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# Synthesized Tripodal Amine as Potential Anti-Cancer Therapeutic

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***Synthesized Tripodal Amine as Potential Anti-Cancer Therapeutic***

An Honors Thesis submitted in partial fulfillment of the requirements for Honors in Chemistry and Biochemistry.

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***ABSTRACT***

Cancer remains a prevalent disease today. This disease may manifest itself in many different ways and affect a variety of tissues with everything from the brain to the blood. With this wide diversity of cancer types, treatment can be complicated since there is not a "one size fits all" treatment for the disease. Surgery, radiation, and chemotherapy are all options that must be weighed with their benefits and side effects. Ultimately though, there are not enough effective treatment options available for every type of cancer. This leaves many with the grim prognosis of never being cured. With this clear need for more anti-cancer medicine, research is warranted into novel treatments and drugs. A novel tris-indolyl iron-chelating drug GS02 was synthesized and tested for its potential anti-cancer properties. The LD50 was determined using MTS assays against human cancer cell lines and apoptosis assays were carried out with the PC3 cell line. Images of the cells were collected using confocal imaging microscopy. Current results show a decrease in cell viability and suggest that GS02 is inducing apoptosis in the PC3 cell line. Furthermore, the compound was shown to be toxic within a specific concentration range which may be of interest for the potential of a new chemotherapy agent. Given these results, GS02 warrants continued investigations into its potential to trigger apoptosis in more human cancer cell lines.

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***Introduction:***

Cancer still remains as a prevalent disease today. This disease may manifest itself in many different ways and affect a variety of tissues. Overall, cancer is a genetic disease and arises with mutations in a cell's DNA.[1] These mutations can occur through genetic inheritance, error in the DNA replication process, or through internal/external mutagens. Oftentimes, these mutations result in uncontrolled cell growth and enable the cancerous cell to bypass typical regulatory checkpoints in the cell cycle.[1] The combination of increased growth with fewer regulatory mechanisms to check for error in DNA synthesis/repair makes it easy for cancer to grow and spread. Treatment can be complicated since there is not a "one size fits all" treatment course and further complications arise once the cancer becomes chemo-resistant. With this need for improved medicine, research is warranted into potential cancer treatments and drugs.

With this clear need for a wider variety of treatment options, research has continued in this field with focuses on different approaches. One area of interest in our lab is using iron chelators as potential anti-cancer therapeutics. Iron chelators already have therapeutic use in treating iron overload disease. These molecules function in "grabbing" iron in the body and the result makes the iron easier for the body to excrete. However, some researchers have turned their attention to exploiting this characteristic for cancer treatment. Iron itself has many important biological functions and is critical to maintaining homeostasis for an organism. Iron may be found in two different oxidation states, $Fe^{2+}$ and $Fe^{3+}$, and is a vital micronutrient for many organisms.[2] Iron is used in biological functions such as DNA synthesis, oxygen transport through hemoglobin,

production of energy, and macromolecule synthesis.[2] Through the role iron has in these vital processes, it is clear to see that iron is essential to any growing organism. The rationale to using iron chelators as an anti-cancer therapeutic thus lies within limiting the cancer cells accessibility to this important micronutrient, thus slowing and ideally preventing cancer cell growth.[3] Various studies have been performed which demonstrate the promising results of using iron chelators as a cancer treatment. One study investigated the effects of deferoxamine and deferasirox, two known iron chelators (Figure 1), on the proliferation and stemness of cancer stem cells.[6] The results gleaned first showed that having an ample supply of iron enhances the proliferation of the cells then, when treated with iron chelators, antiproliferative effects were observed along with a reduction in stemness markers.[6]

