Testing the Ability of Macrocyclic Compounds to Reduce Reactive Oxygen Species (ROS)

by

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Testing the Ability of Macrocyclic Compounds to Reduce Reactive Oxygen Species (ROS)

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ABSTRACT

In the pathogenesis of neurodegenerative inflammatory diseases, such as Alzheimer's disease, there is an abnormal buildup of redox metal ions that associate with β-amyloid plaques and convert oxygen into oxygen radicals. These radicals are highly reactive with cellular components and lead to oxidative stress that induces damage and death of neuronal cells which is associated with the cognitive decline of Alzheimer's disease. Bifunctional macrocyclic compounds with antioxidant properties are a promising potential therapeutic to reduce levels of reactive oxygen species (ROS) and increase neuronal cell survival via the ability to chelate dysregulated metal ions and radical scavenging. In this project, novel macrocyclic compounds were tested for their efficacy in reducing intracellular levels of H_2O_2-induced ROS and H_2O_2-induced cytotoxicity. Intracellular ROS levels and cell survival were quantified in FRDA and BV-2 cells using the DCFH-DA and MTT cytotoxicity assays.

INTRODUCTION

Currently, there are over six million American patients suffering from Alzheimer's disease (AD), a neurodegenerative disease and the most common form of dementia that affects memory, thinking, and behavior. [7] Over the course of the last two years, deaths due to AD and dementia have increased by 16%. [7] Further, between the years of 2000 and 2019, deaths from AD increased by 145% which is in stark contrast to other ailments such as deaths due to heart disease which have decreased by 7.3% during the same time. [7] The rise in cases of AD and the high number of patients currently suffering from the disease has an economic impact as well as Alzheimer's and other dementias will cost the US approximately $355 billion in 2021. [7] This number is only expected to increase over time as current estimates suggest that by 2050 it could cost the nation over $1 trillion. [7] There are several factors that contribute to this expected rise in cases which include the aging of the "baby boomer" generation as well improvements in diagnostics and public awareness of the disease. In addition to the high cost to the nation associated with AD and other dementias, there are currently over 11 million Americans that provide unpaid care to patients suffering from AD with an estimate of 15.3 billion total hours of care which is valued at $257 billion. [7]

When it comes to diagnosing patients with AD, it requires evaluation by a physician where they perform various tests to assess impairments to the patient's memory, thinking, motor skills, and behavior. [8] To test for a patient experiencing AD-like symptoms (including memory impairment, difficulty concentrating, issues completing daily tasks, confusion, language problems, and mood changes), the physician will conduct mental status and neuropsychological tests to assess cognitive skills in addition to interviews with friends and family members that regularly interact with the patient. [8] Additionally, they perform laboratory tests that are helpful in

ruling out other illnesses that may cause similar symptoms such as thyroid disorder or vitamin B-12 deficiency. [8] Lastly, brain-imaging is conducted to identify areas of neuronal cell death, presence of β-amyloid plaques, and hyperphosphorylated tau proteins; the imaging includes magnetic resonance imaging (MRI), computerized tomography (CT), or positron emission tomography (PET). [8] However, scans alone aren't sufficient for diagnosis as there's some ambiguity as to what is considered normal and abnormal signs of aging in the brain. [8]

After diagnosis, the patient outcomes of the disease are quite variable. While no cure for AD exists, early diagnosis is still beneficial as patients may start the course of drug or nondrug interventions at an earlier time which may slow the progress of the disease and the decline in cognitive functions. [8] The rate at which the disease progresses varies among patients, however, the symptoms worsen over time and the average person with AD lives four to eight years after diagnosis. [9] Overall there are three stages of the disease to categorize the severity of symptoms that the patient exhibits which include early-stage (mild), middle-stage (moderate), and late-stage (severe). [9] During early-stage AD, the patient may live independently, but may experience thinking and memory impairments intermittently such as coming up with the right words, forgetting information that was just read, increased trouble with planning/organizing, etc. [9] Middle-stage AD, which is generally the longest stage, is characterized by more pronounced dementia-like symptoms such as forgetting events or personal history, becoming more socially withdrawn, confusion with time or place, bladder/fecal incontinence, etc. [9] Lastly, in late-stage AD, patients lose their ability to respond to the environment, participate in conversations, and controlling their movement and at this point, it may become a necessity for the patient to be put on hospice care for the remainder of their life. [9]

Two of the main hallmarks of AD are the presence of β-amyloid (Aβ) plaques and neurofibrillary tangles consisting of hyperphosphorylated tau proteins. [1] The Aβ plaques arise in the extracellular space and contain misfolded Aβ (either Aβ40 or Aβ42) because of dysregulation in amyloid precursor protein (APP) metabolism; APP gets cleaved into Aβ by the β- and γ-secretase enzymes and the misfolded plaques form due to an overproduction of Aβ in comparison to its degradation by cellular proteases such as cathepsin B.[1] There are several causes of Aβ accumulation, but there's been an increasing amount of evidence that proposes that there are genetic mutations that lead to the overproduction of toxic Aβ. [1] In AD pathology, there's not a universal pattern of Aβ plaque progression, however they typically get deposited in the isocortex and later affect subcortical regions. [1] Additionally, the Aβ plaques also release oligomer forms of Aβ and the abundance of oligomer forms leads to synaptic dysfunction, harms dendritic spines, and causes neuronal death which is associated with the cognitive decline of AD. [1] While accumulation of Aβ plaques in the brain is needed for an individual to be diagnosed with AD, it alone doesn't lead to symptomatic AD as autopsies of deceased elderly individuals have shown that people without dementia can have elevated levels of Aβ. [1]

Further, the Aβ oligomers also stimulate the hyperphosphorylation of tau proteins in the brain. [1] Previous studies have shown that the high levels of hyperphosphorylated tau proteins is associated with neuritic dystrophy and neurodegeneration. [1] Tau proteins are the major microtubule associated protein of a mature neuron and are involved in the stabilization of microtubules for neuronal cell transport. [2,10] The tau proteins are encoded by the microtubule-associated protein tau (MAPT) gene which is found on chromosome seventeen. [10] There are sixteen exons involved that comprise the gene and alternative splicing of exons 2, 3, and 10 leads to six major tau isoforms in the human brain; these different isoforms of tau range from 352 to

441 amino acids in length. [10] As a phosphoprotein, the degree of phosphorylation of tau influences its biological activity. [10] In the normal human brain, tau proteins contain 2-3 moles of phosphate per mole of tau protein, however, in the AD brain the level of phosphorylation of tau is increased 3- to 4-fold from normal which leads to its aggregation and a conformational change to where it can no longer bind to microtubules. [2,10] The hyperphosphorylation of tau is a consequence of the dysregulation of the kinases and phosphatases associated with tau proteins where the kinases either become hyperactivated or the phosphatases downregulated. [2] For example, Cdk5 is a tau kinase that has been found to be upregulated in AD patients and PP2A is a tau phosphatase that gets downregulated. [10] In addition, it has been proposed that abnormally folded tau that has undergone a conformational change can be transferred from one neuron to another trans-synaptically. [2]

Another key component in the pathogenesis of AD is the role that redox metal ion dysregulation has in neuronal cell death that is associated with the cognitive decline of AD. Previous studies on the brains of deceased AD patients show that the Aβ plaques have an extensive buildup of copper, iron, and zinc ions whose concentrations are 5.7, 2.8, and 3.1 times higher than the levels seen in normal brains, respectively. [3] When these redox metals associate with the Aβ plaques and are in the presence of a biological reductant like ascorbate, they catalyze the reduction of dioxygen to generate oxygen radicals which react with cellular components that lead to oxidative damage and thus are termed reactive oxygen species (ROS). [3,4] In the case of AD pathogenesis, the ROS produced is then released onto neuronal cells and the oxidative stress induces neuronal cell death. These transition metal ions in their homeostatic concentrations have important cellular functions including free radical detoxification, oxygen transport, electron transport, neurotransmitter biosynthesis, and neurotransmission. [4] However, the dysregulation of

these ions that causes the production of ROS, oxidative stress, and protein aggregation is found in several other neurodegenerative diseases such as Huntington's disease, Parkinson's disease (PD), and Lou Gehrig's disease. [4] Since there's a dysregulation of the redox metal ions in patients with AD that ultimately leads to death of neuronal cells, metal chelators are a prospective strategy to restore metal ion homeostasis and ultimately reduce the amount of ROS generated and therefore decrease neuronal cell death. [3]

While the number of individuals with AD has been growing annually and is projected to continue its increase, there are only six treatment options on the market that have been approved to treat AD in the United States. Most recently approved by the FDA in June of 2021 is aducanumab which may halt the progression of the disease, but prior to that the most recent drug approved was memantine in 2003. Three of these drugs are cholinesterase inhibitors (donepezil, galantamine, and rivastigmine) which are used to treat symptoms relating to the decline in memory, thinking, language, judgement, and other processes. [5] Cholinesterase typically breaks down the neurotransmitter acetylcholine into choline and acetic acid, but acetylcholine is an important neurotransmitter involved in learning and memory processes; so, these inhibitors function by keeping the levels of acetylcholine signaling at higher levels to aid the communication between neuronal cells and reduce the cognitive decline associate with AD. [5] Another treatment used to treat the cognitive symptoms in moderate to severe AD is memantine as previously mentioned. It functions as a regulator of glutamate which is a neurotransmitter that assists in processing information in the brain. [5] Additionally, one treatment for symptoms associated with cognitive decline is a drug named Namzeric which is a combination of the cholinesterase inhibitor donepezil and the glutamate regulator memantine. [5] However, all these drugs mentioned that function by treating the cognitive symptoms of AD only have a short-term

effect, and it is unknown exactly how beneficial they are in terms of efficiency to cost ratios. [3] Further, between 2002 and 2012, there were clinical trials with 244 drug candidates for AD and they all failed with exception of memantine in 2003. This highlights the importance of developing new treatment strategies capable of stopping or reducing the neurodegeneration that comes with AD. [3]

Most recently approved for treatment is the drug aducanumab which is a monoclonal antibody against the Aβ protein that forms plaques in the brains of patients with AD. [5,6] The formation of these plaques interrupts neuronal communication and hyperactivates microglial cells, triggering the production of ROS that leads to neuroinflammation and neuronal cell death. [5] This is the first treatment that demonstrates an ability to remove Aβ and is also the first treatment targeted to halt the progression of AD rather than just treat its symptoms. [5] However, the FDA has advised that this treatment only be used in patients with mild cognitive damage or are early in the progression of AD. [6] There has also been some conflicting data in the effectiveness of the treatment seen in two clinical trials that measured the effectiveness, side effects, safety, and the practical use of the drug in the clinical setting. One study showed that the drug slowed the decline in thinking, memory, and function in patients with AD, whereas the other one didn't show these results. [6] The sixth drug used in the treatment of AD is suvorexant which is used to treat non-cognitive symptoms, but rather behavioral and psychological symptoms including insomnia, irritability, delusions, and hallucinations. [5] Suvorexant works as an orexin receptor antagonist; this neurotransmitter is involved in the sleep-wake cycle and its inhibition is used to treat insomnia that many individuals with dementia experience. [5]

