional production of free radicals when copper is bound there (Barnham et al., 2003). This oxidative stress environment is also attributable to other facets of AD including production of proinflammatory cytokines like TNF-α, and NO from activated microglia (McGeer & McGeer, 1995). Pro oxidants and reactive oxygen species are implicated in

neuroinflammation, mitochondrial dysfunction, protein oxidation, lipid peroxidation, DNA and RNA oxidation, and ultimately cell death through apoptosis (Butterfield and Lauderback, 2002). These downstream effects impact a variety vital cells in the brain, including the rampant neurodegeneration characterized in AD.

Oxidative stress impacts neuronal cell populations in a variety of ways in AD. Naturally low concentrations of amyloid beta have been shown to have a protective effect against neuronal targeted oxidative stress, while abnormally higher concentrations, such as those seen in AD, can exacerbate oxidative stress and impair neuronal function (Zou et al. 2003). While amyloid beta is shown to cause an increase in oxidative stress in AD, this oxidative stress has been shown to, in turn, increase the neuronal production of pathogenic amyloid beta. Research suggests that oxidative stress stimulates the c-Jun N-terminal kinase (JNK) pathway which induces the activation of beta secretase 1 (BACE1) and gamma secretase which are the enzymes that cleave APP into the pathological form of amyloid beta (Tamagno et al., 2008). Reactive oxygen species often partake in the oxidative degradation of lipids, or lipid peroxidation, where they primarily attack polyunsaturated fatty acids. As neuronal cell membranes have a higher than average level of polyunsaturated fatty acids in their membrane, they are one of the cell populations highly affected by lipid peroxidation resulting in dysfunction and degeneration of the neuronal cells (Markesberry, 1997). Whether it be intracellular or extracellular, oxidative stress is one of the primary culprits for the decline and ultimately death of large populations of neurons in AD.

Research suggests that, in addition to other antioxidant mechanisms, nuclear factor erythroid derived 2- like 2 (Nrf2) plays a vital defense role against oxidative stress in AD. Nrf2 operates as a primary sensor to oxidative stress in the cell, and when stress is sensed this nuclear factor can travel into the nucleus and bind to the antioxidant response element (ARE) to turn on antioxidant, drug transporting, and anti-apoptotic genes (Niture et al., 2014). Literature on Nrf2 suggests that it is the main cellular adaptation in dealing with cellular stress (Hayes et al., 2000). When the cell is not under stress, Nrf2 is retained in the cytoplasm by the inhibitor of Nrf2 (INrf2) or kelch like ECH associated protein 1 (Keap1). Additionally, research has shown that amyloid beta causes lower expression levels of Nrf2 in neuronal cell populations, keeping the cell from properly responding to the resulting oxidative stress (Amin et al., 2017). While Nrf2 is typically endogenously regulated, there have been studies suggesting exogenous sources of regulation as well. Research using antioxidants and tocopherols such as α-tocopherol (vitamin E) have shown that these compounds alone can stabilize and activate Nrf2 (Niture et al., 2014). Additionally, mice injected with amyloid beta directly into the brain and coupled with vanillic acid treatment, a phenolic acid antioxidant, showed increased levels of Nrf2 compared to treatment with amyloid beta alone (Amin et al., 2017). Control of Nrf2 activation by using antioxidants could be a potential therapeutic route for diseases such as AD where rampant oxidative stress in no longer able to be controlled through normal cellular mechanisms.

Oxidative stress, amyloid beta, and the associated neuroinflammation is known to influence the behavior of microglial cells in the brain. Even before the

deposition of amyloid beta plaques, neurons are under stress from intracellular amyloid beta and are releasing proinflammatory markers that influence the behavior of astrocytes and microglia (Hanzel et al., 2014). Microglia are commonly referred to as the immune cells of the brain, and their most well understood role is to survey the brain and phagocytose unwanted materials. In response to stimuli in the brain, microglia can adopt either a proinflammatory or anti-inflammatory phenotype. In AD, the microglia are encouraged to adopt this proinflammatory phenotype, but remain stuck in this phenotype longer than necessary causing overproduction of proinflammatory cytokines which causes tissue damage, additional oxidative stress, and more amyloid beta deposition (Saijo et al., 2011). Research has shown that the neuroinflammation in the AD brain is able to stimulate microglia to become activated, and in this state, they can release excessive glutamate which can be highly excitotoxic to neurons when overexposed (Barger et al., 2007). Microglial cells stuck in this proinflammatory phenotype are unable to process and phagocytose additional threats in the brain including newly forming soluble amyloid beta oligomers.

Currently, more than five million people are faced with Alzheimer's disease, and this number is predicted to triple in size over the next fifty years as the baby boomer generation continues to age (Alzheimer's Facts and Figures, 2016). This increase in AD prevalence will come at a high cost to healthcare, so a significant subset of AD research is focused on the generation of drugs or therapeutic strategies which can alleviate the symptoms of Alzheimer's disease. Despite the efforts of researchers, of the 244 drugs that reached clinical trials between 2002-2014, only

one of the drugs completed the clinical trial and became FDA approved for use. Of the six total FDA approved drugs for AD, none can accomplish more than lessening of the symptoms such as headaches and nausea for a patient for a variable amount of time (Alzheimer's Facts and Figures, 2016). As stated previously, both metal ions and oxidative stress are implicated in several facets in AD. Metal ions aid in the accumulation of amyloid beta and increase levels of oxidative stress, and this oxidative stress will also accumulate causing potent neuroinflammation. Together, these occurrences are highly to toxic to both microglial cells and neurons, and these incidences are all linked to one another. As previously stated, amyloid beta increases the level of oxidative stress, oxidative stress increases and prolongs the proinflammatory state of microglia, and these microglia can cause more amyloid beta deposition and oxidative stress. Furthermore, increases in oxidative stress are also able to directly increase the production of toxic amyloid beta. This suggests that a drug targeting this interaction could have an immensely positive effect on AD pathogenesis.

