EFFECTS OF N-HETEROCYCLIC AMINES

ON CELL VIABILITY

by

Kimberly K. Hyde

Submitted in partial fulfillment of the requirements for Departmental
Honors in the Department of Chemistry
Texas Christian University
Fort Worth, Texas

May 3, 2013

EFFECTS OF N-HETEROCYCLIC AMINES

ON CELL VIABILITY

Project Approved:

Kayla Green, Ph.D.
Department of Chemistry
(Supervising professor)

Giridhar Akkaraju, Ph.D.
Department of Biology

David Minter, Ph.D.
Department of Chemistry

TABLE OF CONTENTS

ACKNOWLEDGMENTS

I acknowledge Dr. Kayla Green, Dr. Giri Akkaraju, and Paulina Gonzalez. Without their tireless efforts, I could not have carried out this research or written this thesis. Their unconditional patience, prioritization of my learning and timeless commitment to my success is appreciated more than they know. Additionally, all the graduate and undergraduate students in Dr. Green's and Dr. Akkaraju's lab were very helpful and supportive throughout this project. I also would like to thank TCU SERC and The Robert A. Welch Foundation (P-1760) for the financial support.

LIST OF FIGURES

LIST OF TABLES

INTRODUCTION

Research Relevance

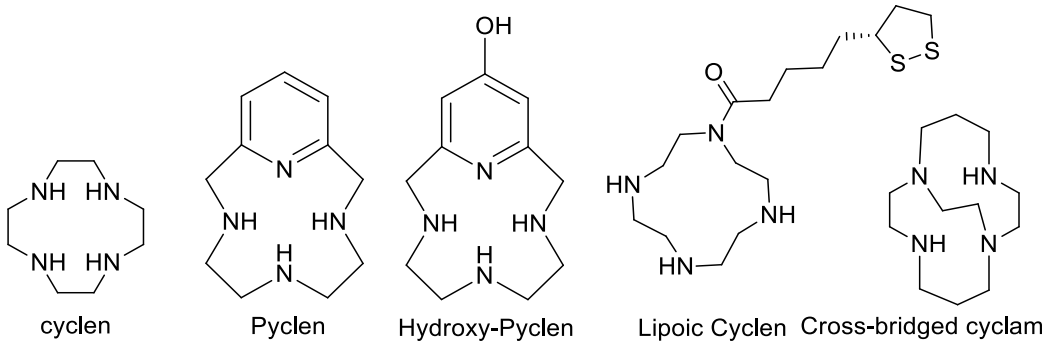
In the United States alone, Alzheimer's Disease (AD) affects greater than 5 million people and this number is expected to double by 2050 qualifying AD as the most common form of dementia[1]. AD is a progressive neurodegenerative disorder characterized by the deposition of beta-amyloid (AB) plaques, elevated levels of transition metals and oxidative stress[1]. Despite more than 100 years of interdisciplinary research on the disease, neither a definitive diagnosis nor a successful treatment has been discovered[1].

Compound Development

Many recent research efforts have focused on the 'Metal Hypothesis of Alzheimer's Disease", which postulates metal ion misregulation in the cascade leading to the physiological and pathological hallmarks of AD[2,3]. Based on this hypothesis, the emphasis of incorporating metal binding capacity into therapeutic agent development is a recent strategy utilized by numerous groups to date[4]. A recent clinical study with clioguinol (CQ), which reported improved cognition in mouse models, has since been terminated due to adverse side affects. This is a prime example of the power of chelation and the challenges associated with this capacity[5,6]. Compounds targeting inhibition of metal ion interaction with Aβ (Beta-amyloid, the aggregated protein found in brain tissue of AD patients) and metal ion homeostasis are often "limited by ion specificity, an inability to cross the blood brain barrier (BBB), and/ or biological compatibility"[4].

Conversely, therapeutic development directed only at lowering oxidant stress and not addressing divalent metal (Cu, Fe, and Zn) accumulation has produced limited efficacy[7]. In fact, chronic oxidative stress is thought to be caused in part by accumulation of divalent metals like iron or copper and a decrease in natural antioxidants[8].

The implication is that both metal chelation and antioxidant capabilities could be instrumental in therapeutic agent development. Therefore, the Green research group at TCU performed a literature search for effective biocompatible chelators capable of seeking out specific metal ions[9]. Similar to several other research groups, the literature search resulted in MRI contrast agent backbones to serve as skeletons for further compound development[9]. These MRI contrast agents are capable of chelating toxic Gadolinium ions with the cyclic amine region of the molecule[10]. When chelated in a ligand core, the toxicity of Gadolinium ions is masked while the compound maintains a level of biocompatibility. Cyclen (Table 1) is the skeleton of several contrast agents including Dotarem and Prohance[11]. Pyclen (Table 1) is the backbone of PCTA (12-dodecyloxy-3,6,9,15-tetraazabicyclo[9.3.1]pentadeca-1(15),11,13-triene-3,6,9-triacetic acid), a recently investigated prospective MRI contrast agent repurposed for therapeutic agent development[11,12]. These molecules serve as the foundation used for molecule development of the compounds discussed in this paper. The modifications to produce hydroxypyclen (HP) and lipoic cyclen (LC) (Table 1) attempt to maintain chelating ability, incorporate or enhance antioxidant properties, and increase biocompatibility of the compound[13].



	Cyclen	Pyclen	Hydroxy-pyclen (HP)	Lipoic Cyclen (LC)	CB cyclam
Metal Chelation	Yes	Yes	Yes	Yes	Yes
ROS * quenching	No	Yes	Yes	Yes	No
Radical absorbing	No	~No	Yes	Yes	No

Table 1. Properties reported for the molecules of interest.
*Reactive Oxygen Species

Both pyclen and HP have displayed reactive oxygen species (ROS) quenching ability due to the aromaticity of the pyridine ring[13]. The conversion of the pyridine into a pyridol was facilitated by lab-mate Kimberly Lincoln to create hydroxypyclen[13]. The pyridol functional group resembles tannins, a well-known, powerful antioxidant, and not surprisingly enables HP to absorb hydroxyl radicals[13].

Paulina Gonzalez, another lab-mate, developed and synthesized lipoic cyclen (LC), which added lipoic acid to cyclen[14]. The cyclen portion offered the N-heterocyclic amine, a strong metal chelator specific for metals associated with AD[9]. Lipoic Acid (LA) is not only a good ligand set for divalent metals found *in vitro* but also is capable of enhancing endogenous antioxidants[15]. LA has been shown to increase expression of γ-glutamylcysteine ligase, the rate-controlling enzyme for

glutathione (GSH) synthesis, as well as recycle antioxidants like ascorbate and GSH by reduction as DHLA[15]. Suh and colleagues showed dietary LA to restore normal iron status in aged mice as well as reverse the decline in tissue ascorbate and GSH levels associated with old age[15]. Suh's study and that of others offered a rationale for the construction of a hybrid molecule composed of lipoic acid and cyclen. Post development, studies were carried out to compare the antioxidant ability of cyclen and lipoic acid versus the hybrid molecule lipoic cyclen (LC). Results show that LC maintained the metal chelation abilities of cyclen as well as the antioxidant abilities of lipoic acid[14].

Finally, CB Cyclam (Table 1) is another MRI contrast agent skeleton used as a treatment in the HEK 293 cell line in this study. It served as another control to cyclen and pyclen abilities.

Cell Line Background

The use of Human Embryonic Kidney (HEK) 293 cells was a primitive step for this project. HEK 293 cells are human, epithelial in morphology and relatively tolerant for exploring *in vitro* sensitivity to drug treatment. This was a good opportunity to develop a live culture technique and troubleshoot the challenges associated with the MTT (3-(4,5-dimethylthiazolyl-2)-2, 5-diphenyltetrazolium bromide) assay, which is used in determination of cell line sensitivity to drug treatment.

HT22 cells are murine neuronal cells isolated from the hippocampus. The hippocampus regulates spatial orientation and both short and long term memory.

This cell line was used because of its hippocampal origin, which is a major target of physiological effects associated with Alzheimer's disease[16,17,18].

Finally, Friedreich's Ataxia (FRDA) is an autosomal recessive neurodegenerative disease[19]. FRDA fibroblasts contain a misexpressed frataxin gene of the mitochondria in which an expansion of the first intron occurs and subsequently inhibits gene transcription and replication[19]. FRDA fibroblasts can be characterized by an increased sensitivity to oxidative stress induced by ROS. Additionally, misregulated metal ion pathways result in an accumulation of Fe and Cu with up to 40% greater iron content than normal fibroblasts[19]. Wong and colleagues propose this cell line as a potential model system for sensitivity of cells to oxidant-induced death[19]. Currently, a similar series of cellular studies imposed on both HEK 293 and HT22 cell lines is in progress.

MATERIALS AND METHODS

Cell Culture

The mouse neuronal hippocampal cell line (HT22) and the fibroblasts from Friedreich's Ataxia patients were obtained from the UNT Health Science Research Center. The human embryonic kidney cell line (HEK 293) was obtained from ATCC (Manassas, VA). HT22 and HEK 293 cells were cultured in DMEM (Dulbecco's Modified Eagle Medium) supplemented with 10% fetal bovine serum, 2 mM glutamine, 100 μg/mL streptomycin, 100 μg/mL penicillin, and 0.1 mM non-essential amino acids. Both cell lines were grown in a water-jacketed incubator at 37°C with 5% CO_2 and 95% air atmospheric conditions.

Determination of Cell Viability*

To perform this assay $5x10^3$ cells/well were plated into 96 well plates for 24 hours. They were then treated with increasing concentrations of cyclen, pyclen, LC, HP, CB cyclam, BSO (Buthionine sulfoximine), or etoposide in replicates of 4 or 8 and incubated for 24 hours at 37°C. After incubation, the assay was used to determine the extent of cell death. Medium was replaced by 100 μL of 1 mg/mL MTT (Sigma Aldrich) in serum free medium. The cells were then incubated for 4 hours at 37°C, after which MTT was replaced by 100 μL of DMSO to solubilize the precipitate. The plate was placed in a shaker for 5 minutes and absorbance was measured at 540nm using a plate reader (Molecular Dynamics). Statistical analysis was used to eliminate outliers and a graph of the data was produced as a percentage of cell viability with respect to drug concentration. EC_{50} values were calculated using the MasterPlex program. **This protocol was adapted from the work of Gladys Jebiwot Keitany, TCU '02.*[20]

CYTOTOXICITY PLATES

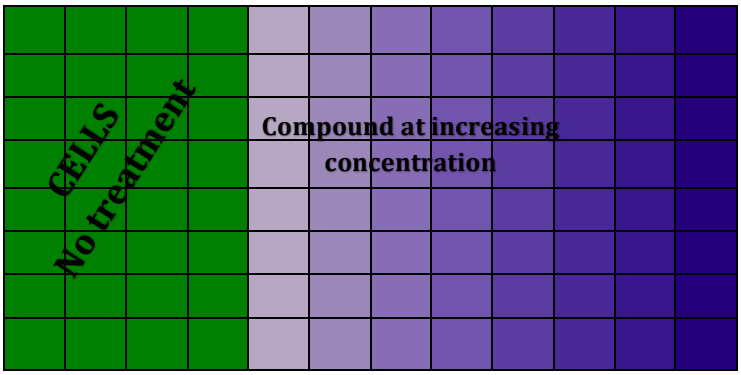


Figure 1. Cytotoxicity plate design for HEK 293 and HT22 cellular studies. Results in figures 4-11.