Since some of these current drugs for AD treatment only have a short-term symptomatic effect, it's important for further research aimed at designing therapies that can halt the

neurodegenerative progression of the disease. [3] As mentioned earlier, metal chelators and antioxidants have the potential to be effective therapeutic treatments due to the dysregulation of redox metal ion levels in AD pathogenesis that lead to the production of ROS and subsequently causes neuronal cell death. [3] There have been recent studies done with such compounds using *in vitro* and *in vivo* models. For example, one study analyzed the effect of α-Cyperone on H_2O_2-induced ROS levels in SH-SY5Y neuronal cells. [11] This compound is extracted from the *Cyperus rotundus* plant and prior studies had shown its ability to serve as an antioxidant. [12] In the study they show utilize the DCFH-DA assay to show that α-Cyperone reduces H_2O_2-induced intracellular ROS levels and in-turn attenuates H_2O_2-induced ROS-mediated cytotoxicity in these neuronal cells. [11] Further, by using western blot analysis they showed that this reduction in intracellular ROS was achieved through activation of the *Nrf2* signaling pathway that serves as a transcription factor for downstream antioxidant genes. [11] Another study measured the effect of cyanidin-3-glucoside (C3G) on glutamate-induced ROS levels using the DCFH-DA and MTT cytotoxicity assays. [13] Their results showed that C3G, a known antioxidant anthocyanin present in berries, can reduce glutamate-induced intracellular ROS which attenuates glutamate-induced ROS-mediated cytotoxicity in HT22 mouse neuronal cells. [13] Similar to the study done using α-Cyperone, C3G has this neuroprotective effect through activation of the *Nrf2* signaling pathway. [13]

In addition to antioxidants having the ability to reduce intracellular ROS levels and increase neuronal cell survival, metal chelators have been shown to exhibit similar effects. For example, one study tested the ability of lactoferrin, an iron chelator, on intracellular ROS levels utilizing the DCFH-DA assay and the ability of lactoferrin to reduce levels of pro-inflammatory cytokines like tumor necrosis factor alpha (TNFα) and interferon-beta (IFN-β). [14] For this study,

they used 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridin (MPTP)-induced mice that serve as a model of Parkinson's Disease. [14] They found that MPTP-mice had heightened levels of intracellular ROS baseline and that intracellular ROS levels were reduced when treated with lactoferrin. [14] Further, western blot analysis showed that lactoferrin reduced levels of pro-inflammatory cytokines such as TNFα and IFN-β. [14] In addition to the reduction of these cytokines, treatment with lactoferrin also was shown to attenuate the motor deficits found in MPTP mice. [14]

The use of metal chelators has also been shown to have beneficial behavioral effects in non-transgenic AD mice studies. Transgenic AD mouse models correlate to late stages of the disease which isn't as helpful in mimicking the progression of the disease and testing compounds that can halt the pathogenesis. [3] So, one study induced early stages of AD with episodic memory loss in mice via intracerebroventricular injection of Aβ-oligomers and measured the effect of a N4-tetradentate 8-aminoquinoline ligand copper chelator, PA1637, on episodic memory in AD-induced mice. [3] They found that oral administration of PA1637 attenuated the episodic memory loss seen in AD-induced mice and restored episodic memory to just as strong as in healthy mice. [3] Further, there have been *in vitro* studies utilizing copper chelators that show their ability to scavenge oxygen radical and reduce intracellular ROS. One study that did so synthesized a pyridol compound, known as L2, as pyridols are known to react with hydroxyl radical through addition at the C3 or C5 position of the pyridol ring and thus having antioxidant capabilities. [4] In addition to antioxidant properties, L2 exhibits the ability to chelate metal ions such as Cu (II) and Zn (II). [4] This bifunctionality of L2 was correlated to its ability to reduce oxygen radicals as seen in the DPPH assay in addition to reducing L-buthionine-S,R-sulfoximine butanoic acid (BSO)-induced intracellular ROS levels in FRDA cells as seen in the DCFH-DA assay. [4]

METHODS

Cell culture

The FRDA fibroblast (Friedrich's Ataxia, Coriell Institute) cells were grown in 75 cm^2 cell culture flasks in RPMI media containing 15% FBS (fetal bovine serum), 1% penicillin, 1% streptomycin, 1% L-glutamine, and 1% non-essential amino acids, at 37°C in an atmosphere of 5% CO_2 and 95% air. At the point when the cells were around 85% confluent on the flask, they were passaged by washing with 1X phosphate buffered saline (PBS), trypsinization (0.05%), and placed in new 75 cm^2 flasks with fresh media.

The BV-2 cells (microglia) were grown in 75 cm^2 cell culture flasks in DMEM media containing 15% FBS, 1% penicillin, 1% streptomycin, 1% L-glutamine, and 1% non-essential amino acids, at 37°C in an atmosphere of 5% CO_2 and 95% air. At the point when the cells were around 85% confluent on the flask, they were passaged by washing with 1X PBS, trypsinization (0.05%), and placed in new 75 cm^2 flasks with fresh media.

DCFH-DA Assay

FRDA/BV-2 cells were seeded into 96-well plates (20,000 cells per well) for approximately 18 hours. They were then pre-treated with increasing concentrations of the compound of interest (PK60, L2, PK95) and placed in the 5% CO_2 incubator for 8 hours. The media supernatant was then replaced by 25% dichlorofluorescein diacetate (DCF-DA, Sigma) and 75% serum-free medium and put back in the 5% CO_2 incubator for 45 minutes. The dichlorofluorescein diacetate

solution was poured out and replaced with RPMI/DMEM media containing 15% FBS, (?) Pen-Strep, (?) L-glutamine (depending on the cells used). The cells were then treated with 90 μM H_2O_2 and placed back in the 5% CO_2 incubator. After 16 hours, the plates were analyzed for fluorescence at excitation and emission wavelengths of 485 and 520 nm, respectively to measure intracellular levels of ROS.

MTT Assay

FRDA/BV-2 cells were seeded into 96-well plates (5,000 cells per well) for approximately 18 hours. Next, they were pre-treated with increasing concentrations of the compound of interest (PK60, L2, PK95) and placed in the 5% CO_2 incubator for 8 hours. They were then treated with 90 μM H_2O_2 and returned to the 5% CO_2 incubator. After 16 hours, the media supernatant was replaced by 3-(3,4-Dimethylthiazole-2-yl)-2,5-diphenyltetrazoliumbromide (MTT, Sigma) at 1 mg/mL with serum-free medium and incubated for an additional 4 hours. The MTT solution was then replaced by 100% dimethyl sulfoxide (DMSO) and the plates were shaken at room temperature for 5 minutes. The absorbance of the plates was then analyzed at a wavelength of 540 nm to measure cell viability.

RESULTS

Before testing the macrocyclic compounds used in the study, we wanted to ensure that we are able to measure an increase in intracellular ROS levels when cells were exposed to varying concentrations of H_2O_2. To carry out the assay, we used FRDA cells which are fibroblast cells from patients with Friedrich's Ataxia that have a heightened sensitivity to changes in redox state due to a compromised antioxidant system. The pathology of these cells comes from mutations in the nuclear *FRDA* gene that encodes the iron-binding protein frataxin; a protein that is important for mitochondrial iron metabolism, antioxidant protection, and cellular iron homeostasis. Cells were plated in a 96 well tray at a density of 20,000 cells/well and treated with increasing concentrations of H_2O_2. Next, intracellular ROS was measured using the DCFH-DA assay.

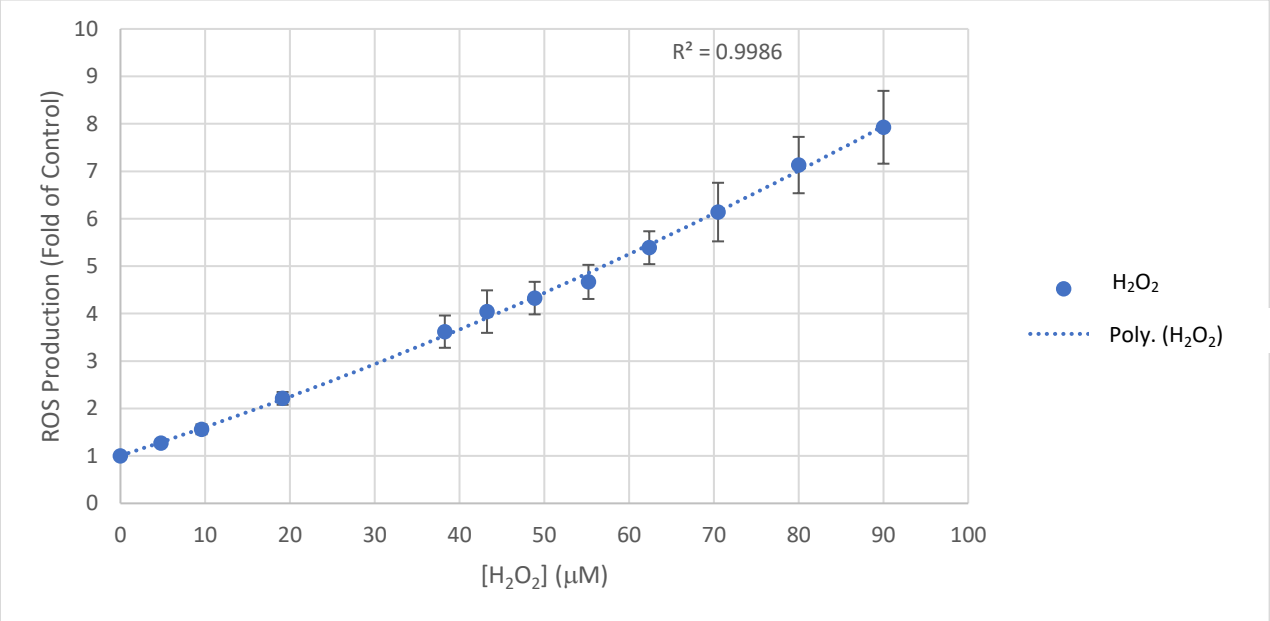


Fig. 1 ROS Production in FRDA Cells Treated with H_2O_2.

We could measure an H_2O_2-dependent increase in ROS levels (Fig. 1) up to the maximum concentration where we saw an 8-fold increase.

Having shown that we are able to measure an increase ROS levels in the cell, we next wanted to assess the effect of ROS on cell cytotoxicity. High levels of ROS would be expected to induce cytotoxicity. FRDA cells were treated with increasing concentrations of H_2O_2, and cytotoxicity measured using the MTT cytotoxicity assay.

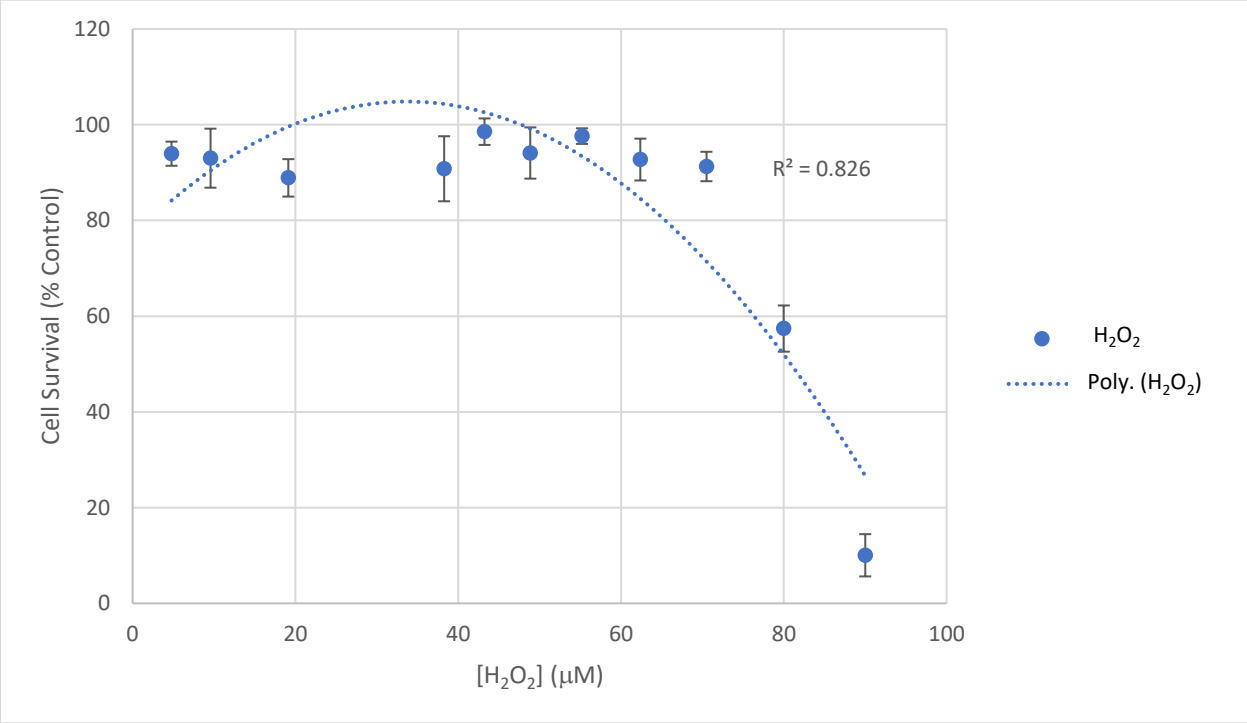


Fig. 2 Survival of FRDA Cells Treated with H_2O_2

Up to 70 μM of H_2O_2, there was no cytotoxicity observed (Fig. 2), but at the two highest concentrations used (80 μM and 90 μM) there was a decrease in cell survival.