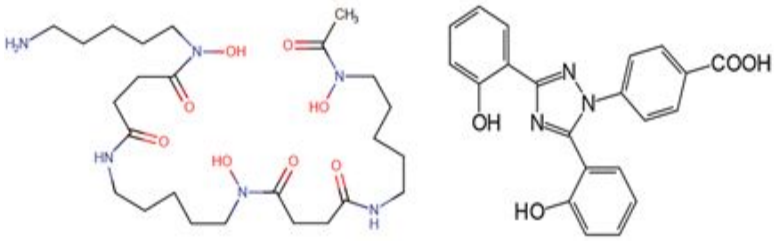


**Figure 1.** Structure of iron chelators deferoxamine and deferasirox on left and right respectively.[4,5]

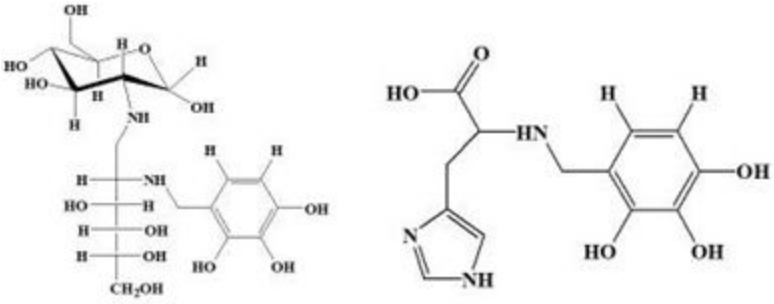


**Figure 2.** Structure of iron chelators SP6 and SP10 on left and right respectively.[7]

Another experimental investigation into iron chelators found that a novel chelator, super-polyphenol (SP), in two forms (Figure 2) was effective at inducing apoptosis in various cancer cell lines.[7] Furthermore, their findings suggested

that the novel SP compound was safer for use than clinically available deferoxamine, thus providing a potentially less toxic option for therapeutic use.[7]

Other researchers have discovered the antiproliferative effects of an iron chelator VLX600 (Figure 3) on cancer cell lines and the compound even proceeded to a phase-1 clinical trial.[8]

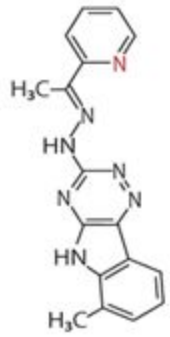


**Figure 3.** Structure of iron chelator VLX600.[7]

With designing testing for the anti-cancer properties of GS02, apoptosis was the desired method of inducing cell death. Apoptosis is pre-programmed cell death and can occur due to some external signal or caused by the cell itself.[9] Hallmarks of this type of death include the expenditure of energy, thus it is an active form of dying, and the breakdown of all membranes, including the nuclear membrane.[9] Apoptosis is an important part of homeostasis for an organism and disturbances in its regulation can lead to serious diseases such as cancer.[10,11] Often as seen in cancer, there is some sort of inhibition of apoptosis which leads to the rapid, uncontrolled growth of cancerous cells. The importance of apoptosis in cancer leads to a potential target, especially since targeting apoptosis does not lead to inflammation like necrosis does (passive cell death).[9,12]

The aims of this project are to test the potential anti-cancer properties of a novelly synthesizied tris-indolyl amine, GS02. This compound's structure is based on iron chelators and is a TRENSOX derivative with indoles in place of quinolines.[13] Cytotoxicity of GS02 will be tested against the PC3 cell line which is human prostate cancer. Apoptosis is the targeted method of cell death and will be tested for using a

cytotoxicity assay which may be used for measuring cell death.[14] Further analysis will be done using spectrophotometry and imaging techniques.

***Methods and Materials:***

*Synthesis of GS02:*

GS02 (tris(2-[indole-3-glyoxylamido]ethyl)amine), was synthesized using a previously established procedure.[13] Ether (200 mL) was added to indole (10.10 g) at 0 ℃, then over a period of 15 minutes, oxalyl chloride (14.6 g) was added dropwise. The reaction mixture stirred for 30 minutes, then was filtered and a yellow solid was collected. THF (50 mL) was used to dissolve the yellow solid and once dissolved, the mixture was placed in an ice bath. TREN (6.12 g, 42.0 mmol), TEA (4.27 g, 42.3 mmol), and THF (10 mL) were combined and added to the 0 ℃ solution of dissolved solid. The solution was allowed to stir for 1 hour at 0 ℃ . Afterward, it was filtered and the filtrate allowed to evaporate (Figure 1). A beige solid was collected (0.52 g, 0.79 mmol, 2.7%) after recrystallization from MeOH.

**Figure 4.** A colorful synthesis of GS02. From left to right: stirring the reaction for 30 min, filtering the solid, stirring for 1 hour, and filtering out the desired filtrate.