There is continuing literature discussing the generation and testing of variations on antioxidant and metal chelation combination therapy. The general concept behind these treatments is that the therapy could draw out metal ions from the amyloid beta plaques, solubilizing them, and the antioxidant will both diminish the current oxidative stress present and help prevent additional stress from occurring. Additionally, research has shown that chelation of the metal ions can help to restore metal ion homeostasis, attenuating the negative effects associated with the imbalance, and leading to greater amyloid beta clearance (Lannfelt et al., 2008,

and Faux et al., 2010). Clioquinol and its second generation derivative PBT2 are two metal associated therapeutics which reached the clinical trial stage of testing. Originally used as an anti-fungal drug, clioquinol was repurposed as a potential Alzheimer therapeutic because it is a hydroxyquinoline ionophore with the ability to act as a zinc and copper chelator (Bareggi and Cornelli, 2012). Initial studies showed that clioquinol was capable of cutting brain amyloid deposition in half in a mouse model of Alzheimer's disease, but while in phase 2/3 clinical trial clioquinol studies were halted by their production company Prana because the production of the drug generated a toxic contaminant (a di-iodo form of clioquinol) (Cherny et al., 2001, Prana website statement, 2005). The same company began work on the second generation clioquinol, PBT2, because it doesn't have the same chemical makeup and production does not yield the same toxic contaminant. PBT2 also acts as a copper and zinc ionophore, but following a long-term Phase 2 study, Prana reported no significant differences in brain amyloid deposition between individuals given PBT2 or the placebo (Alzforum article, 2014). In both cases, the metal ionophores ultimately failed in the clinical trial stage. Food constituents epigallocatechin gallate (EGCG) and curcumin were shown to have both chelation and antioxidant abilities, but were also suggested to have trouble crossing the blood brain barrier as efficiently as other multifunctional therapeutics tested (Chan et al., 2015). There are a variety of other multifunctional compounds being generated and tested, but the fact remains that for one reason or another these efforts are not moving to clinical trials, or having significant effects in clinical trials if they make it there. Understanding what has proven successful and where these compounds have fallen

short will help researchers to work towards generating a therapeutic that will have the desired significant effects.

At TCU, these multifunctional compounds are being designed and created in the lab of Dr. Kayla Green. In 2012, the Green lab constructed pyclen using the backbone of an MRI contrasting agent PCTA. This base has already been well researched and is small enough to cross the blood brain barrier. Using TEM/SEM imaging, pyclen was shown to be a copper and zinc ion chelator, and it caused the solubilizing of amyloid beta plaques and stopped the formation of plaques when metals were introduced to the peptide in solution. Additionally, cell culture assays showed that pyclen possesses antioxidant and preventative protection capabilities. The Green lab attributes the antioxidant capabilities to the pyridine backbone in the compound (Lincoln et al., 2012). Further research by the Green lab looked to enhance the antioxidant capabilities of the pyclen compound without sacrificing the metal chelation capacity. To accomplish, the pyridine backbone was converted to a pyridol, and the resulting compound was called Hydroxylpyclen. Hydroxylpyclen maintained its chelation capacity, but significantly increased its antioxidant capacity in cell culture when compared to pyclen. One additional promising note for Hydroxylpyclen is that it can enter cells without altering the function of cytosolic metalloenzymes (Lincoln et al., 2013). This is important because the chelation capabilities of the compound are designed to help restore the metal ion homeostasis without interfering with the metal ions working in their designated roles. Results from the studies on Hydroxylpyclen suggest that it could be a potent therapeutic for AD. Unpublished data showed that intraperitoneal injections of Hydroxylpyclen

resulted in a non-significant, but trending decrease in plaque size and number between saline and compound treated 5xFAD mice, a mouse model of Alzheimer's disease (Gurney, unpublished TCU Honor's Thesis). Based on these results, more work needs to be done on the compound *in vitro* to better understand how varying concentrations of the compound interact and effect key cell populations in the brain, namely microglia and neurons.

Both neurons and microglia are vital cells in the brain for its day to day health. AD targets and disrupts these cells which causes neurodegeneration and worsening of the pathology. The goal of this set of studies was to establish the methodology to test Hydroxylpyclen *in vitro*, and to use the methodology, once established, to determine the therapeutic value of Hydroxylpyclen.

2. Chapter 1, Establishing a method to test Hydroxylpyclen in neuronal cultures

As discussed above, neurons are heavily assaulted in AD. This first study looked solely at neurons to establish a methodology for testing the compound, Hydroxylpyclen, and its effects *in vitro*. Cultured HT22 neurons are assaulted with known oxidative stress inducers hydrogen peroxide, glutamate, or lipopolysaccharide (LPS). The DCFHDA assay is used to measure levels of oxidative stress, whilst a VitaBlue assay is run on similar samples to detect cell viability. These results yield the concentration and time point at which the assault causes the most oxidative stress without causing an unacceptable level of cell death. Then following the same methodology, the compound alone can be tested to determine its optimal therapeutic window, maximizing its antioxidant properties while minimizing cell

death. Using the compound and assault together, this same set of tests can be run to determine if the compound is able to protect the neurons from oxidative stress. An effective compound can then be tested in primary neurons, and synapses can be directly counted to see if the compound is able to protect synaptic density in the neurons. This project is trying to show that if we can chelate metal ions out of plaques, and/or reduce oxidative stress using this compound, then we may be able to attenuate symptoms of the disease.

3. Materials & Methods

3.1 HT22 Cell Maintenance

HT22 immortal neuronal murine hippocampal cells (kind gift of Dr. Kayla Green, Texas Christian University) were maintained in a cell incubator at 37 degrees Celsius at 5% CO_2. Cells were grown in 10cm tissue culture dishes in 10mL DMEM (Sigma-Aldrich, St. Louis, MO) supplemented with 5% Pen/Strep, 5% L-Glutamine, and 10% Fetal Bovine Serum (FBS). When the cells reach 75-85% confluency, cells were passaged in a sterile culture hood as follows. The media was aspirated off and cells were detached from the bottom of the dish using a cell scraper. The dish was then rinsed with 5mL of fresh media, and the cell-media mixture was moved into a 15mL conical tube and spun down at 0.3 rcf for 10 minutes to pellet the cells. Then cells were counted on a hemocytometer and plated at the correct cell number for the plate well number and assay being run. All cells were used between passage 28-30.

3.2 Experimental Treatment of Cells

Following the counting and plating of the HT22 cells as described above, cells were treated with one of three experimental conditions: H_2O_2, LPS, or glutamate. Hydrogen peroxide was applied to the cells at 150μM, 100μM, 50μM, or 25μM, and LPS was added at 25μg/mL and 12.5μg/mL. Glutamate was added at a concentration of 100, 8, 4, or 2 mM. Glutamate and LPS were tested at 30 minutes and 1.5, 4, 8, 12, 24, 30, and 48 hours. Hydrogen peroxide was tested at 30 minutes, 1 and 6 hours. Cells treated with the compound Hydroxylpyclen were given 1mM or 250, 62.5, 15.625, 3.91, or 1μM for 12 hours prior to treatment with 8mM glutamate for an additional 8 hours.