Next, we wanted to ensure that we are able to measure an increase in intracellular ROS levels in BV-2 cells (an immortalized mouse microglial cell line) were exposed to varying concentrations of H_2O_2. BV-2 cells were treated with increasing concentrations H_2O_2 and intracellular ROS levels were subsequently measured using the DCFH-DA assay.

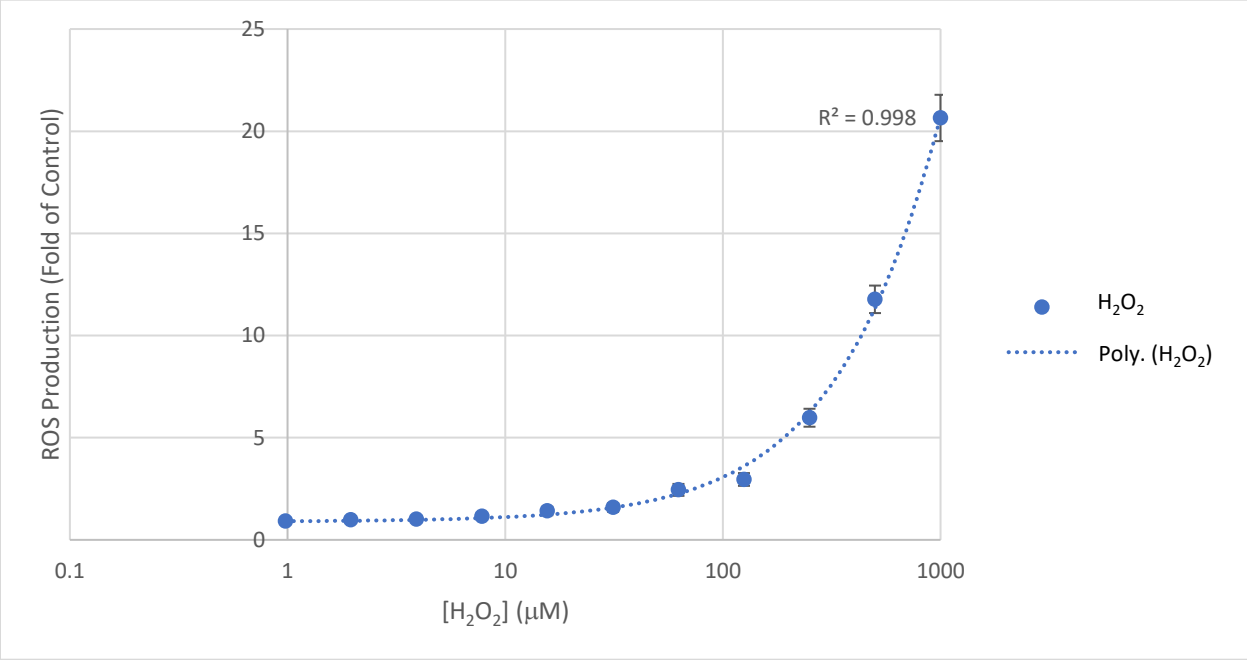


Fig. 3 ROS Production in BV-2 Cells Treated with H_2O_2

As seen in Fig. 1, intracellular ROS levels increased in an H_2O_2-dependent manner and peaked at 2.5-fold increase from untreated cells (Fig. 3).

Next, we wanted to ensure that increased levels of H_2O_2-induced intracellular ROS was correlated with decreased BV-2 cell survival through ROS-mediated cytotoxicity. To test this, cells were treated with increasing concentrations of H_2O_2 and cell survival was subsequently measured utilizing the MTT cytotoxicity assay.

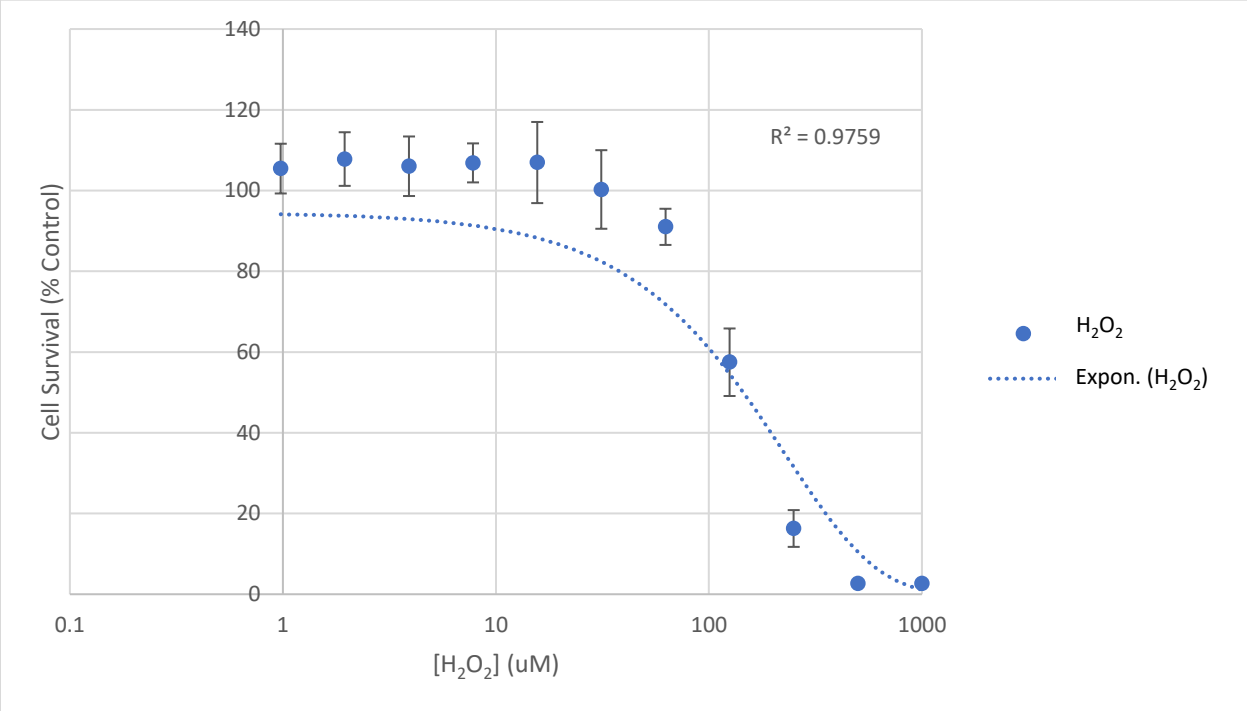
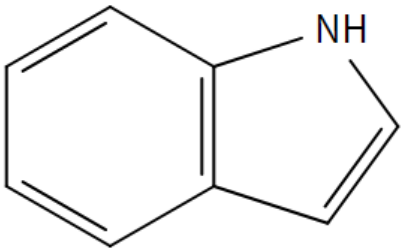


Fig. 4 Survival of BV-2 Cells Treated with H_2O_2

As concentrations of H_2O_2 were increased, there was a decrease in cell survival with near 100% cell survival up until 31.25 µM H_2O_2 and no cell survival at 500 µM and beyond (Fig. 4).

After showing that intracellular levels of ROS can effectively be manipulated and measured through the DCFH-DA assay (Fig. 1, 3), we next tested the effect of Indole, a commercially available antioxidant, on intracellular H_2O_2-induced ROS by treating the cells with increasing concentrations of H_2O_2 either alone or in the presence of a fixed concentration of indole.



Indole

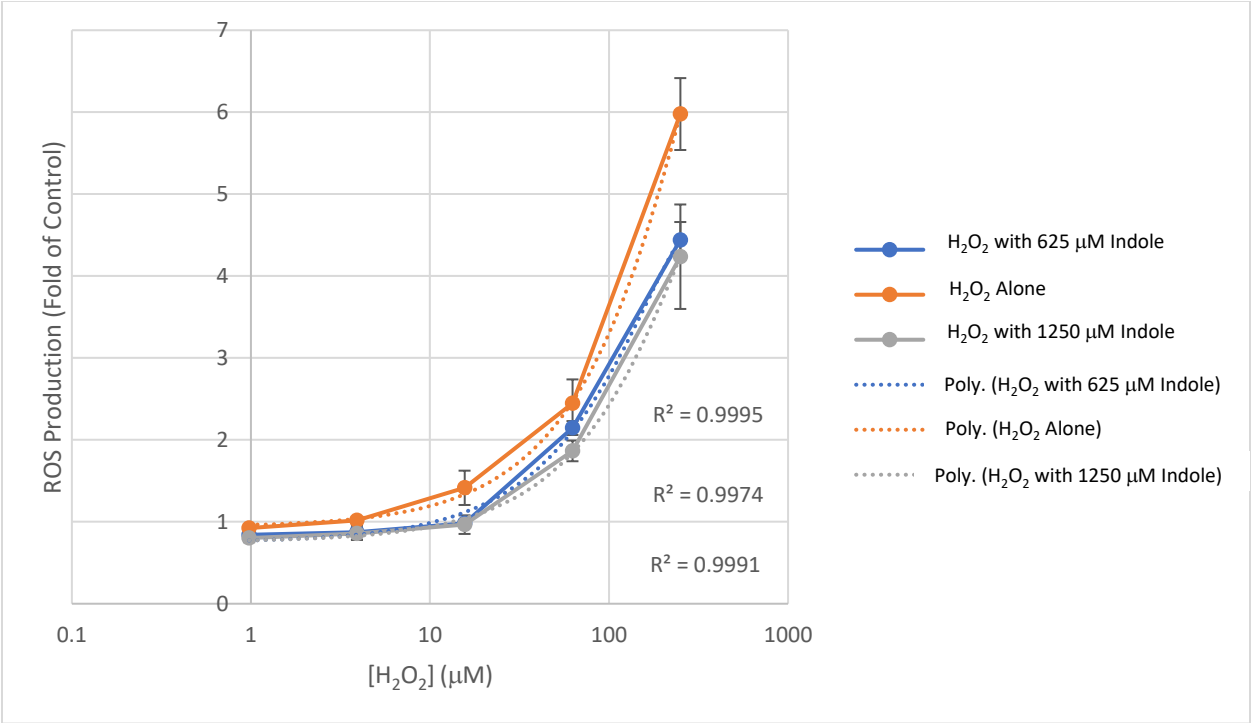
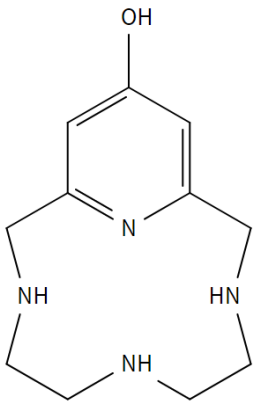


Fig. 5 ROS Production in BV-2 Cells Treated with H_2O_2 in the Presence of Indole Compared to H_2O_2 Alone

Cells that were treated with Indole had decreased levels of intracellular ROS in comparison to cells treated with H_2O_2 alone. For example, cells treated with H_2O_2 alone at 250 μM had a 6-fold increase in intracellular ROS while this was decreased by approximately 30% in cells treated with 625 μM and 1250 μM indole (Fig. 5).