*Apoptosis Assay:*

Cells were plated into a 96 well plate at a concentration of 7,500 cells per well and incubated for 24 hours at 37°C. Mitochondrial membrane was labeled via incubation with Mito-tracker red for 30 minutes at 37°C. The cells were then washed and resuspended in cell culture media. Control cells were incubated in the normal cell culture media. Other cells were treated with either 1% DMSO, 2 mM staurosporine, or 74.8 μM GS02 with the DMSO and staurosporine treatments serving as negative and positive controls respectively. Three samples were taken from each experimental condition at 2, 4, 8, and 24 hour time points. Acridine Orange was used to fluorescently label cells previously stained with Mito-tracker red. Cells were also fluorescently labeled with either Caspase 3, 7 green detection reagent and Sytox Aadvanced. A BD Accuri C6 flow cytometer was used for cytometric analysis.

*Capturing Images of Cells:*

PC3 cells were plated onto microscope slide cell culture disks and treated with 74.8 μM GS02 and labeled with MitoTracker Red and Hoechst 422. Cells were fixed at the 12 hour time point and images collected using a Zeiss confocal imaging microscope. Images were collected showing only the fluorescence of one dye at a time, then images were collected simultaneously showing both dyes.

***Data and Results:***

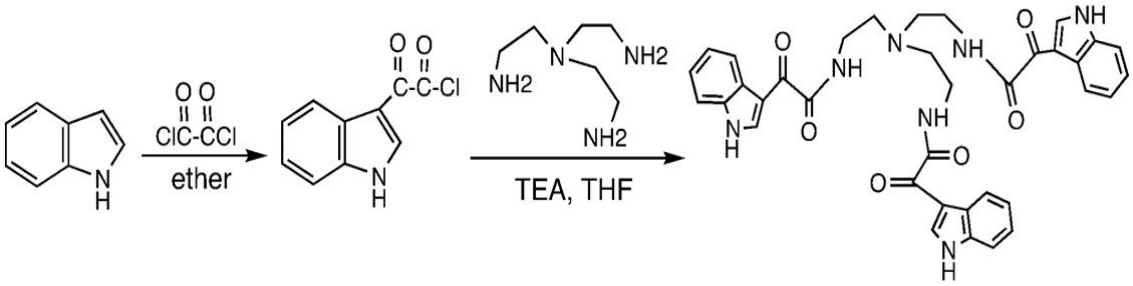


**Figure 5. Synthesis of GS02.** Pictorial representation of the synthesis of potential cancer therapeutic GS02 showing structures of materials and end product.

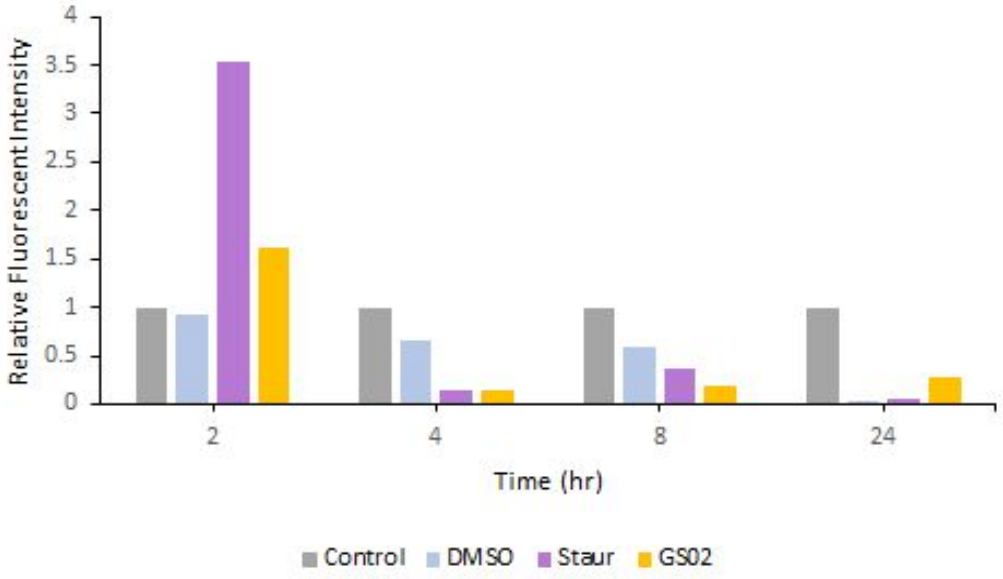


**Figure 6.** Cells were incubated with CellEvent Caspase 3, 7 green detection reagent, a four-amino acid peptide (DEVD) conjugated to a nucleic acid-binding dye which is non-fluorescent until cleaved from the peptide and bound to DNA. Fluorescent intensity was measured at 2, 4, 8, and 24 hours. Control, DMSO, Staurosporine (Staur), and GS02 were the various treatments.