3.3 DCFHDA Assay

To measure reactive oxygen species in the neuronal cells, 2', 7' dichlorofluorescin diacetate (DCFHDA) was used. Briefly, HT22 cells were counted as stated above and 5000 cells per well were plated into 96 well plates, and allowed to adhere for at least 30 minutes. Following adhesion, either H_2O_2, LPS, or glutamate was added to the cells at the concentrations stated in the previous section. Each concentration of the specific assault was either run with an n of three or five. The assault sat on the cells for the desired treatment time before the media was removed and 100 microliters of the DCFHDA (either 1μM or 10μM dissolved in media or PBS) was added to the cells and incubated for 20 minutes. The incubated cells were then washed in PBS, and read on a plate reader (BMG LabTech FLUOstar Omega, Cary, NC) at 495 nm absorbance and 529 nm emission.

3.4 VitaBlue Assay

Cell viability was measured using the VitaBlue Cell Viability reagent and protocol from BioTool (BioTool, Houston, TX). Briefly, at the end of the allotted experimental time for assault treated cells, the VitaBlue reagent was added and tapped to thoroughly distribute. Cell viability was tested after 4, 8, 12, and 24 hours of treating cells with glutamate and LPS. The reagent incubates with the cells for four hours before being read on the plate reader (BMG LabTech FLUOstar Omega, Cary, NC) at 530-570nm excitation and 590-620 nm emission.

3.5 Primary Neuron Extraction and Culture

C57BL/J postnatal day 0-2 pups were utilized for primary neuron cultures. One day prior to the culture process, square glass coverslips were fire polished in a sterile culture hood, and then coated with poly-D-lysine for one hour. Coated coverslips were then washed in sterile water before being seeded in 6 well tissue culture dishes to dry overnight.

For each extraction, four pups underwent rapid decapitation after anesthetization using ice, in accordance with IACUC approved methods. Brains were then isolated into an ice-cold dish with HBSS (Gibco-Thermofisher, MA), and the meninges were removed after separating the two hemispheres from the remainder of the brain. The hemispheres were then collected into a 15mL conical tube, and the tissue was digested by allowing it to rock in Trypsin/EDTA at 37 degrees Celsius for approximately 20 minutes (1 mL of trypsin for each animal used). Once sufficient tissue break down had occurred, the digestion process was stopped by the addition of twice as much DMEM as trypsin, and the suspension was spun down at 0.2 rcf for

10 minutes. Cells were re-suspended in Neurobasal Media supplemented with 2% B-27 and 1% L-glutamine. Cells were counted, and then plated at the desired concentration in the six well tissue culture dishes on the poly-D-lysine cover slips. After one hour on sitting directly on the cover slips to achieve the greatest amount of adhesion, the remainder of the growth media was added. The plates were allowed to grow for 14 days, exchanging half of the media every 3-5 days.

3.6 Primary Neuron Staining

Primary neuronal cultures were stained to observe synapse morphology and number. To accomplish this, neuronal coated coverslips were removed from the tissue culture wells and placed on parafilm in a sterile culture hood. Cells were fixed with 4% paraformaldehyde (PFA) for ten minutes. After fixation, the cells were blocked in 5% donkey serum for thirty minutes, and then incubated with the pre- and post-synaptic primary antibodies against the targets Bassoon (1:500, Novus Biologicals, CO) and PSD95(1:500, Novus Biologicals, CO) respectively for one hour. After the first incubation, the coverslips were washed and the incubated with fluorescent secondary antibodies for another hour. After incubation with the primary and secondary antibodies, the coverslips were washed once more before being mounted cell side down onto a glass microscope slide. Bassoon and PSD95 positive neurons were analyzed using a Zeiss LSM 710 laser-scanning confocal microscope (Thornwood, NY) at 60x optic. Image analysis was performed using Zeiss Zen software.

3.7 Statistical Tests

Statistical analyses were performed using one-way analysis of variance (ANOVA) following the DCFHDA and VitaBlue assays. Alpha level was set at 0.05. Tukey post hoc analysis was completed on DCFHDA and VitaBlue data to assess significant group differences when appropriate.

4. Results

The purpose of this set of studies was to establish a methodology to test the efficacy of the compound Hydroxylpyclen in protecting both HT22 and primary neurons from the deleterious effects of oxidative stress. As shown in **Table 1**, only the 30-minute glutamate and LPS assaulted group showed significant group differences ($p=0.014$). Post hoc analysis showed that the 100mM dose's production of ROS was significantly lower than all other groups. None of the other groups were significantly different from one another. No other time block showed significant differences between the groups. As shown in **Table 2**, no hydrogen peroxide assault time points showed significant differences between any of the groups.

The Vita-Blue cell viability trial results, shown in **Table 3**, show that both the 12 and 24 hour trials showed significant group differences. For the 12 hour timepoint, post hoc analysis showed that the 100mM and 8mM doses of glutamate had significantly lower levels of cell death compared to the 2mM dose of glutamate ($p=0.046$ and $p= 0.018$ respectively), the 25μg/mL dose of LPS ($p=0.032$ and $p=0.012$), as well as both the untreated cells with VitaBlue control ($p<0.001$ for both assaults), and the VitaBlue with media control ($p<0.001$ for both assaults). The 100mM and 8mM doses were not significantly different from one another

(p=0.998). Both of our controls, untreated cells with Vita-Blue, and Vita-Blue with media, were both significantly lower in their fluorescence values than all the glutamate and LPS dosed cells, typically indicative of cell death. Overall, the plate readout values for the 12-hour assay were noticeably larger than the 24-hour assay. Post hoc analysis on the 24-hour time point shows that all the assault dose groups were significantly higher than the two controls, untreated cells with VitaBlue (100mM p=0.008, 8mM p=0.001, 4mM p<0.001, 2mM p=0.002) and VitaBlue with media (100mM p=0.0.008, 8mM p=0.001, 4mM p<0.001, 2mM p=0.002), but were not significantly different in their cell death from one another. No other timepoints showed significant group differences.

Table 4 shows the DCFHDA assay results after co-treating cells with Hydroxylpyclen and 8mM glutamate. This dosage was chosen because of the results of a single 8-hour technical replicate where it appeared that 8mM glutamate was close to being significantly different from the 4mM and 2mM glutamate treated groups (p=0.068). The group differences are as follows. The 1mM compound treated group showed significantly less ROS than all three controls: the media with the compound and DCFHDA, the media with the glutamate and DCFHDA, and the media with the compound, glutamate and DCFHDA. The 250μM, 62.5μM, 15.62μM, 1μM, and untreated cell control groups had significantly less ROS production than the control with the glutamate, DCFHDA, compound, and media. The 3.91μM compound treated group was not significantly from any of the groups, control or assault treated.

Figure 1 shows a representative image of the Bassoon and PSD95 stained primary neurons (14 days post collection). The protocol for culturing primary neurons yields successful neuronal growth on coverslips each time an attempt is made, signifying a successful protocol has been established for this lab to utilize. Extraneous staining signifies neurons or other glial cells which lifted off the coverslip during the staining process.