L2

After showing that intracellular levels of H_2O_2-induced ROS can be reduced using a known antioxidant (Fig. 5), we next tested the effect of L2, a macrocyclic compound that is a known radical scavenger[4], on intracellular H_2O_2-induced ROS by treating the cells with increasing concentrations of L2 in the presence of 90 μM H_2O_2.

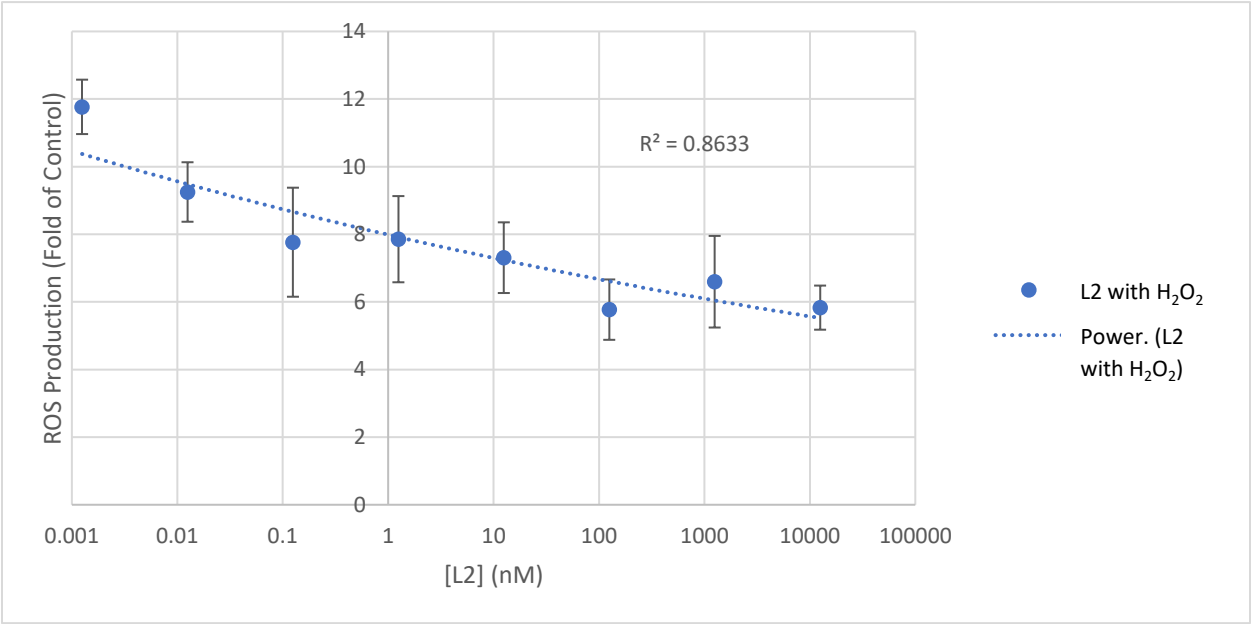


Fig. 6 H_2O_2-Induced ROS Production in FRDA Cells Treated with L2

At the lowest concentration of L2, there was a 12-fold increase of the control in H_2O_2-induced ROS production that was reduced by 50% (to 6-fold) at the highest concentration of L2 tested.

After showing that L2 can reduce H_2O_2-induced ROS, we next looked at the effect of L2 on ROS-mediated cytotoxicity. If L2 reduced H_2O_2-induced ROS, we expect that it would reduce H_2O_2-induced cytotoxicity. FRDA cells were treated with increasing concentrations of L2 followed by treatment with 90 μM H_2O_2 and cell survival was measured utilizing the MTT cytotoxicity assay.

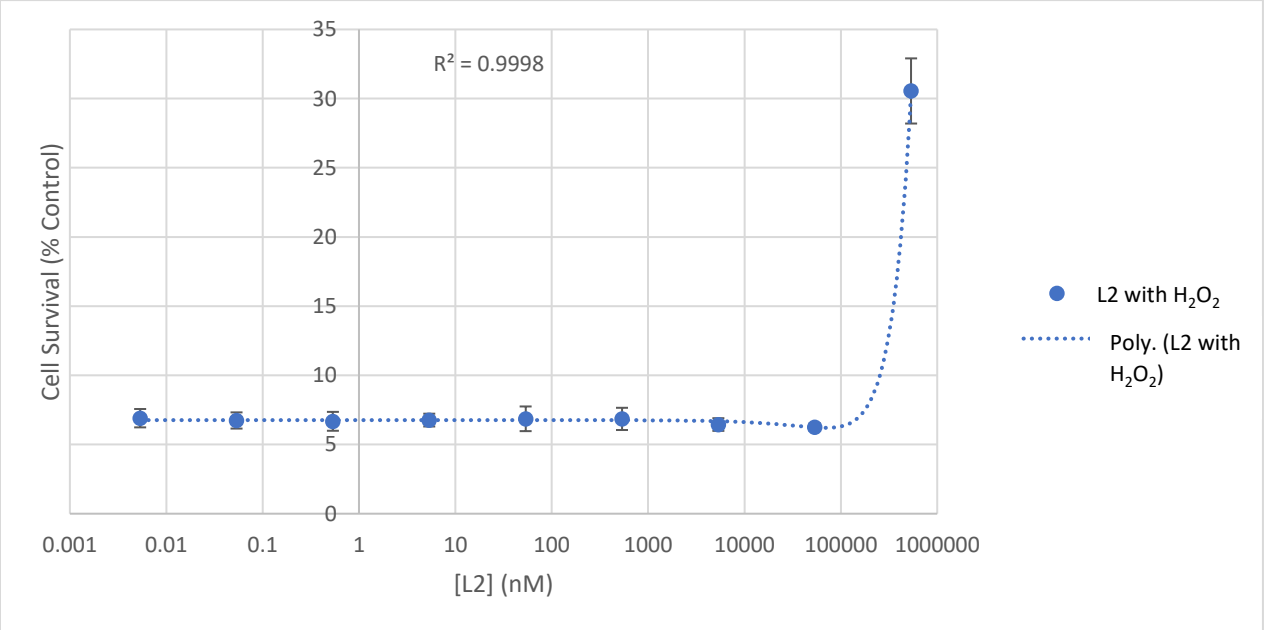


Fig. 7 Survival of FRDA Cells Treated with L2 in the Presence of 90 μM H_2O_2

A 7% cell survival was observed at all concentrations up until the highest concentration of 536 μM where 30% of the cells survived (Fig. 7).

In the next experiment, the intracellular ROS levels of FRDA cells was measured in cells treated with L2 alone using the DCFH-DA assay. We wanted to ensure that L2 alone didn't induce intracellular ROS.

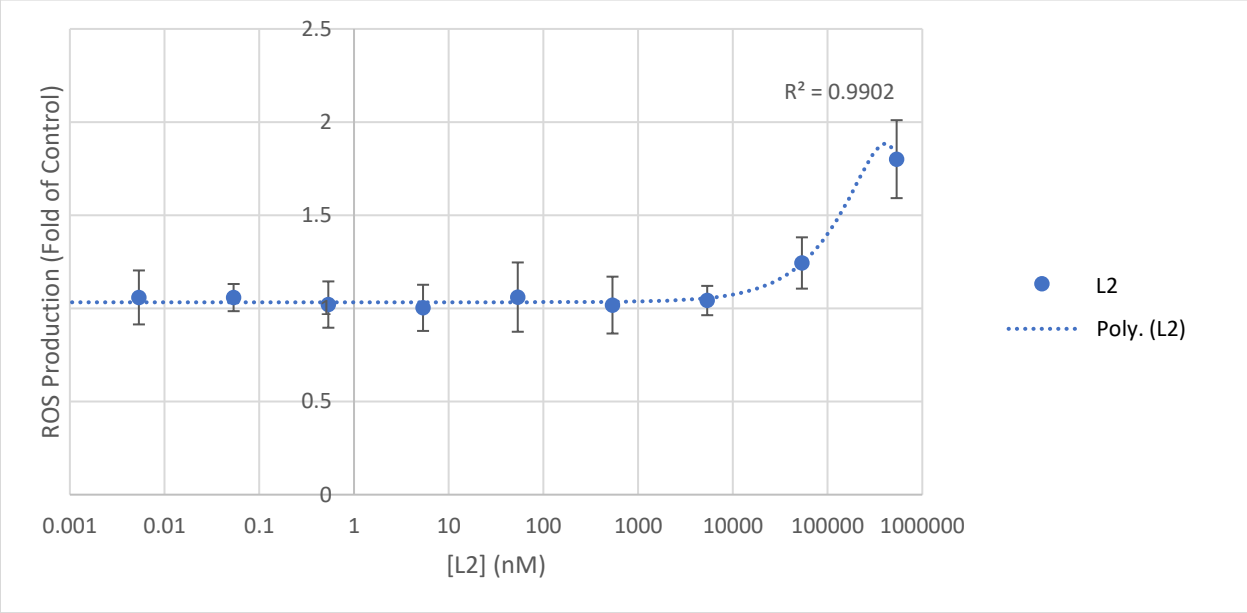


Fig. 8 ROS Production in FRDA Cells Treated with L2

No significant changes in intracellular ROS levels were seen for all the concentrations of L2 used (Fig. 8).

An ideal drug would be effective in concentrations below cytotoxic levels, so in this experiment the cell survival of FRDA cells treated with L2 alone was measured using the MTT cytotoxicity assay.

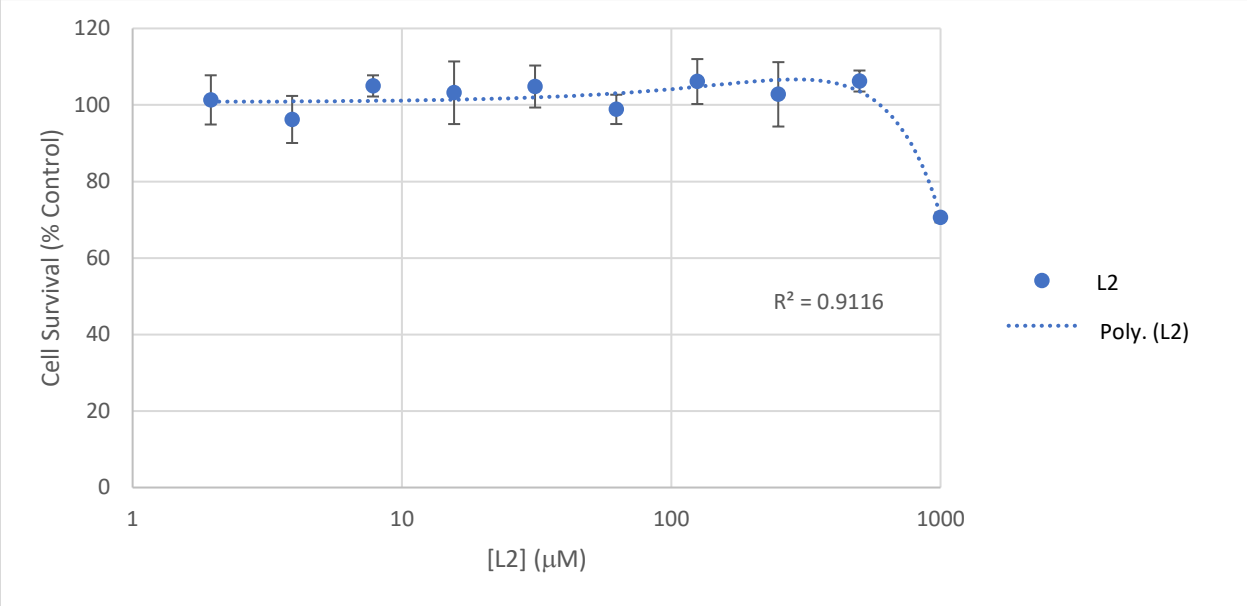
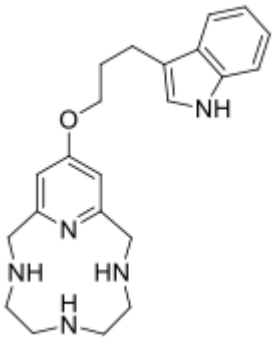


Fig. 9 Survival of FRDA Cells Treated with L2

There was 100% cell survival up until the highest concentration of L2 was used, 1mM, where there was approximately 70% survival.

