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Killing Cancer with Light

Kaitlyn Thomas
Ouachita Baptist University

SENIOR THESIS APPROVAL

This Honors thesis entitled

"Killing Cancer with Light"

written by

Kaitlyn Thomas

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the requirements for completion of
the Carl Goodson Honors Program
meets the criteria for acceptance
and has been approved by the undersigned readers.

Dr. Joseph Bradshaw, thesis director

Dr. Allyson Phillips, second reader

Dr. Johnny Wink, third reader

Dr. Barbara Pemberton, Honors Program director

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Introduction

Photodynamic therapy (PDT) is a relatively new treatment option in medicine including treating some types of cancers. PDT utilizes light to activate small molecules for treatment. Some of these molecules used as PDT agents are porphyrin derivatives. While PDT is currently being used in the fight against cancer, it has several limitations and is currently being used in conjunction with chemotherapy and/or radiation in order to be effective. This research focused on synthesizing a new zinc (II) porphyrin and incorporating a fluorescent sulfocycline and testing the compound on the MB 231 breast cancer cell line.

Once the metallated porphyrin was synthesized and purified, IR, UV-vis, fluorescence, and NMR spectroscopies were run to characterize the compound. Once this data was collected, the compound was tested on the MB231 cell line. The MB231 cell line is also referred to as triple negative breast cancer cells. After cells were treated with the metallated porphyrin an MTT assay was run to look at cell viability in both light and dark conditions.

Along with the testing of the synthesized metallated porphyrin, research was done on how patient attitude has been shown to effect patient outcome. While looking at this, cancer patients were focused on. In order to increase the number of available cases, other diseases were looked at as well.

Background

Photodynamic Therapy (PDT)

Photodynamic therapy is a medical procedure that utilizes a photosensitive molecule and a light source that activates the molecule (Cole). This treatment is currently used to treat some kinds of cancers and even acne. Researchers began to look at photodynamic therapy in the 1960's after Lipson and Baldes discovered that neoplastic tissues that had been injected with a porphyrin mixture that was a photosensitizer would fluoresce under a black light (Cole). Schwartz had originally synthesized this compound and classified it as a hematoporphyrin (Cole). Once tested, it was discovered that this hematoporphyrin had a higher affinity for and stronger phototoxicity for tumor cells than hematoporphyrins. Photofrin was the first FDA approved drug from this group, and it was approved for clinical use in the 1970's. This also led to multiple light sources being created that could deliver specific wavelengths of light to more centralized areas of the body. Further research led to more approvals of various PDT's in the 1990's that could be used in the treatment of malignant and non-malignant cancers.

When looking at photodynamic therapy there are three major steps in the process (Cole). The first step is the application of the photosensitizer drug. The second step involves an incubation period that allows the photosensitizer drug to locate and imbed itself in the tumor tissue. The final step is to deliver the wavelength of light to the area that will activate the compound.

How the photosensitizer drug is applied depends on what area of the body that the tumor in question is located. Typically, the drug will be injected into the bloodstream so it can travel through the bloodstream to reach the target tumor tissue ("Photodynamic

Therapy"). In other cases, the drug will be applied to the skin. Because of the composition of the blood, it is imperative that the drug be water-soluble. The photosensitizer drug is synthesized so it is able to locate specific cell markers (Huang et al., 2008). These markers need to be located on the tumor cells themselves or other cells that are throughout the tumor. This will enable the photosensitizer drug to embed itself throughout the tumor tissue. The photosensitizing drugs have some criteria that they should meet to be ideal (Huang et al., 2008). To be ideal, the photosensitizer should: be made of a commercially available and pure chemical, high photocytotoxicity but low dark cytotoxicity, good selectivity for tumor cells, a longer wavelength that will allow for deeper penetration of the light waves, be rapidly removed from the body, and have multiple administration routes.

The incubation period is the second step of photodynamic therapy (Huang et al., 2008). Depending on the photosensitizer drug administered and the composition of the tumor being targeted, the incubation period varies in time. The length of time for the incubation period is a critical component for success. The incubation period needs to be long enough for the maximum amount of the photosensitizer drug to be able to spread throughout the tumor but not so long that the drug begins to leave the body through natural processes. To determine the optimum incubation period of the photosensitizer, it must be ensured that the photosensitizer drug has fluorescence emission. When the photosensitizer drug has fluorescence emission, the progress of the embedding of the PDT agent into the tumor cells can be monitored *in vivo*. By measuring the incubation period in an *in vivo* scenario, the incubation period in a clinical setting can be estimated fairly accurately (Huang et al., 2008).

Once the photosensitizer drug has incubated for an appropriate amount of time a light is administered to the affected area (Cole). This light source must be at the range that the photosensitizer drug will become activated at (Huang et al., 2008). In the beginning, these light sources were noncoherent. Noncoherent light sources are safe for the patient, cheap to use, and are easy to operate. Unfortunately, these light sources have "significant thermal effect, low light intensity, and difficulty in controlling light dose" (Huang, 2005). Recently these drawbacks have been overcome with the introduction of light sources using light emitting diode (LED). LED lights are beneficial because they are able to deliver the desired wavelength of light at a high energy level. Two photon excitation (TPE) is also a current option (Huang, 2005). TPE involves the "simultaneous absorption of two photons generated from ultrafast laser pulses of high flux in the near-infrared region" (Huang et. al., 2008). TPE has been shown to be beneficial for deeper lesions and is able to be more precise with a smaller area. The most recent discovery has been that of the diode laser. These lasers are able to produce "high energy monochromatic light of a specific wavelength with a narrow bandwidth" (Huang et. al., 2008). The last thing to consider when looking at the light application is whether the light should be administered superficially or interstitially. Light application mainly depends on the location of the tumor. Probes allow doctors to administer the light interstitially for esophageal cancers and others.

Once the photosensitizer drug is activated it must have oxygen in the environment or there will not be any antitumor effects (Huang et. al., 2008). For this reason, tumors that are hypoxic don't usually respond to PDT. A huge obstacle that can be presented is when the PDT causes hypoxia in the tumor because of the rapid

oxygen depletion that can happen. Experiments have been conducted that look at the effect of hyper oxygenating the tumor environment (Huang et. al., 2008). The clinical results have not yet been reported. Oxygen changing from its triplet form to its singlet form is what causes free radical reactions within the tumor cells (Pilawa et. al., 2006). These free radical reactions then lead to the death of tumor cells.

PDT has its drawbacks of course ("Photodynamic Therapy"). One of the main disadvantages is that the light source, no matter which of the many options you pick, can only reach so far. This is why only some tumors can even feasibly be treated with PDT based on where they are located in the body. Because of how precise the light placement has to be, PDT cannot be used on cancer that has spread and is in multiple areas throughout the body. Additionally, the photosensitizer drugs that can be used for PDT make the patient sensitive to all light for an amount of time after the drug is injected. This requires the patient to take extra precautions in light.