Assault Time	100mM glutamate	8mM glutamate	4mM glutamate	2mM glutamate	25µg/mL LPS	12.5µg/mL LPS	Untreated Control	P value
30 minutes								
	1226.20 ± 71.02	1792.20 ± 22.62	1893.40 ± 73.75	1885.40 ± 23.96	1887.20 ± 37.29	1981.00 ± 58.39	1881.50 ± 32.82	0.014
1.5 hours								
		2068.00 ± 112.87	1934.80 ± 86.00	1859.20 ± 47.66	1780.80 ± 11.80	1824.00 ± 14.12	1869.40 ± 18.57	0.096
4 hours								
	2119.33 ± 119.17	2021.12 ± 85.45	2024.50 ± 66.29	1802.01 ± 214.00	1980.00 ± 61.48	2005.00 ± 29.94	2031.61 ± 108.05	0.99
8 hours								
	2520.17 ± 184.76	2028.44 ± 84.86	1841.44 ± 48.90	2045.00 ± 140.08	1733.33 ± 49.30		2310.89 ± 229.61	0.802
12 hours								
	2316.89 ± 152.55	2094.27 ± 257.30	1845.79 ± 87.91	1918.49 ± 87.59	1754.84 ± 35.55	1879.60 ± 50.45	2014.29 ± 153.67	0.568
24 hours								
	4749.83 ± 746.88	3960.50 ± 790.00	3734.67 ± 419.19	5193.33 ± 433.80	4594.00 ± 163.39	4767.00 ± 657.55	5148.00 ± 1162.36	0.899
30 hours								
	8080.33 ± 1365.43	5037.00 ± 175.39	5129.67 ± 713.54	6075.00 ± 107.48			15760.00 ± 5313.59	0.142
48 hours								
		1724.33 ± 23.85	1789.33 ± 17.11	2213.67 ± 383.95	1732.67 ± 12.16	1788.33 ± 45.78	1958.67 ± 88.67	0.509

Table 1: DCFHDA assay results using glutamate and lipopolysaccharide as assaults show an overall lack of effect of time or assault concentration on ROS production. The thirty-minute time point resulted in significant group differences. Each time point shows the average of the technical replicates with the corresponding standard error for each assault. Each technical replicate is comprised of an n of three or five for all assaults, and the experimental group average is the average of all of the technical replicates. Alpha was set at 0.05.

Assault Time	150µM	100µM	50µM	25µM	Control (0µM)	P Value
30 minutes						
	1664.40 ± 45.44	1870.37 ± 88.59	1894.37 ± 76.86	1866.17 ± 64.23	1819.70 ±54.58	0.801
1 hour						
	1665.20 ± 107.85	2105.60 ± 165.32	2294.00 ± 326.03	2713.20 ± 552.83	2095.80 ± 55.62	0.316
6 hours						
	1660.40 ± 133.28	1781.80 ± 63.64	1768.20 ± 57.40	1833.60 ± 48.02	1793.00 ± 74.79	0.645

Table 2: DCFHDA assay results using hydrogen peroxide as the assault shows no effect of time or assault concentration on ROS production. No time points resulted in significant group differences. Each time point shows the average of the technical replicates with the corresponding standard error for each assault. Each technical replicate is comprised of an n of three of five for all assaults, and the experimental group average is the average of all of the technical replicates. Alpha was set at 0.05.

VitaBlue Time Point Trials	100mM glutamate	8mM glutamate	4mM glutamate	2mM glutamate	25µg/mL LPS	VitaBlue and Untreated Cells Control	Media and VitaBlue Only	P Value
4 Hours								
Trial 1	7260.33 ± 701.45	6091.67 ± 576.15	7328.00 ± 1034.95	21948.33 ± 12236.46	5402.67 ± 111.90	4287.00 ± 5.72	2508.33 ± 7.95	0.327
8 Hours								
Trial 1	7627.00 ± 822.21	7959.00 ± 620.50	6199.67 ± 200.79	21739.00 ± 11280.49	6939.67 ± 491.71	3559.00 ± 25.15	2113.00 ± 34.42	0.249
12 Hours								
Trial 1	14996.00 ± 1805.78	15754.67 ± 396.28	12566.67 ± 944.90	10528.67 ± 1161.97	9726.33 ± 548.02	3595.33 ± 51.54	2588.67 ± 112.21	<0.001
24 Hours								
Trial 1	2900.00 ± 305.95	3680.67 ± 506.57	4144.67 ± 319.83	3304.00 ± 431.15		377.00 ± 5.91	370.00 ± 2.62	<0.001

Table 3. HT22 cell viability was dependent upon time and assault concentration. Significant group differences in cell viability were seen at the 12 and 24-hour time points. Table shows one technical replicate plus or minus the standard error for each assault. The value for each assault in the individual trials is an average of an n of 5. Alpha was set at 0.05.

1mM	250µM	62.5µM	15.625µM	3.91µM	1µM	No Compound Added
14396.67 ± 394.06	21836.33 ± 8056.19	21859.00 ± 3822.23	22143.67 ± 2484.69	28607.33 ± 2923.97	24036.33 ± 1688.84	20003.33 ± 1536.37

Media, Compound, DCFHDA	Media, Glutamate, DCFHDA	Media, Compound, Glutamate, DCFHDA	P Value
36396.00 ± 3183.97	45932.67 ± 4833.18	32561.33 ± 7293.37	0.001

Table 4. ROS production was significantly different between groups when HT22 cells are treated with both glutamate and Hydroxylpyclen. The top row shows the compound treatment groups, and the bottom row shows the control groups which did not include cells. The table is the result of a single technical replicate, with each assault value being the average of an n of five. Each assault value is shown with its corresponding standard error. Alpha was set at 0.05.

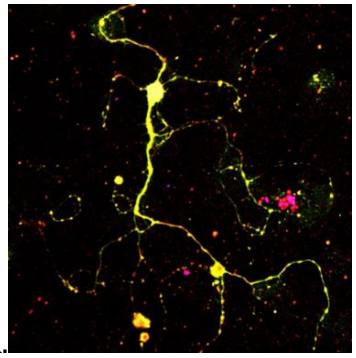
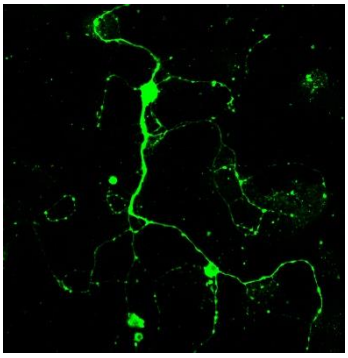
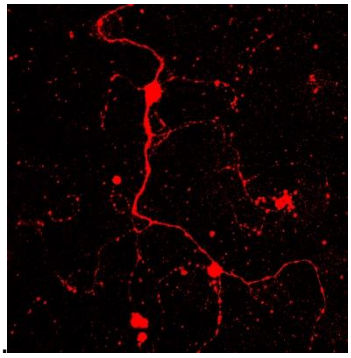
A.  B.  C. 

