TESTING THE EFFECT OF NOVEL ANTIOXIDANT COMPOUNDS ON THE ACTIVATION OF THE ANTIOXIDANT GENE ACTIVATOR NRF2

by

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ABSTRACT

The presence of Reactive Oxidative Species (ROS) in the brain have been linked to the etiology of Alzheimer's disease and neurodegeneration. In this project, novel antioxidant Indole derivative drugs were tested on BV-2 microglial cells using RT-qPCR to assess their ability to activate antioxidant gene expression. Nuclear factor erythroid 2–related factor 2 (Nrf2) is a gene transcription factor that is activated by oxidative stress and binds to a sequence called the Antioxidant Response Element (ARE), a region upstream of the DNA promoter sequence. Nrf2 activates transcription of antioxidative genes. Based on theoretical docking studies, we hypothesize that the novel compounds will disrupt the interaction between Nrf2 and its inhibitor KEAP, releasing Nrf-2 and enabling it to translocate to the nucleus. The novel antioxidant drugs should either increase the transcription of Nrf2-activated genes or reduce overall levels of antioxidative stress within cells. We tested for antioxidant properties by measuring Hemeoxygenase-1 (HO-1) and Nrf2 mRNA levels in BV-2 cells in the presence of these compounds.

ACKNOWLEDGEMENTS

I would first like to thank Dr. Giridhar Akkaraju, my Principal Investigator, who has helped guide me and show me the beauty of the scientific process. These past 2 years in the lab have been the most enlightening and formative moments within my time at TCU. From eating on the streets in Rome to walking the trails at Dinosaur Valley, Dr. Akkaraju has unequivocally changed my life for the better. I would also like to Dr. Kayla Green and her laboratory for providing the molecules I used within this project. Dr. Marlo Jeffries and Dr. Matt Hale, thank you for helping me in running my qPCRs and with the statistical analysis of my data. Additionally, I thank Dr. Laura Luque and Dr. Daniel Gil for agreeing to be a part of my honors thesis committee.

INTRODUCTIONS

Alzheimer's disease (AD) is a leading cause of dementia and neurodegeneration in the United States (Harrington 2012). Affecting around 6.7 million Americans, Alzheimer's primarily impacts individuals 65 years or older (Harrington 2012). Around 10.8% of all people above the age of 65 are diagnosed with Alzheimer's disease (Harrington 2012). Past studies have shown that the presence of ROS leads to neuroinflammation (Harrington 2012, Qaisiya 2014, Pey 2019). Beta-Amyloid plaques and Tau-Fibers cause oxidative stress, leading to chronic neuroinflammation (Harrington 2012). Abnormal levels of Beta-Amyloid (ß-Amyloid) can activate the neuronal immune cells, microglia, to target neurons and induce a cycle of degenerative neuroinflammation (Harrington 2012). ß-amyloid is derived from amyloid

precursor protein, which collects in between neuronal cells and clumps together (Harrington 2012). Functional ß-Amyloid acts as a membrane-bound protein that assists in neuronal growth and repair (Harrington 2012). Tau is a microtubule-stabilizing protein within neurons (Harrington 2012). In unhealthy cells, Tau proteins clump and grow into fibers, which destabilize neuronal tissues (Harrington 2012). Both of these protein clumps have been shown to increase oxidative stress and further destabilize neuronal connections in AD brains (Harrington 2012, Ma 2013). APOE is a cholesterol transport molecule, with three different forms (Harrington 2012). APOE-e4 has been genetically linked to late-onset Alzheimer's and is the strongest indicator/risk-factor of Alzheimer's disease (Harrington 2012).

Oxidative stress has been shown to influence and cause Alzheimer's disease (Ma 2013). ß-amyloid plaques and hyperphosphorylated Tau fibers are usually degraded via a cell's natural immune response. However, oxidative stress impedes a cells immune response leading to a buildup of these plaques (Ma 2013). One way in which the immune system responds to reduce the effects of oxidation is by activating the Nrf2 pathway. Nrf2 is a gene activator that induces antioxidative gene transcription (Ma 2013). In the absence of oxidative stress, Nrf2 is bound to Kelch-like erythroid cell-derived protein with CNC homology-associated protein 1 (KEAP1) and tagged for ubiquitination and proteasomal degradation (Ma 2013). KEAP1 binds to Nrf2 with the Cul3/Rbx1 based E3-ubiquitin ligase complex (Wu 2019). This complex tags Nrf2 for proteasomal degradation. The degradation of Nrf2 results in low levels of Nrf2 in the cell and reduced antioxidant gene transcription. Oxidative stress modifies cysteine residues within the protein sequence of KEAP1, causing it to release Nrf2 (Wu 2019, Ma 2013). As a result, Nrf2 levels increase. It is subsequently translocated into the nucleus, activating increased transcription of downstream antioxidative genes. After translocation Nrf2 binds to a sequence on DNA known

as the Antioxidant Responsive Element (ARE) (Prawan 2008, Qaisiya 2014). Nrf2 binding to this ARE effector site allows for increased transcription of antioxidative genes.

Currently, downstream genes activated by Nrf2 release and translocation are being studied as potential models showcasing an increased antioxidative response in brain tissue (Qaisiya 2014, Prawan 2008, Johnston 2019). Among these many antioxidative genes, this study will isolate and look at a few specific genes downstream of the ARE. These include Hemeoxygenase-1 (HO-1) and NAD(P)H quinone oxidoreductase 1 (NQO1) (Pey 2019, Parawan 2008, Araujo 2012). HO-1, the inducible isoform of Hemeoxygenase, is responsible for the reduction of oxidative stress by the cleavage of heme groups leading to the production of biliverdin, CO, and Ferrous iron (Araujo 2012). HO-1 is constitutively expressed in low levels in basal conditions, but antioxidative stress increases expression through activation of the ARE by Nrf2 (Araujo 2012). NQO1 is an electron-reductase protein responsible for the reduction of quinones and other organic compounds. In cells undergoing oxidative stress, the NQO1 gene is expressed and the protein, scavenges free radicals and reduces overall oxidative damage (Pey 2019).

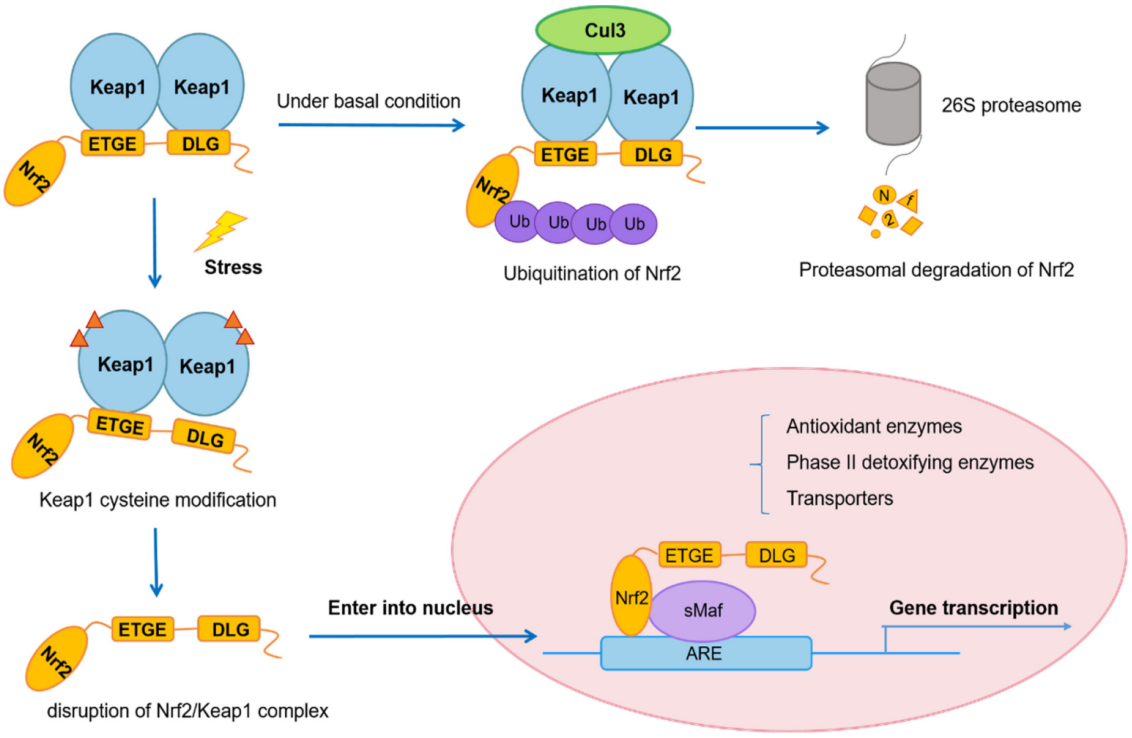

Figure 1. The Nrf2 Pathway (Wu et. al)

In this study, we examine the antioxidant properties of a variety of compounds synthesized in the lab of Dr. Kayla Green in the Department of Chemistry at TCU. These compounds were designed to have antioxidative properties, potentially reducing the need for cells to transcribe antioxidant genes (Johnston 2019). If true, Nrf2 translocation and gene transcription should be reduced in cells treated with these compounds. This was quantified by the mRNA levels of antioxidant gene transcripts in cells derived from different tissues, such as BV2, HT22, and FRDA. The drugs developed and tested in this study were designed to be multimodal, containing both metal chelation and radical scavenging moieties. Two competing hypotheses were tested in this study. These compounds reduce the activation of Nrf2 and the transcription of HO-1 mRNA by acting as radical scavengers and reducing the level of ROS in the cell. Alternatively, the drugs interfere with the interaction between KEAP1 and Nrf2, and it would activate Nrf2.