Currently, PDT is used to treat many diseases. Actinic keratosis is one disease that PDT seems to help with, but it is very difficult to truly know how much PDT is helping with treating the disease because affected areas can clear up spontaneously without treatment (Huang, 2005). Actinic keratosis are dangerous because of their tendency to transform into squamous cell carcinomas. Although it is difficult to know if observed changes are solely due to PDT, many studies show a higher rate of disappearance of actinic keratosis when PDT is used as the treatment option. This was compared with placebo PDT treatments to account for spontaneous clear-ups. PDT is also used to treat squamous cell carcinomas. When treating squamous cell carcinomas, cryotherapy is an option. PDT has been shown to show significantly less scarring

compared to cryotherapy. On top of this, PDT was shown to be more effective and achieve quicker results than cryotherapy. PDT is also considered when the surgical removal of the affected areas would result in death. PDT has also shown promise in treating basal cell carcinomas. PDT is typically recommended for basal cell carcinomas that are superficial or very thin. Cutaneous T cell lymphoma can also be treated with PDT. Like the other diseases mentioned, examination of location and thickness are determining factors for if this treatment is a viable option. PDT is also used to treat wet macular degeneration. Macular degeneration can either be wet or dry ("Wet Macular Degeneration"). Wet macular degeneration is much more severe and only accounts for 10-15% of cases. It is usually caused by fluid or blood leaking into the macula which is a part of the retina that is responsible for central vision ("Wet Macular Degeneration"). PDT closes the abnormal blood vessels when it is activated.

For this research, the end metallated porphyrin azide can be found in the figure below.

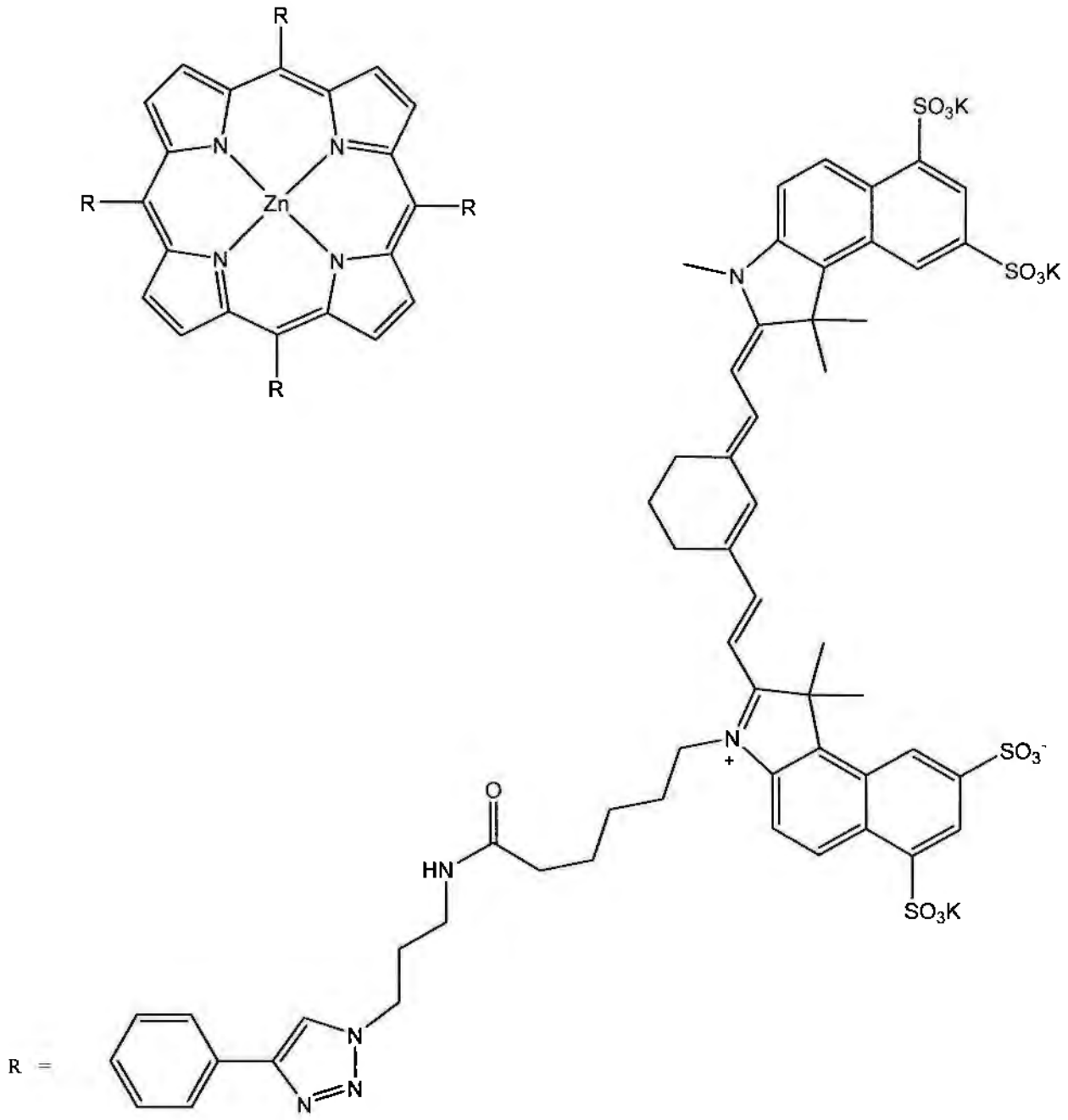


Figure 1.

Fluorescence Versus Phosphorescence

Fluorescence and phosphorescence are very closely related. There is one major difference between the two concerning the length of emission radiation ("Phosphorescence"). Phosphorescent molecules are able to emit light for a longer

amount of time because they emit light at a lower metastable state compared to fluorescence. Fluorescence emits light almost immediately once excited.

Phosphorescence molecules are typically solid materials that have a crystalline structure ("Phosphorescence"). This structure allows for the conservation of energy that allows the molecules to stay in the metastable state that they emit light in. This is part of the reason why phosphorescence is used when making things like emergency exit signs. Phosphorescent paints are so strong that they can be painted on all of the walls of an underground room and will continue to glow for several minutes after all of the lights go out and the walls will glow for several minutes. The amount of light given off after it has absorbed excitation radiation and the amount of absorbed excitation radiation are directly linked.

Fluorescence is a subcategory of cold-light emission ("Fluorescence"). Once a fluorescent molecule has absorbed electromagnetic radiation it goes to a high energy state. Once it falls from this energy state back to the ground state, a photon is released and a light is observed. When looking at the emission radiation that is given off when this happens, it is always longer than the excitation radiation. This shows that the emission radiation has less energy than the excitation radiation. Fluorescent molecules contain alternating covalent single and double bonds. This system of alternating single and double covalent bonds can be seen in the metallated zinc porphyrin. Although fluorescent molecules do not retain the energy for as long as phosphorescent molecules, they are still used in several different areas. Fluorescence is seen in energy saving lamps and observed in special dye molecules. As a special dye, it can be added onto special documents like banknotes to make it harder for forging.

In this research, the metallated porphyrin and azide product fluoresce when exposed to a 365 nm light as shown by the figures below.

Figure 2. Final product in white light.

Figure 3. Final product exposed to light of 365.