Figure 1: Primary neuron removal and staining procedures are successful, but are not pure enough for synapse quantification. Neurons were labeled for PSD95 and Bassoon at a concentration of 1:500. Images were taken on a confocal microscope at a 60x optic. Image A shows the overlay of both labeled targets, PSD95 and bassoon. Image B shows the labelling of only PSD95. Image C shows the labelling of bassoon only.

5. Discussion

The combined results from this set of studies suggest more work needs to be done to create an effective methodology to test compounds, such as Hydroxylpyclen, in neuronal cell populations. Only assault trials for the DCFHDA assay showed significant differences between the groups. The 100mM glutamate dosage is much larger than the originally chosen high dose of 8mM, and was chosen after the initial glutamate DCFHDA assays provided no significant group differences at several time points. This dose was chosen to see if even an extreme dosage of glutamate would be able to prompt a significant group difference. It has been shown that approximately 5mM dosage of glutamate is cytotoxic to HT22 neuronal cells, causing both high levels of ROS production and activation of the death executioner caspases 1 and 3 (Ha and Park, 2006). Additionally, glutamate concentrations can naturally range from 1-10mM in the synaptic cleft and intraneuronal compartments (Dzubay and Jahr, 1999). Aside from our 100mM dose, this puts all our other doses

(2, 4, and 8mM) in a biologically relevant range. Post hoc analysis of the 30-minute group showed that the 100mM glutamate treated group showed significantly less ROS production than all of the other groups. The could be because at the 30-minute time point, this high dose of glutamate caused cytotoxicity in the HT22 cells causing cell death before sufficient ROS production could be generated. A more likely explanation is that the 100mM glutamate dose value is only a single technical replicate, while the remainder of the treatment groups are the average of three technical replicates. The 100mM glutamate dose was not always used because it is so far outside of the biological relevance range. This single replicate may not be representative of the actual behavior of the HT22 cells, and an average of three like the other trials, would most likely show no significant differences between groups.

The hydrogen peroxide assault resulted in no significant differences between any of the groups at any of the time points. Taken together with the glutamate and LPS assault assay results, it is likely that there was an inherent problem with the HT22 cells that we used. HT22 cells have been used with the DCFHDA assay successfully, similar in methodology to the way they were used in this set of studies. The cells we received came to us at a very high passage (passage 26), which is approximately the passage recommended for HT22 cells if you are looking for them to behave in a stable manor. To look at oxidative stress and ROS production, the HT22 cells should be at a much less stable passage, and this could be one of the reasons we were unable to find significant group differences. Additionally, a higher dosage of the DCHFDA reagent could potentially yield more accurate results. Recent research utilizing HT22 cells found significant differences using a 600μM

concentration of the DCFHDA reagent, which is at least 60 times more concentrated than the doses used in this research (Amin et al., 2017). Our lab has not done DCFHDA assays before, so the doses of the DCFHDA reagent chosen for this paper (1μM and 10μM) were chosen from the literature using the Hydroxylpyclen compound which used 1μM DCFHDA reagent, but this was done in FRDA fibroblast cells which produce a very high level of cytosolic ROS (Lincoln et al., 2012). Any future work with the HT22 cells will require a fresh set of cells. We hypothesize that the initial cells provided to our lab were not at full health, and this led to cultivating a group of HT22 cells at too high of a passage which were not completely healthy. Those factors together likely contributed to the technical issues in this set of studies.

The VitaBlue Cell Viability assay measured significant differences at both the 12 and 24-hour time points using varying concentrations of glutamate assaults. At the 12-hour time point, the two highest glutamate doses had significantly lower cell death than all other treated cells, as indicated by the higher fluorescence value. This VitaBlue data was the result of a single technical replicate, so the data may not represent an actual trend in the HT22 cells behavior. Additionally, as these are the same cells that were used in the DCFHDA assay, it is believed that they were not healthy and behaving in an expected way as the literature would suggest. The 12-hour time point produced higher overall fluorescence values than any of the other timepoints, which were all in a similar range to one another. This would suggest that the 12-hour time point should be studied further, as there was the most cell viability noticed during this experimental treatment window. After 24 hours of varying glutamate concentration assaults, the controls showed significantly lower

fluorescence from the treatment groups. This was also the case for the 12-hour time point. More research should be done to determine if the media is interacting with the VitaBlue to cause this readout. While the protocol did not specifically call for phenol free media, there may be an interaction between then phenol and the reagent which could be causing this fluorescence. Moving forward, this background fluorescence will need to be accounted for in order to achieve the most accurate results. Achieving more consistent and expected results will help to determine the time point and dose of glutamate which generated high levels of ROS with the least amount of overall cell death. This assault concentration and time point can then be used with the compound to assess its overall effects.

Co-treating the cells with Hydroxylpyclen and 8mM glutamate yielded significant group differences, but the results warrant further research. None of the compound treated groups were significantly different from each other, suggesting that either the wrong assault or compound dose was chosen, the wrong time point was chosen, or more clarified results may have been generated from using the 600µM DCFHDA dose used by Amin et al. The 1mM, 250µM, 62.5µM, 15.62µM, 1µM, and untreated cell groups were all significantly lower in their ROS levels than the controls. This result was not expected because there were no cells included in these controls, and their original purpose was to ensure all the reagents did not interact with each other to yield false positives for ROS. This suggests that there may be an interaction between two or more of the reagents used, and this result yields ROS which may be the reason that none of the treatment groups are significantly different. Future work should focus on the interaction between these reagents, and

to eliminate this interference for ongoing testing. Additionally, the future work done with the DCFHDA assays should help to clarify the dosage and time point that should be used in conjunction with the compound.