BSO

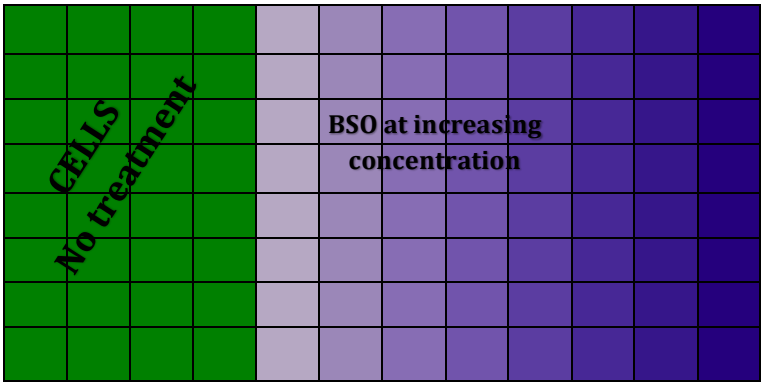


Figure 2. BSO as an insult plate design for HEK 293 and HT22 cellular studies. Results in figures 13-19.
Corresponding number of increments varied between experiments.

Rescue Capacity

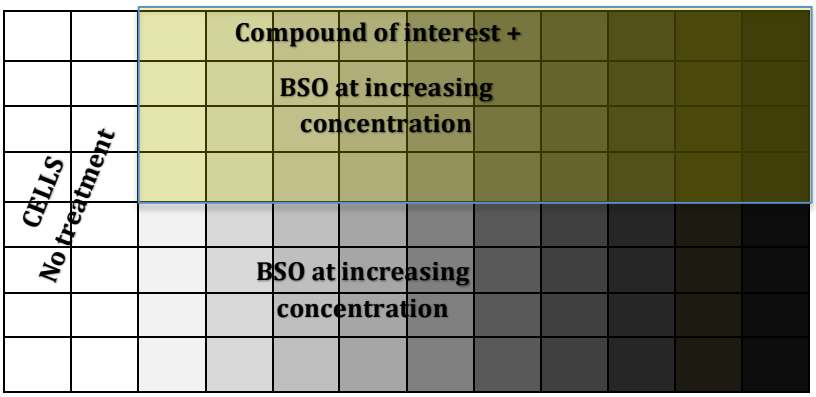


Figure 3. Rescue capacity plate design for HEK 293 cellular studies. Results in figures 20-25.

Compounds of Interest

Pyclen and CB-cyclam were gifts from Zoltan Kovacs at UT Southwestern Medical Center (Dallas, TX). Lipoic cyclen was produced by lab-mate Paulina Gonzalez[14]. Hydroxy-pyclen was produced by lab-mate Kimberly Lincoln[13]. All other reagents were purchased from commercial sources and used as received unless noted otherwise.

RESULTS AND DISCUSSION

The results of this study aimed to investigate HEK 293 and HT22 susceptibility to drug treatment and characterize the MRI contrast agents and their derivatives in each cell line. Experiments were directed at obtaining cytotoxicity of the drugs, sensitivity to BSO, and rescue capacity of the drugs from BSO induced oxidative stress. The MTT assay was used to determine cell line sensitivity to the drugs used in this study[20]. This assay utilizes the ability of live cells to metabolically reduce yellow tetrazolium MTT in part by the action of dehydrogenase enzymes to generate NADH and NADPH[20]. The final product is a purple formazan, which can be solubilized by solvents like DMSO and quantified using a spectrophotometer[20]. The intensity of the purple color is directly related to the number of live cells[20].

Cytotoxicity studies were carried out by treating a plate of cells with increasing concentrations of each drug of interest. In the HEK 293 cell line, cytotoxicity studies were completed with cyclen, LC, CB cyclam and etoposide. It should be noted that etoposide is a DNA damaging agent, which induces cell death by a general caspase cascade and serves as a control for cell death in most cell lines[20]. In the HT22 cell line, cytotoxicity studies were performed with cyclen, LC, pyclen, and HP. Drug choice was largely limited by availability during the timeframe of this thesis. The concentrations reported reflect the final concentration in each well. Data were normalized to untreated cells.

Cytotoxicity

HEK 293

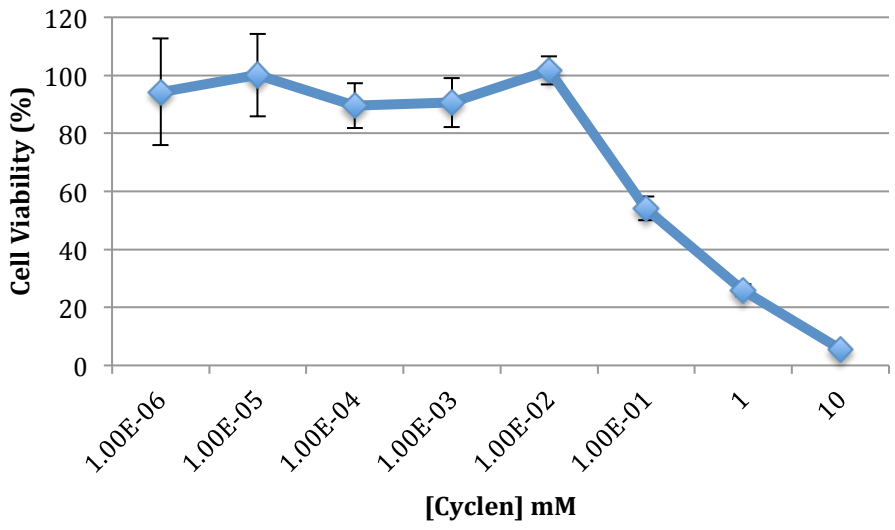


Figure 4. MTT colorimetric response in HEK 293 cells after 24 hours of exposure to cyclen [1 nM-10 mM].

The cytotoxicity of cyclen was studied in the HEK 293 cell line. Results are shown in figure 4. HEK 293 cells were treated with a cyclen concentration range of 1 nm to 10 mM. From 1 nM to 10 μM of cyclen less than 10% cell death was evoked while the 100 μM to 10 mM range exhibited a moderately inverse relationship between cyclen concentration and cell viability. This result indicates the μM cyclen range is pivotal in the transition from less than 40% cell death. It appears that cyclen does not strongly affect cell viability until it approaches 100 μM. At this point, it has been hypothesized that cyclen begins interrupting normal cellular function likely in the form of metal ion pathway interference due to its inherent metal chelation ability. The HEK 293 cell line demonstrates fully functional metabolic pathways and is likely able to subdue cyclen via natural defense mechanisms until a

threshold of 100 μM is reached, when cyclen becomes a detriment to normal cell function. This implies that in order for cyclen to be used in HEK 293 cells without inflicting greater than 40% cell death, concentrations must be kept below 100 μM.

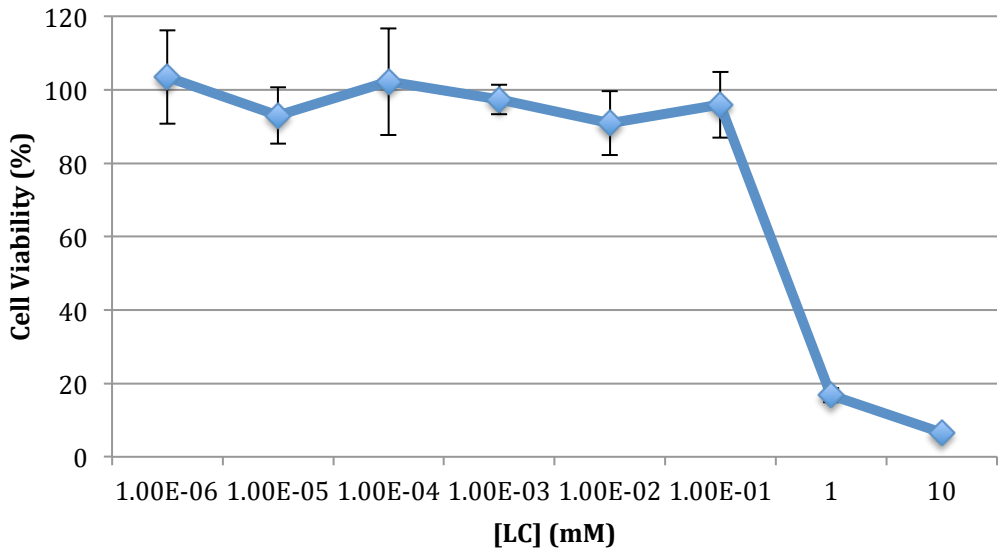


Figure 5. MTT colorimetric response in HEK 293 cells after 24 hours of exposure to LC [1 nM-10 mM].

The cytotoxicity of LC was studied in the HEK 293 cell line. Results are shown in figure 5 where HEK 293 cells were treated with an LC concentration range of 1 nm to 10 mM. From 1 nM to 100 μM of LC approximately 10% cell death was evoked while a sudden decline in viability occurred between 100 μM and 1 mM where less than 20% of cells survived the treatment. It appears that LC does not strongly disrupt normal cell function until a concentration of 1 mM is reached. The abrupt decline in viability insinuates some sort of threshold mechanism. Either, LC suddenly outcompetes some other substrate in a metal ion pathway for metal

chelation, or LC reaches the concentration where any drug will cause disruption to normal cell function. It should be noted that HEK 293 cells seem to tolerate LC at a concentration magnitude higher than cyclen. This supports the hypothesis that the lipoic acid addition either conveys some advantage in the cell line or to some extent masks toxicity of cyclen. This result implies that in order for LC to be used in HEK 293 cells without inflicting greater than 40% cell death, concentrations must be kept below 1 mM. Further experimentation attempting to define closer the threshold concentration by introducing increments between 100 μM and 1 mM has been considered.

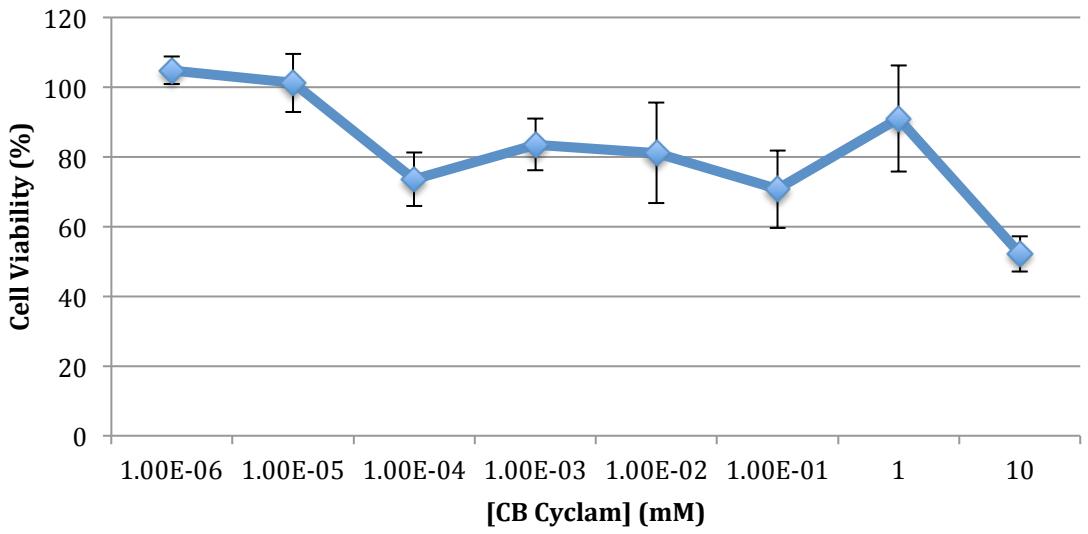
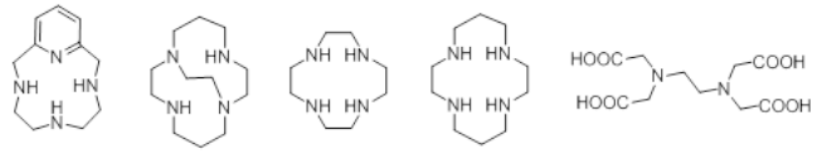


Figure 6. MTT colorimetric response in HEK 293 cells after 24 hours of exposure to CB Cyclam [1 nM-10 mM].