METHODS

Cell Culture

BV2 Microglial cells were grown in an atmosphere of 5% CO_2, 95% Air at 37ºC in a Solution of Complete Medium (containing Dulbecco's Modified Eagle Medium, 10% Fetal Bovine Serum, 100 units Penicillin, 100 µg Streptomycin per mL, 2mM L-glutamine, and 1X MEM non-essential amino acid solution [Millipore Sigma]). Cells were plated in 2mL 12-well plates at a density of 100,000 BV2 cells per well. The following day, cells were treated as described for 24 hours before RNA extraction.

Gene Expression Analysis

RNA was extracted from BV2 cells 24 hours post-treatment using double-column silica membrane spin technology. Cells were lysed and RNA isolated using the Macherey-Nagel Nucleospin RNA kits following the manufacturer's protocol. RNA was eluted with H_2O and concentrations were analyzed using a NanoDrop 1 UV-Vis Spectrophotometer (ng/µL). RNA was stored at -80ºC for further analysis. Each sample was diluted to equal concentrations for reverse transcription.

RNA was converted to cDNA using QuantaBio qScript cDNA Supermix following the manufacturer's protocol in a BIO-RAD t100 Thermal Cycler (Cycling conditions: 25ºC 5:00 min, 46ºC 20:00 min, 95ºC 1:00 min, 4ºC hold). cDNA was stored at -80 ºC until the next step. A Real-Time quantitative Polymerase Chain Reaction (qPCR) was carried out on the cDNA using DNA Primers for Hemeoxygenase-1 (HO-1) or ß-actin (Table 1) and QuantaBio PerfeCTa SYBR Green FastMix with three replicates of each sample in a 96-well PCR plate (Cycling conditions: 95ºC 30s , [95ºC 10s, 60ºC 15s, 39 cycles]) in a BIO-RAD CFX Connect Real-Time System. The Cycle Threshold (Ct) of each replicate was recorded and the Comparative Ct method (DDCt) was used to analyze the fold gene expression. mRNA Ct was normalized with ß-actin mRNA and directly compared to untreated sample mRNA.

MTT Assay

BV2 cells were plated at a density of 5,000 cells per well in 100 µL Complete Medium in 96-well plates. The following day, cells were treated as described for 24 hours. Medium was aspirated from each well and replaced with 100µL of 1 mg per mL of 3-(3,4-Dimethylthiazole-2-yl)-2,5-diphenyltetrazoliumbromide (MTT) dissolved in Serum-Free Medium (SFM). Cells were incubated in MTT for 4 hours. MTT solution was aspirated and replaced with 100 µL of Dimethyl Sulfoxide (DMSO). The plate was shaken for 5 minutes, and the absorbance was measured at 540 nm.

Table 1. DNA primers used in the Reverse Transcription

Gene	**Primer Forward**	**Primer Reverse**
Hemeoxygenase -1	5'-TGACAGAAGAGGCTAAGACCG-3'	5'-GTGAGGACCCACTGGAGGA-3'
NAD(P)H quinone oxidoreductase 1 (NQO1)	5'-AGCCCAGATATTGTGGCC-3'	5'-CCTTTCAGAATGGCTGGCAC-3'
Nuclear factor erythroid 2-related factor 2	5'-CTGAACTCCTGGACGGGACTA-3'	5'-CGGTGGGTCTCCGTAAATGG-3'
ß-actin	5'-CATGTACGTTGCTATCCAGGC-3'	5'-CTCCTTAATGTCACGCACGAT-3'

RESULTS

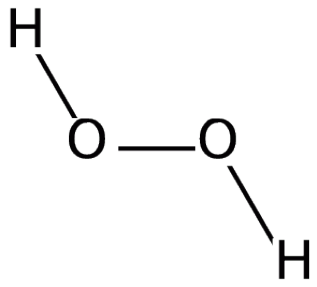

Figure 2A. The chemical structure of H_2O_2.

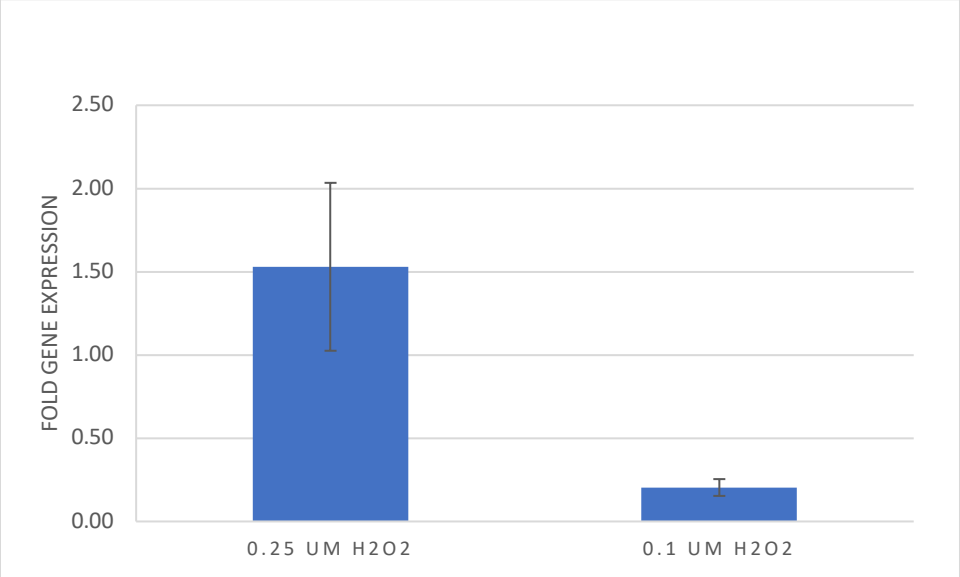

Figure 2B. Effect of H_2O_2 on HO-1 gene expression in BV2 cells. BV2 cells were treated for 24 hours with the concentrations indicated and mRNA was extracted and quantified.

To see if the antioxidative response in BV2 mice microglia cells could be quantified via RT-qPCR, by analyzing levels of HO-1 mRNA. BV2 cells were treated with two concentrations of Hydrogen Peroxide (H_2O_2, shown in Fig. 1A) . H_2O_2 is a known inducer of oxidative stress. A dose-dependent increase in HO-1 mRNA levels was seen.

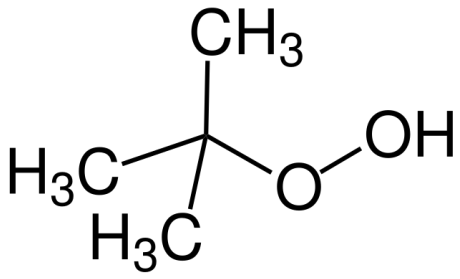

Figure 3A. The chemical structure of TBHP.