After showing that L2 had the ability to reduce intracellular H_2O_2-induced ROS and increase cell survival (Fig. 6, 7), we next tested the effect of PK60, a novel compound, that is a derivative of L2 on intracellular H_2O_2-induced ROS levels. Based on its structure, PK60 is expected to be a metal chelator and radical scavenger. FRDA cells were first treated with increasing concentrations of PK60 followed by 70 μM H_2O_2 following which intracellular ROS was measured using the DCFH-DA assay.



PK60

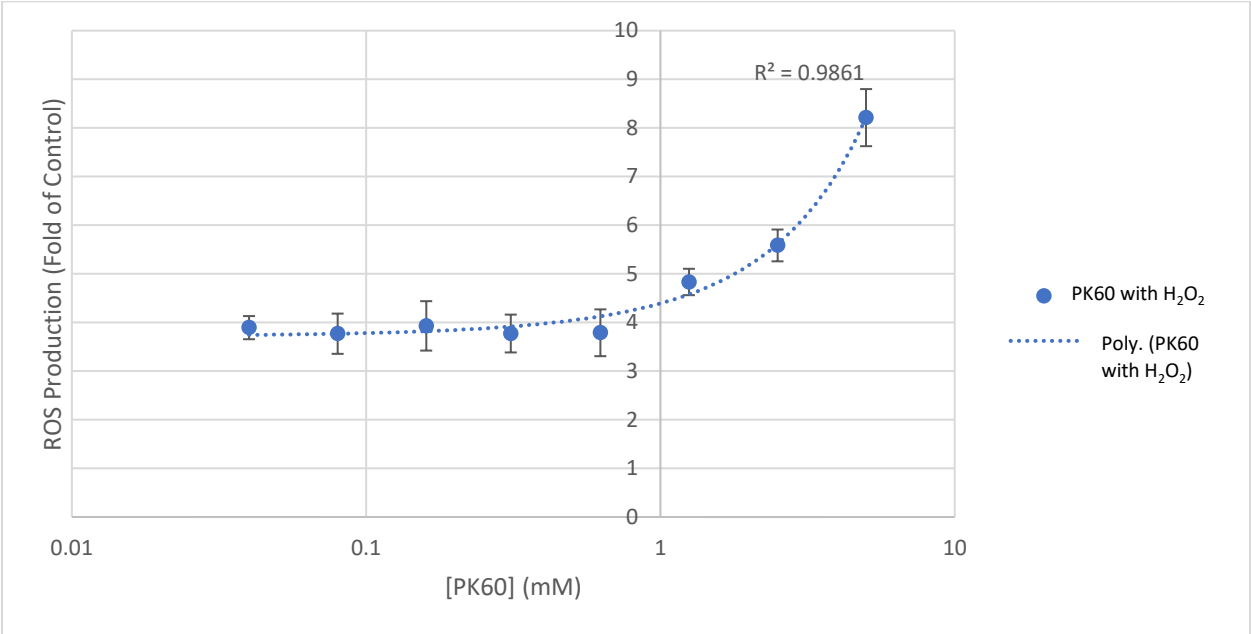


Fig. 10 H_2O_2-induced ROS Production in FRDA Cells Treated with PK60

Treatment with PK60 led to increased levels of intracellular ROS as concentrations of PK60 increased.

In the next experiment, we tested the effect of PK60 on H_2O_2-induced ROS-mediated cytotoxicity in FRDA cells utilizing the MTT cytotoxicity assay. The cells were treated with increasing concentrations of PK60 followed by 70 µM H_2O_2 and cell survival was measured.

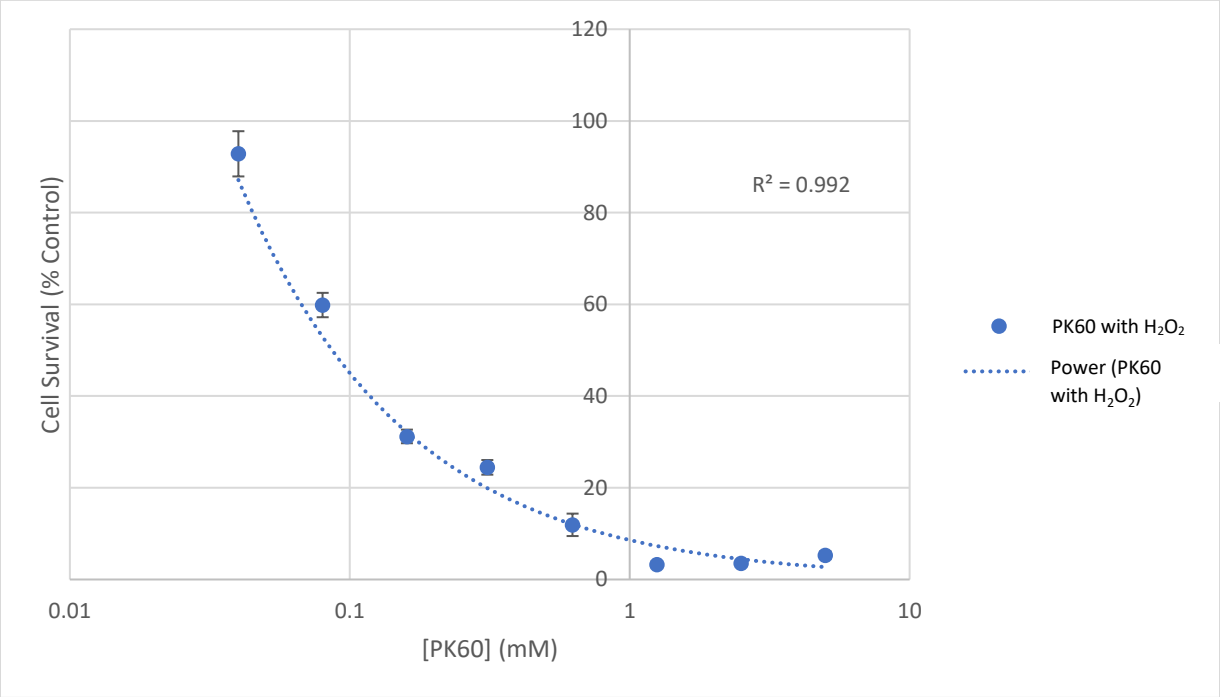


Fig. 11 Survival of FRDA Cells Treated with PK60 in the Presence of 70 µM H_2O_2

Cell survival steadily decreased as concentrations of PK60 were increased.

In this experiment, we tested the effect of PK60 alone on intracellular ROS levels in FRDA cells utilizing the DCFH-DA assay as we wanted to explore if PK60 was the cause of increased intracellular ROS levels as seen in Fig. 10. Cells were treated with increasing concentrations of PK60 and intracellular ROS levels were subsequently measured.

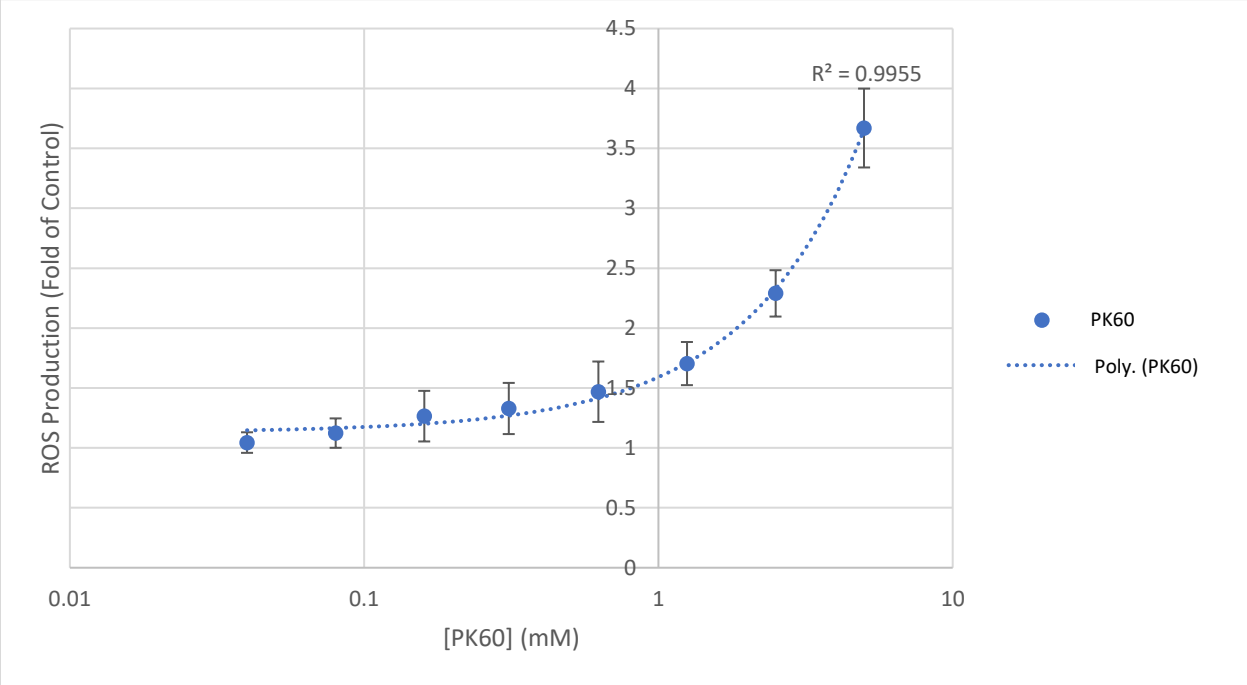


Fig. 12 ROS Production in FRDA Cells Treated with PK60

As concentrations of PK60 increased, intracellular ROS levels were increased as well.

An ideal drug would be effective in concentrations below cytotoxic levels, so in this experiment the cytotoxicity of PK60 alone in FRDA cells was measured utilizing the MTT cytotoxicity assay. Cells were treated with PK60 alone and cell survival was subsequently measured.

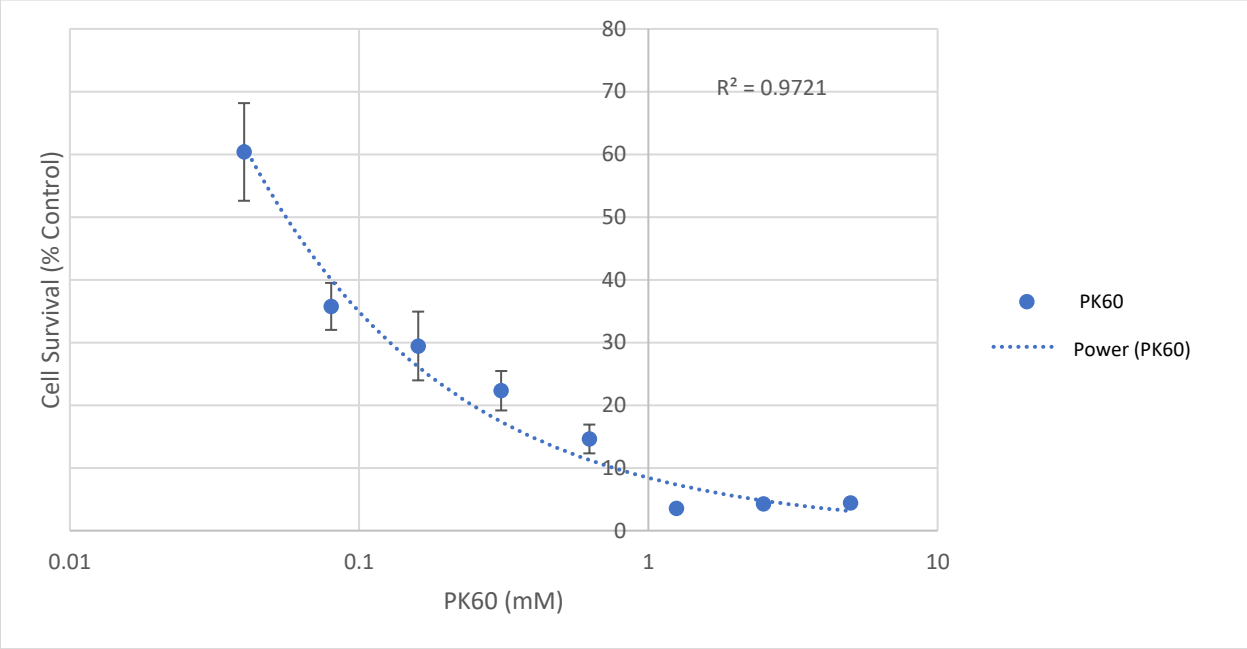


Fig. 13 Survival of FRDA Cells Treated with PK60

As concentrations of PK60 were increased, there was decreased cell survival and at concentrations 1.25 mM and higher, all the cells were killed.