The cytotoxicity of CB cyclam was studied in the HEK 293 cell line. Results are shown in figure 6 where HEK 293 cells were treated with a CB cyclam concentration range of 1 nm to 10 mM. Concentrations of 1 nM to 1 mM of CB

cyclam elicited less than 40% cell death while 10 mM of CB cyclam approaches approximately 50% cell death. It appears that CB cyclam does not strongly interrupt normal cell function at any concentrations in the experimental range besides 10 mM, which is still weak (~52% cell viability). CB cyclam was included in this experiment set as a sort of control. It's reasonable that treatment evoked little cell death overall considering is a commercially available macrocyclic ligand used in contrast imaging. It should be noted that the binding constant for CB cyclam is slightly larger than cyclen when measured with Zn^{II} and Cu^{II} (Table 2). Based on this information, it is expected that CB cyclam would interfere with metal ion pathways more strongly than cyclen and subsequently cause a similar stronger response in cell death. This challenges the mechanism proposed for cylcen's impact on viability in the HEK 293 cell line. There is a response observed which has some correlation with increased concentration, but the exact mechanism by which it is impacting viability is still unknown. Further experimentation to confirm cyclen and the other drugs are entering the cells and characterization of the mechanism for increasing cell death would be beneficial. This could possibly be done by addition of a fluorescent marker to the compounds of interest. It's also plausible and likely in fact that almost any drug in the mM concentration range can cause problems with normal cell functioning. The experimental implication of this result is that CB cyclam concentrations for treatment on HEK 293 cells should not exceed 10 mM to achieve greater than 60% cell viability consistently.

Chart 1



Ka binding constants					
Cu^{II}	20	>27	23	27	9.18
Zn^{II}	14	> 15	16	15	8.41

TABLE 2. K_A binding constants for a selection of compounds.

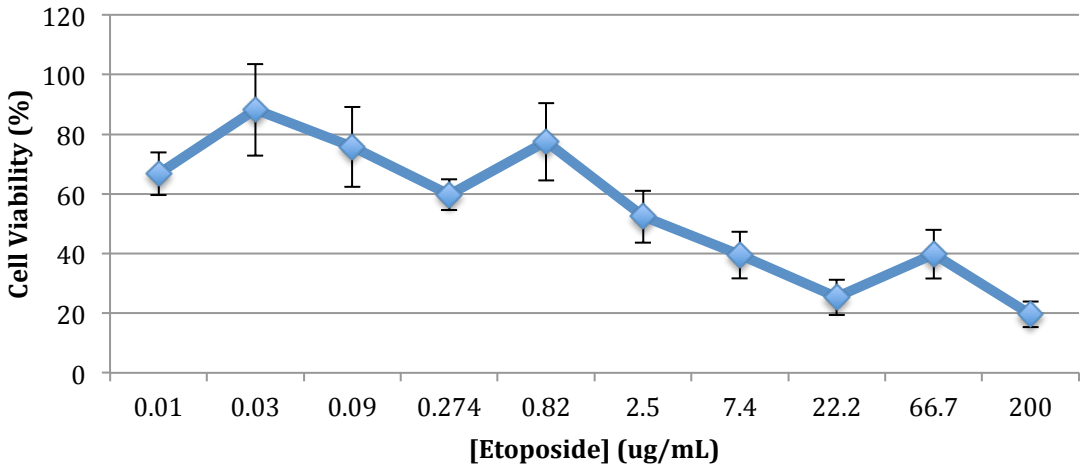


Figure 7. MTT colorimetric response in HEK 293 cells after 24 hours of exposure to etoposide [0.01 μg/mL – 200 μg/mL].

Finally, the cytotoxicity of etoposide was studied in the HEK 293 cell line as a control for cell death and assay success. Results are shown in figure 7. HEK 293 cells were treated with an etoposide concentration range of 0.01 μg/mL – 200 μg/mL Etoposide elicited a response in HEK 293 cells and trended an indirect relationship between etoposide concentration and cell viability. Distinguishing a concentration range that consistently achieved less than 40% cell death is not carried out for this

study because the mechanism by which etoposide decreases cell viability is unrelated to metal chelation and antioxidant capabilities which the compounds of interest exhibit.

HT22

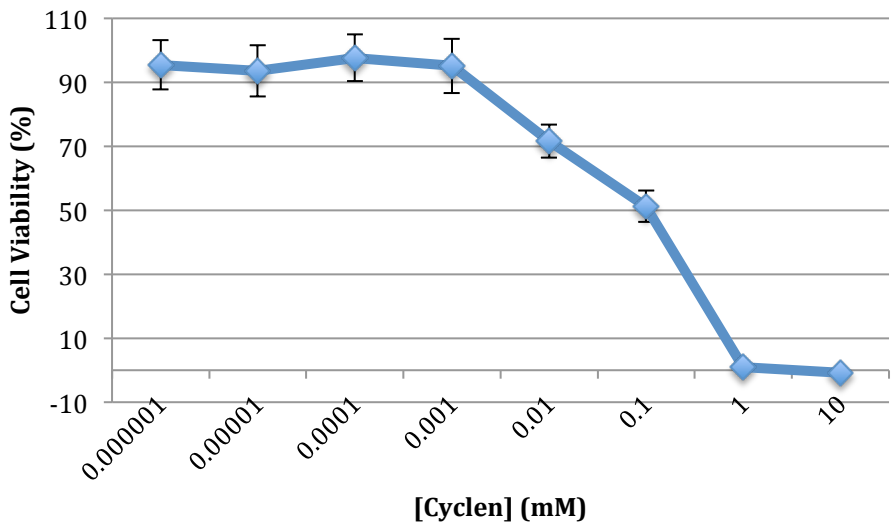


Figure 8. MTT colorimetric response of HT22 cells after 24 hours of exposure to cyclen [1 nM-10 mM].

The cytotoxicity of cyclen was studied in the HT22 cell line. Results are shown in figure 8 where HT22 cells were treated with a cyclen concentration range of 1 nm to 10 mM. From 1 nM to 10 μM of cyclen less than 40% cell death was evoked while the 100 μM cyclen concentration represents the transition to less than 60% cell viability. Additionally, the mM concentration range is characterized by complete cell death. This infers cyclen does not interfere strongly with normal HT22 cellular function until 100 uM. At this point, it's hypothesized that cyclen begins interrupting cellular function most likely in the form of metal ion pathway interference due to its inherent metal chelation capabilities. This results in the

decline in viability shown between 10 μM and 100 μM. It should be noted that similar to HEK 293 cells, the HT22 cell line demonstrates fully functional metabolic pathways and is likely able to subdue cyclen via natural defense mechanisms until a threshold of 100 μM is reached at which point cyclen begins to impair normal cell function. This implies that in order for cyclen to be used in HT22 cells without eliciting greater than 40% cell death, concentrations must be kept below 100 μM. Further experimentation to classify the mechanism by which cyclen elicits cell death might include treatment with CB cyclam as performed in the HEK 293 cell line. If CB cyclam results were similar to the HT22 response to increasing cyclen concentration, the claim identifying metal ion pathway interference would be supported. Additionally, as suggested in the HEK 293 cell line, efforts to closely track the compounds of interest could help answer questions regarding the mechanism of cell death within each cell line.

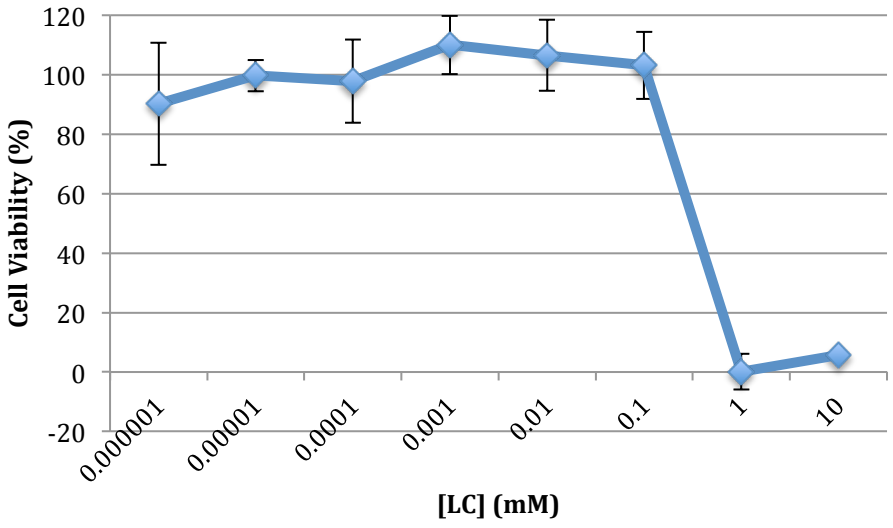


Figure 9. MTT colorimetric response of HT22 cells after 24 hours of exposure to LC [1nM-10mM].

The cytotoxicity of LC was studied in the HT22 cell line. Results are shown in figure 9 where HT22 cells were treated with an LC concentration range of 1 nm to 10 mM. From 1 nM to 100 μM of LC less than 20% cell death was evoked. In fact, HT22 cells treated in the μM range actually display an approximately 10% increase in viability compared to untreated cells. This observation supports the hypothesis that the lipoic acid addition either conveys some advantage in the cell line or to some extent masks toxicity of cyclen. LC may be better tolerated because of its antioxidant capabilities, which could improve cellular wellbeing, outweighing the metal pathway interference until 1 mM. This increased tolerance is a similar trend to the results found in the HEK 293 cell line. It will be worthwhile in future studies to replicate this experiment in more cell lines to categorize LC as more biocompatible than cyclen and maybe the other compounds developed. It would be interesting to treat HT22 cells with only lipoic acid to determine if the addition

presents an increase in viability compared to untreated cells. This could help in identifying the mechanism by which LC seems to improve normal functioning cells' viability. Beyond the μM range, the transition to 1 mM was accompanied by a significant decline in viability where less than 10% of cells survived the treatment. Similar to the HEK 293 cell line reaction to LC (figure 5), the staggering decline in viability insinuates some sort of threshold mechanism. It's possible that the metabolic pathway LC interferes with is able to compensate until 1 mM when LC outcompetes some other substrate for metal chelation. Alternatively, 1 mM LC may be the concentration at which any drug impairs normal cell function. This implies that in order for LC to be used in HT22 cells without inflicting greater than 40% cell death, concentrations must be kept below 1 mM. It should also be noted that cells seem to tolerate LC at a concentration magnitude higher than cyclen.