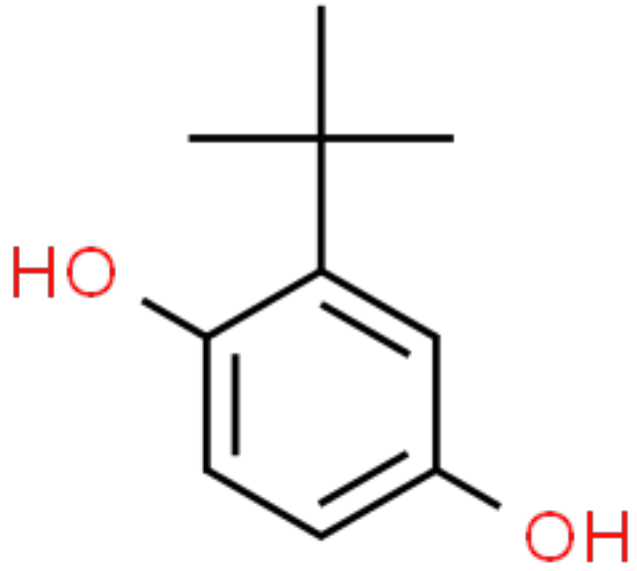

Figure 3B. The chemical structure of TBHQ

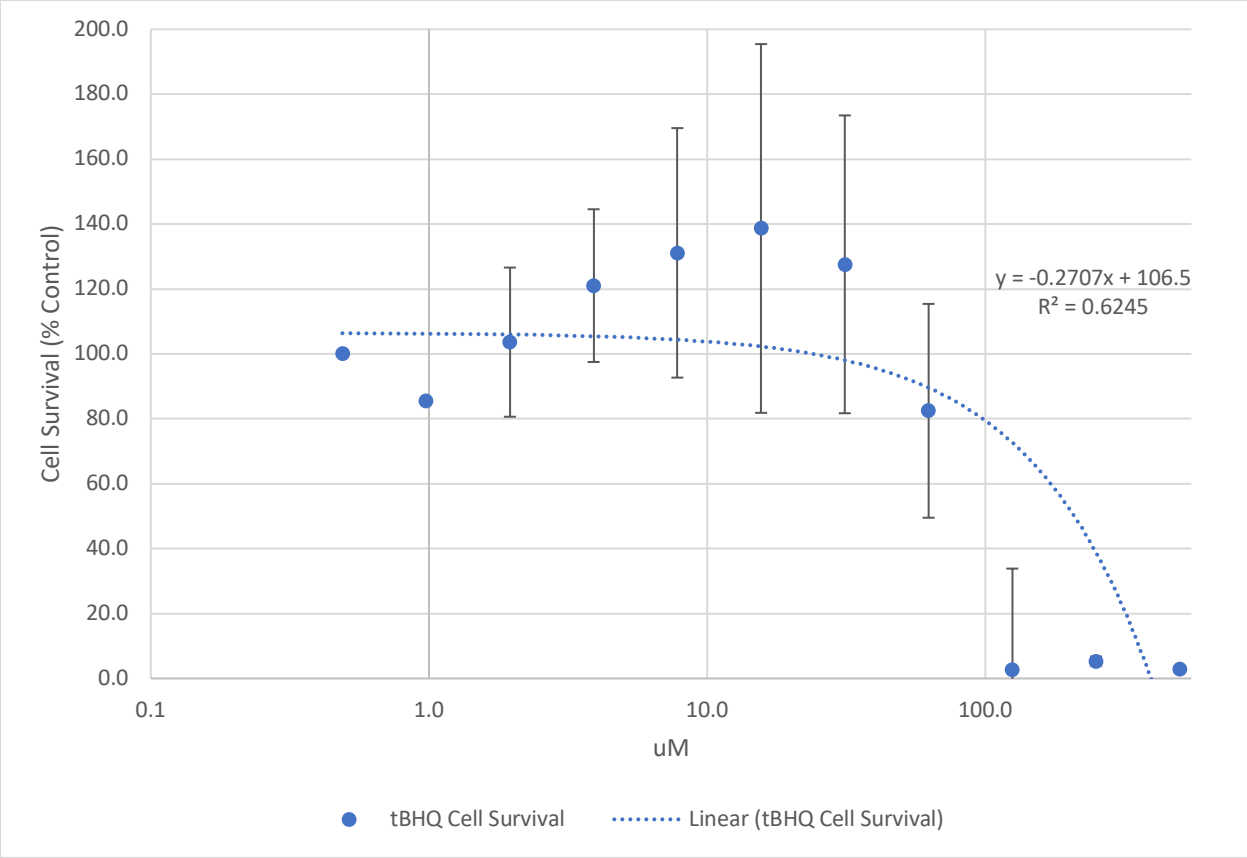

Figure 3C. Effect of TBHQ on cell survival in BV2 cells. BV2 cells were treated with the concentrations indicated and cell survival was analyzed via MTT assay.

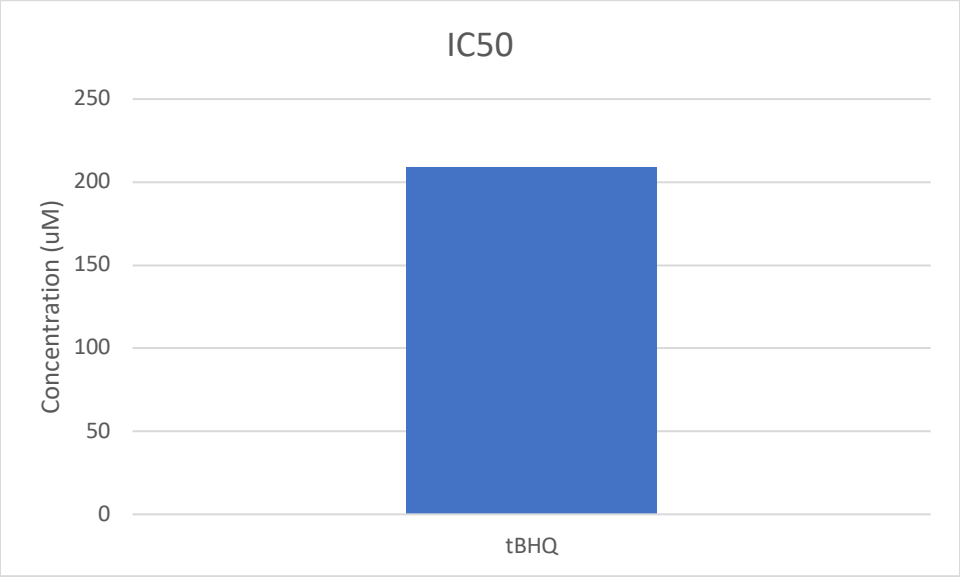

Figure 3D. Half Maximal Inhibitory Concentration of TBHQ.

TBHQ is a known inhibitor of the KEAP1/Nrf2 interaction. In subsequent experiments, this was used as a positive control for Nrf2 activation. TBHQ-induced cellular death was quantified via MTT Assay with increasing concentrations of TBHQ to find suitable concentrations for future use.

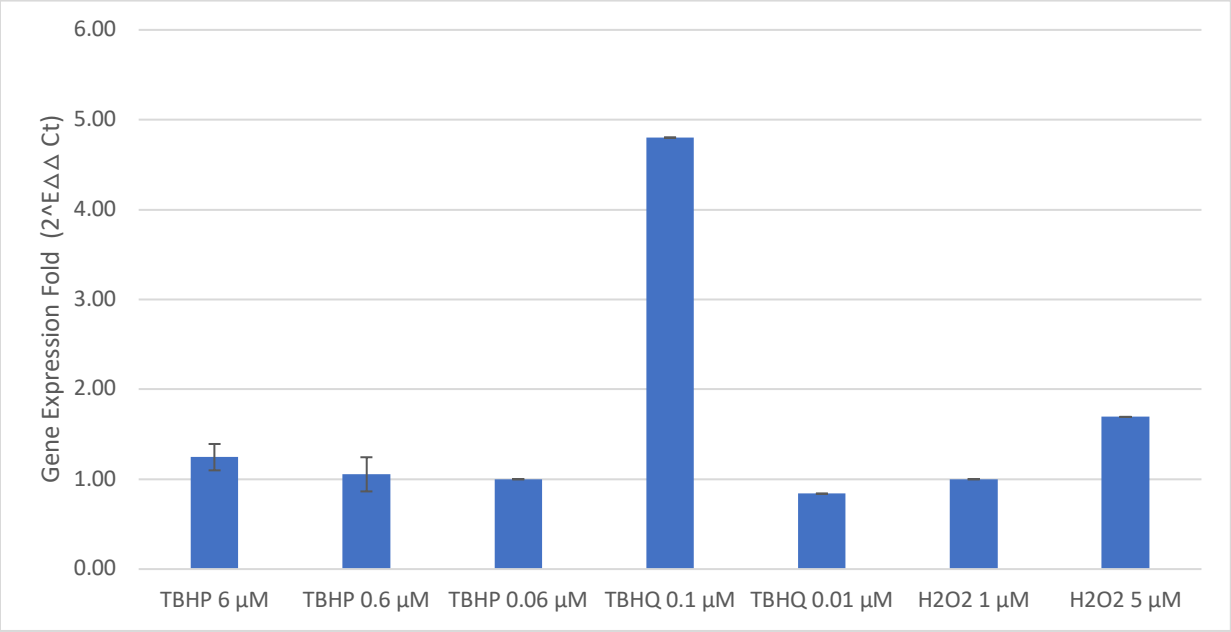

Figure 4. Effect of TBHP, TBHQ, and H_2O_2 on HO-1 gene expression in BV2 cells. BV2 cells were treated for 24 hours with the concentrations indicated and mRNA was extracted and quantified.

BV2 cells were treated with other known activators of oxidative stress to observe if Nrf2 activation could be quantified and signal as potential positive controls for future experiments. Tert-Butyl Hydroperoxide (TBHP, shown in Fig. 2A), a known inducer of oxidative stress; Tert-Butyl Hydroquinone (TBHQ, shown in Fig. 2B), a known disrupter of KEAP1/Nrf2 interaction; and H_2O_2 were used to analyze activation of Nrf2-induced HO-1 expression.