Porphyrin

Porphyrins can be described as "highly coloured cyclic tetrapyrrolic pigments formed by the linkage of four pyrrole rings through methene bridges" (Rastogi, 2006). Porphyrins are insensitive to steric hindrance which means it reacts well to having bulky side groups added to it. Porphyrins can also be metallated. Zinc is a great option for this because it is easy to insert but it also easy to remove. The zinc, or whatever metal is used, allows the porphyrin to retain fluorescence and singlet oxygen production. The retention of fluorescence and singlet oxygen production is critical when porphyrins are used in photodynamic therapy. Below is an example of the starting porphyrin used in this research with and without the zinc core.

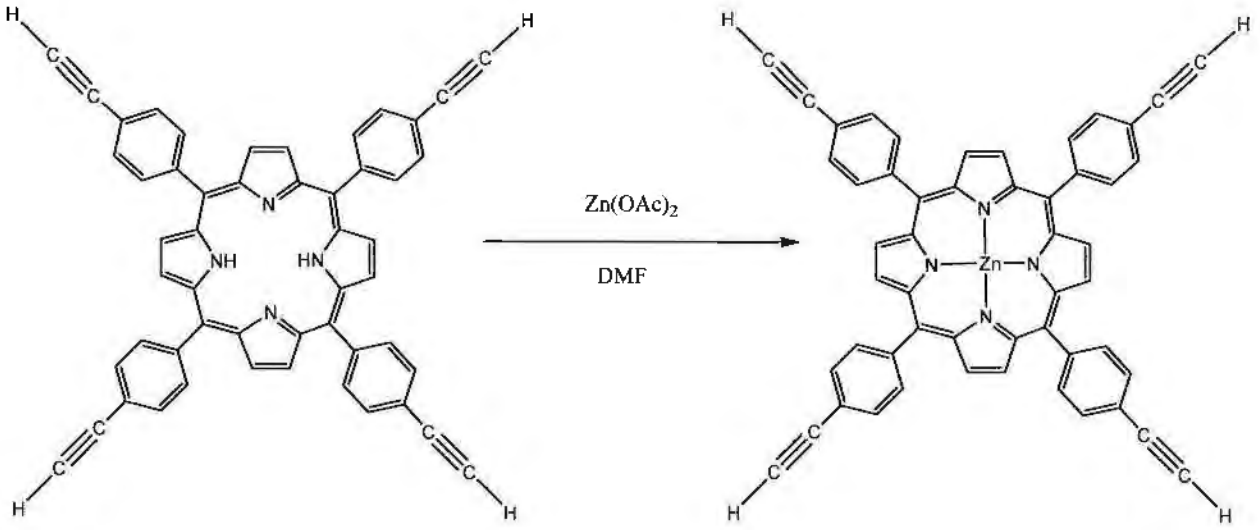


Figure 4.

Click Chemistry

Click chemistry describes reactions that are "high yielding, wide in scope, create only byproducts that can be removed without chromatography, are stereospecific, simple to perform, and can be conducted in easily removable or benign solvents" ("Click Chemistry). It is a relatively new process that was coined by K.B. Sharpless in 2001 ("Click Chemistry"). Click chemistry is used in reactions such as cycloadditions, nucleophilic ring openings, carbonyl chemistry of the non-aldol type, and additions to carbon-carbon multiple bonds (Hein, 2008).

When looking at click chemistry, there is one clear example that perfectly embodies the process. Copper-catalyzed azide-alkyne cycloaddition is this example. This type of click chemistry is especially useful when trying to add R groups to large molecules like porphyrins (Bryden). These reactions require a high temperature in order

to overcome the large activation energy. This is the type of reaction that was used in this research. The figure below (Figure 5) shows a simplified version of this type of click chemistry.

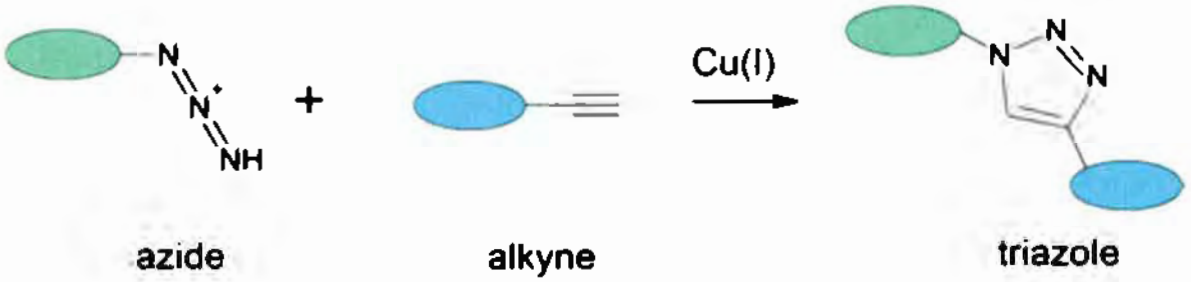
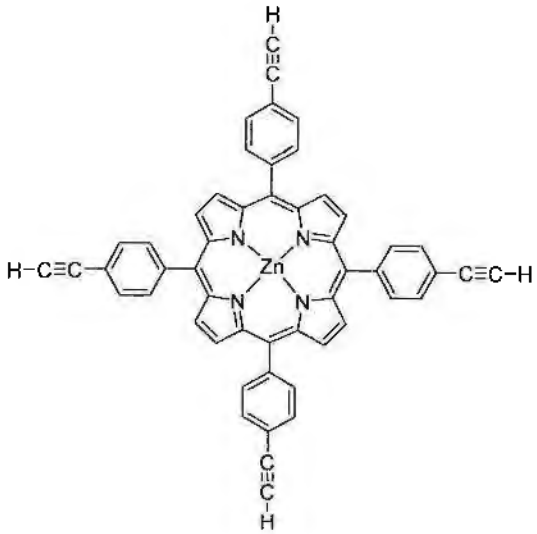
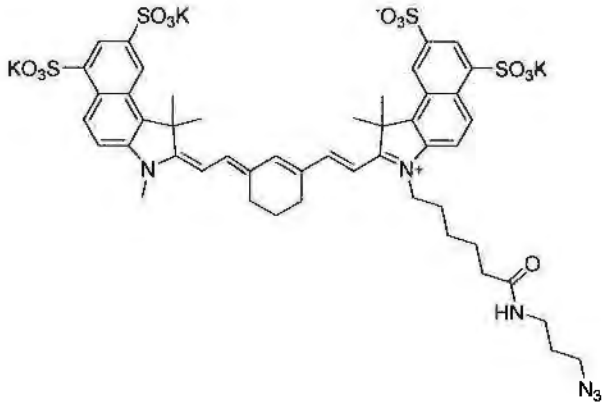


Figure 5.

When looking at the metallated porphyrin that was used in this research, it is a large molecule. The azide that was added on is also a large molecule that is acting as the R group. In figure 6 below, the metallated porphyrin and azide are shown separately. This combination led to a very large ending molecule. As seen in the figure above (Figure 5), the combination forms an aromatic ring containing three nitrogen atoms. Coupling of the azide with the alkyne can be proven from various methods including NMR spectroscopy.



5,10,15,20-tetrakis(4-alkynylphenyl)porphyrinatozinc(II)



Sulfo-Cyanine7.5 azide

Figure 6.