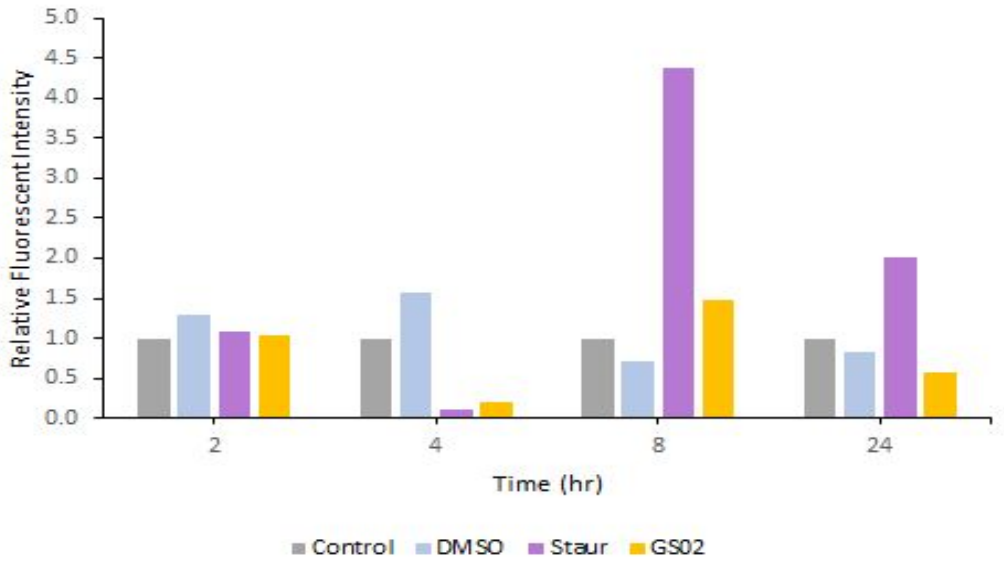


**Figure 7. Relative fluorescent intensity of Sytox across varying time points**. Cells were incubated with Sytox Aadvanced, a DNA binding dye which becomes fluorescent upon binding to DNA. As such fluorescence indicates cell death. Treatments were Control, DMSO, Staur, and GS02 with data gathered at 2, 4, 8, and 24 hours.

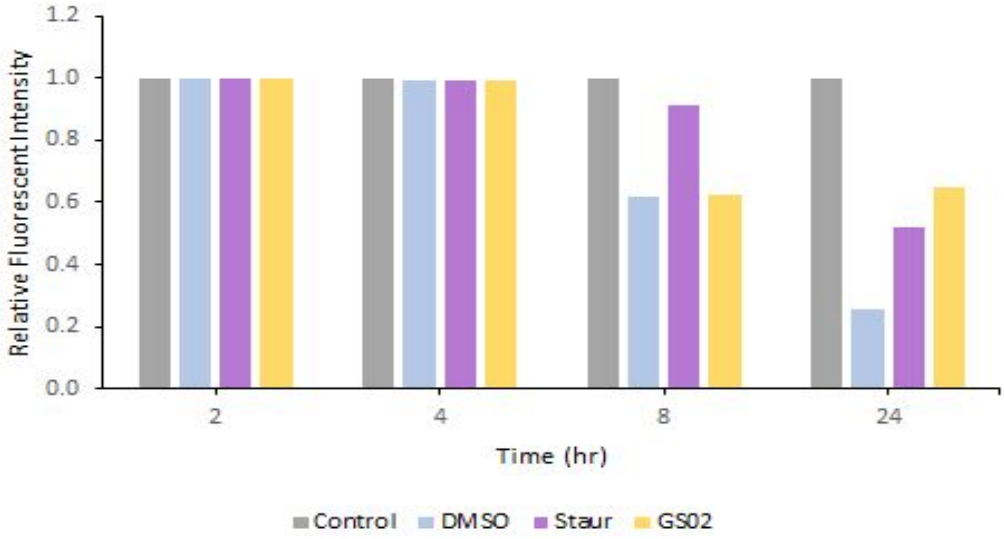


**Figure 8. Relative fluorescent intensity of Mito-Tracker Red across varying time points.** Cells were fluorescently labeled with Mito-tracker red which fluoresces red when bound to intact mitochondrial membranes. Samples of Control, DMSO, Staur, and GS02 were read at time points of 2, 4, 8, and 24 hours.