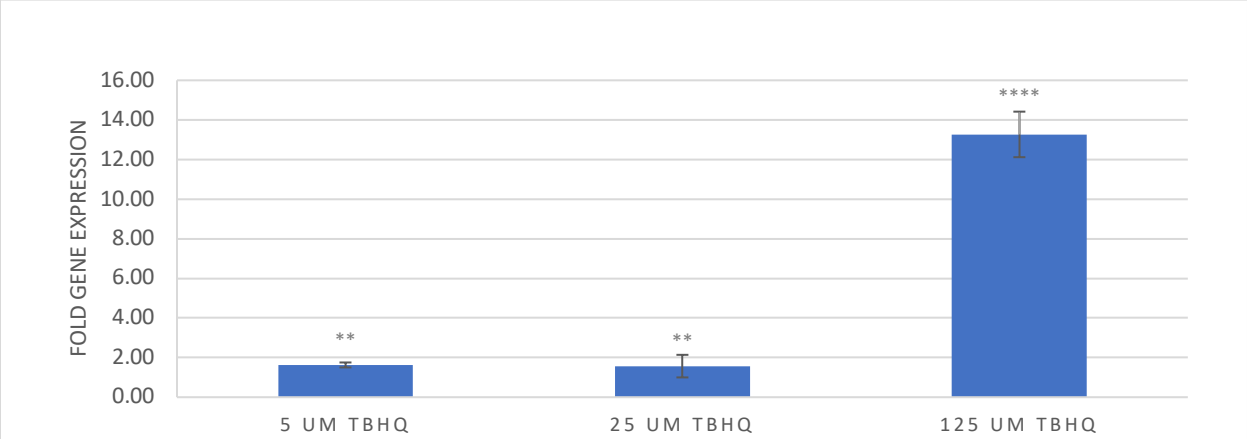

Figure 5. Effect of TBHQ on HO-1 gene expression in BV2 cells. BV2 cells were treated for 24 hours with the concentrations indicated and mRNA was extracted and quantified.

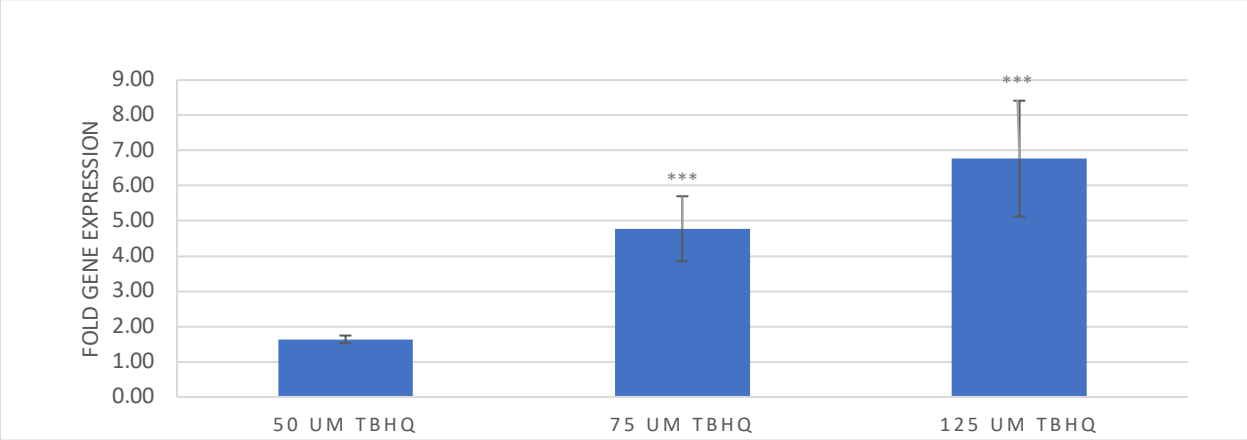

Figure 6. Effect of TBHP, TBHQ, and H_2O_2 on HO-1 gene expression in BV2 cells. BV2 cells were treated for 24 hours with the concentrations indicated and mRNA was extracted and quantified.

Next, to identify a suitable concentration of TBHQ to induce a significant level of Nrf2 activation, cells were treated with different concentrations of TBHQ. TBHQ interferes with the interaction between KEAP and Nrf2, leading to the translocation of Nrf2 into the nucleus and the activation of Nrf2-sensitive genes, such as HO-1. However, it doesn't increase cellular levels of

oxidative stress. At 75 µM we saw a 5-fold increase in HO-1 mRNA. We decided to use 75 µM and 125 µM of TBHQ for future experiments as a positive control for Nrf2-activation.

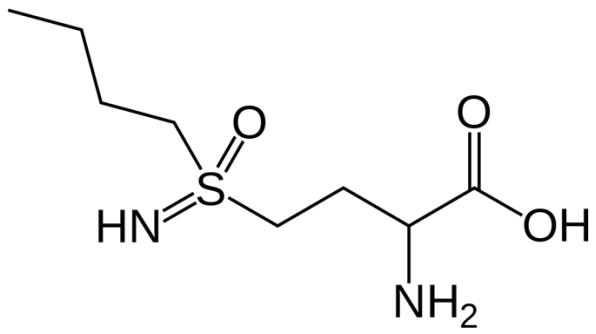

Figure 7A. The chemical structure of BSO.

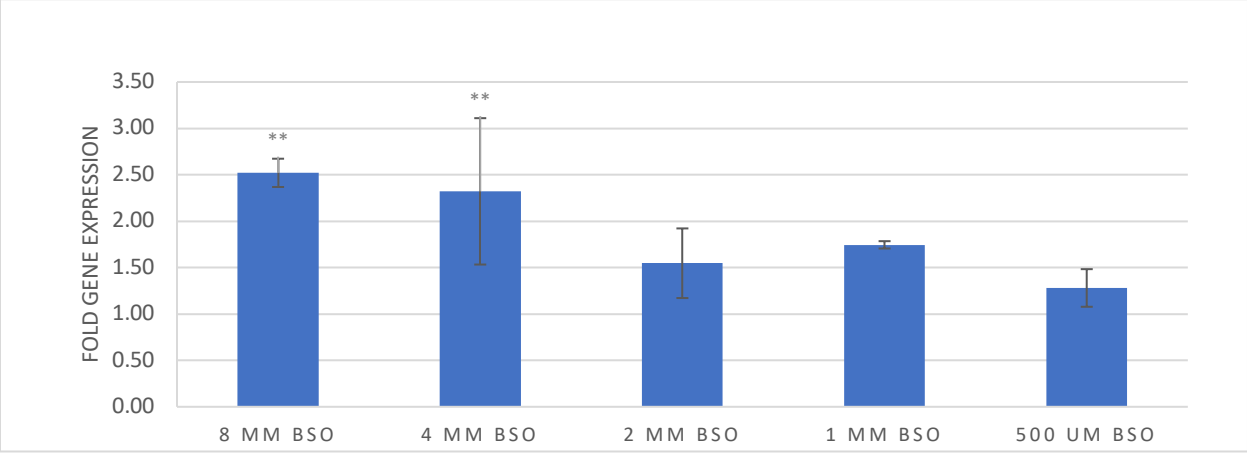

Figure 7B. Effect of BSO on HO-1 gene expression in BV2 cells. BV2 cells were treated for 24 hours with the concentrations indicated and mRNA was extracted and quantified.

Buthionine Sulfoximine (BSO, shown in Fig. 7A), a direct inhibitor of Glutathione, an antioxidant, was used to see if oxidative stress-induced Nrf2 activation could be quantified. BSO-directed inhibition of Glutathione leads to the build-up of ROS in the cell. In turn, this should lead to an increase of Nrf2 activation. BSO was shown to increase the levels of HO-1 mRNA in a dose-dependent manner.