MB231 Cell Line

The MB231 cell line is the most commonly used breast cancer cell line in laboratory studies ("MDA-MB-231"). This cell line was isolated from a 51-year-old Caucasian woman's pleural effusion. This patient was diagnosed with metastatic mammary adenocarcinoma. An adenocarcinoma is a type of cancer that arises in a mucus secreting gland in the body ("Adenocarcinoma"). In this case, it was a mucus secreting gland found in epithelial breast tissue.

The MB231 cell line can be identified because of how highly aggressive, invasive, and poorly differentiated ("MDA-MB-231"). All of these factors make this particular cell line very difficult to kill even in a laboratory setting. The invasiveness of this cell line comes from the proteolytic degeneration of the extracellular matrices of cells. The triple negative aspect of the name comes from the fact that it lacks estrogen receptors, progesterone receptors, and human epidermal growth factor 2 receptors. The lack of these receptors means that the presence of any of these hormones does not

affect the growth of this cancer. At first this sounds like a good characteristic. In reality, many cancer treatments focus on how depriving the cancer cells of some or all of these hormones can prevent the growth of the cancer. Since these hormones do not affect the success of the cancer, treatment options are limited. When working with this cell line in a laboratory setting, if these cells are killed off then whatever you are testing should have great success in killing many different types of cancer cells.

Procedures

Porphyrin Synthesis

To synthesize the starting porphyrin (H_2TPP-4ET), 1.0 g of 4-ethylbenzaldehyde, 0.5 mL of pyrrole, and 100 mL of propionic acid were mixed together in a 250 mL round bottom flask. The reaction was stirred under reflux for 2 hours. The reaction was then allowed to cool to room temperature. And then it was placed into a -20°C freezer overnight. This allowed crystallization to occur. After crystallization, vacuum filtration was performed to isolate the crystallized product. Hexanes was then used to wash the solid. The solid was allowed to air dry overnight. The solid was later purified by silica gel column chromatography using dichloromethane to separate out impurities from the porphyrin. This reaction is shown in Figure 7 below.

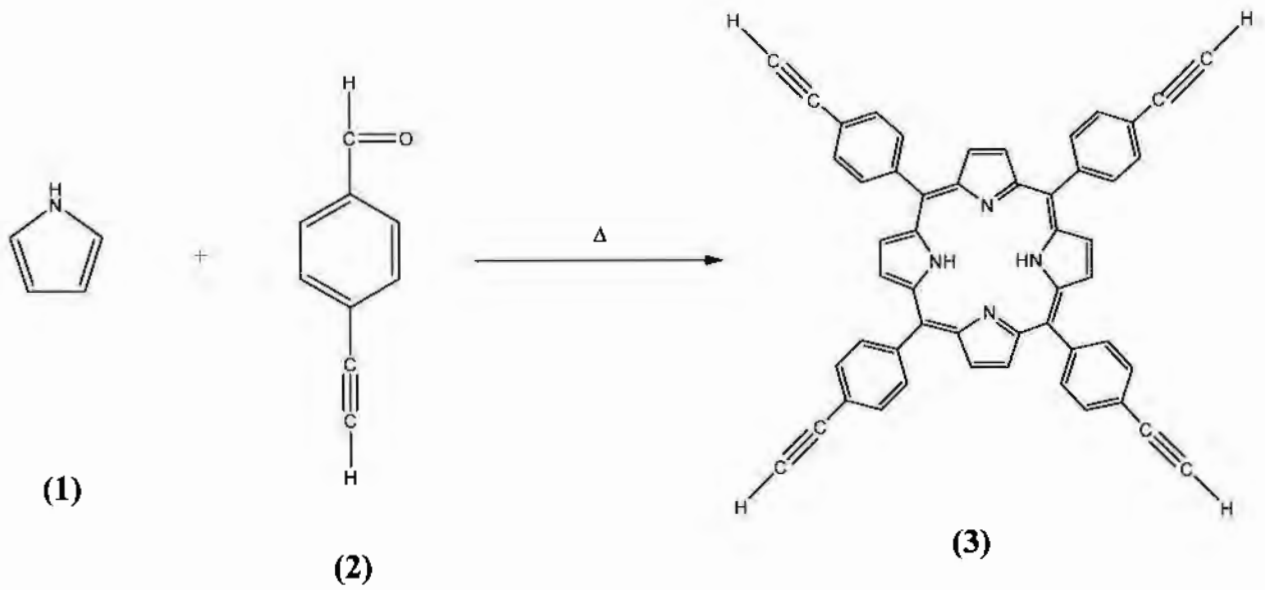


Figure 7.

Metallation of the Porphyrin

The final amount of unmetallated porphyrin produced determines the amount of zinc acetate dehydrate that should be added. In this research, 0.5 g of the unmetallated porphyrin and 0.32 g of the zinc acetate dihydrate were used with an excess of DMF. The zinc acetate dihydrate, porphyrin, and an excess of DMF were placed in a 250 mL round bottom flask and left to reflux and stir for about 2 hours. Next, the mixture was cooled to room temperature and run through a silica gel column with using dichloromethane as the element. The product was then dried using a rotary evaporator. This reaction is shown below in Figure 8.

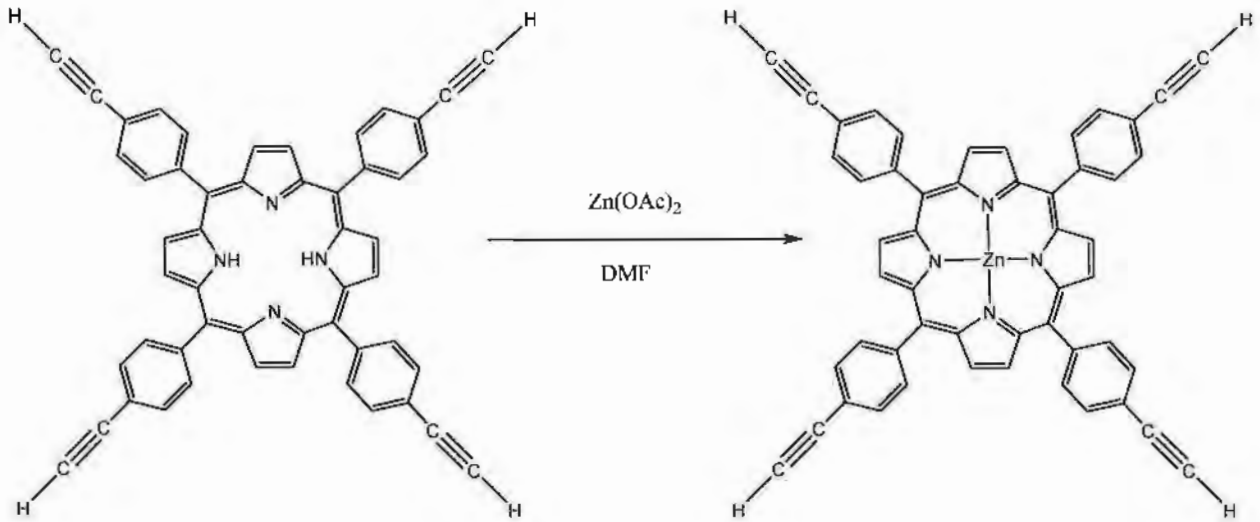


Figure 8.