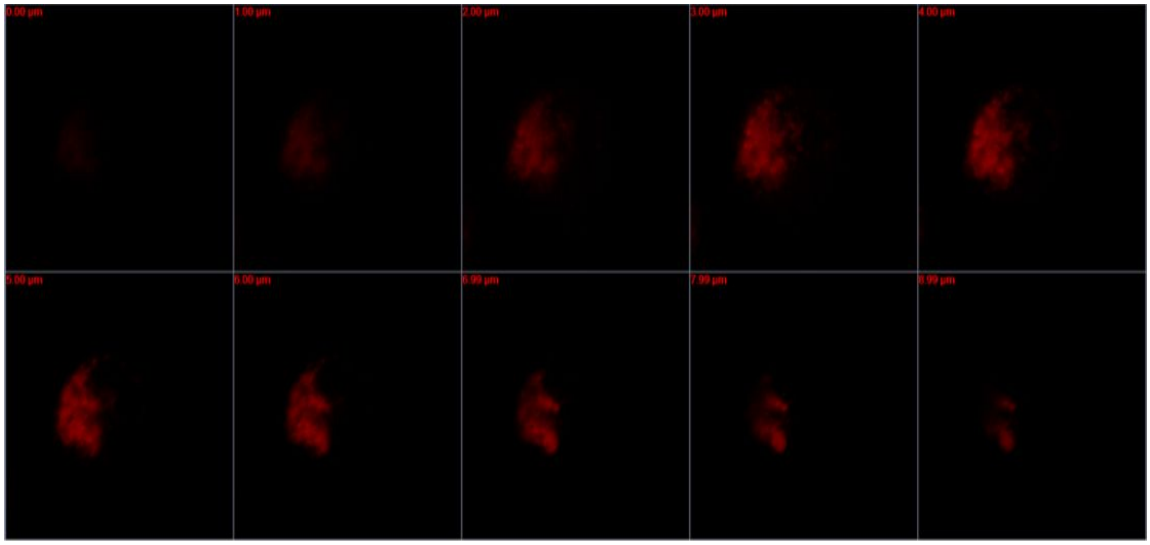

**Figure 9. MitoTracker Red stained PC3 cells at 12 hour time point.** PC3 cells were treated with GS02 and labeled with the fluorescent indicator MitoTracker Red. This stains intact mitochondrial membrane. At the 12 hour time point, the PC3 cells were fixed. Z-stacked images were collected using a Zeiss confocal microscope.