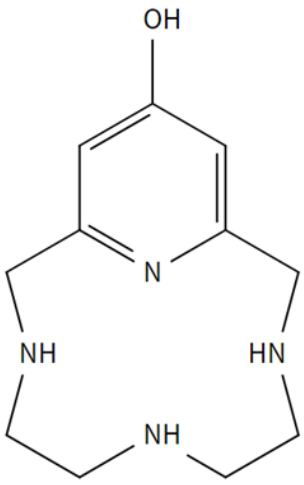

Figure 8A. The chemical structure of L2

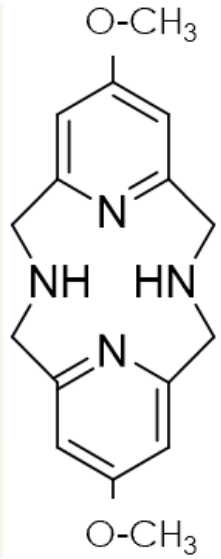

Figure 8B. The chemical structure of $OMePy_2N_2$

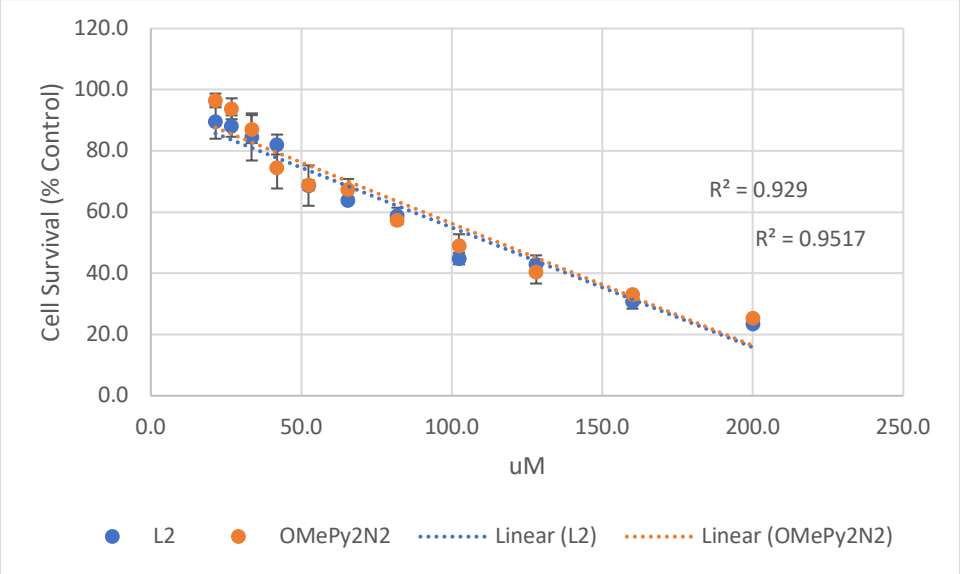

Figure 8C. Effect of PK97 and PK60 on cell survival in BV2 cells. BV2 cells were treated with the concentrations indicated and cell survival was analyzed via MTT assay.

Next, we looked at the cytotoxicity of L2 and $OMePy_2N_2$, two compounds designed in the lab of Dr. Kayla Green in the Department of Chemistry (Shown in Fig. 5A and 6A). Both drugs were designed to reduce ROS levels in the cell. Cytotoxicity of L2 and $OMePy_2N_2$ were both tested on

BV2 cells to find suitable concentrations for future experiments.

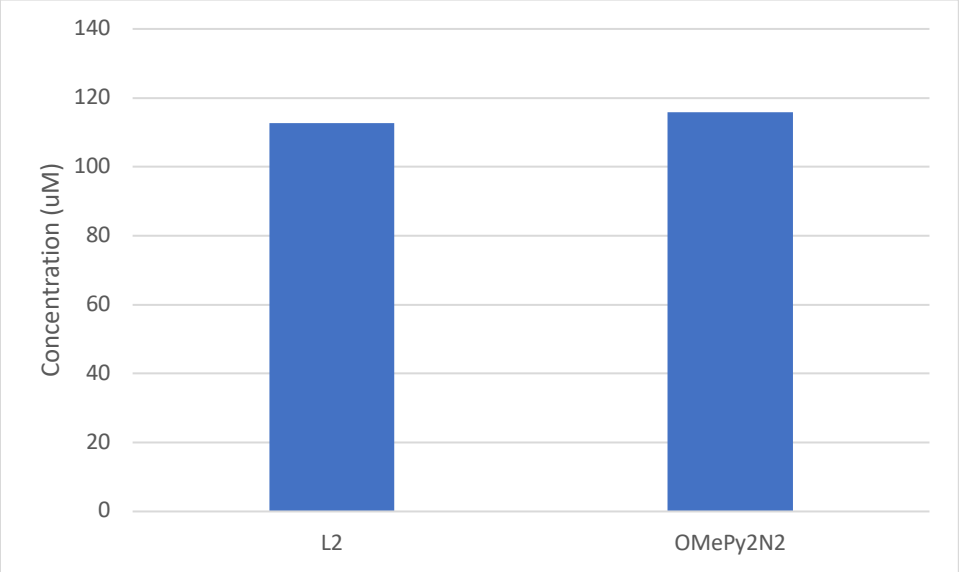

Figure 8D. Half Maximal Inhibitory Concentration of L2 and $OMePy_2N_2$.

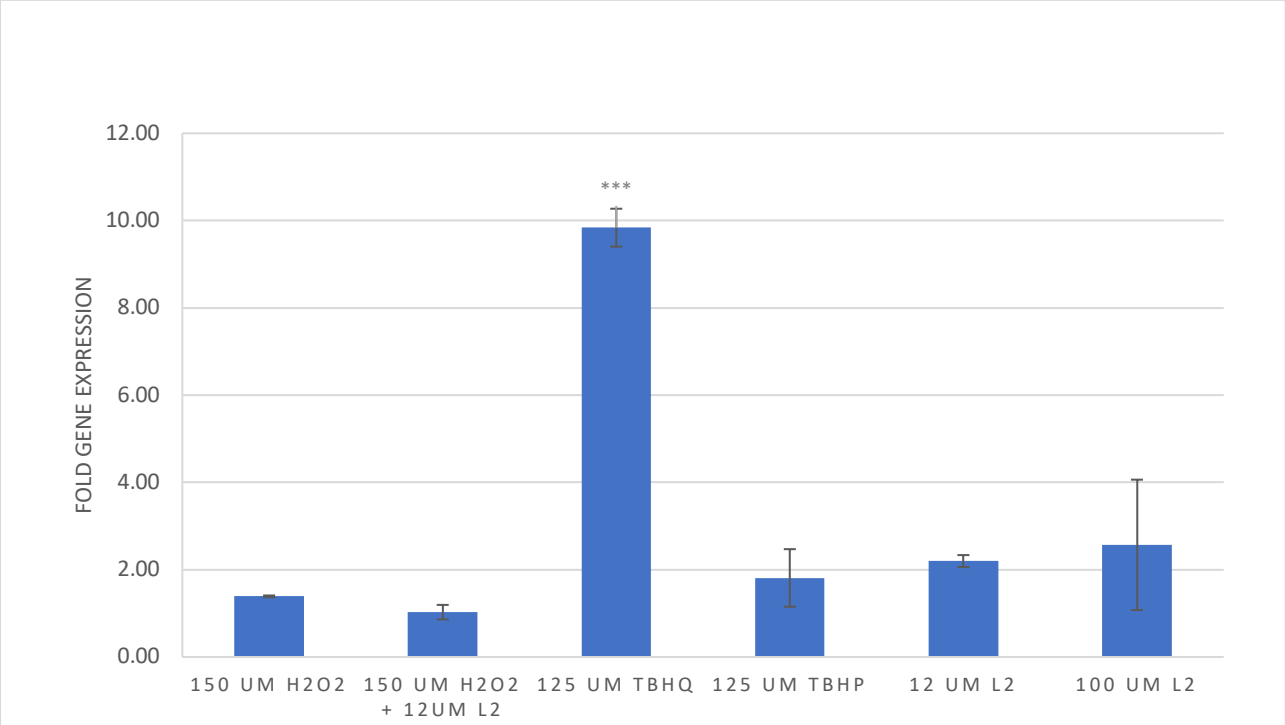

Figure 9. Effect of TBHP, TBHQ, H_2O_2, and L2 on HO-1 gene expression in BV2 cells. BV2 cells were treated for 24 hours with the concentrations indicated and mRNA was extracted and quantified.

Cells were treated with L2 alone and in combination with H_2O_2 to measure its effects on H_2O_2-induced activation of Nrf2. TBHQ was used as a positive control. The results showed an increase in the activation of Nrf2 in cells treated with L2 alone, indicating that L2 was either increasing oxidative stress or blocking the binding of Nrf2 and KEAP1, inducing Nrf2 nuclear localization.