Coupling of the Zn TPP-4ET with Sulfur-Cyanine 7.5 Azide

The zinc porphyrin ZnTPP-4ET (.0004 g) and .0005 g of the azide were added to 16 mL of THF in a 25 mL round bottom flask. The mixture was degassed in nitrogen for 10 minutes. 0.005 g of $CuSO_4 \cdot 5H_2O$ in 325 µL of H_2O was added to 0.006 g of TBTA in 325 µL of DMSO. This combined mixture was then added to 0.007 g of tertbutylhydroquinone in 325 µL of THF. This mixture was shaken and added to the zinc porphyrin and azide mixture. The mixture was left to stir and heat in a 50 °C water bath overnight. The solution was then run through a silica gel column to purify using dichloromethane as the eluent. The purified material was isolated and characterized by IR, UV-vis, fluorescence, and NMR.

Treatment of MB231 Cell Line

The MB231 cell line was plated on 100 cm^2 plates at 15,000 cells/cm^2. The cells were plated with growth serum medium. The cells were maintained and split for a few weeks before testing began. Once it was time for the experiment, the number of cells on each plate was calculated so the proper dilutions could be done to get to 2,000 cells/ well in 100 µL liquid. The dilution was performed with serum-free growth medium. Two 96-well plates were obtained and the cells were plated onto these. The two plates were covered in foil and placed in the incubator for two days to allow the cells to adhere.

The liquid was aspirated out of the wells and replaced with different concentrations of the metallated porphyrin azide dissolved in DMSO and serum-free growth medium. The concentrations were 1 mg/µL, 5 mg/µL, 10 mg/µL, 25 mg/µL, 50 mg/µL, and 100 mg/µL. Other wells had pure DMSO dissolved in serum-free growth medium and some had just new serum-free growth serum added.

The next day the liquid was aspirated out of the wells and 100 µl of serum-free growth medium was added. One of the plates was then exposed to light for 20 minutes while the other was placed back into the incubator with the foil. The plate that was exposed to light was also added to the incubator after the 20 minutes. The next day, the liquid was aspirated out of the wells and replaced with MTT solution. The plates were then placed on the Belly Dancer for 3 hours. An MTT assay was then run on both plates.

Results

Ultraviolet Visible Spectroscopy

UV-vis showed at what wavelength the metallated porphyrin azide is activated. Based on the results shown in the figure below, the compound has an absorption maxima at 424 nanometers.

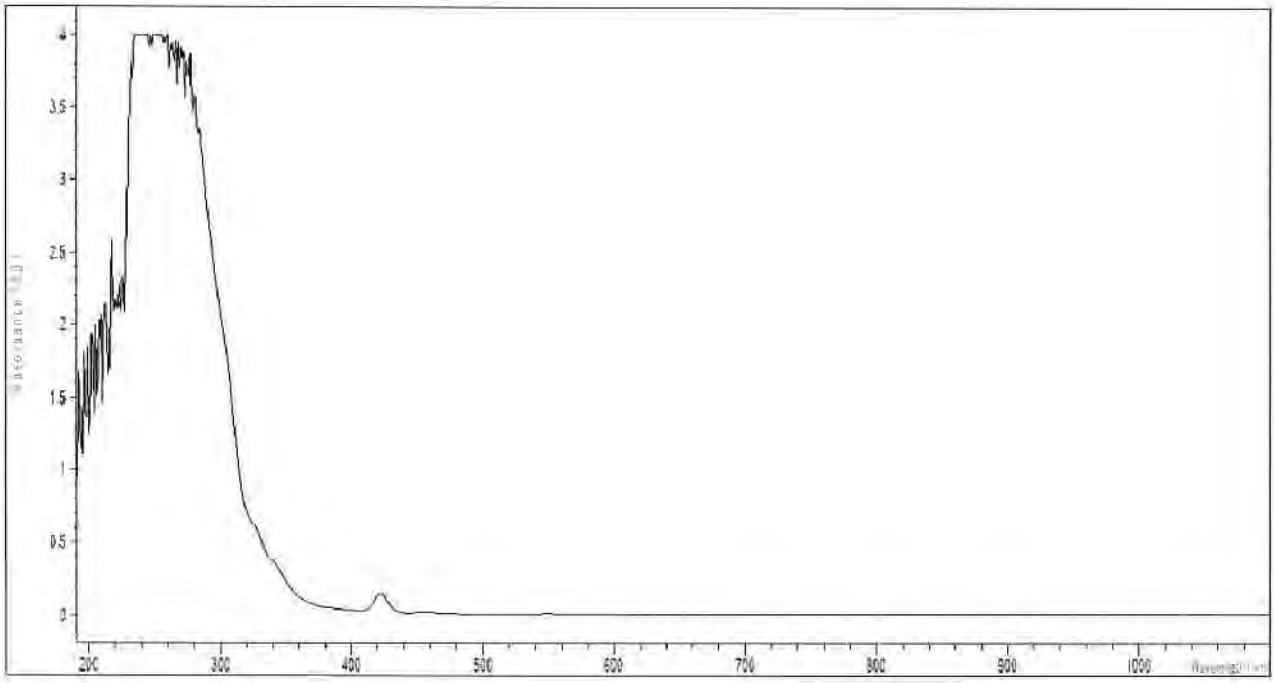


Figure 9. UV-vis results for the metallated porphyrin azide.

UV-vis was also run to calculate the molar extinction coefficient. This value was needed in calculations to determine how much of the metallated porphyrin azide was needed to get to the desired 25 mM concentration for the stock solution for MTT Assay. The molar extinction coefficient is .004193 mM^{-1} cm^{-1} at 424 nm.