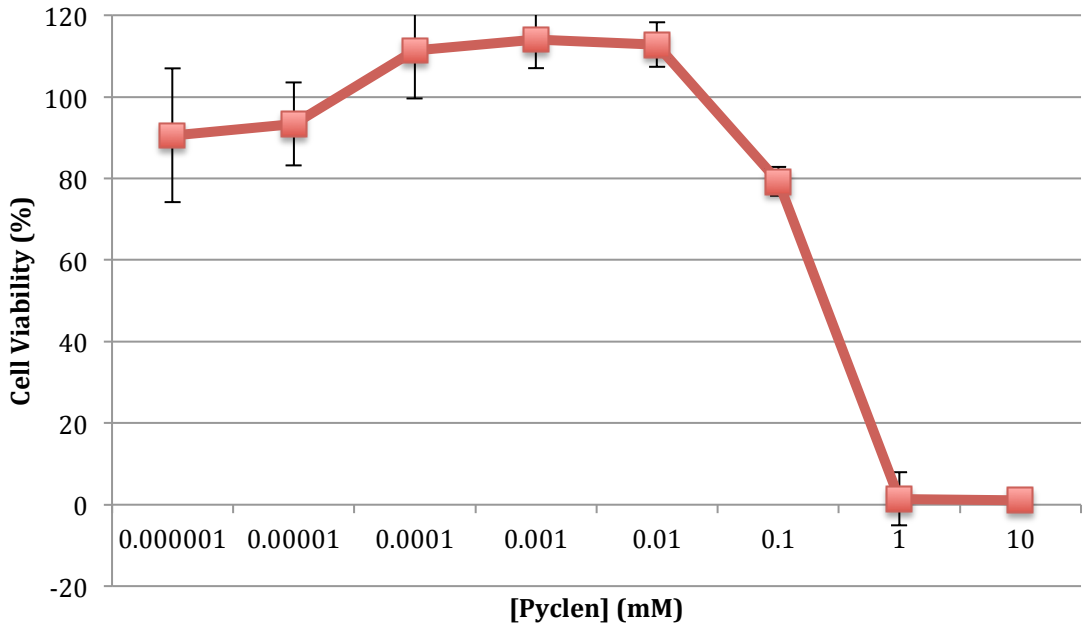


Figure 10. MTT colorimetric response of HT22 cells after 24 hours of exposure to pyclen [1 nM-10 mM].

The cytotoxicity of pyclen was studied in the HT22 cell line. Results are shown in figure 10. HT22 cells were treated with a pyclen concentration range of 1 nm to 10 mM. From 1 nM to 100 μM of pyclen less than 20% cell death was evoked. In fact, the 100 nM–10 μM concentration range actually shows an approximately 10% increase in viability compared to the untreated cells similar to the trend observed in LC treatment on HT22 cells. It's possible the ROS quenching nature of the aromatic portion of pyclen serves to improve viability of cells treated within the 100 nM-10 μM range, but the metal chelating nature of the cyclic amine begins to disrupt metal ion pathways and contribute to cell death between 100 μM and 1 mM where less than 10% of cells survived. This drastic response implies that pyclen affects HT22 cell viability in some sort of threshold mechanism. Either it suddenly

out-competes some other substrate for binding, or it reaches the concentration where any drug will cause disruption to normal cell function. Regardless, the advantage conveyed between 100 nM and 10 μM becomes outweighed at 1 mM. This suggests that in order for pyclen to be used in HT22 cells without inflicting greater than 40% cell death, concentrations should be kept below 1 mM. It should be noted that like LC, pyclen is better tolerated than cyclen by a concentration magnitude.

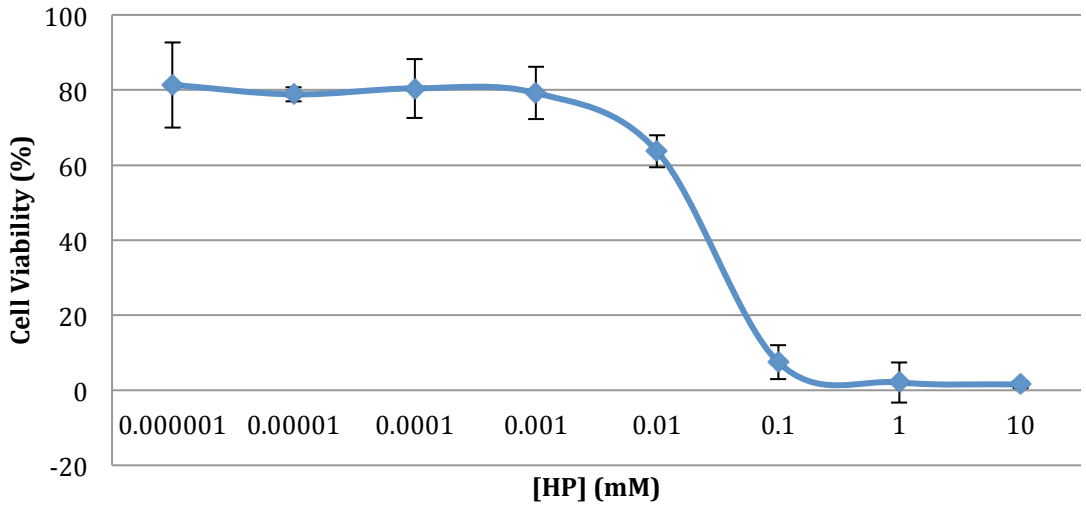


Figure 11. MTT colorimetric response of HT22 cells after 24 hours of exposure to HP [1 nM-10 mM].

The cytotoxicity of HP was studied in the HT22 cell line. Results are shown in figure 11 where HT22 cells were treated with an HP concentration range of 1 nm to 10 mM. Concentrations between 1 nM and 1 μM elicited approximately 20% cell death, while 10 μM HP approaches 60% cell viability. It appears that HP affects cell viability more strongly than pyclen, cyclen, and CB cyclam in the HT22 cell line. HP

is less tolerated overall and reaches greater than 40% cell death at a concentration magnitude lower than the other compounds. Additionally, it confers no advantage compared to untreated cells. This is surprising considering HP has been shown to quench ROS and even radicals more effectively than pyclen. This suggests that the antioxidant capacity of pyclen may not be the only characteristic that confers an advantage in viability compared to untreated cells. It would be interesting to track ROS presence via the DCFH-DA (Dichloro-dihydro-fluorescein-diacetate) assay to further characterize the mechanism by which HP and the other compounds of interest elicit cell death. The experimental implication of this result is that concentrations of HP should be kept below 100 μM if attaining less than 40% cell death is desirable.

The next set of experiments was carried out to characterize HEK 293 and HT22 sensitivity to BSO. BSO is chemotherapeutic agent which reduces intracellular levels of glutathione (GSH), a natural antioxidant, by inhibiting the synthetic pathway as shown below in Figure 12. BSO directly affects the cellular levels of ROS.

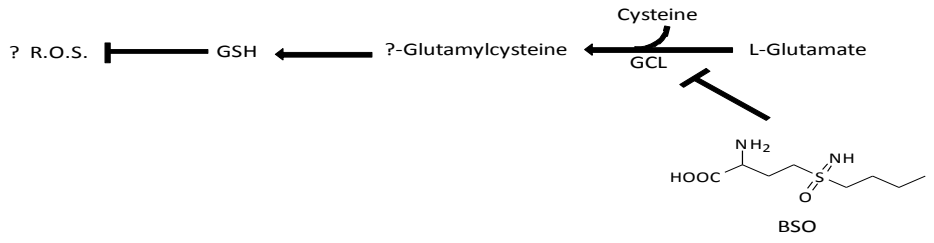


Figure 12. Scheme of BSO inhibition of glutathione synthesis.

BSO insult

HEK 293

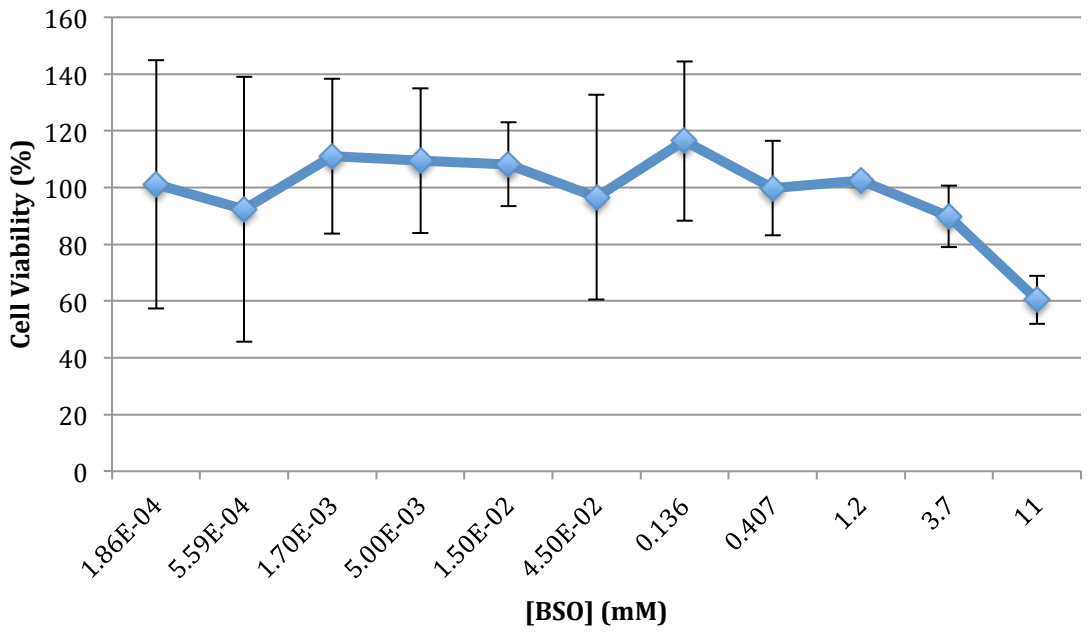


Figure 13. MTT colorimetric response of HEK 293 cells after 24 hours of exposure to BSO [186 nM-11 mM].

The toxicity of BSO was studied in the HEK 293 cell line. Results are shown in figures 13 and 14. In figure 13, the cells were treated with a concentration range of BSO from 186 nM – 11 mM. From 186nM to 3.7mM, less than 40% cell death were elicited. The viability between 3.7 mM and 11 mM (a more focused concentration range) approached less than 60% cell viability, though additional data was desired to assure BSO could elicit cell death in HEK 293 cells. These results prompted an additional experiment with a BSO concentration range from 137 μM to 100 mM. These results can be found in Figure 14.