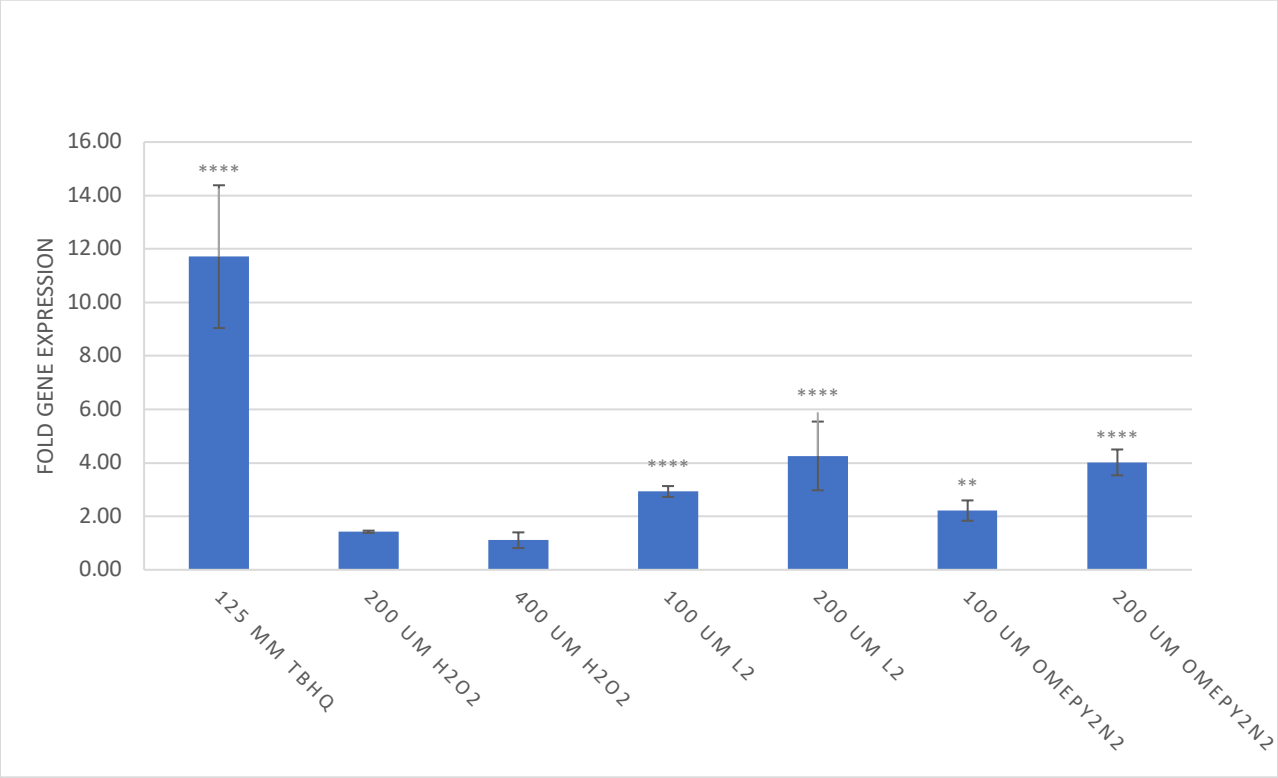

Figure 10. Effect of TBHQ, H_2O_2, L2 and $MeOPy_2N_2$ on HO-1 gene expression in BV2 cells. BV2 cells were treated for 24 hours with the concentrations indicated and mRNA was extracted and quantified.

Because no notable effect was seen using the concentrations of L2 seen in Fig. 5, Two higher concentrations were used. $OMePy_2N_2$, a second drug developed by Dr. Green's lab (shown in Fig. 6A), was included to see if other Green Drugs could elicit an activation of Nrf2. The results showed a dose-dependent increase of HO-1 levels when increasing the concentration of the L2 and $OMePy_2N_2$.

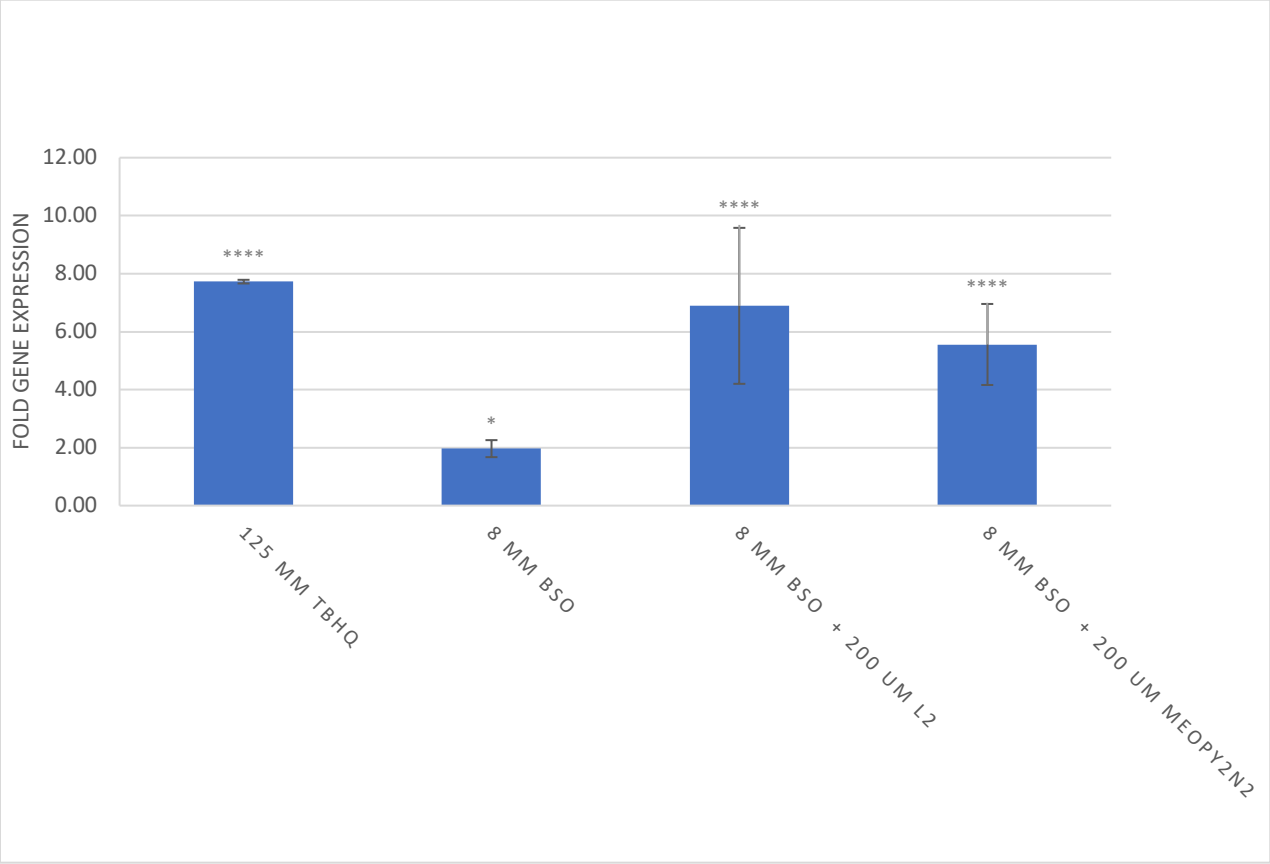

Figure 11. Effect of TBHQ, BSO, L2, and $MeOPy_2N_2$ on HO-1 gene expression in BV2 cells. BV2 cells were treated for 24 hours with the concentrations indicated and mRNA was extracted and quantified.

Next, Drug-induced activation of Nrf2 in the presence of oxidative stress induced by BSO was assessed. BSO increases intracellular ROS levels. If the compounds reduce ROS, it should reverse BSO induced HO-1 mRNA expression. In this experiment, BSO was combined with either L2 or $OMePy_2N_2$. Both L2 and $OMePy_2N_2$ increased BSO-induced expression of HO-1 mRNA compared to BSO alone. This indicated that the drugs were activating Nrf2 activation in the presence of oxidative stress.

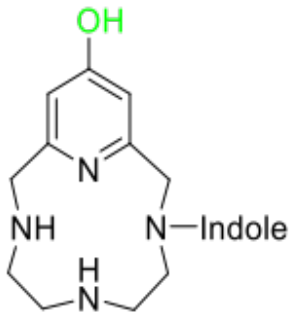

Figure 12A. The chemical structure of PK97.