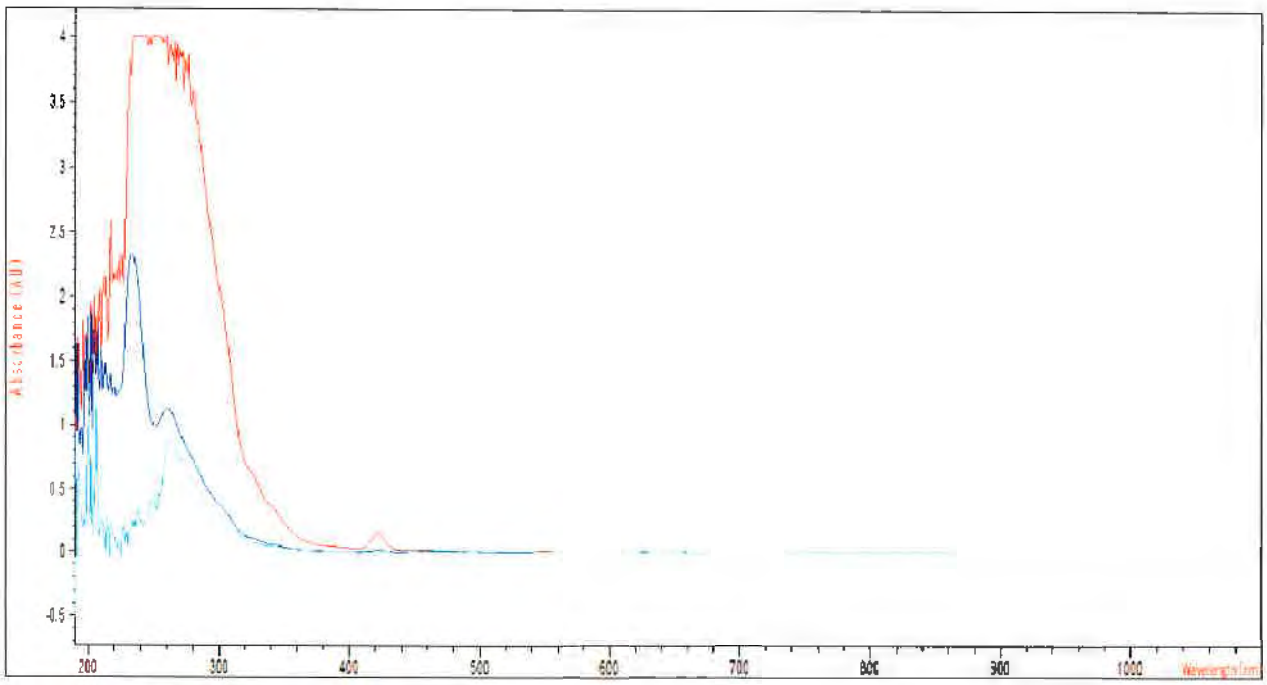


Figure 10.

The red line (Figure 10) is the non-diluted metallated porphyrin azide. The dark blue line is the 25 mM stock solution of the metallated porphyrin azide. The light blue line is the 10 mM stock solution of the metallated porphyrin azide.

Fluorescence Spectroscopy

Fluorescence results show that 373 nm with an excitation wavelength for the final coupled porphyrin azide product (Figure 1) produced an emission at 458 nm. The metallated porphyrin azide emitted at 650 nm at the same excitation wavelength of 373 nm. These results show that the addition of the sulfur-cyanine 7.5 azide causes the metallated porphyrin to emit at a wavelength closer to the ultraviolet range.

Nuclear Magnetic Resonance

The NMR results showed that the metallated porphyrin and the azide did combine. This is shown by comparing the metallated porphyrin and the metallated porphyrin azide. When looking at the NMR of the metallated porphyrin in Figure 11,

there are signals that point towards the following structures being present: TMS with the signal at 0, chloroform with the signal around 7, and benzene and pyrrole rings with the signals at 8. When looking at the metallated porphyrin azide in Figure 12, the above signals are present as well as the signal around 1 that shows the presence of triple bonds on alkene groups. The NMR for the metallated porphyrin azide in Figure 12 shows signals that point to the same structures as with the metallated porphyrin.

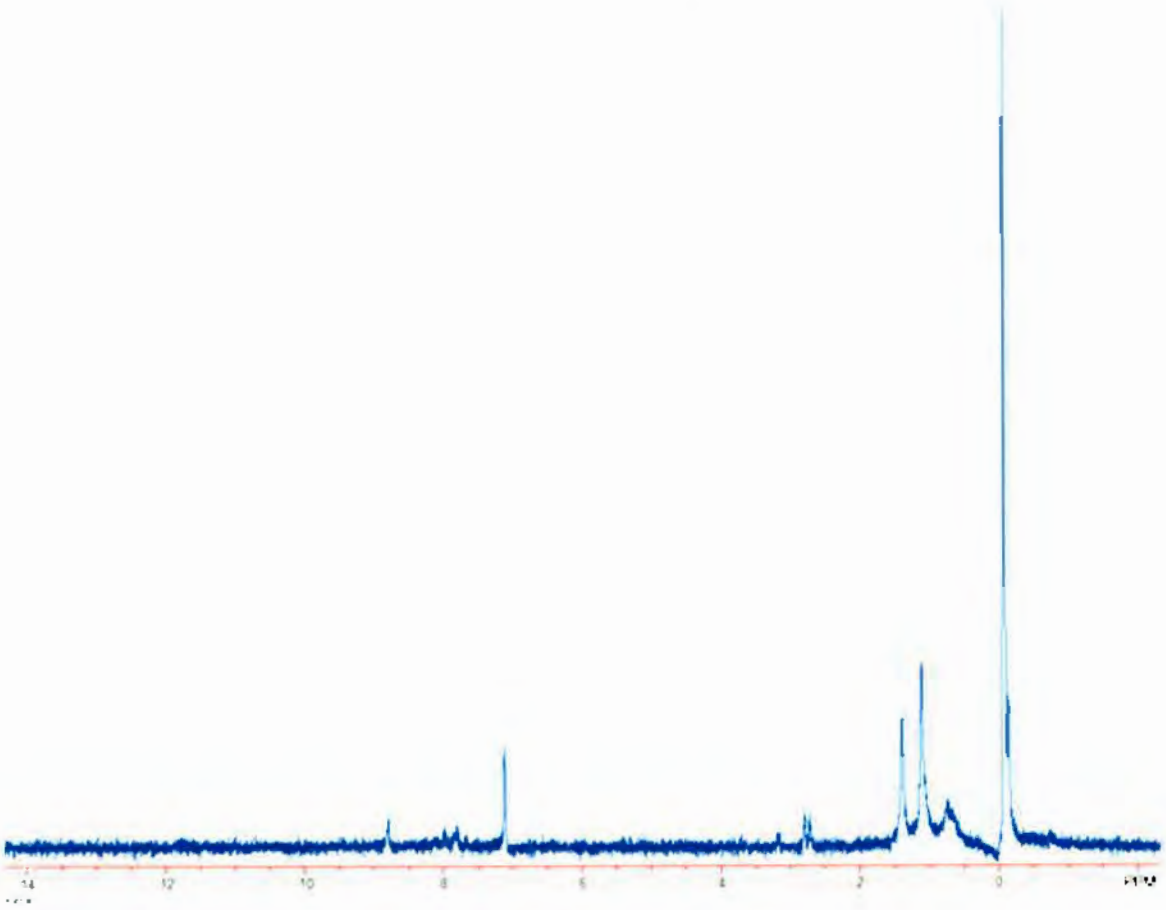


Figure 11. NMR of metallated porphyrin.