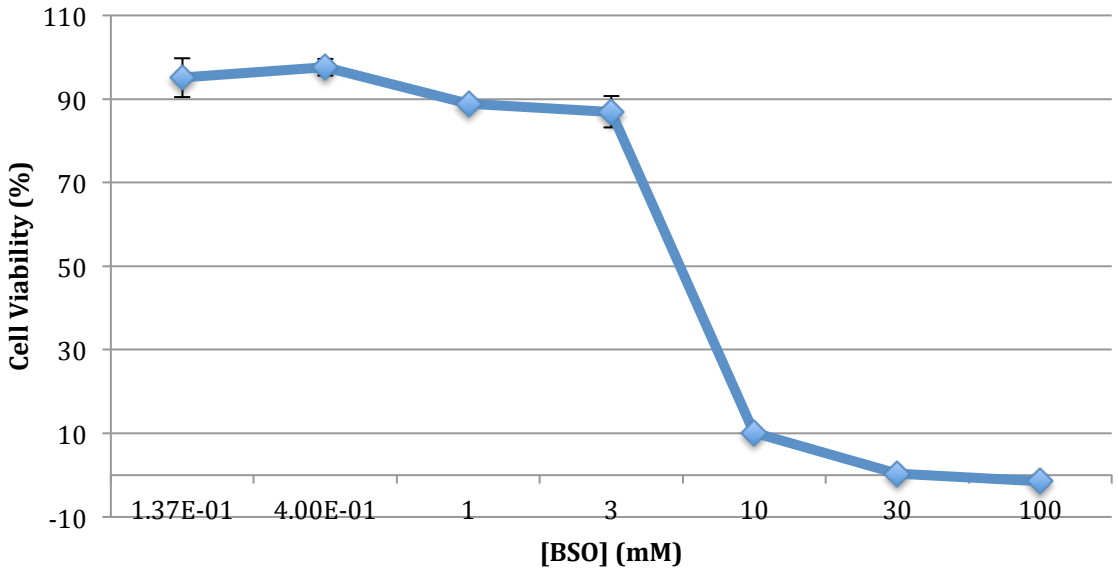


Figure 14. MTT colorimetric response of HEK 293 cells after 24 hours of exposure to BSO [137 μM-100 mM].

The treatment of HEK 293 cells with a BSO concentration range from 137 μM to 3 mM (figure 14) resulted in less than 40% cell death, but is followed by a staggering decline in viability between 3 mM and 10 mM where less than 10% of cells treated survived. The sharp decline in viability could be a problem of increment size. It's possible that smaller increments would produce a more linear indirect relationship between cell viability and BSO concentration. Alternatively, this trend could be the result of a threshold breech where inhibited production of GSH catches up to the cell and ROS accumulation contributes to an abrupt transition to almost complete cell death. The metabolic pathways in the HEK 293 cell line are unaltered and fully functional to our knowledge. This supports the claim that natural cellular defenses countered the GSH synthesis inhibition up to a particular concentration of BSO where the ROS concentration outweighs the cells ability to

counter the effect on cell viability without GSH. This suggests that approximately 3 mM BSO is the highest concentration that maintains greater than 60% cell viability. Further experimentation to characterize the region of the staggering decline in viability could be helpful. The reason this experiment has not already been carried out is as follows. Further experimentation regarding the compounds of interests' ability to improve cell viability from BSO induced oxidative stress was carried out with a range of BSO concentrations from 1.7 μM to 33 mM. This encompasses the staggering decrease in viability. The results in figures 13 and 14 support the hypothesis that the HEK 293 cell viability is affected by increasing BSO concentration. We suspect the increased cell death is the result of ROS accumulation, and hope to support this suspicion further via DCFH Assay, which allows quantification of ROS levels via fluorescence.

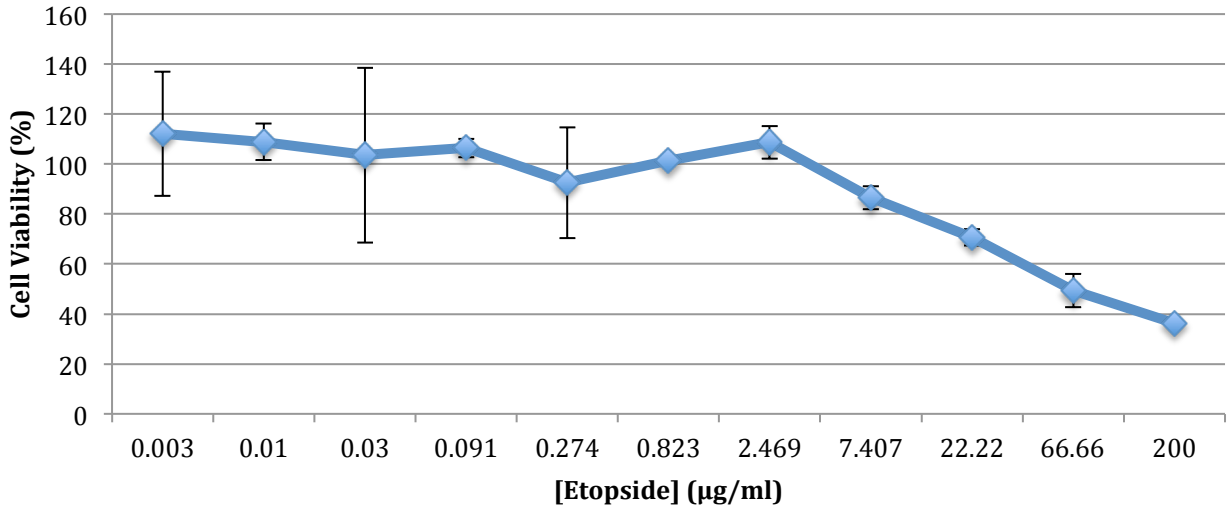


Figure 15. MTT colorimetric response of HEK 293 cells after 24 hours of exposure to Etoposide [0.003 μg/mL- 200 μg/mL].

Figure 15 is a control experiment in which HEK 293 cells are treated with a range of etoposide from 0.003 μg/mL to 200 μg/mL. The experiment is included to assure the assay conveys a response of the HEK 293 cells to increasing etoposide concentration. The results indicate that there was in fact a reaction to the DNA damaging agent and the assay, which was used in the same experiment set as the results in figure 13, was successful in reflecting results of the treatment.

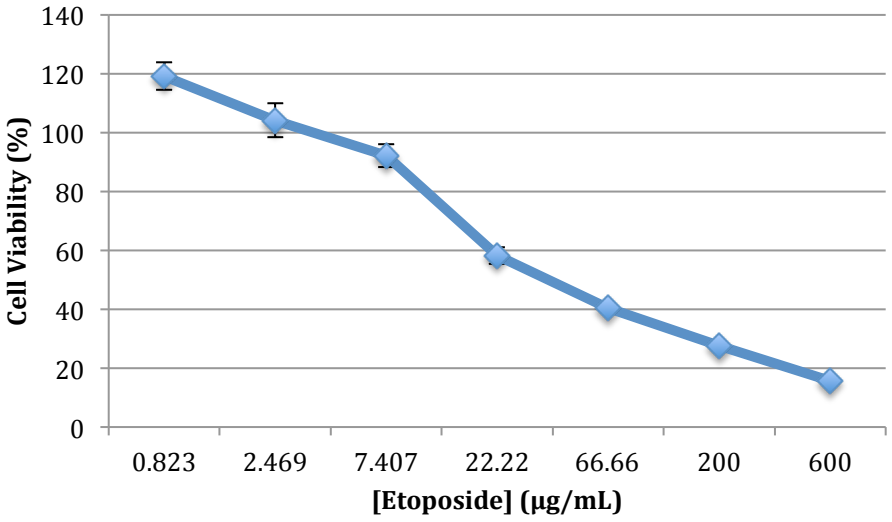


Figure 16. MTT colorimetric response of HEK 293 cells after 24 hours of exposure to Etoposide [0.823 μg/mL – 600 μg/mL].

Figure 16 exhibits the results from a control experiment in which HEK 293 cells are treated with a range of etoposide from 0.823 μg/mL to 600 μg/mL. This concentration range was shifted from the previous etoposide experiment in attempts to reach complete cell death. The experiment is included for general characterization purposes and to assure the assay conveys a response of the HEK 293 cells to increasing etoposide concentration. The results indicate that there was

in fact a reaction and the assay, which was used in the same experiment set as the results in figure 14, was successful in representing the results of the treatment.

HT22

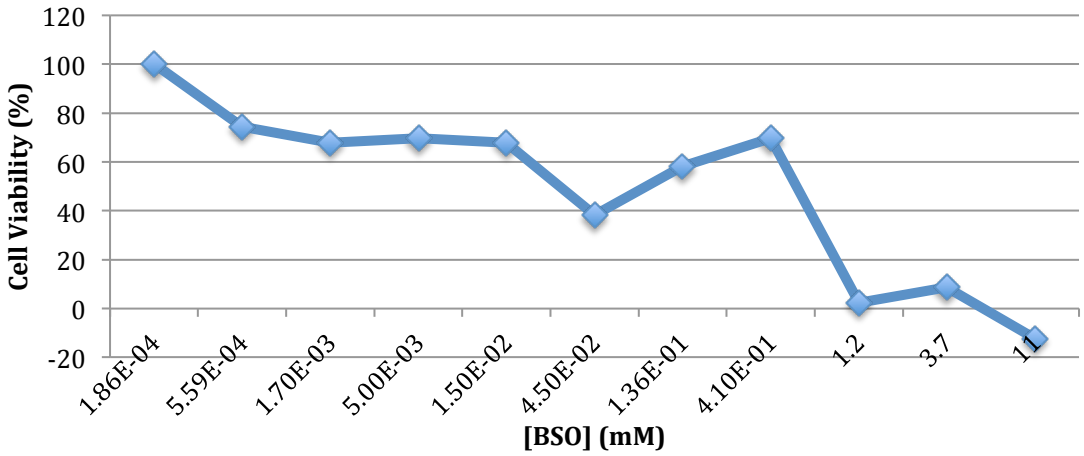


Figure 17. MTT colorimetric response of HT22 cells after 24 hours of exposure to BSO [186 nM-11 mM].

The toxicity of BSO was studied in the HT22 cell line. Results are shown in Figures 17, 18, and 19. In figure 17, the cells were treated with a BSO concentration range of 186 nM – 11 mM. BSO treatment resulted in less than 40% cell death between 186 nM and 15 uM. From this concentration, it's most valuable to consider the general trend where greater than 40% cell death is attained and surpassed by complete cell death at 1.2 mM. This response supports the hypothesis that HT22 cell viability is affected by increasing BSO concentration. We suspect the increased cell death is the result of ROS accumulation and hope to support this suspicion further via DCFH Assay, which allows quantification of ROS levels via fluorescence. The lack

of fit to linearity prompted further experimentation to characterize the concentration range in question.

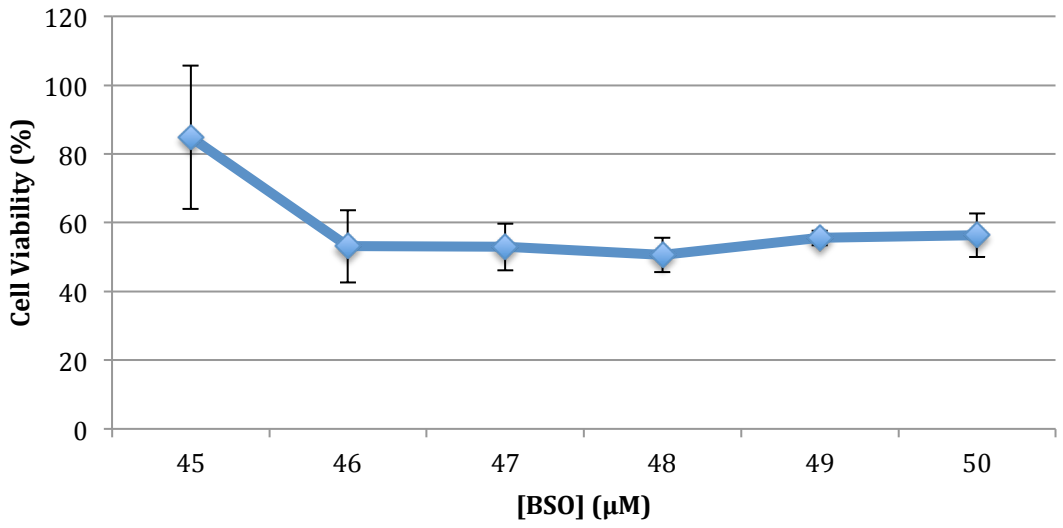


Figure 18. MTT colorimetric response of HT22 cells after 24 hours of exposure to BSO [45 μM-50 μM].