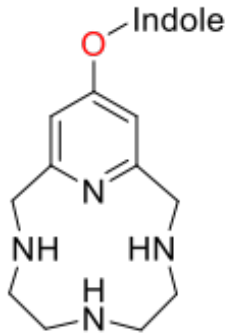

Figure 12B. The chemical structure of PK60

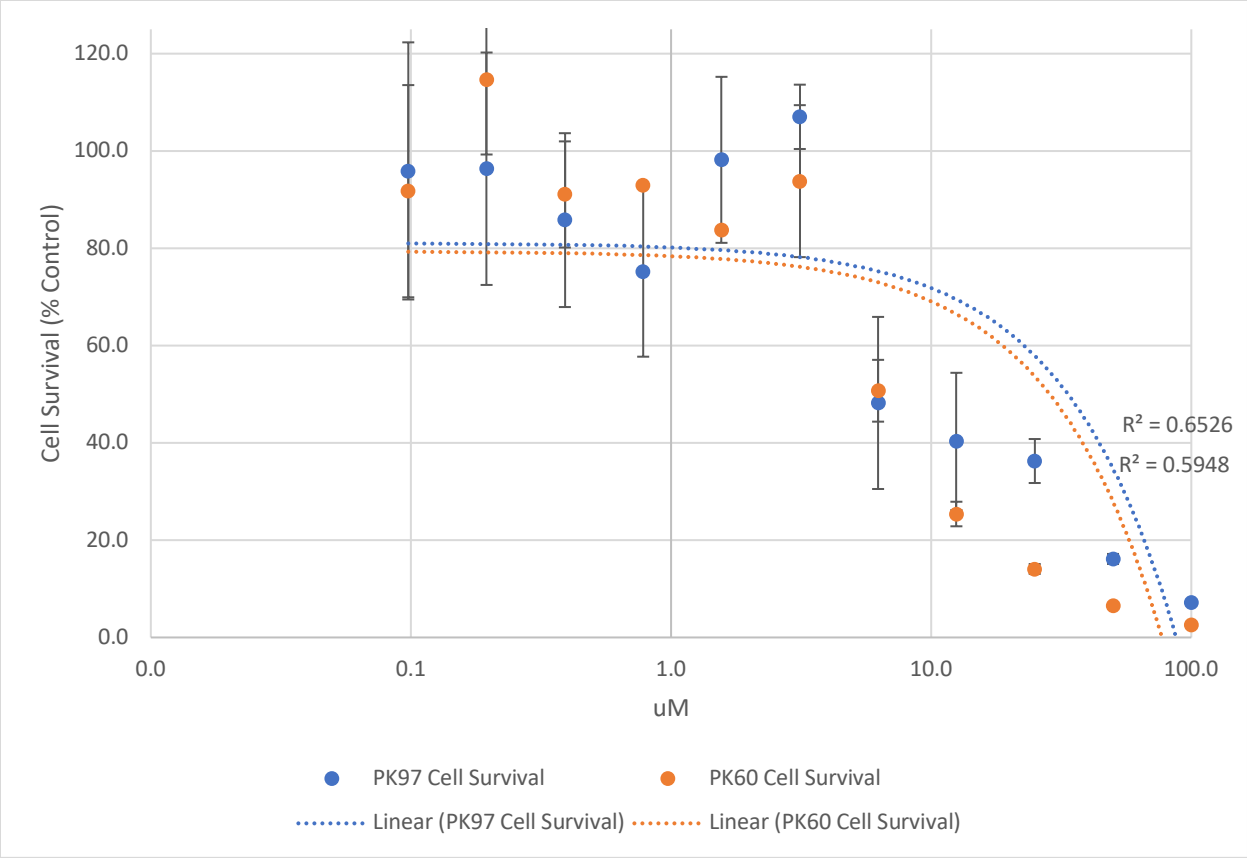

Figure 12C. Effect of PK97 and PK60 on cell survival in BV2 cells. BV2 cells were treated with the concentrations indicated and cell survival was analyzed via MTT assay.

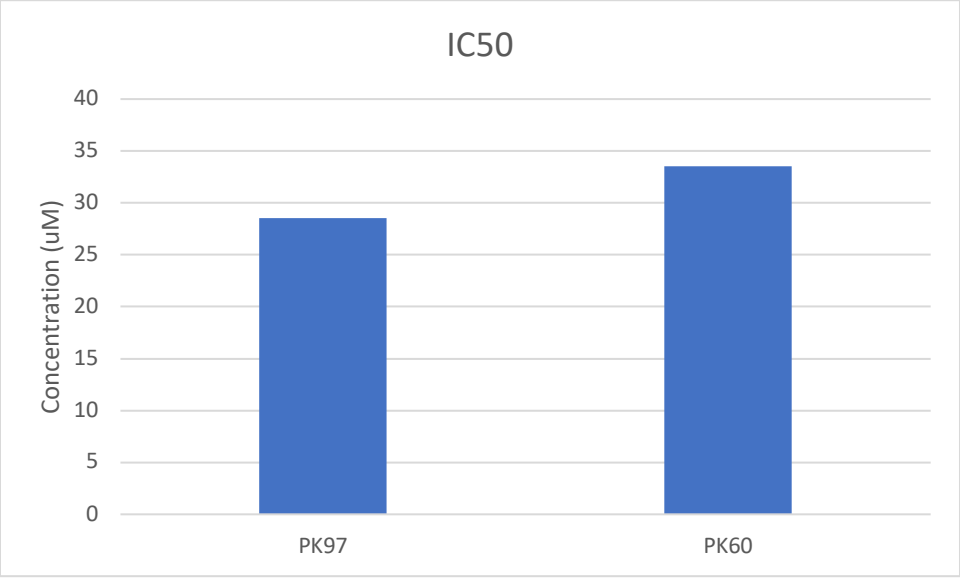

Figure 12D. Half Maximal Inhibitory Concentration of PK97 and PK60.

The cytotoxicity of two new drugs, PK97 and PK60 (developed in Dr. Green's lab), were analyzed via MTT assay to look for adequate concentrations for future experiments.

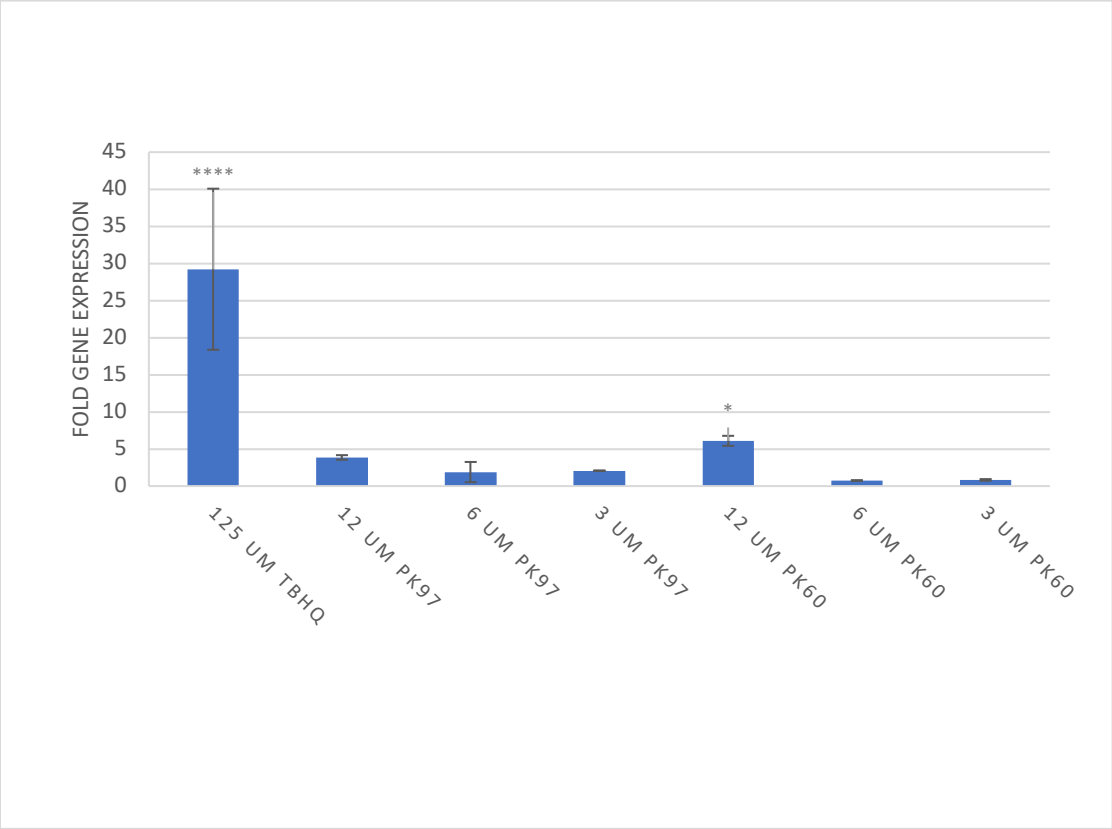

Figure 13. Effect of TBHQ, PK97, and PK60 on HO-1 gene expression in BV2 cells. BV2 cells were treated with the concentrations indicated and mRNA was extracted and quantified.

BV2 cells were treated with PK97 and PK60. mRNA was extracted and gene expression of HO-1 was quantified. Cellular death was observed at high concentrations, with no mRNA quantification. At lower concentrations, PK60 was able to increase levels of HO-1 mRNA, suggesting an increased activation of Nrf2.

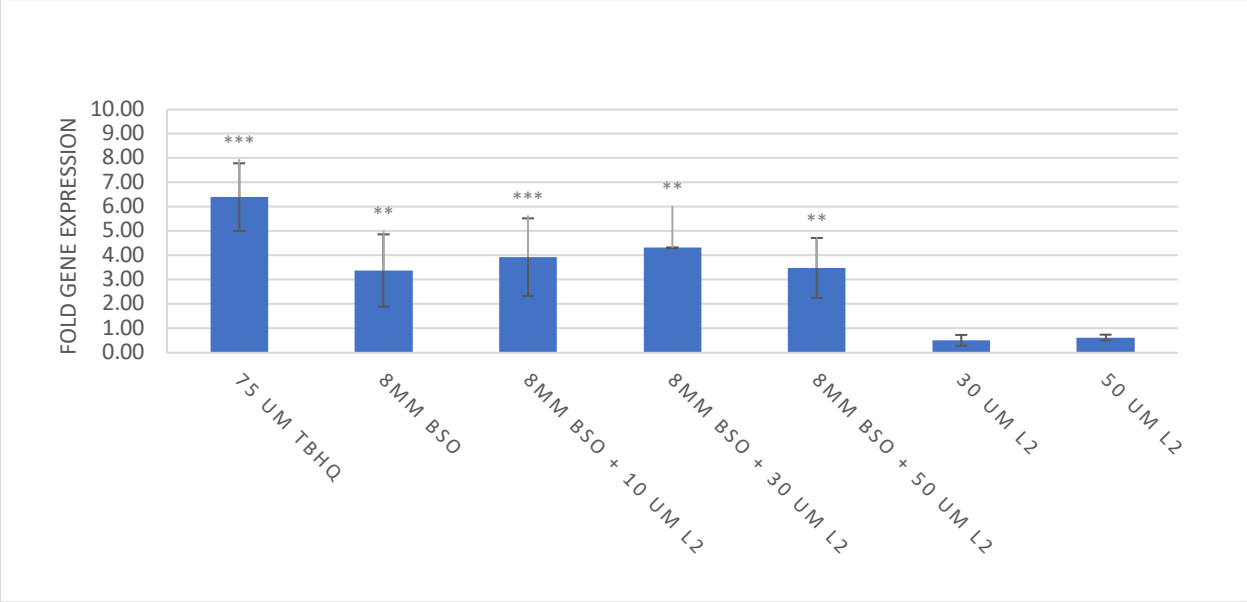

Figure 14. Effect of TBHQ, BSO, and L2 on HO-1 gene expression in BV2 cells. BV2 cells were treated with the concentrations indicated and mRNA was extracted and quantified.