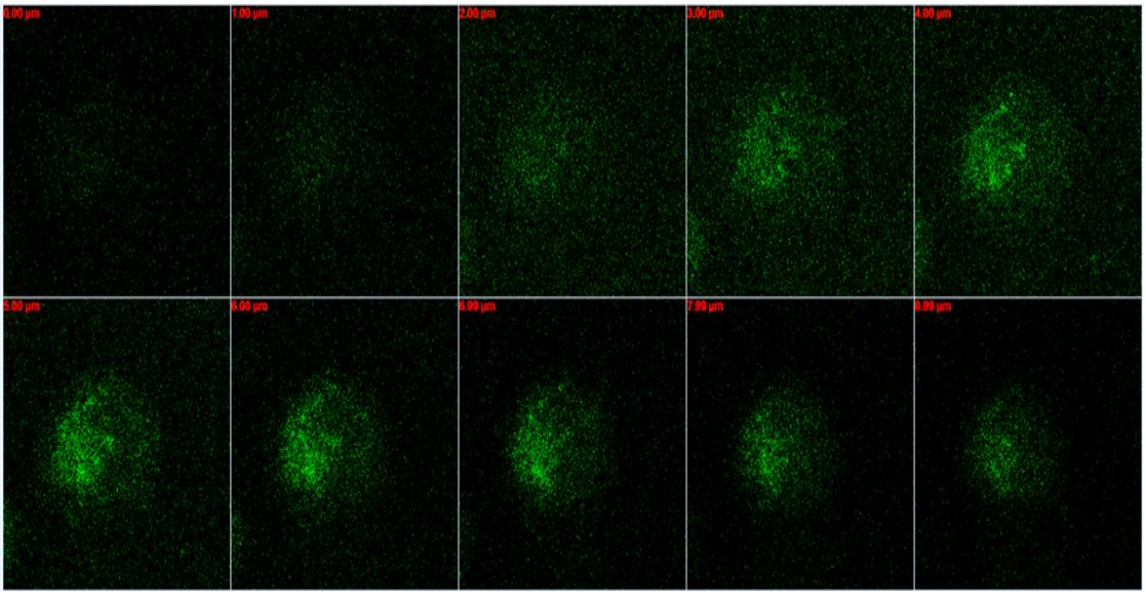

**Figure 10. Hoechst 422 stained PC3 cells at 12 hour timepoint.** PC3 cells were treated with GS02 and labeled with the fluorescent indicator Hoechst 422. Hoechst 422 is a DNA intercalator that fluoresces when bound to DNA. At the 12 hour time point, the PC3 cells were fixed. Z-stacked images were collected using a Zeiss confocal microscope.

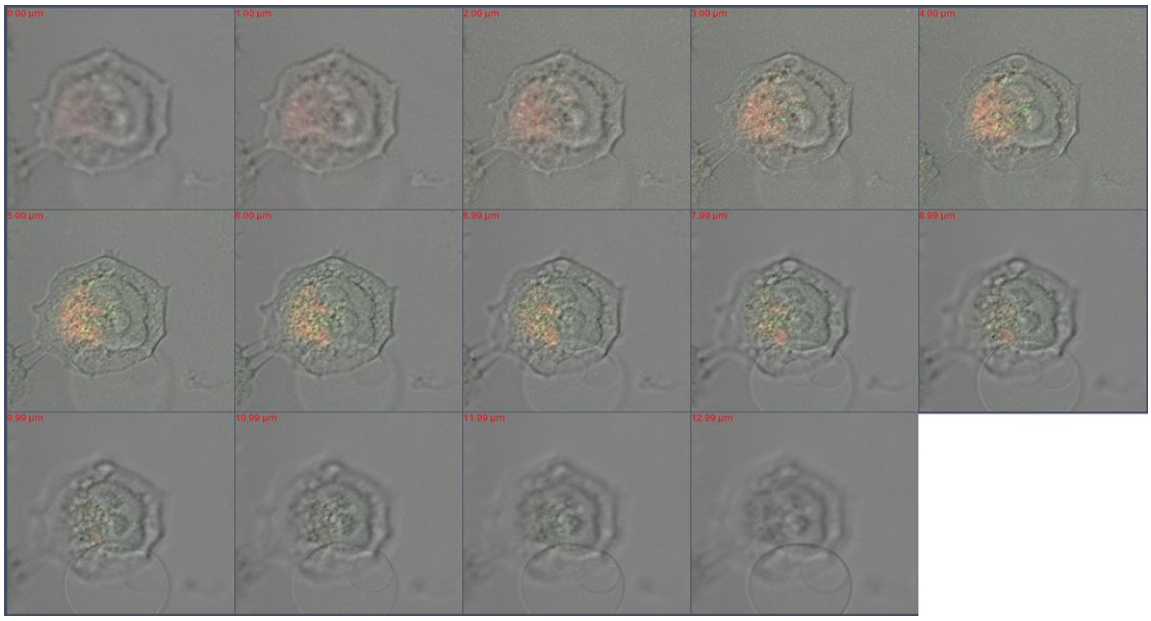

**Figure 11. PC3 cell treated with GS02 at 12 hour time point.** PC3 cells were treated with GS02 and fluorescently labeled with Hoechst 422 stain and MitoTracker Red. At the 12 hour time point, the PC3 cells were fixed. Z-stacked images were collected using a Zeiss confocal microscope. The figure shows fluorescent activity from both fluorescent indicators of the Hoechst 422 (green) and MitoTracker Red (red) stains.