We wanted to determine whether the increases in intracellular ROS levels seen in Fig. 12 in BV-2 cells were caused by PK60. Cells were treated with increasing concentrations of PK60 alone and subsequently measured intracellular ROS levels utilizing the DCFH-DA assay.

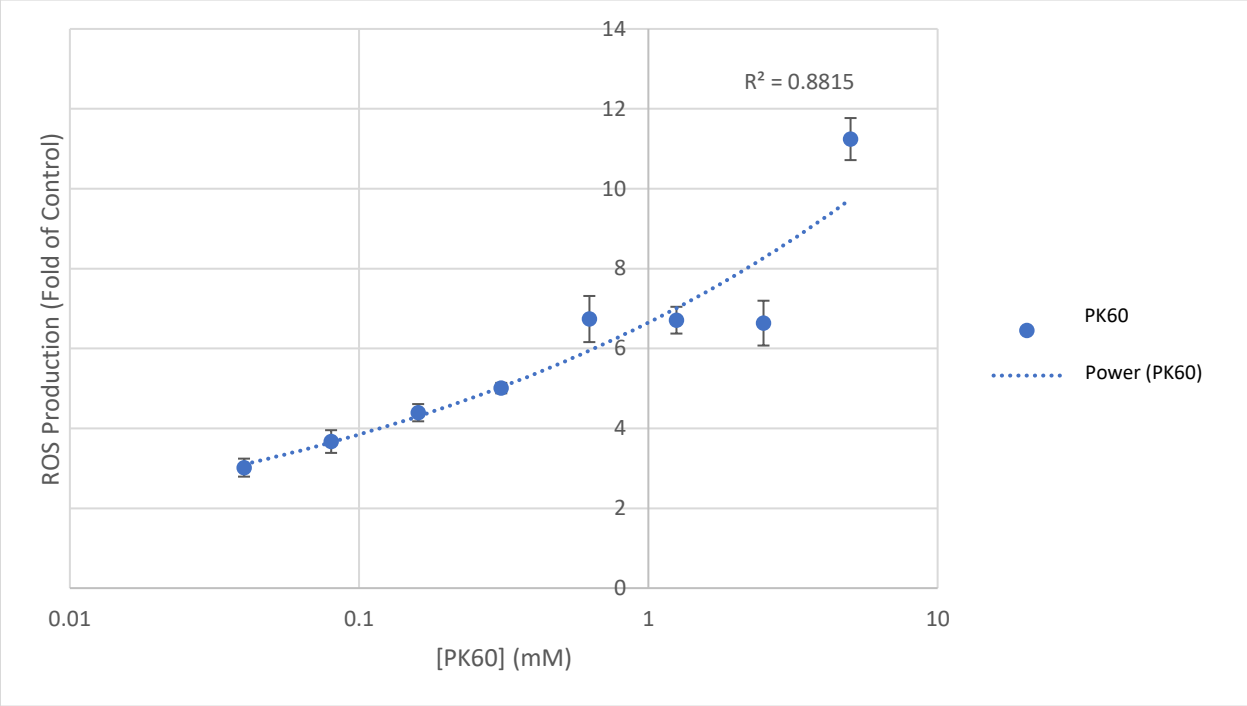


Fig. 14 ROS Production in BV-2 Cells Treated with PK60

As concentrations of PK60 were increased, intracellular ROS levels were steadily increased.

After showing that treatment with PK60 alone resulted in ROS-mediated cytotoxicity (Fig. 12, 13) in FRDA cells, we wanted to analyze this effect on BV-2 cells. So, cells were treated with increasing concentrations of PK60 alone and subsequently cell survival was measured utilizing the MTT cytotoxicity assay.

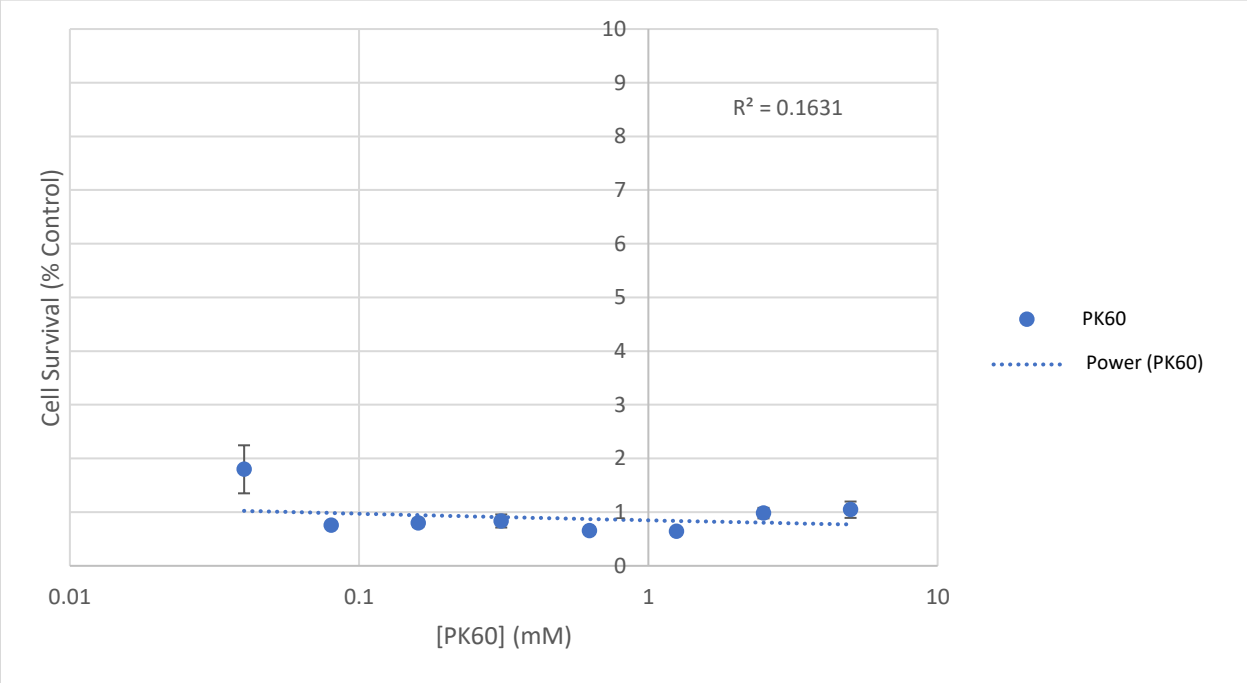
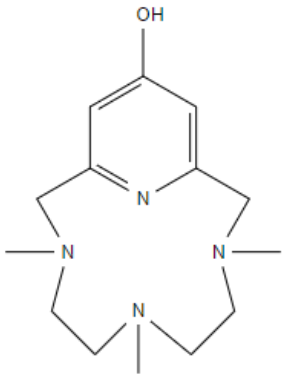


Fig. 15 Survival of BV-2 Cells Treated with PK60

There was nearly no cell survival seen for all concentrations of PK60 used.

An ideal drug should be effective below cytotoxic concentrations, so in this experiment, the cytotoxicity of PK95 alone in BV-2 cells was measured utilizing the MTT cytotoxicity assay. This is important for a novel compound in order to know which concentrations of the drug to test for its ability to reduce intracellular levels of ROS. Cells were treated with increasing concentrations of PK95 and cell survival was subsequently measured.



PK95

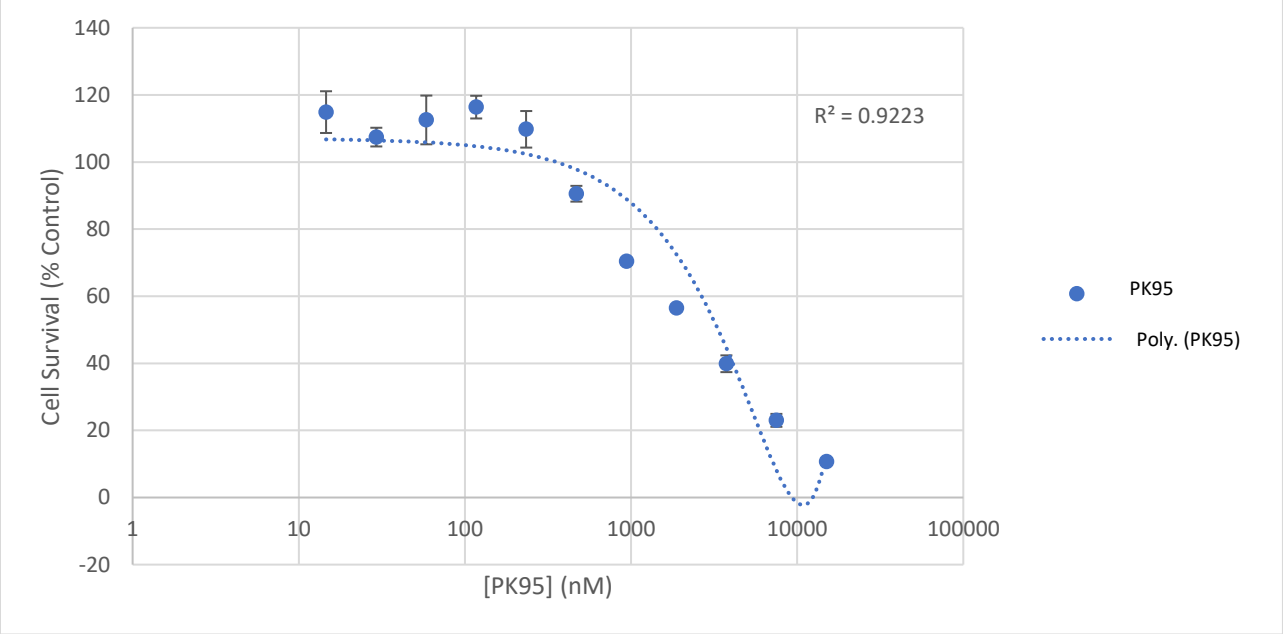


Fig. 16 Survival of BV-2 Cells Treated with PK95

100% of the cells survived up to a concentration of 234 nM following which it steadily declined until there was around 10% cell survival at a concentration of 15 µM.

Since we showed that L2 had the ability to reduce H_2O_2-induced intracellular ROS, we tested a novel compound that is a derivative of L2, PK95, and its ability to reduce H_2O_2-induced intracellular ROS. Based on its structure, PK95 is expected to be a metal chelator and radical scavenger. BV-2 cells were treated with increasing concentrations of PK95 followed by 90 μM H_2O_2 and subsequently measured intracellular ROS utilizing the DCFH-DA assay.