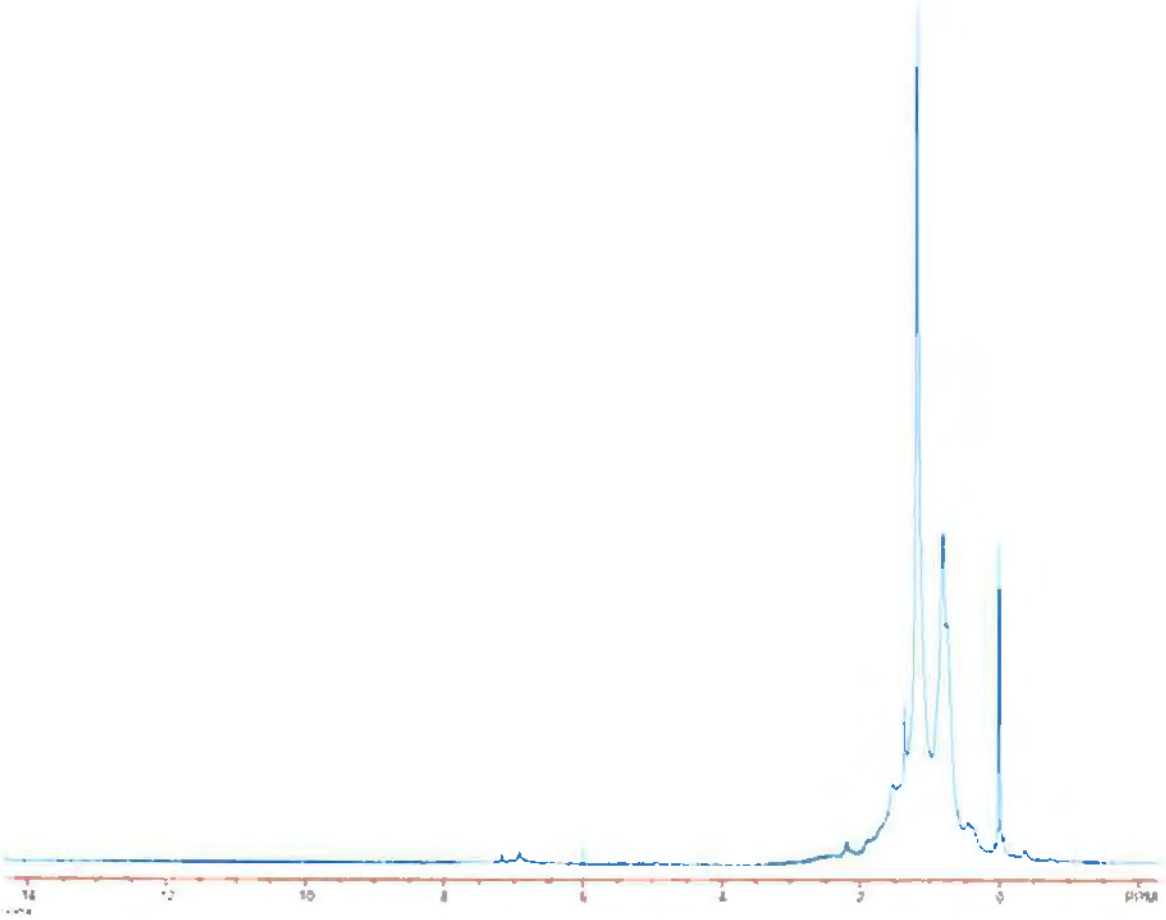


Figure 12. NMR of metallated porphyrin azide.

MTT Assay

The results of the MTT assay show that the metallated porphyrin azide is not apparently toxic to the cells at the concentrations investigated. This was demonstrated when the different concentrations on the plate not exposed to light had relatively the same cell viability as that of the plate exposed to the light in Figures 13 and 14. Unfortunately, the results also show that the light that the one plate was exposed to did not activate the metallated porphyrin azide.

When performing an MTT assay, you can look at the plate and get an idea about cell viability. The darker the shade of purple, the more cell viability. Looking at the two

plates this way, it looked as if the cell viability was the same as seen in Figures 13 and 14. A plate reader was used to calculate true cell viability as seen in Figures 15 and 16.

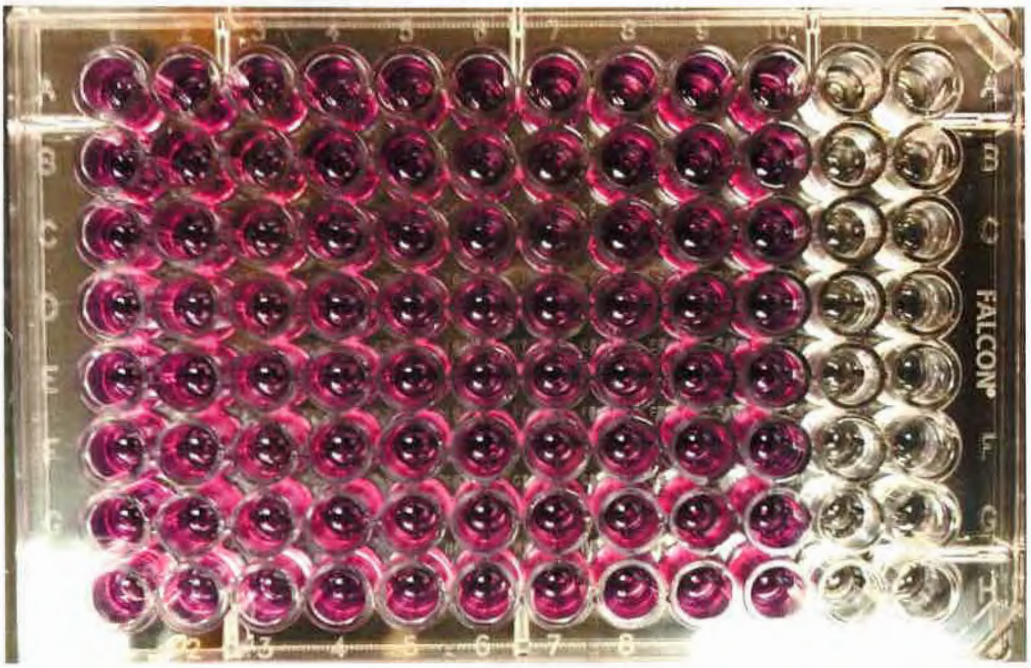


Figure 13. Plate that was not exposed to light.

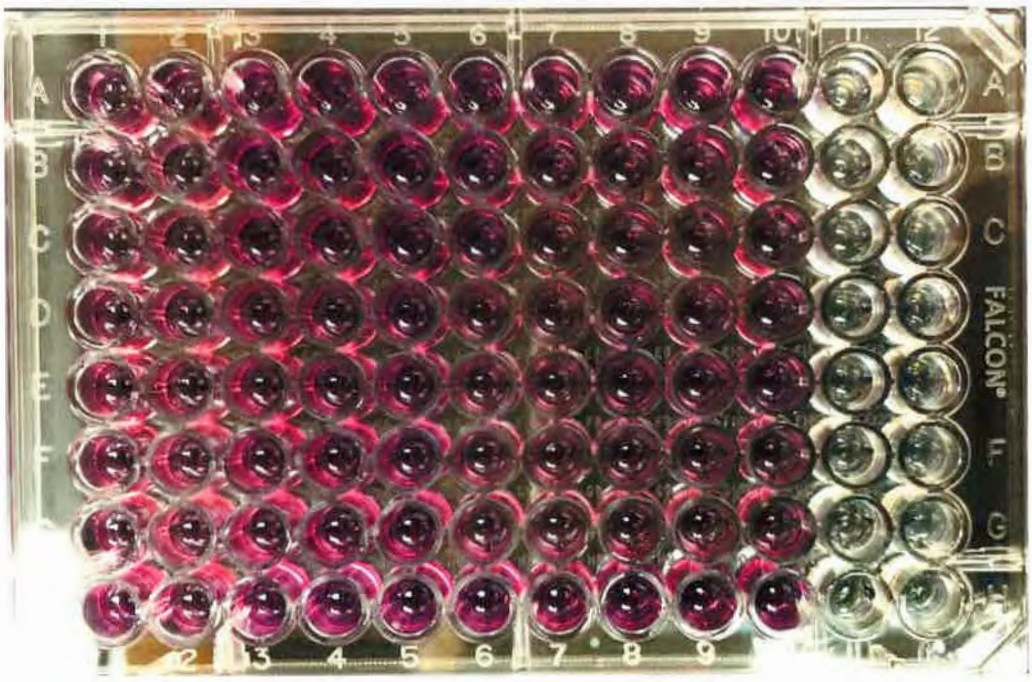


Figure 14. Plate that was exposed to light.