To test a newer batch of L2-induced Nrf2 activation alone and combined with BSO-induced oxidative stress, the assay was repeating with L2 and BSO. The L2 at concentrations lower than 100 μM seemed to reduce the overall activation of Nrf2 and HO-1 transcription. These results suggest a dose-dependent mode of action in the molecules. At lower concentrations, there is not enough drug to competitively inhibit KEAP1/Nrf2 binding, thus it solely reduced oxidative stress. However, at higher concentrations, the molecule can competitively inhibit KEAP1, increasing Nrf2-induced gene transcription.

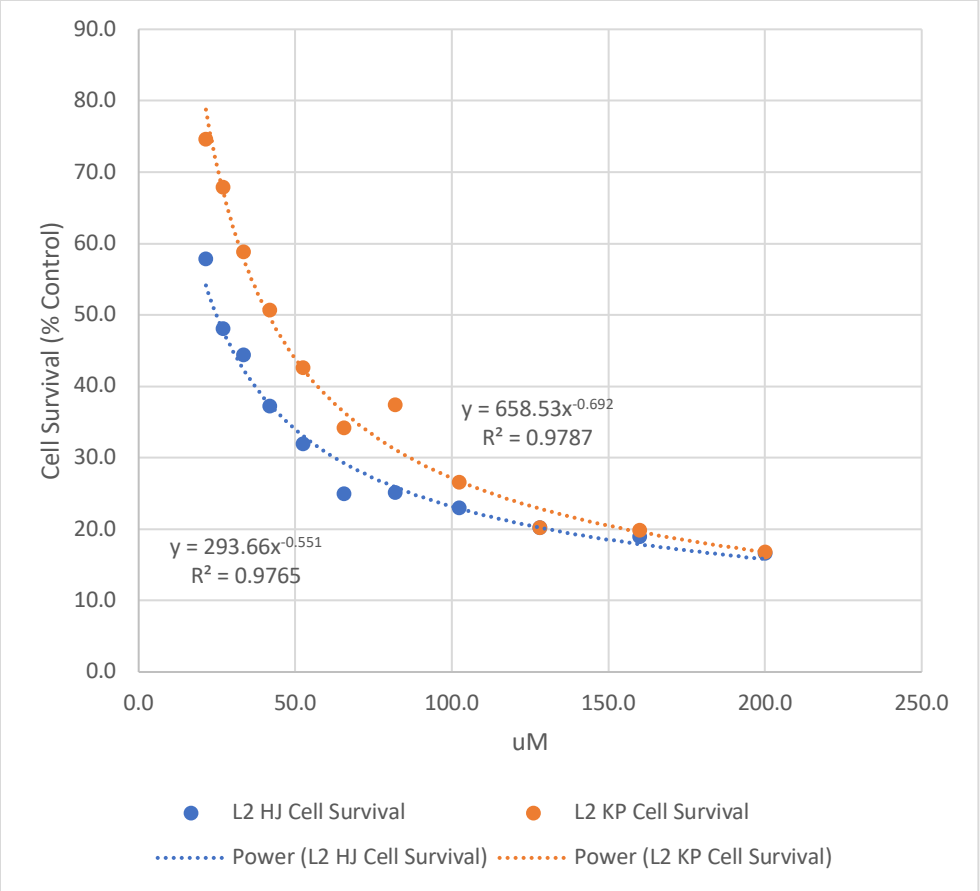

Figure 15A. Effect of PK97 and PK60 on cell survival in BV2 cells. BV2 cells were treated with the concentrations indicated and cell survival was analyzed via MTT assay.

Cytotoxicity asssays were carried out on compounds PK97 and PK60 to identify concentrationns that could be used in future experimemnts. that could be used in future experimemnts.

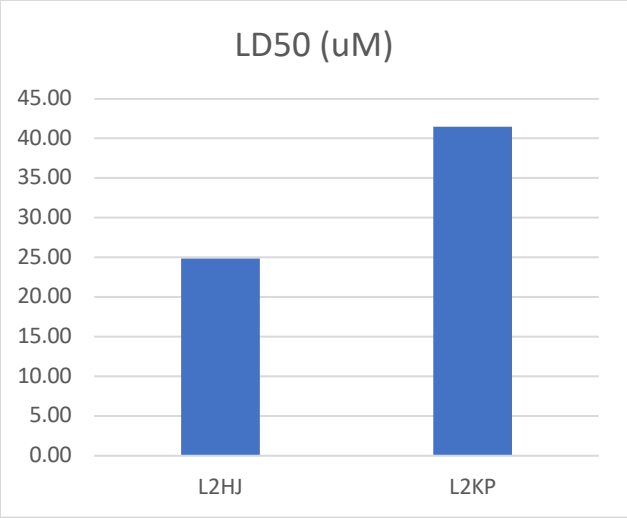

Figure 15B. Effect of TBHP, TBHQ, and H_2O_2 on HO-1 gene expression in BV2 cells. BV2 cells were treated with the concentrations indicated and mRNA was extracted and quantified.

Using the results from the MTT Assay in Fig. 15, the lethal dose, of L2HJ or L2KP, for 50% of all cells treated was calculated.

DISCUSSION AND CONCLUSIONS

The antioxidant effect of the multi-modal drugs were analyzed by measuring the -levels of Nrf2-activated antioxidative genes. These drugs were hypothesized to have a multi-modal mechanism of action: i) by exerting direct-antioxidant effects by virtue of the radical scavenging indole-like groups and the chelating center of each compound and ii) by interfering with the Keap1/Nrf2 binding complex. Docking studies conducted in Dr. Kayla Green's lab suggested that some of the compounds used in the study were capable of docking with Keap1 at its interface with Nrf2, which suggested a possible antagonistic effect between the Keap1/Nrf2 complex. This study showed that real-time Quantitative Polymerase Chain Reaction (RT-qPCR) is an effective method for analyzing the potential of novel compound by looking at downstream effects of Nrf2

activation. Our results suggest that BSO and TBHQ are consistent activators of Nrf2-induced gene activation and can be used as positive controls. TBHQ, a disruptor of the Keap1/Nrf2 binding complex, was used as a positive control in multiple experiments, showing an overall increase in HO-1 mRNA levels in fold expression levels consistently higher than 6. BSO, a direct creator of oxidative stress, was shown to induce a significant increase in HO-1 gene fold expression of 3 (Fig. 7B)

The chosen methods of experimental analysis showed promise in assessing the effectiveness of novel drugs. TBHQ, a known activator of Nrf2 translocation consistently induced HO-1 expression in a dose-dependent manner as quantified via mRNA qPCR (Fig 5 and 6). TBHQ was used in future experiments as a positive control, . Treatment with L2 induced an overall increase in the cellular production of HO-1 mRNA, indicating that the drug was able to disrupt the KEAP1/Nrf2 complex. Previous docking studies by Dr. Green hypothesized that certain drugs, including L2, could bind with higher affinity to the KEAP1 protein, disrupting the inactivation of Nrf2 and increasing gene activation. The data from the experiments using L2 and $OMePy_2N_2$ alone showed a dose-dependent increase in Nrf2 activation (Fig 10). PK60, another multi-modal drug developed by Dr. Green shows promise in activating Nrf2-dependent antioxidant response in BV2 cells. A 5-fold increase in HO-1 mRNA was seen in cells treated with PK60. (Fig. 13). Future studies should utilize other methods to quantify nuclear translocation of Nrf2 to further support the predictions from the docking studies. Other methods, like immunostaining and nuclear fluorescence, could be used to visualize and quantify the intensity of Nrf2 in cytosolic versus nuclear locations. Additionally, BSO proved to be a consistent inducer of direct oxidative

stress. This drug should be used going forward as a test of cell responses to oxidative stress alone, in comparison to H_2O_2 which did not give consistent results.

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