Figure 18 displays HT22 cellular response when treated with a BSO concentration range from 45 μM to 50 μM. This experiment was prompted by the poor fit to linearity observed in Figure 17 that surrounded the 45 μM range. This study emphasized the efforts to find a BSO concentration range that consistently achieves approximately 40% cell death. We hypothesized that a compound with antioxidant capabilities could improve viability of cells in this condition. The experimental implication of these results is that a concentration range established from an approximately 50 μM midpoint of BSO would result in a reasonable range to pursue for observing rescue by an antioxidant compound.

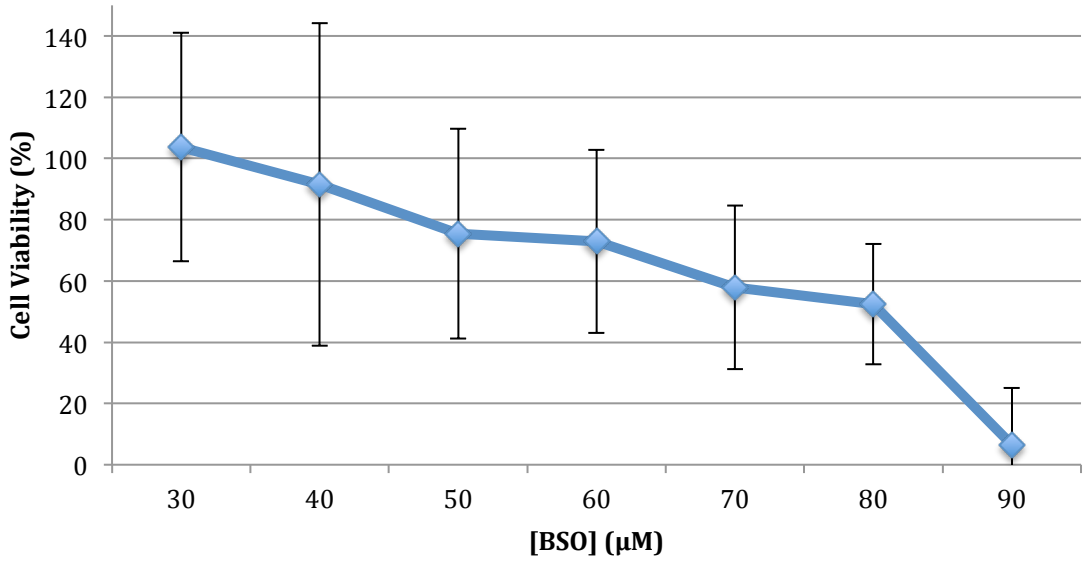


Figure 19. MTT colorimetric response HT22 cells after 24 hours of exposure to BSO [30 μM-90 μM].

Figure 19 exhibits the HT22 cellular response when treated with a BSO concentration range of 30 μM – 90 μM for 24 hours of incubation before the MTT assay was utilized to process the cells. Although the error bars are not ideal, the overall trend of this system can be extrapolated and thus establishes the ability of BSO to elicit cellular response in HT22 cells. Additionally, it designates the μM BSO range as pivotal for transition to greater than 40% cell death in the HT22 cell line.

This final section describes the rescue capacity of cyclen, LC, lipoic acid, and etoposide from BSO induced oxidative stress in HEK 293 cells. In these experiments, half the cells were treated with BSO and the other half were treated with both BSO and a drug of interest. This experiment was carried out with the HT22 cell line, but was unsuccessful in obtaining responsive data. It's likely that the cell passage number was too high. When this occurs, cells can become nonresponsive to

treatment. The experiment was intended to be repeated, but has not since been carried out due to cell availability and timing problems. These challenges highlight a few of the limitations of *in vitro* studies.

Rescue Capacity

HEK 293

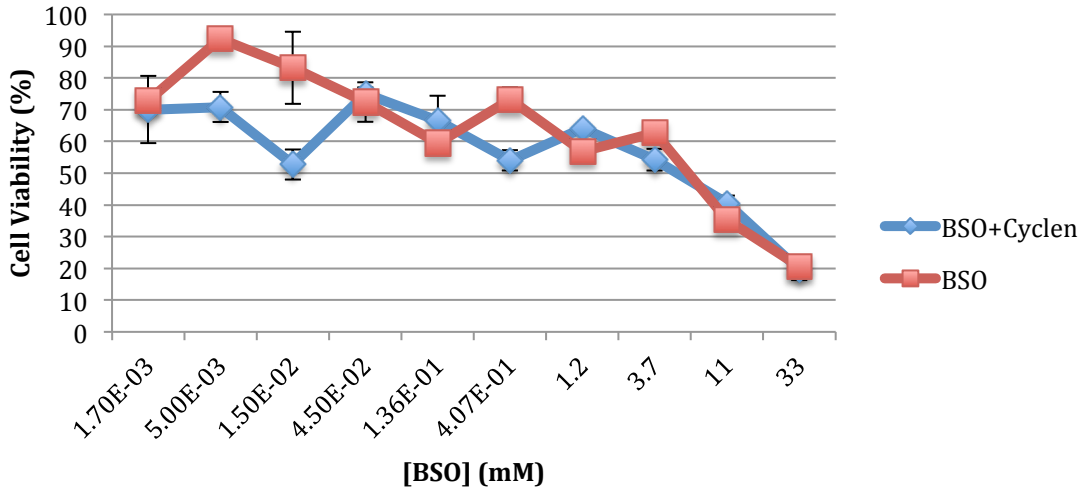


Figure 20. MTT colorimetric response HEK 293 cells after 24 hours of exposure to BSO [1.7 μM-33 mM] and Cyclen [10 μM].

The Green research group has shown previously that HP is capable of rescuing BSO induced cell death by the addition of HP while cyclen had no effect[1, 2]. The ability of cyclen to affect viability of cells treated with BSO was studied in HEK 293 cells. Results are shown in Figure 20. As a control, half the plated cells were treated with a BSO concentration range of 1.7 μM to 33 mM. In contrast, another plate was treated with the same BSO concentration range and 10 μM cyclen. The

cells treated with BSO show the same trends as observed in previous studies. The addition of cyclen at 10 μM does not produce a distinguishable difference in cell viability. This result was supported by the studies showing cyclen unable to quench ROS to an appreciable extent[7]. Further, cyclen and BSO do not appear to increase cell death synergistically. The implication of this study is that cyclen is not capable of improving HEK 293 cell viability when also treated with BSO, which subsequently increases ROS and cell death.

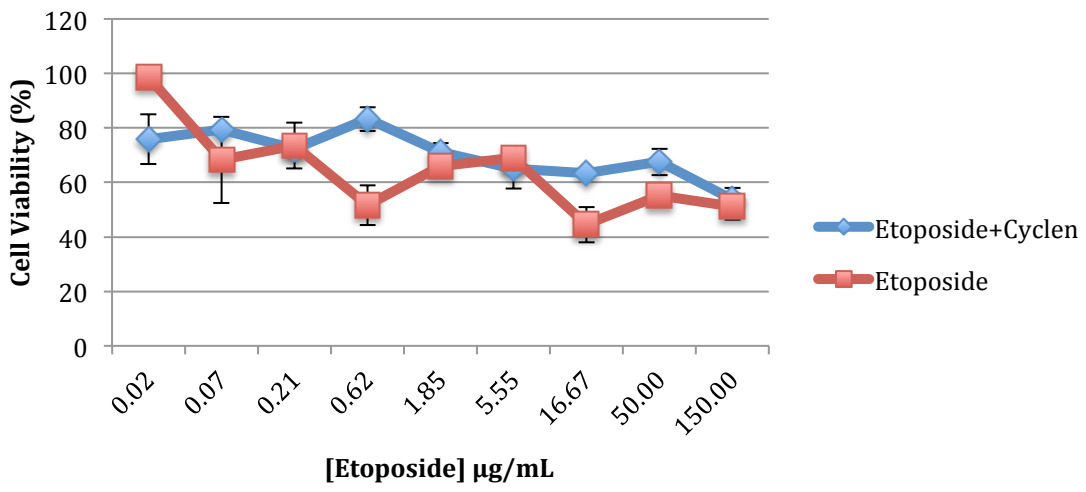


Figure 21. MTT colorimetric response of HEK 293 cells after 24 hours of exposure to etoposide [0.02 μg/mL-150 μg/mL] and cyclen [10 μM].

As a control to determine how cyclen affects the viability of HEK 293 cells, etoposide at a concentration range from 0.02 μg/mL to 150 μg/mL was also used in treatment. The results can be found in figure 21. HEK 293 cells treated with only etoposide did not present a distinguishable difference in cell viability from those cells treated with etoposide and cyclen. Again, cyclen has not been shown to

interfere with DNA damaging mechanisms and therefore should not impact the viability of cells treated with both etoposide and cyclen.

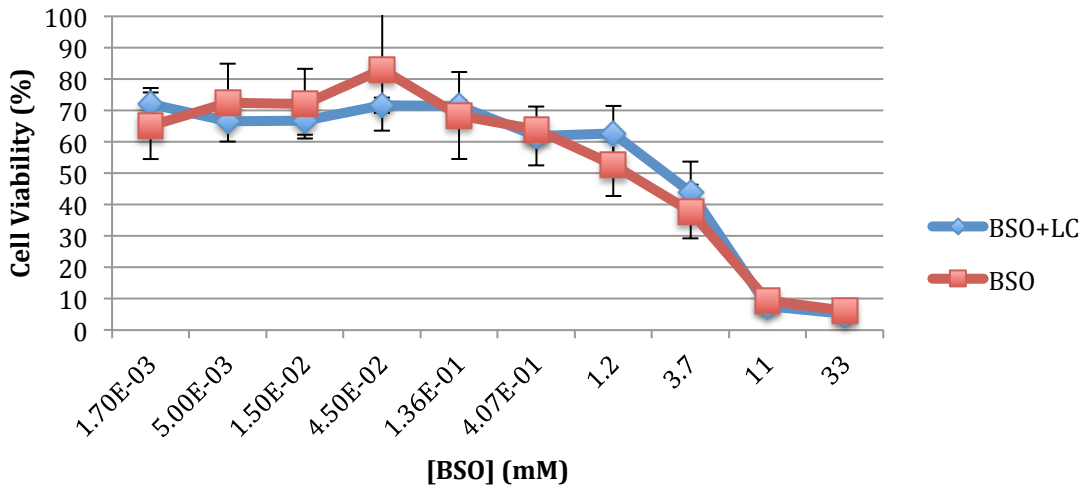


Figure 22. MTT colorimetric response HEK 293 cells after 24 hours of exposure to BSO [1.7 μM-33 mM] and LC [10 μM].