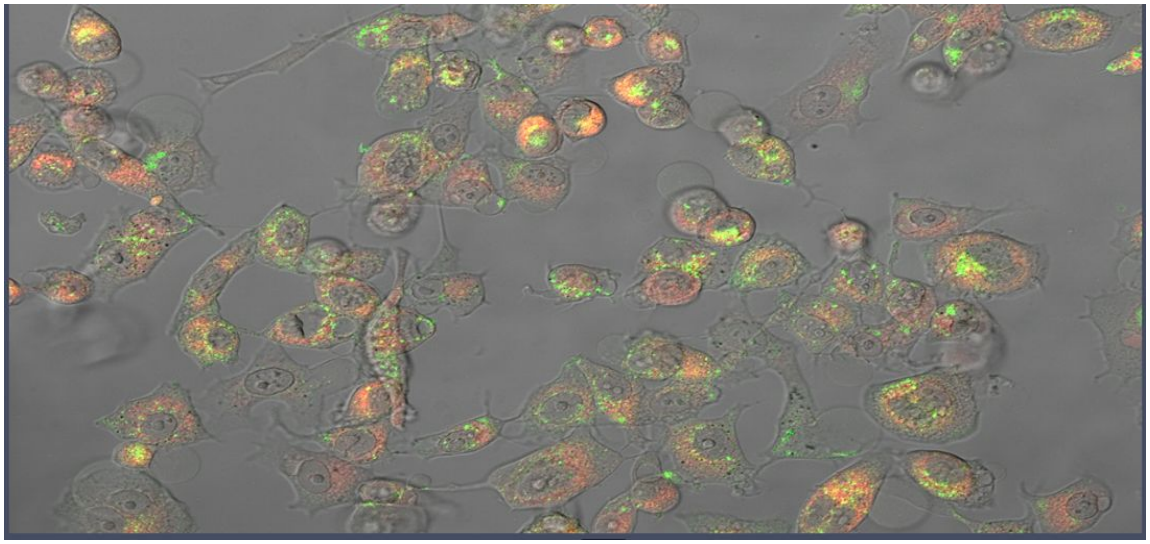

**Figure 12. GS02 treated PC3 cells with overlapping of fluorescent indicators at 12 hours.** Multiple PC3 cells shown with both indicators of Hoechst 422 and MitoTracker Red indicators present. Overlapping of the red MitoTracker Red indicator and the green Hoechst 422 stain show where both are present with an orange/yellow color.

***Discussion:***

Synthesis of GS02 was carried out and previous analysis with NMR and IR spectroscopy was executed to confirm the structure (Figure 4,5).[13] Various assays were performed to determine the potential anti-cancer properties of GS02. The flow cytometry data yielded interesting results. The first fluorescent dye used was Caspase 3,7 detection reagent which is activated in the presence of caspases 3 and 7. Once activated, the dye will fluoresce once bound to DNA.The presence of fluorescence indicates that cells are dying and potentially dying from apoptosis since the dye is able to reach DNA through the nuclear membrane and there are caspase enzymes present in a high enough concentration to activate the detection reagent. Apoptosis is characterized by a presence of the caspase enzymes 3 and 7 and the nuclear membrane does breakdown which is in contrast with other methods of cell death. GS02 had a spike in fluorescence at 2 hours lowered by the 4 hour time point and remained near constant afterwards. Similarly, the positive control staurosporine shows a spike, much higher than GS02, at the 2 hour time point which diminishes as time continues. These spikes for both staurosporine and GS02 are higher than the levels of control and it is inferred that cells are dying and potentially through apoptosis (Figure 6).

Confirmation of cell death was determined using another fluorescent dye, Sytox Aadvanced. The presence of Sytox fluorescence indicates cell death, but does not indicate which method of cell death is occuring. The control level of relative fluorescent intensity remained near constant across time points. Both the staurosporine and GS02 treatments had similar trends in observing a decrease in fluorescent intensity at 2 hours followed by

an increase later on. Staurosporin did have higher levels of relative fluorescent intensity and thus is potentially more cytotoxic than GS02, but both treatments were shown to increase cell death. Overall, the decrease in relative fluorescence intensity seen in the treatment with GS02 provides support that apoptosis is the cause of cell death (Figure 7).