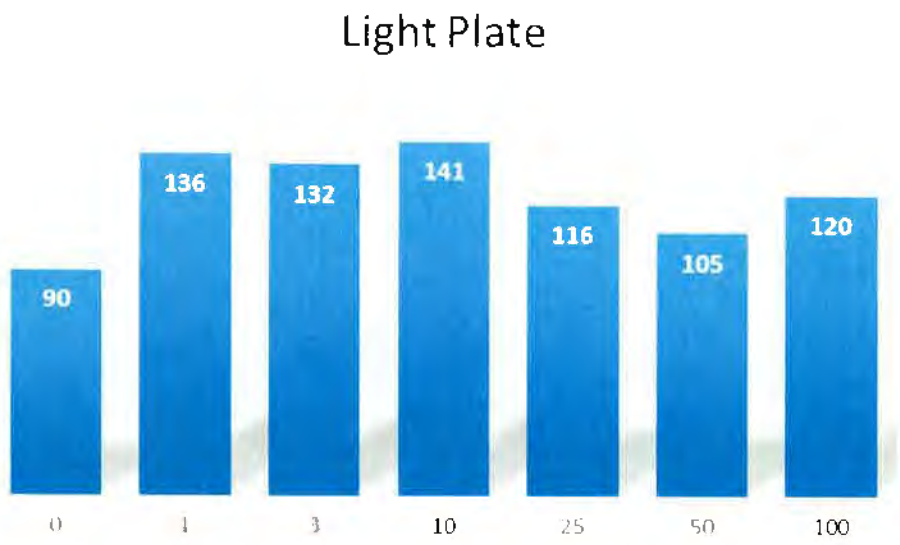


Figure 15. MTT assay results from the plate exposed to light. The x-axis is the concentration of metallated porphyrin (in μM) and the number on each bar is the cell viability percentage.

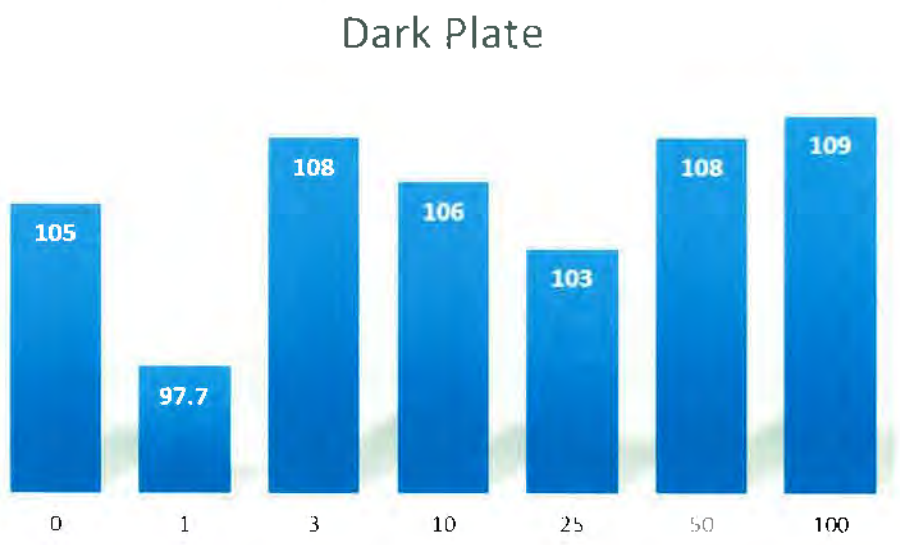


Figure 16. MTT assay results from plate not exposed to light. The x-axis is the concentration of metallated porphyrin (in μM) and the number on each bar is the cell viability percentage.

Conclusion

Based on results from examining the compound under UV light, running UV-vis, fluorescence, and NMR spectroscopies, the metallated porphyrin and azide did combine. However, the results of the MTT assay show that there was no true difference in cell viability between the two plates exposed to light or dark conditions or between the different concentrations. These results show that although the metallated porphyrin was able to combine with the azide, the two together were not an effective agent for PDT. If it was, the MTT assay would've shown less cell viability on the plate exposed to light because the light should've activated the porphyrin and led to cell death. The high levels of cell viability also shows that the metallated porphyrin azide is not toxic to the cells.

In future research, I would suggest testing the two plates again in a similar fashion but using a different light source. When looking to see if the metallated porphyrin azide exhibited fluorescence, the compound is excited at a wavelength of 365 nanometers. It would be beneficial to expose one of the plates to a light source that is at or close to this wavelength to examine cytotoxicity in an MTT assay.

Additional Research

As was mentioned earlier, patient attitude towards treatment was examined and compared to treatment effectiveness. Before looking at the research, it was hypothesized that there should be a correlation between an optimistic attitude and better treatment outcomes. Looking at research, the results are inconclusive.

There have been several studies performed on patients with various types of cancers. Many of these studies show a correlation between attitude and treatment effectiveness. Some studies, however, didn't show a correlation between attitude and treatment effectiveness but did show that positive attitudes helped with coping ("Attitudes and Cancer"). In order to maintain these positive attitudes, patients took part in activities like group therapy with other people that had cancer diagnoses. These studies showed that therapy and relaxation could help with reducing distress. While it is disheartening to see from these studies that attitude didn't affect outcome, attitude can help with mental health. An improvement in mental health makes the duration of treatment more bearable and help quality of life.

The studies that showed a correlation between attitude and outcome are less conclusive. In one of these studies, patients with more optimistic attitudes were less likely to be hospitalized for complications (Fischer). These complications were shown to lead to worse overall outcomes and lower survival rates. These results are less conclusive because it is hard to know what is the cause and what is the effect. One option is that these patients' attitudes are to blame for the level of effectiveness and the higher likelihood of a poor overall prognosis. A second option is that these patients had

bad attitudes because the treatment had an adverse effect that made them feel unwell. These adverse effects would lead to the poor outcome and the pessimistic attitude.

Gathering data for this part of the research was difficult because of how contradictory the research studies are. For every study that found no correlation, there was another study that showed that there was one. The problem seemed to be that the sample sizes were small and seemed to be picked with some bias according to reviews of the research studies. In the future, more randomized studies would be beneficial to organize. In order to determine the effects, the studies should be longer in length and have qualified health professionals interview the patients and determine their mental state and attitude. These studies are important to do because if attitude can affect effectiveness, more work should be put into ensuring patients stay optimistic throughout treatment. If it is the case, this could lead to patients being able to have positive outcomes with less surgery and chemotherapy.

Acknowledgments

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