The ability of LC to affect cell viability of cells treated with BSO was studied in HEK 293 cells. Results are shown in Figure 22. As a control, half the plated cells were treated with a BSO concentration range from 1.7 μM to 33 mM. The other half were treated with the same BSO concentration range and 10 μM LC. Similar to the results in cyclen, the cells treated with BSO and LC did not produce a distinguishable difference in viability compared to those treated only with BSO. This result requires consideration of several factors. First of all, it is important to note that both lipoic acid and BSO impact the GSH synthetic pathway. It is possible that BSO is decreasing cell viability by an unidentified mechanism and is unaffected by LC's potential counteractive efforts to amplify the production of GSH. Alternatively, BSO is

decreasing cell viability by inhibiting the metabolic pathway to produce GSH, but the cyclen portion of LC obstructs the lipoic acid portion from countering inhibition. Finally, it is possible LC is not entering the cell altogether and therefore not inhibiting BSO's efforts to prohibit glutathione production. Further experimentation with a DCFH assay capable of quantifying ROS via fluorescence could aid in characterizing a mechanism for the affect of LC and BSO on HEK 293 viability.

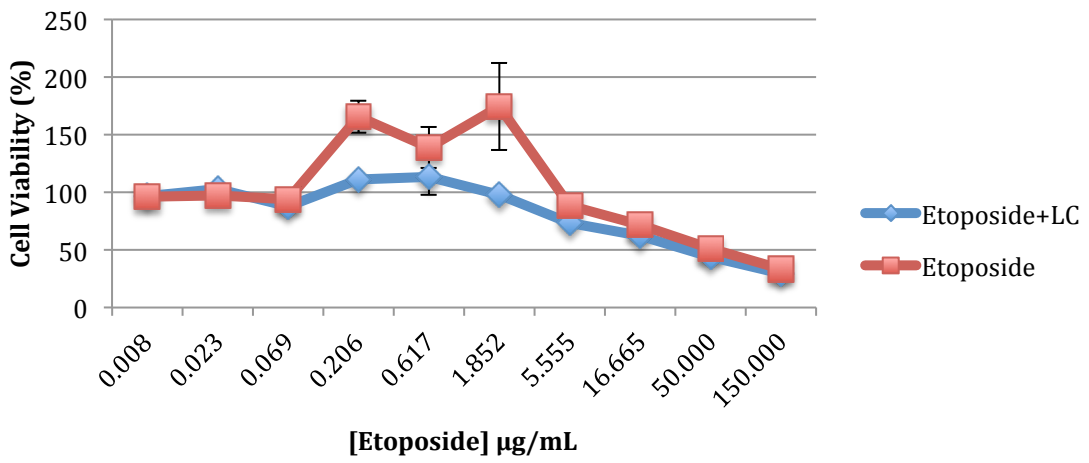


Figure 23. MTT colorimetric response HEK 293 cells after 24 hours of exposure to etoposide [0.008 µg/mL-150 µg/mL] and LC [10 µM].

As a control to determine how LC affects the viability of cells, etoposide at a concentration range of 0.008 µg/mL to 150 µg/mL was also treated on HEK 293 cells. The results can be found in figure 23. The error bars are not ideal, but the general trend can still be extrapolated. There is no distinguishable difference in cell viability between cells treated with LC and etoposide from those treated with only etoposide. LC's metal chelation and antioxidant properties support this result

considering it is not expected that LC would interfere with DNA damage caused by etoposide.

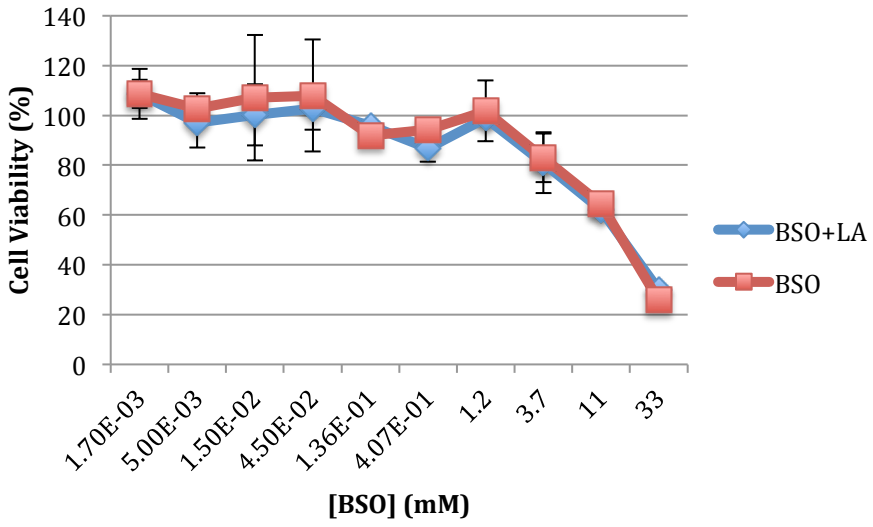


Figure 24. MTT colorimetric response HEK 293 cells after 24 hours of exposure to BSO [1.7 μM-33 mM] and Lipoic Acid [10 μM].

In an attempt to characterize the result in Figure 22, another experiment was carried out in which the effect on cell viability of lipoic acid salt was investigated. The results can be found in figure 24. For a control, half the cells were treated with the same BSO concentration as above, 1.7 μM – 33 mM, and the other half was treated with the same BSO concentration range and the lipoic acid salt at 10 μM. The results indicate no distinguishable difference in cell viability between cells treated with only BSO and those treated with BSO and lipoic acid. It should be noted that both curves are shifted up in comparison to the previous study carried out with LC. The fact that the two curves representing BSO treatment vary by about 20% cell viability indicates that it might be beneficial to repeat the 1.7 μM to 33 mM BSO

concentration range to identify which curve is most accurate for HEK 293 cells. Despite this, the general trend is the same. This variability points out an important challenge to working with live culture. Depending on the cell line, variability in response to a drug can be common. To counteract this tendency, repetition is key. Due to time constraints, repeat trials of some of the experiments performed in this study were not possible. It will be considered for future study especially if more detailed data than trend extrapolation is desired. It's possible that lipoic acid is not entering the cell and therefore countering the effect of BSO to inhibit GSH production. Alternatively, it is possible that BSO is affecting cell viability by a different mechanism than the one proposed. These studies should be complimented with DCFH-DA study, which monitors the concentration of ROS in the cell to show that BSO is indeed increasing ROS levels as a means to cell death. The likelihood of this hypothesis is probably fairly small considering the ubiquitous nature of the pathway, but it should be considered. Again, DCFH assays are currently in progress to quantify the levels of ROS present at variable concentrations of BSO to attempt to link decrease in cell viability to accumulation of ROS. A third hypothesis could be directed by the binding affinities of BSO and lipoic acid to the part of the metabolic pathway they respectively influence. It's possible BSO binds more strongly than lipoic acid and therefore dominates the cellular reaction. Further, BSO could inhibit the metabolic pathway at an earlier stage marginalizing or even eliminating the chance for lipoic acid to amplify glutathione production. Additional experimentation would be necessary to further characterize this result. The fact that the lipoic acid

trend is generally the same as LC prevents us from ruling out LC as an antioxidant in cells because lipoic acid is a well-known antioxidant *in vitro*.

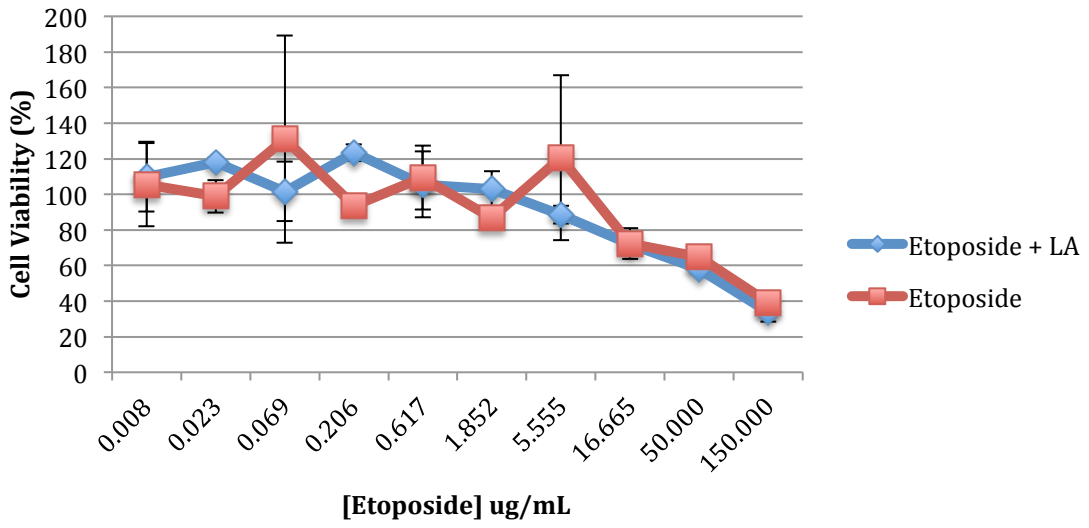
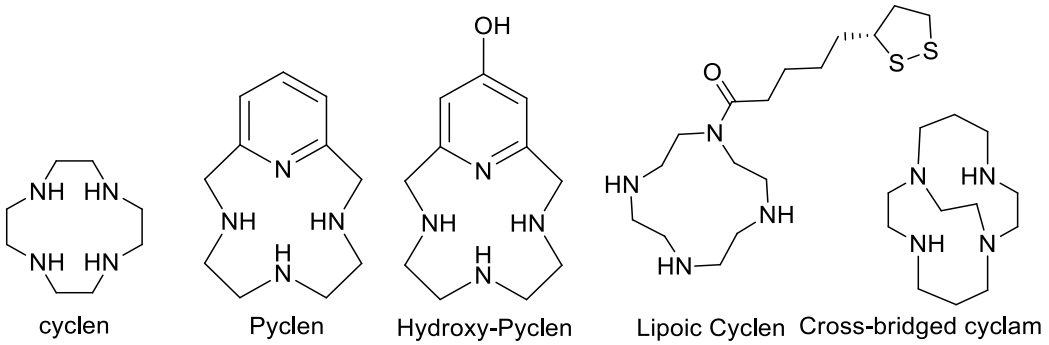


Figure 25. MTT colorimetric response HEK 293 cells after 24 hours of exposure to etoposide [0.008 μg/mL-150 μg/mL] and Lipoic Acid [10 μM].

As a control to potentially characterize a mechanism for lipoic acid's affect on cell viability, etoposide at a concentration range of 0.008 μg/mL to 150 μg/mL was treated on HEK 293 cells. The results can be found in figure 25. The error bars are not ideal, but the general trend can still be extrapolated. There is no distinguishable difference in cell viability between cells treated with lipoic acid and etoposide from those treated with only etoposide. The understanding that lipoic acid is a metal chelator and antioxidant supports this result. It was not expected that lipoic acid would restrict the general mechanism by which etoposide elicits cell death.