The results from the primary neuronal cultures show that a successful protocol has been established for this lab, however the cultures still need to be cleaned up such that synapse numbers can more easily be counted. Targeting bassoon and PSD95 with primary antibodies yielded images on the confocal which showed both pre- and post-synaptic regions, however there was still excess staining around the neuron from the bassoon antibody (**Figure 1, Image C**). To alleviate this problem, we will change pre-synaptic primary antibody to use synaptophysin as a target protein, which is a popular and reliable presynaptic marker that can be used for synapse number counting in the JOVE protocol for similar experiments (Ippolito and Eroglu, 2010). Another issue with these cultures is that they are not purely primary neurons. Visualization under the confocal microscope shows primary astrocytes and microglia as well. One of the suspected reasons for this is that the excess glial cells are adhering to the poly-d-lysine coating on the coverslip. One possibility to alleviate this problem would be to try another coating, laminin, to see if less glial cells adhere. Another possible solution could be to use AraC to remove non-neuronal cells from the culture, as this protocol has been shown to be successfully used with murine primary neuronal cells (Hopkins Medicine Protocol). In addition to the aforementioned methods, there are two additional measures that can be taken in the future to purify the cultures. More time can be allowed before staining the primary neurons. This will allow for more clearance of excess glial cells

because the neurobasal media used is supplemented with neuron specific nutrients. Additionally, there are now medias being made which have the specific role of clearing glial cells from neuronal cultures in order to have pure cultures for staining. These strategies will create clean neuronal cultures for this lab to use for synapse counting.

6. Chapter 2, Combating microglial stress with Hydroxylpyclen

While neuronal cell populations are often the target for AD therapeutics, any therapeutic must additionally be beneficial to the immune cell of the brain, microglia. Their role in the brain is to phagocytose unwanted items, including amyloid beta. It has already been shown that the microglia in their proinflammatory state partake in numerous detrimental activities in AD including the production of proinflammatory cytokines and an enhanced oxidative stress environment. These microglia are heavily influenced by neuroinflammation that can come from amyloid beta and oxidative stress. This study aims to establish methodology to assess the therapeutic value of the compound, Hydroxylpyclen, on microglial cell cultures. To accomplish this, microglia cultures will be assaulted with amyloid beta, and levels of TNF-α will be measured. Additionally, a VitaBlue assay will be conducted to ensure the results seen in the TNF alpha ELISA are not due to cell death. The working hypothesis is that the compound will reduce oxidative stress which will result in a decrease in the production of the proinflammatory cytokine TNF-α. By reducing the stressful environment, the microglia could be influenced to resume their role as surveyors of the brain, and phagocytose the soluble amyloid beta oligomers being

generated through the metal chelation of the compound. Together, this effect could decrease the detrimental symptoms of the disease.

7. Materials & Methods

7.1 BV2 Cell Maintenance

BV2 murine microglia cells (kind gift of Dr. Sonny Singh, University of North Texas Health Sciences Center) were maintained in a cell incubator at 37 degrees Celsius at 5% CO_2. Cells were grown in 10cm tissue culture dishes in 10mL DMEM (Sigma-Aldrich, St. Louis, MO) supplemented with 5% Pen/Strep, 5% L-Glutamine, and 10% Fetal Bovine Serum (FBS). When the cells reached 75-85% confluency, cells were passaged in a sterile culture hood as follows. The media was aspirated off and cells are detached from the bottom of the dish using 1.5mL trypsin/EDTA. The dish was then rinsed with 10mL of fresh media, and the cell-media mixture is moved into a 15mL conical tube and spun down at 0.3 rcf for 10 minutes to pellet the cells. Then cells were counted on a hemocytometer, and 500,000 cells per 10cm dish were plated and allowed to sit overnight to achieve 80% confluency. All cells were used between passage 28-32.

7.2 Amyloid Beta and Compound Treatments

Cell plates which have reached the 80% confluency point were ready to be treated with human oligomeric amyloid beta 1-42 (GenicBio, Shanghai, China), Hydroxylpyclen compound (Kayla Green Lab, TCU), or both. Amyloid beta was given to the cells at 5μM for 12 hours which is a dose used in the literature with cultured and primary microglia (Walker et al., 2001, Park et al., 2013). The compound was

given to the cells at 33.3 mg/mL or 8.625 mg/mL for 6 hours prior to treatment with amyloid beta for an additional 12 hours.

7.3 BV2 Cell Lysate and Supernatant Preparation

Following treatment of the BV2 cells, the supernatant was removed and spun down at 0.3 rcf for 5 minutes to remove cell debris before saving at -20 degrees Celsius. The cells adhered to the plate were then treated with protein extraction solution (RIPA, Research Products International Corp., Prospect, IL) and protease inhibitor, and the cells were scraped off the dish and collected in microfuge tubes before being processed into the usable cell lysate. To finish preparing the lysates, the microfuge tubes containing the cells and RIPA buffer were spun down for 40 minutes at 13.3 rpm at 4 degrees Celsius. Following the centrifugation, the supernatant of the spun down lysate sample was moved into clean microfuge tubes and stored at -20 degrees Celsius until further use. The lysate protein levels were determined with a DC Protein Assay (Bio-Rad Laboratories, Hercules, CA).

7.4 TNF-α Cell Supernatant ELISA

The cell supernatant was used to assess TNF-α production across the different treatment conditions. The protocol was followed exactly from BioLegend Mouse TNF-α ELISA MAX Deluxe kit (BioLegend, San Diego, CA).

7.5 VitaBlue Assay

Cell viability was measured using the VitaBlue Cell Viability reagent and protocol from BioTool (BioTool, Houston, TX). Briefly, at the end of the allotted experimental time for amyloid beta assault treated cells, the Vita-Blue reagent was added and tapped to thoroughly distribute. Vita-Blue was tested after 12 hours of treating cells

with the different amyloid beta and compound treatment groups. The reagent incubates with the cells for four hours before being read on the plate reader (BMG LabTech FLUOstar Omega, Cary, NC) at 530-570nm excitation and 590-620 nm emission.

7.6 Statistical Tests

Statistical analyses were performed using a univariate analysis of variance (UNIANOVA) for the TNF alpha ELISA and VitaBlue assay. Alpha level was set at 0.05. Tukey post hoc analyses were completed on the TNF alpha ELISA and Vita-Blue data to assess significant group differences.

8. Results

The purpose of this set of studies was to establish a methodology to test the efficacy of the compound Hydroxylpyclen in protecting and rescuing BV2 microglial cells from the neuroinflammation and oxidative stress induced by amyloid beta. **Figure 2** shows the results from the TNF alpha cell supernatant ELISA. There are significant group differences ($p<0.001$), and post hoc analysis was completed to analyze individual group differences. Post hoc analysis shows that the untreated control had significantly less TNF alpha production than the 5mM amyloid beta treated group ($p=0.007$), and significantly more TNF alpha production than the compound treatment groups (Low dose compound $p=0.001$, high dose compound $p=0.001$, Tx1 $p=0.001$, Tx2 $p=0.001$, Tx3 $p=0.003$, Tx4 $p=0.002$). The 5mM amyloid beta treated group was also significantly higher than all compound treatment groups ($p<0.001$ for all groups).