To further gain insight into the type of cell death, the Mito-Tracker Red dye was used. This is useful in that it fluoresces red when bound to intact mitochondrial membrane. At the 2 hour time point, all treatments remain at similar levels of relative fluorescence intensity. As time proceeds, the control treatment remains at steady levels which would be expected. Once the 8 hour time point is reached, a decrease in fluorescent intensity is observed in the other three treatments. Interestingly, GS02 shows a swifter decrease in fluorescence than does staurosporine, potentially indicating a swifter breakdown of mitochondrial membranes. The overall decrease in relative fluorescent intensity of GS02 suggests that the mitochondrial membrane is breaking down, a hallmark present in apoptosis which is characterized by the breakdown of various membranes in the cell (Figure 8).

Further insights into the effects of GS02 were gained through confocal imaging of the cells. As previously done, Mito-Tracker Red was used to stain for intact mitochondrial membrane. Once the cell was fixed, the z-stacked image obtained showed fluorescent activity through varying levels of the cell. This showed that there was intact mitochondrial membrane at the 12 hour time point (Figure 9). Another dye, Hoechst 422, was used as well. This dye was chosen since it fluoresces green once bound to DNA. Imaging of the fixed cell at the 12 hour time point showed clear fluorescent activity

across the z-stacked images. The incidence of green fluorescence indicates that the nuclear membrane is breaking down and apoptosis is likely occurring (Figure 10). Taken together, both sets of imaging data provide insight that at the 12 hour time point, the cell is still undergoing apoptosis and has not been completely destroyed. The simultaneous presence of both intact mitochondrial membrane and the ability for the Hoechst 422 dye to travel through the nucleus and bind to DNA help provide a reference point as to when the activity of GS02 begins.

An overlay of the previous two images was done to investigate any other insights that the date might provide. Interestingly, once the z-stacked images were overlaid of the same cell, green and red fluorescence was seen, but also an orange color was suddenly present. This indicated a colocalization occuring in the cell. The presence of both intact mitochondrial membrane and Hoechst 422 dye bound to DNA showed that the mitochondria of the cell was slowly having holes poked through it, likely due to the caspase enzyme cascade in apoptosis (Figure 11). A broader image was gathered to analyze whether this was local phenomena to the individual cell and found that at the 12 hour time point, many other PC3 cells also had colocalization of the dyes and as such, indicated the presence of intact mitochondrial membrane along with holes being present in the membrane (Figure 12). This further indicated that apoptosis was occurring and also showed that the Hoechst 422 would bind not just to nuclear DNA, but also to mitochondrial DNA. Taken together, the data supports the claim that GS02 does possess anti-cancer properties and is at least cytotoxic to human prostate cancer. Along with being toxic to cancer cells, it also is highly likely that the mode of cell death induced is

apoptosis as seen from the breakdown of mitochondrial membrane and increase in the presence of caspase enzymes 3 and 7.

***Conclusion and Future Work:***

A novel, tripodal amine GS02 was synthesized and used in testing against the PC3 cell line as a potential anti-cancer therapeutic. Combining the data from various fluorescent dyes and confocal imaging of cells supports the claim that GS02 is cytotoxic to PC3 cells and is inducing apoptosis. This set of data offers a promising foundation for further research with GS02 and other iron chelators of similar structure. Other derivatives of GS02 may have similar or even more potent effects against cancer cell lines and it would be of great interest to synthesize a variety of related compounds for testing. The experiments done above could be repeated except with more time points to analyze exactly when GS02 induces apoptosis and the time it takes for it to completely kill the population. This will likely be before the 12 hour time point since the confocal images show progression of apoptosis, but not the completion. To continue developing GS02, more testing against other cancerous cell lines needs to be completed to identify whether the cytotoxic effects of GS02 are unique to the PC3 or if there is another cancer against which it might be a more effective treatment option. In particular, it would be noteworthy to see the effect against a lung cancer cell line since the lungs would be a location of much iron due to the gas exchange that occurs with the transportation of oxygen in hemoglobin, a iron-containing protein. Also, there is currently no data for GS02's effect on healthy cells. A study done on various primary cells should be completed to determine

whether GS02 is more or less toxic to healthy cells than cancerous cells. Still another avenue for investigation is the mechanism by which GS02 induces apoptosis. Western Blots and immunoblots would be of interest as to identify which proteins might be getting interrupted and which pathway in the cell is triggering the signal for apoptosis. Overall, the results of this experiment will help provide the foundation for further analysis and development of potential anti-cancer agents. This is a necessary step as other researchers continue to investigate potential treatments for those afflicted with cancer.

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