EC_{50} (effective concentration for 50% survival) values for the studies discussed above were calculated and tabulated in Table 3. The EC_{50} value is a

standard measure of the concentration required to obtain 50% response, which can be gathered from all cytotoxicity studies and compared on a common scale. In HEK 293 cells, the EC_{50} of LC is three times that of cyclen. This supports the hypothesis that the addition of lipoic acid to the cyclen skeleton serves to increase the biocompatibility of the compound in this cell line. An EC_{50} value for CB cyclam could not be obtained in HEK 293 cells because only 40% cell death occurred at the highest concentration. Considering CB cyclam is commercially used, it is reasonable that it has little deleterious effect *in vitro*. In HT22 cells, the EC_{50} values for both pyclen and LC are a magnitude greater than those in HP and cyclen. This insinuates that the pyridine to pyridol conversion to create HP does not increase compound biocompatibility in this cell line. Conversely the increased biocompatibility of LC compared to cyclen observed in HEK 293 cells is also found in HT22 cells. It will be interesting to monitor EC_{50} values of LC and cyclen in the FRDA cell line and others to further classify LC as the most tolerated of the cyclen derivatives.



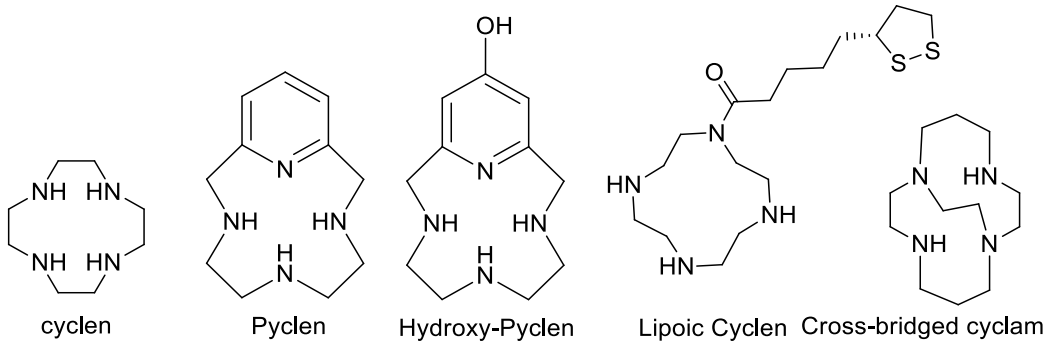
	Cyclen	Pyclen	Hydroxy-pyclen (HP)	Lipoic Cyclen (LC)	CB cyclam
HEK 293	155 μM	‡	‡	460 μM	*
HT22	91 μM	123 μM	22 μM	312 μM *	‡

Table 3. EC50 values for the compounds of interest in both HEK 293 and HT22 cells.
*Concentration range did not breach 50% cell death.
‡ Studies in progress

CONCLUSION

Metal chelation ability, antioxidant properties, and biocompatibility are potential characteristics that may play a strong role in the development of a drug capable of treating and/or preventing Alzheimer's Disease. This study was aimed at characterizing the cytotoxicity of several MRI contrast agent skeletons (cyclen, pyclen, CB-cyclam) and their derivatives (LC, HP) developed by the Green research group.

The results of this study indicate both cell lines as most sensitive in the μM concentration region only with the exception of CB cylcam, which didn't show much reaction at all in HEK 293 cell line and was not tested in HT22 cells. Additionally, with the exception of CB cyclam, the mM region was characterized by nearly complete cell death for both cell lines and all compounds of interest. It's plausible that the treatment with most drugs in the mM region would evoke complete cell death just by overloading the cell systems. The specific limitations of each compound per cell line are described in Table 4. Interestingly, LC was tolerated by HEK 293 and HT22 cells at a magnitude greater then cyclen. This correlates the EC_{50} values tabulated in Table 3 where the EC_{50} of LC was a magnitude greater than cyclen in HT22 cells and three times the value for cyclen in HEK 293. Lipoic acid addition does in fact confer some advantage, which translates to increased viability. Characterization of that mechanism is of interest for further study. Additionally, HP was tolerated at a magnitude lower concentration than it's skeleton pyclen. This suggests the conversion to pyridol does not confer an advantage to the HT22 cell line utilized in this study compared to pyclen.



	Cyclen	Pyclen	Hydroxy-pyclen	Lipoic Cyclen (LC)	CB cyclam
HEK 293	< 100 μM	N/A	N/A	< 1 mM	< 10 mM
HT22	< 100 μM	< 1 mM	< 100 μM	< 1 mM	N/A

Table 4. Compound of interest concentration limitations to elicit less than 40% cell death in HEK 293 and HT22 cell lines.

It will be interesting to compare these results to those found in FRDA cells. It's possible that because FRDA cells are experiencing metal ion misregulation and increased oxidative stress, HP and LC might be able to confer some advantage in the form of metal ion chaperoning or decreasing ROS levels. Additionally, results obtained from completing the studies in both HT22 and HEK 293 cells could reveal more information about mechanisms for cell death. DCFH-DA assays that track ROS levels could also aid in determination of mechanism.

The data from this study serve as the beginning blocks, which could continue to advance the study of these molecules into other cells lines and *in vivo* studies. Additionally, compound development based on these results will continue to occur within the Green research group. In a broader context, we expect to see a resurgence of prioritization of the characteristics we've investigated in this thesis in

therapeutic agent development. Previous studies discounting the ability of chelation and antioxidant therapies seem to be flawed in that subjects admitted to clinical trials likely represented an advanced disease state. We suspect this would be the case considering a definitive diagnosis of AD is nonexistent. Patients admitted most likely exhibited a clear symptom set that likely was accompanied by significant plaque build up and metal ion misregulation. Today, with advanced imaging abilities and early detection mechanisms, chelation and antioxidant therapy trials might present greater success on patients characterized by an early stage of the disease.

REFERENCES

1. Alzheimer's Disease Facts and Figures, Alzheimer's & Dementia. *Alzheimer's Assoc.* **2011**, *7*.
2. Bush, A. I., *Trends Neurosci.* **2003,** *26*, 207-214.
3. Bush, A. I.; Tanzi, R. E., *Neurotherapeutics* **2008,** *5*, 421-432.
4. Braymer, J.J.; DeToma, J. S.; Choi, J. S.; Ko, K. S.; Lim, M-H., *Int. J. Alzheimer's Dis.* **2011**, 1–9.
5. Ritchie, C. W.; Bush, A. I.; Mackinnon, A.; Macfarlane, S.; Mastwyk, M.; MacGregor, L.; Kiers, L.; Cherny, R.; Li, Q. X.; Tammer, A.; Carrington, D.; Mavros, C.; Volitakis, I.; Xilinas, M.; Ames, D.; Davis, S.; Beyreuther, K.; Tanzi, R. E.; Masters, C. L., *Arch. Neurol.* **2003,** *60*, 1685-91.
6. Barnham, K. J.; Masters, C. L.; Bush, A. I., *Nat. Rev. Drug Discov.* **2004,** *3*, 205-14.
7. Que, E. L.; Domaille, D. W.; Chang, C. J., *Chem. Rev.* **2008,** *108*, 1517-1549.
8. Fodero-Tavoletti, M. T.; Villemagne, V. L.; Rowe, C. C.; Masters, C. L.; Barnham, K. J.; Cappai, R., *Int. J. Biochem. Cell B.* **2011,** *43*, 1247-51.
9. Izatt, R. M.; Pawlak, K.; Bradshaw, J. S.; Bruening, R. L., *Chem. Rev.* **1991,** *91*, 1721-85.
10. Sherry, A. D.; Caravan, P.; Lenkinski, R. E., *J. Magn. Reson. Imaging* **2009,** *3*, 1240-1248.
11. Accardo, A.; Tesauro, D.; Aloj, L.; Pedone, C.; Morelli, G., *Coord. Chem. Rev.* **2009**, *253*, 2193-2213.

12. Rojas-Quijano, F. A.; Benyo, E. T.; Tircso, G.; Kalman, F. K.; Baranyai, Z.; Aime, S.; Sherry, A. D.; Kovacs, Z., *Chem-Eur. J.* **2009,** *15*, 13188-13200.

13. Lincoln, K. M.; Gonzalez, P.; Richardson, T. E.; Julovich, D. A.; Saunders, R.; Simpkins, J. W.; Green, K. N., *Chem. Commun.* **2013,** *49*, 2712-2714.

14. Gonzalez, P.; Hyde, K.; Green, K. N. *manuscript in preparation.*

15. Suh, J. H.; Moreau, R.; Heath, S. D.; Hagen, T. M., *Redox Rep.* **2005**, *10*, 52-60.

16. Lynch, M. A., *Physiol. Rev.* **2004,** *84*, 87-136.

17. Adlard, P. A.; Cherny, R. A.; Finkelstein, D. I.; Gautier, E.; Robb, E.; Cortes, M.; Volitakis, I.; Liu, X.; Smith, J. P.; Perez, K.; Laughton, K.; Li, Q. X.; Charman, S. A.; Nicolazzo, J. A.; Wilkins, S.; Deleva, K.; Lynch, T.; Kok, G.; Ritchie, C. W.; Tanzi, R. E.; Cappai, R.; Masters, C. L.; Barnham, K. J.; Bush, A. I., *Neuron* **2008,** *59*, 43-55.

18. Strozyk, D.; Launer, L. J.; Adlard, P. A.; Cherny, R. A.; Tsatsanis, A.; Volitakis, I.; Blennow, K.; Petrovitch, H.; White, L. R.; Bush, A. I., *Neurobiol. Aging* **2009,** *30*, 1069-1077.

19. Wong, A.; Yang, J.; Cavadini, P.; Gellera, C.; Lonnerdal, B.; Taroni, F.; Cortopassi, G., *Hum. Mol. Gen.* **1999**, *8*, 425-430.

20. Keitany, G. J., M.S. Thesis, Texas Christian University, Fort Worth, TX, 2004.

21. Lincoln, K. M.; Richardson, T. E.; Rutter, L.; Gonzalez, P.; Simpkins, J. W.; Green, K. N., *ACS chem. neurosci.* **2012**, *3*, 919-27.

ABSTRACT

Alzheimer's Disease, a progressive neurodegenerative disorder affecting greater than 5 million people in the United States, still lacks a definitive diagnosis and reliable treatment protocol (1). Oxidative stress and metal ion misregulation have been associated with the molecular markers of the disease. As such, modifications to MRI contrast agent skeletons attempt to maintain metal chelating ability, incorporate or enhance antioxidant properties and increase biocompatibility of the molecule. This was carried out by the conversion of pyclen and cyclen to hydroxypyclen (HP) and lipoic cyclen (LC), respectively. This study investigates compound toxicity *in vitro* in HEK 293 (Human Embryonic Kidney cells) and HT22 (hippocampal mouse cells). Results showed that HT22 cells tolerated LC at a concentration magnitude greater than cyclen, while HEK 293 cells tolerated LC at almost 3 times greater concentration than cyclen. HT22 cells tolerated HP at a concentration magnitude lower than pyclen. Further studies focused on antioxidant abilities of the ligands using BSO (buthione sulfoximine) as an ROS inducing assault. Experimental implications of these results are discussed.

1. Alzheimer's Disease Facts and Figures, Alzheimer's & Dementia. *Alzheimer's Association* **2011**, *7*.
2. Lincoln, K. M.; Gonzalez, P.; Richardson, T. E.; Julovich, D. A.; Saunders, R.; Simpkins, J. W.; Green, K. N., *Chem. Commun.* **2013**, *49,* 2712-2714.