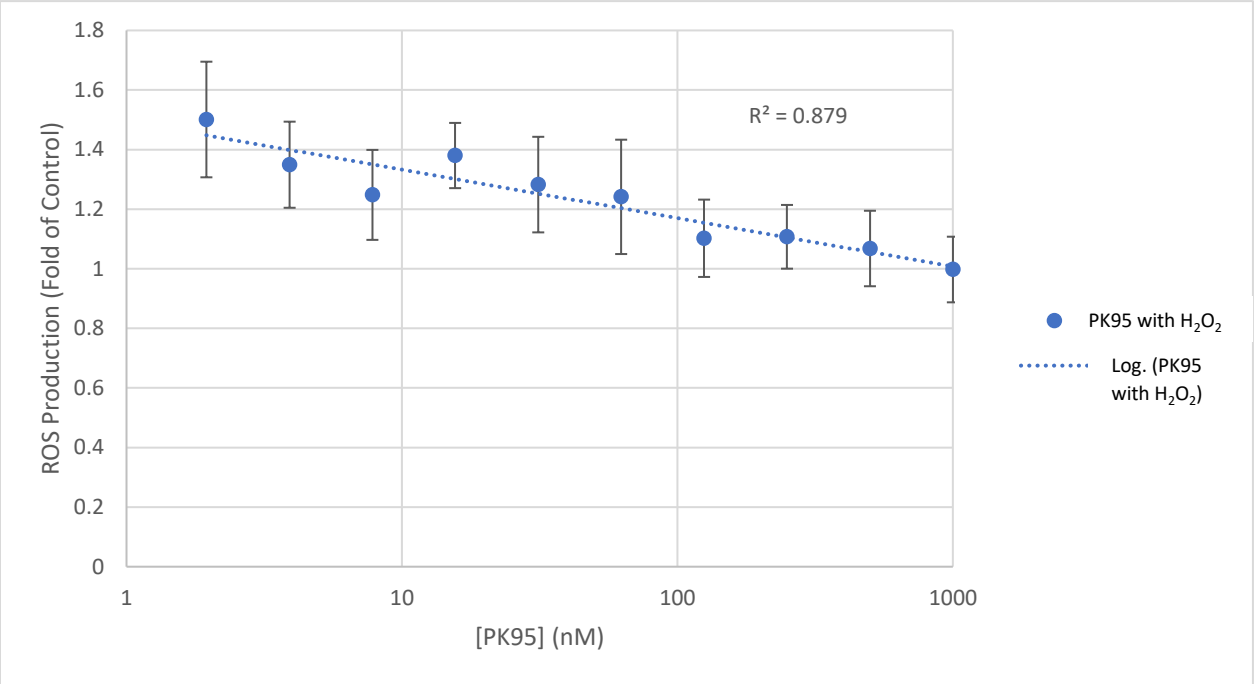


Fig. 17 H_2O_2-induced ROS Production in BV-2 Cells Treated with PK95

As concentrations of PK95 were increased, H_2O_2-induced intracellular ROS levels didn't change.

In this experiment, the effect of PK95 on H_2O_2-induced ROS-mediated cytotoxicity in BV-2 cells was measured utilizing the MTT cytotoxicity assay. The cells were treated with increasing concentrations of L2 followed by 90 μM and cell survival was subsequently measured.

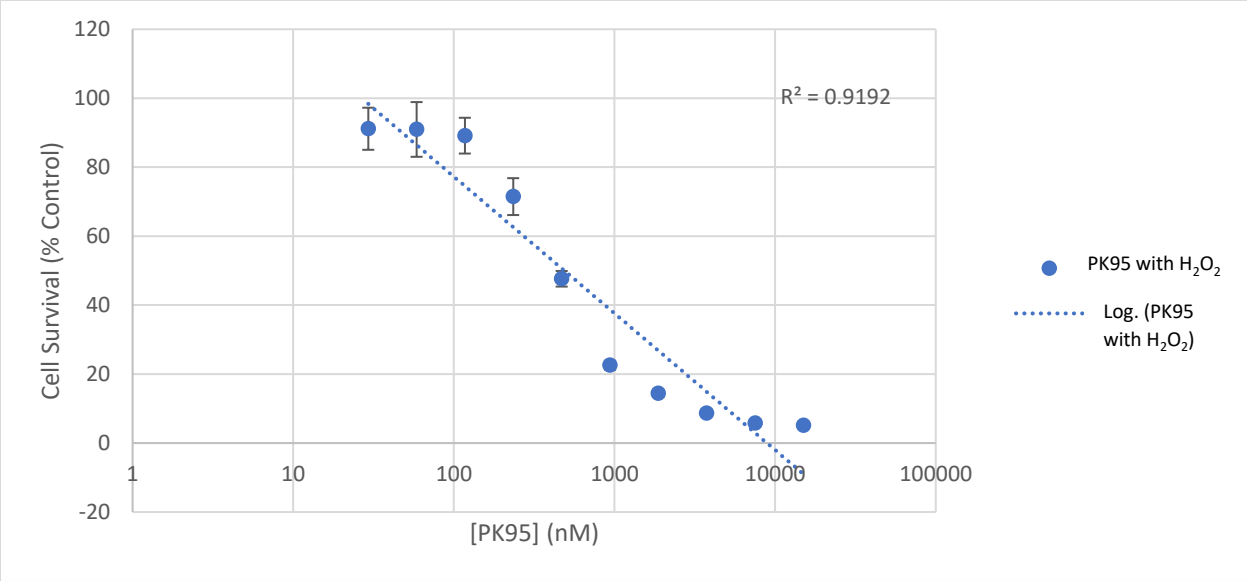


Fig. 18 Survival of BV-2 Cells Treated with PK95 in the Presence of 90 μM H_2O_2

As concentrations of PK95 were increased, cell survival decreased steadily until there was essentially no cell survival at a concentration of 7.5 μM.

In this experiment, the effect of PK95 alone on intracellular ROS levels in BV-2 cells was measured utilizing the DCFH-DA assay. We wanted to ensure that PK95 alone doesn't increase intracellular ROS levels. Cells were treated with increasing concentrations of PK95 and subsequently measured intracellular ROS levels.

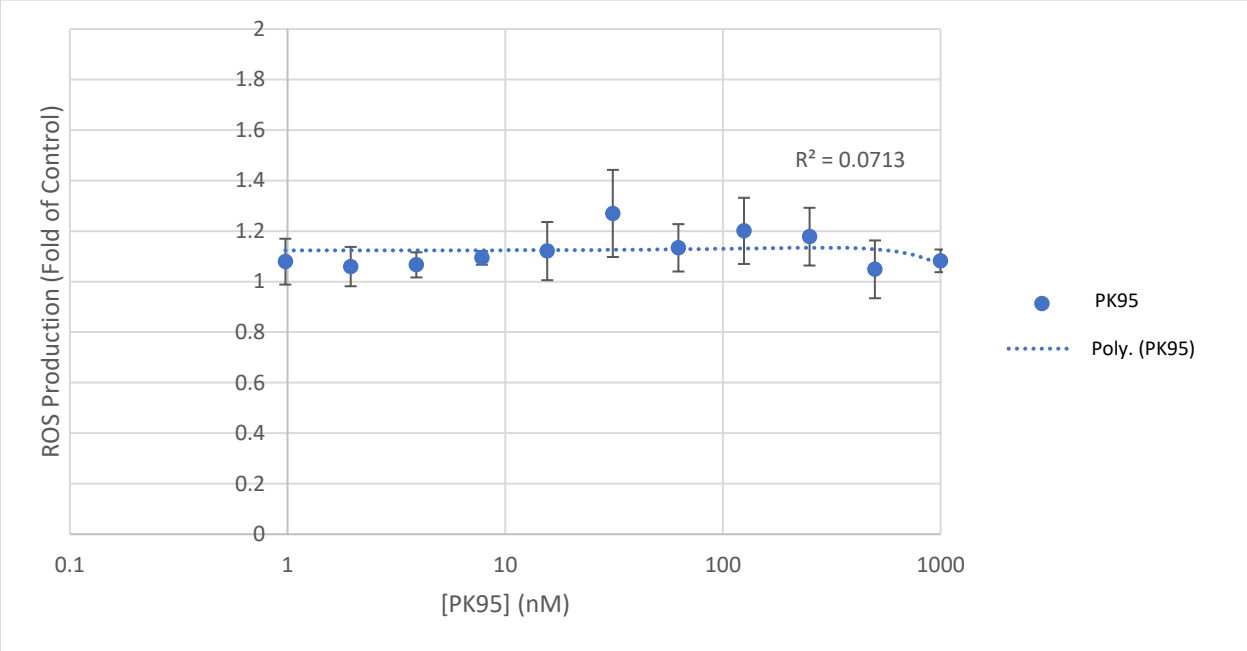


Fig. 19 ROS Production in BV-2 Cells Treated with PK95

At the varying concentrations of PK95, there were no significant changes in intracellular ROS levels.

DISCUSSION

Since prior studies have shown that oxidative stress contributes to the pathogenesis of neurodegenerative inflammatory diseases through toxicity to neuronal cells, such as in AD, it's important to discover compounds that can mitigate intracellular ROS as potential therapeutics. Additionally, metal ions play a role in the development of AD as redox metals such as iron, copper, and zinc are found to be in elevated levels in patients with AD; this is correlated with an increase in Aβ plaque deposition and catalyzing the reduction of oxygen to oxygen radical through Fenton chemical reactions. Further, it's known that microglial cells, immune cells of the central nervous system (CNS), are hyperactivated in response to Aβ plaques that results in them generating and releasing ROS onto neuronal cells, leading to the death of neuronal cells. Thus, in theory, a compound that can reduce intracellular ROS in microglial cells would be correlated with decreased ROS-mediated cytotoxicity in neuronal cells; with less death of neuronal cells, there would be increased cognitive function and thus able hinder the cognitive decline seen in AD. In this project, the first step in this process was the synthesis of compounds with the potential to reduce ROS. This is done by creating and identifying compounds that have bifunction qualities of chelation and radical scavenging. These properties should make for an ideal therapeutic molecule as it will reduce the burden of metal ion dysregulation and reduce oxygen radicals which should lead to a decrease in oxidative stress. The tetraazamacrocyles used in this project are theoretically an ideal compound to reduce H_2O_2-induced ROS as the nitrogen groups in the macrocycle form coordinate covalent bonds with metal ions and the hydroxyl groups on the pyridine group of the macrocycle provides radical scavenging activity. The combination of these properties makes them theoretically promising as they combat the issues of metal ion dysregulation and oxidative stress seen in neurodegenerative diseases.

Prior to analyzing the effect of macrocyclic compounds on H_2O_2-induced ROS and ROS-mediated cytotoxicity, it was first important to test the suitability of the DCFH-DA and MTT cytotoxicity assays to accurately measure increases in intracellular ROS levels and in ROS-mediated cytotoxicity, respectively when treated with H_2O_2 alone. So, it was necessary to conduct experiments with these assays showing that increasing concentrations of H_2O_2 correlates with elevated levels of intracellular ROS and this increase is then associated with increased ROS-mediated cytotoxicity. FRDA cells were used first to validate these assays as they are fibroblast cells from patients with Friedrich's Ataxia that have heightened sensitivity to changes in redox state due to a compromised antioxidant system. Therefore, these cells serve as a good model for measuring intracellular ROS due to their increased sensitivity. As expected, as concentrations of H_2O_2 are increased, we were able to measure a direct increase in intracellular ROS levels with the greatest effect seen at the highest concentration used of 90 μM that has an 8-fold increase in intracellular ROS in comparison to untreated cells (Fig. 1). This increase in intracellular ROS induced by H_2O_2 is correlated with ROS-mediated cytotoxicity as there is a decrease in cell viability as concentrations of H_2O_2 are increased (Fig. 2). At 90 μM H_2O_2 where there was an 8-fold increase in ROS, there was approximately 10% cell survival. The same trend was then seen in BV-2 cells, as when there was an increase in concentrations of H_2O_2, this was correlated to elevated levels of intracellular ROS and decreased cell survival (Fig. 3, 4). These results showed that the DCFH-DA assay can effectively measure changes in intracellular ROS levels and that this is correlated to ROS-mediated cytotoxicity in the MTT cytotoxicity assay.