The Vita-Blue assay with the BV2 cells suggested that there was an effect of both amyloid beta and the compound on cell viability. Results from a one-way ANOVA showed that there was a significant main effect of treatment ($p<0.001$). Post hoc analysis showed that the cells treated with amyloid beta alone had significantly higher cell viability than all other groups. Additionally, there was significantly lower cell death in the untreated control group than all the compound and compound with amyloid beta treatment groups. None of the compound treated groups, whether with amyloid beta or by itself, showed significant individual group differences. The media alone group was significantly lower than all groups.

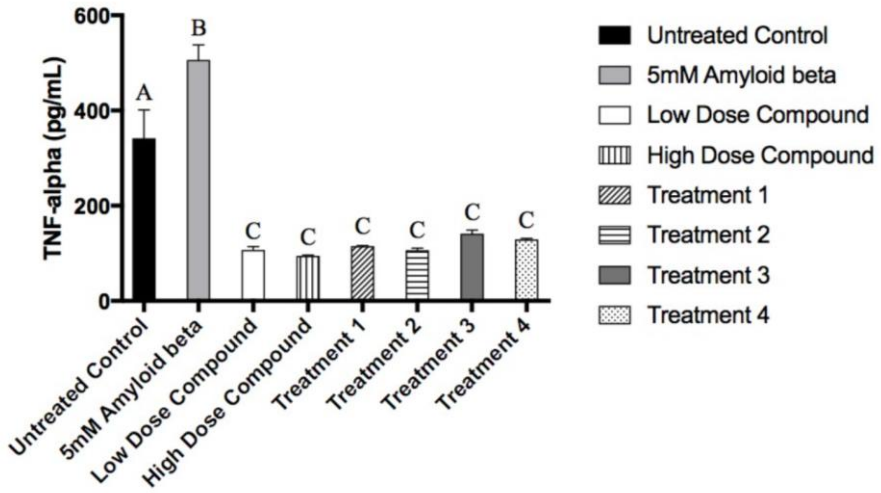


Figure 2. TNF alpha production is significantly reduced in BV2 cells treated with Hydroxylpyclen. The low dose of the Hydroxylpyclen compound is 8.325µg/mL, and the high dose of the Hydroxylpyclen compound is 33.3µg/mL. Treatment 1 is pretreatment with low dose compound for 6 hours before amyloid beta treatment for an additional 12 hours. Treatment 2 is pretreatment with high dose compound for 6 hours before amyloid beta treatment for an additional 12 hours. Treatment 3 is post treatment with low dose compound after 6 hours of treatment with amyloid beta alone. Treatment 4 is post treatment with high dose compound after 6 hours of treatment with amyloid beta alone. Standard error is shown by the bars and alpha was set at 0.05.

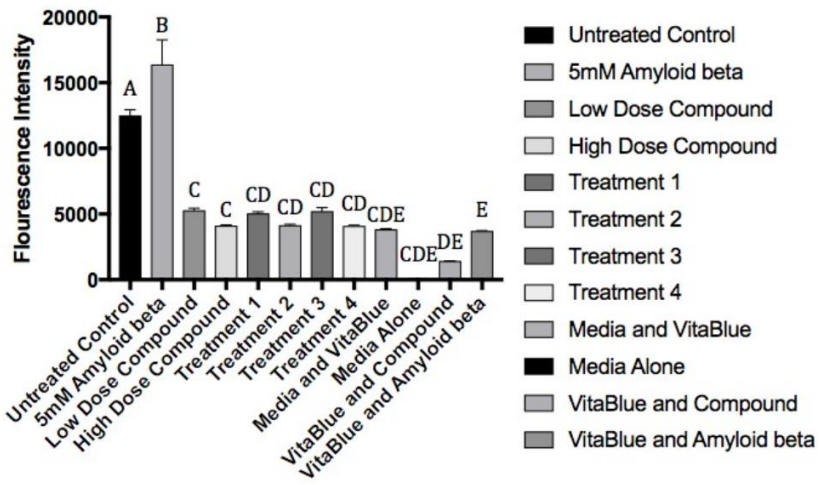


Figure 3. Hydroxylpyclen, but not amyloid beta, significantly reduces cell viability in BV2 cells. The low dose of the Hydroxylpyclen compound is 8.325µg/mL, and the high dose of the Hydroxylpyclen compound is 33.3µg/mL. Treatment 1 is pretreatment with low dose compound for 6 hours before amyloid beta treatment for an additional 12 hours. Treatment 2 is pretreatment with high dose compound for 6 hours before amyloid beta treatment for an additional 12 hours. Treatment 3 is post treatment with low dose compound after 6 hours of treatment with amyloid beta alone. Treatment 4 is post treatment with high dose compound after 6 hours of treatment with amyloid beta alone. Standard error is shown by the bars and alpha was set at 0.05. Fluorescence intensity is a direct measure of cell viability, meaning that more fluorescence is indicative of more cell viability.

9. Discussion

The results from this second set of experiments suggests that Hydroxylpyclen may have a role in decreasing the neuroinflammation and oxidative stress associated with the presence of amyloid beta, both in a protective and rescuing capacity. As expected, BV2 microglial cell exposure to amyloid beta causes a significant increase in TNF alpha production in comparison to the untreated control group. The high dose of this compound (33.3µg/mL) was chosen because it was the dose that was injected into mice in the initial *in vivo* study done to assess

Hydroxylpyclen's effects in our lab's Alzheimer's disease mouse model. That study showed a trend towards smaller and fewer overall plaques, but the differences were not significant. The low dose (8.325μg/mL) was chosen because it is a four-fold dilution of the high dose, enough to be able to estimate the therapeutic window for this compound with the BV2 microglial cells. As there is not an efficient diagnostic technique to detect Alzheimer's disease right when it begins, it was important to see if the compound could not only protect from the effects of amyloid beta before their initial onset (represented by the pretreatment groups), but also to rescue microglial cells that have already been under stress from the amyloid beta (represented by the posttreatment groups). In both cases, pretreatment and posttreatment with the compound, there was a significant decrease in TNF alpha production across all compound treatment groups when compared to the 5mM amyloid beta treatment group. This is vitally important because microglia are the primary producers of TNF alpha in the brain, and this TNF alpha, when over expressed, is toxic to neurons (Block and Hong, 2005). This decrease in TNF alpha production was also significantly lower than the untreated control, including the groups that were treated with the compound alone. This would suggest that the dosage can be lowered further and still be efficient. The high dose chosen was too high for the cells, likely because choosing the injected dose of Hydroxylpyclen was unwise. The amount injected into the peritoneal cavity is not the amount that actually makes it into the brain, meaning the BV2 cells were exposed to an amount of compound equal to if 100% of the injected dose made it across the blood brain barrier. A further experiment should be to do titrations of the compound and look at TNF

alpha production to find the dose of compound where treatment with the compound alone has a similar TNF alpha production to the untreated control.