After showing that increasing concentrations of H_2O_2 leads to increased levels of intracellular ROS and decreased cell survival, the next step was to analyze the effect of a known antioxidant on H_2O_2-induced ROS. Indole is the parent molecule of a commercially available

antioxidant that people take in supplements such as melatonin. The goal with using indole was to have a positive control as a known antioxidant should reduce levels of intracellular ROS. The BV-2 cells that were treated with a constant concentration of Indole in the presence of increasing concentrations of H_2O_2 showed a reduction in levels of intracellular ROS in comparison to the cells treated with H_2O_2 alone (Fig. 5). There was a 30% reduction in ROS levels in the cells treated with both 625 μM and 1250 μM indole in the presence of 250 μM H_2O_2 in comparison to the cells treated with the 250 μM H_2O_2 alone and ultimately Indole reduced the rate at which H_2O_2 increases intracellular ROS. These results confirmed that the DCFH-DA assay would serve as a reliable measure for the ability of compounds to reduce intracellular levels of ROS since as expected, there was a decrease in H_2O_2-induced levels of ROS when cells were treated with a known antioxidant.

After using indole as a positive control to show that antioxidant compounds can reduce intracellular levels of H_2O_2-induced ROS, the next step was to test macrocyclic compounds that have bifunctionality of chelation and radical scavenging for their efficacy in reducing H_2O_2-induced ROS and ROS-mediated cytotoxicity. The first compound tested was L2 which was synthesized by Kimberly Lincoln in Dr. Kayla Green's lab. Previous published studies done with L2 showed its ability to scavenge oxygen radicals and to bind metal ions.[4] With these properties, it would be expected that as concentrations of L2 are increased, that there will be a decrease in intracellular H_2O_2-induced ROS and an increase in cell survival for cells treated with L2 in comparison to cells treated with H_2O_2 alone. When cells were treated with increasing concentrations of L2 followed by 90 μM H_2O_2, there was a steady decline in intracellular ROS levels as concentrations of L2 increased and there was 50% reduction of ROS levels between the lowest concentration tested and the highest at 125 μM of L2 and beyond (Fig. 6). Since L2

reduced intracellular ROS levels by 50%, it would then be expected that it would attenuate ROS-mediated cytotoxicity and increase cell survival. However, for all concentrations of L2 used, there was approximately 7% cell survival up until the highest concentration of 536 μM where cell survival improved to 30% (Fig. 7). A potential reason that cell survival was still low for all concentrations of L2 even though it had been seen to reduce levels of intracellular ROS is that the concentration of H_2O_2 used was too high. As seen in Figure 2, FRDA cell survival when treated with 90 μM of H_2O_2 is around 10% which may be too cytotoxic to be able to measure a protective effect of L2.

In addition to testing L2 in the presence of H_2O_2 for its ability to reduce intracellular H_2O_2-induced ROS and mitigate ROS-mediated cytotoxicity, it's also important to test the effect of L2 alone on intracellular ROS levels and its cytotoxicity. It would be expected that L2 by itself would have no effect on intracellular levels of ROS in comparison to untreated cells as there's no agonist of ROS and the compound itself should not induce an increase in ROS either. As expected, L2 alone had no effect on intracellular ROS levels as there was approximately the same levels of intracellular ROS for all concentrations of L2 used compared to untreated cells (Fig. 8). Additionally, an ideal drug would be effective at concentrations where it has low toxicity, so testing for the cytotoxicity of the compound itself is important to know which concentrations could effectively reduce levels of intracellular ROS while not being toxic to the cells. From concentrations of 1.95 μM to 500 μM of L2, there was no cytotoxicity and at the highest concentration tested at 1 mM, there was 70% cell survival compared to untreated cells (Fig. 9). Since the concentrations of L2 that reduced H_2O_2-induced ROS by 50% as seen in Figure 1 were 125 nM, 1.25 μM, and 125 μM, this means that they were at concentrations below

cytotoxic levels. This is promising as L2 was effective in reducing levels of intracellular H_2O_2-induced ROS at nontoxic concentrations.

The results showing that L2 was able to mitigate H_2O_2-induced ROS and ROS-mediated cytotoxicity at nontoxic concentrations further confirmed the results of prior studies that showed that L2 is an effective antioxidant due to it's radical scavenging and metal ion binding capabilities. [4] We then examined a derivative of L2 for its ability to reduce intracellular levels of ROS and increase cell survival in comparison to cells treated with H_2O_2 alone. The compound PK60, synthesized by Kristof Pota in Dr. Kayla Green's lab, resembles L2 with the addition of an Indole group at the top of the pyridine on the compound (Fig. 10). Since L2 and Indole separately showed the ability to reduce intracellular levels of ROS (Fig. 5, 6), it would be reasonable to expect that a macrocyclic compound containing the combined moieties of both compounds would have a similar effect. However, the opposite result was seen as when cells were treated with increasing concentrations of PK60 in the presence of 70 μM, there was an increase in intracellular levels of ROS and a decrease in cell survival (Fig. 10, 11). This may be explained by the fact that PK60 alone led to an increase in intracellular levels of ROS which was correlated with high cytotoxicity at low concentrations in both FRDA and BV-2 cells (Fig. 12-15). Ultimately, after multiple experiments testing PK60, it was concluded that due to its high toxicity and ability to increase intracellular levels of ROS when cells were treated with PK60 alone, it wasn't an effective antioxidant compound. One possible explanation for the reduction in efficacy of PK60 could be that the addition of the indole group replaces the hydroxyl group on the pyridine of the macrocycle, and it's known that the hydroxyl group is responsible for oxygen radical scavenging. [4] However, the indole group is also supposed to possess antioxidant properties, so the exact mechanism for high toxicity and induction of ROS by PK60 is unknown.

After conducting multiple experiments that showed that PK60 itself was an inducer of ROS and was highly toxic to cells and thus not a useful therapeutic antioxidant, we turned to a novel compound that is another derivative of L2, named PK95 that was also synthesized by Kristof Pota in Dr. Kayla Green's lab. The structure of PK95 differs from that of L2 as the nitrogen groups in the macrocyclic ring are bound to a methyl group, whereas is in L2 they are bound to a single hydrogen. However, the mechanism of action should be the same as that of L2 as the nitrogen still contains an extra pair of electrons to forms coordinate covalent bonds with metal ions and contains the hydroxyl group atop of the pyridine group for radical scavenging. Prior to testing the ability of PK95 to reduce intracellular levels of ROS, it was first necessary to test the cytotoxicity of the compound alone; the reason being that an ideal drug would be effective at nontoxic concentrations and therefore the cytotoxicity profile needs to be determined prior to picking concentrations to test its efficacy in mitigating H_2O_2-induced ROS and toxicity from H_2O_2. When BV-2 cells were treated with PK95 alone, it was found to be nontoxic below concentrations of 234 nM (Fig. 16). Cells were then treated with increasing concentrations of PK95 in the presence of 90 μM H_2O_2 with the prediction that intracellular levels of ROS would decrease, and cell survival would increase as concentrations of PK95 are increased. However, as concentrations of PK95 were increased, there was no change in intracellular levels of ROS between the lowest and highest concentrations of PK95 tested (Fig. 17). Additionally, the opposite effect as predicted was seen for cell survival as concentrations of PK95 were increased in the presence of a constant concentration of H_2O_2, cell survival decreased (Fig. 18). To assess if this unanticipated result of PK95 was like that of PK60, it was also important to see if PK95 induced ROS in BV-2 cells. So, cells were treated with increasing concentrations of PK95 alone, but in contrast to PK60, increasing concentrations of PK95 had no effect on intracellular levels

of ROS (Fig. 19). Further, *in vitro* studies done in Dr. Kayla Green's lab with PK95 showed that it doesn't possess radical scavenging capabilities which can help explain as to why it was not an effective antioxidant in the cell line used.

In conclusion, we have shown that the DCFH-DA and MTT cytotoxicity assays are reliable ways of measuring H_2O_2-induced levels of ROS and that being correlated with cytotoxicity. Additionally, these assays are effective in showing that antioxidant compounds can reduce intracellular levels of ROS as indole, a known antioxidant, reduced the rate at which H_2O_2 induces ROS. Macrocyclic compounds with bifunctionality of chelation and oxygen radical scavenging properties also show promise as a potential therapeutic for neurodegenerative diseases, like AD, as L2 reduced H_2O_2-induced ROS by 50% and ROS-mediated cytotoxicity by 23% in the highest concentration tested. However, novel compounds (PK60 and PK95) tested in this study that are derivates of L2, have yet to show the ability to serve as effective antioxidants. Based on these findings, the next steps going forward should be to continue testing the efficacy of novel compounds, that are derivatives of L2, for their ability to reduce intracellular ROS levels and ROS-mediated cytotoxicity.

REFERENCES

(1) Lane, A. C., Hardy, J., and Schott, M. J. (2018) Alzheimer's Disease. European Journal of Neurology. 25, 59-70

(2) Jouanne, M., Rault, S., Voisin-Chiret, A. (2017) Tau protein aggregation in Alzheimer's disease: An attractive target for the development of novel therapeutic agents. European Journal of Medicinal Chemistry. 139, 153-167

(3) Liu, Y., Nguyen, M., Robert, A., Meunier, B. (2019) Metal Ions in Alzheimer's Disease: A Key Role or Not? Acc. Chem. Res. 52, 2026-2035

(4) Lincoln, K. M., Gonzalez, P., Richardson, T. E., Julovich, D. A., Saunders, R., Simpkins, J. W., Green, K. N. (2013) A potent antioxidant small molecule aimed at targeting metal-based oxidative stress in neurodegenerative disorders. Chem. Commun. 49, 2712-2714

(5) (2021) Medications for Memory, Cognition and Dementia-Related Behaviors. Alzheimer's Association. https://www.alz.org/alzheimers-dementia/treatments/medications-for-memory

(6) Budson, A. E. (2021) A new Alzheimer's drug has been approved. But should you take it? Harvard Health Publishing. https://www.health.harvard.edu/blog/a-new-alzheimers-drug-has-been-approved-but-should-you-take-it-202106082483

(7) (2021) Facts and Figures. Alzheimer's Association. https://www.alz.org/alzheimers-dementia/facts-figures

(8) (2021) Diagnosing Alzheimer's: How Alzheimer's is diagnosed. Mayo Clinic. https://www.mayoclinic.org/diseases-conditions/alzheimers-disease/in-depth/alzheimers/art-20048075

(9) (2021) Stages of Alzheimer's. Alzheimer's Association. https://www.alz.org/alzheimers-dementia/stages

(10) Pirscoveanu, D., Pirici, I., Tudorica, V., Balseanu, T., Albu, V., Bondari, S., Bumbea, A., Pirscoveanu M. (2017) Tau protein in neurodegenerative diseases – a review. Romanian Journal of Morphology and Embryology. 58(4), 1141-1150

(11) Huang B., et al. (2020) α-Cyperone Attenuates H_2O_2-Induced Oxidative Stress and Apoptosis in SH-SY5Y Cells *via* Activation of Nrf2. Frontiers in Pharmacology. 281(11), 1-10

(12) Hu, Q., Cao, X., Hao, D., Zhang, L. (2017) Chemical Composition, Antioxidant, DNA Damage Protective, Cytotoxic and Antibacterial Activities of Cyperus rotundus Rhizomes Essential Oil against Foodborne Pathogens. Scientific Reports. 7:45231

(13) Sukprasansap, M., Chanvorachote, P., Tencomnao, T. (2020) Cyanidin-3-glucoside activates Nrf2- antioxidant response element and protects against glutamate-induced oxidative and endoplasmic reticulum stress in HT22 hippocampal neuronal cells. BMC Complementary Medicine and Therapies. 20:46 1-12

(14) Xu et al. (2019) Lactoferrin ameliorates dopaminergic neurodegeneration and motor deficits in MPTP-treated mice. Redox Biology. 21:101090 1-13