One possible explanation for the overall compound related significant decrease in TNF alpha production could be cell death. If the cells died due to the compound exposure at both doses, then the cells may not have had time to respond and produce TNF alpha during the experimental treatment time. VitaBlue cell viability assays done on the BV2 cells using the same treatment groups as in the TNF alpha ELISA suggest that cell death is the culprit for the reduction in TNF alpha. The amyloid beta treated BV2 cells had significantly higher cell viability than all of the groups, including the untreated control. This increased cell viability is most likely because the microglial cells proliferated in response to the amyloid beta meaning that there were more cells to take up the VitaBlue reagent and more overall fluorescence. Additionally, proinflammatory microglia, such as those exposed to amyloid beta, are larger in size than resting microglia, like in the untreated control. These larger cells could take up more of the VitaBlue reagent which could also result in more overall fluorescence. The untreated control showed significantly more cell viability than all the other treatment groups, both compound alone and compound co-treated with amyloid beta. Further analysis of the data shows both the high and low dose compound had no significant difference in cell death, like in their TNF alpha production. As stated before, these doses are both higher than needed, and future work should look at the dose of compound which shows the same TNF alpha production as the untreated control, and see if the cell death is also at the untreated control's level. This set of data suggests that when the compound, both high and low

dose, was given to the cells, it resulted in mass cell death. The cell death with the compound resulted in fluorescence that was not significantly different from the control groups that included Vita-Blue with the compound and Vita-Blue with the amyloid beta, where there were no cells present. This suggests that the cell death that occurred was so rapid and complete that there may have been no cells remaining alive after the treatment. The compound alone treated groups were not significantly different from the amyloid beta and compound co-treated groups which continues to support the idea that all cells that treated with the compound may have been overtreated and died. It will be important to find a lower dose of the compound that matches the untreated control when used alone, because even if there are some cells still alive, the data suggests that these doses of the compound are diminishing the cell's natural ability to respond to threats. TNF alpha is utilized in a non-diseased central nervous system as a mediator of synaptic plasticity and learning and memory (Olmos and Lladó, 2014). For Hydroxylpyclen to be used in a therapeutic setting, it needs to be confirmed that its use alone does not dampen the normal microglial TNF alpha response, and provides cell viability in line with the untreated control.

Despite overt cell death being a likely candidate for the decrease in TNF alpha production, it is still important to consider the mechanism for the potential reductions in TNF alpha production, given the right concentration of the compound is found. One potential mechanism for this reduced TNF alpha production would be an increase in Nrf2 brought about exogenously by the compound. This is in line with research mentioned earlier where exogenous antioxidant agents have been shown

to restore or increase Nrf2 levels that are diminished by amyloid beta (Niture et al., 2014). BV2 cell lysates were collected for all the groups used for the TNF alpha ELISA, however the protein concentration in the lysates was too low to do Nrf2 western blotting. The hypothesis was that amyloid beta alone will decrease Nrf2 expression, and the treatment with the compound will increase those levels at or above the expression level of the untreated control. Moving forward, another experiment should be run with the lower concentrations of the compound so lysates can be collected again for western blotting, and supernatants can be recollected for TNF alpha ELISAs. The best explanation for this absence of protein in the lysates is that the compound killed the BV2 cells, which caused them to detach from the plate during the treatment period. This would lead to the cells being collected in the supernatant, and discarded after spinning down the supernatant sample. The cells that remained alive and collected as the lysate were too few to generate enough protein for western blotting, despite plating a high enough number of cells on the plate at the beginning of the experiment. Additionally, aliquots should be made after spinning down the lysates to avoid multiple freeze thaw cycles as this could be another reason for the diminished protein concentration. In addition to looking at Nrf2 expression, it will also be beneficial to look at downstream products of Nrf2 like heme oxygenase 1 (HMOX1) and glutathione-s-transferase (GST) which are detoxifying and antioxidant enzymes (Xiong et al., 2015). This would show that not only is Nrf2 expression restored, but it also restores the downstream effector products of this nuclear factor. This would also help to indicate that the restored expression of Nrf2 is moving into the nucleus to turn on downstream genes to deal

with the amyloid beta induced neuroinflammation and oxidative stress. Positive results would suggest a mechanism attributable to the overall decrease in the production of the inflammatory marker, TNF alpha.

Overall, both studies highlight the need for more research. The original goal with the neuronal cells was to find an appropriate dose, assault, and time point to use with the HT22 cells, co-treat them with Hydroxylpyclen, and then move into primary neurons with successful treatment combinations to look at the synaptic effects. This goal was not achieved, however much of the groundwork has been laid for future research with Hydroxylpyclen, or other compounds. Once a successful treatment combination has been attained, neuronal culturing techniques have already been proven to work, which will aid in the primary culture testing. Working with the microglia elicited a more promising result. This data suggested that there could be both a protective and rescuing effect of the Hydroxylpyclen if the dose was much lower than even the lowest dose used in these experiments. As microglia are the primary immune cell in the brain, and can influence the behavior of neurons, it is vital that any potential therapeutic show positive results in this cell model as well. Hydroxylpyclen has already been shown to break up amyloid plaques, and if we can better show its ability to protect and rescue neurons and microglia from oxidative stress and neuroinflammation in the future, it will be a novel compound to try in a murine model of Alzheimer's disease, and work towards establishing Hydroxylpyclen as a novel AD therapeutic.

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ABSTRACT

HYDROXYLPYCLEN AS A POTENTIAL THERAPEUTIC FOR ALZHEIMER'S DISEASE

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Alzheimer's disease (AD) diagnoses are on an exponential rise with no current cure or therapeutic options. Amyloid beta is implicated in the pathology of AD through its interaction with neuronal cells and its propagation of a toxic environment in the brain. Metal ions aid in amyloid beta accumulation and their levels become unbalanced in the process. These occurrences lead to a toxic oxidative stress environment which harms neurons and microglial cells, both of which are important for healthy brain function. This interaction is an optimal target for AD therapeutics. Hydroxylpyclen is a compound which can help restore the normal balance of metal ions and reduce oxidative stress. By assessing markers of oxidative stress and cell viability, Hydroxylpyclen can be assessed *in vitro* in neuron and microglia cells. Results suggests more work needs to be done to find the appropriate dose of the compound to use with these cells, but the methodology is well established for future research to be completed. Successful findings with Hydroxylpyclen could establish it as a potential AD therapeutic.