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Killing Breast Cancer One Porphyrin at a Time

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I would like to dedicate this thesis to my dad, Brad Lymburner, as well as Dr. Tim Knight and Dr. Joe Bradshaw. Thank you for always pushing me to do my best and succeed in everything I do.

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Abstract

New treatments for cancer are continuously being developed and improved. One such treatment is Photodynamic Therapy, more commonly referred to as PDT. PDT is quickly becoming more popular due to its relative lack of side effects that are present in other treatments. In PDT, light-sensitive agents are required and are activated by light in the targeted cells. There are many types of PDT agents but the one focused on in this research is a four-pyrrole ring structure known as a porphyrin[1]. The combination of H_2TPPC with 3-amino-oxetane-3-yl-methanol created the final product of H_2TPP-Oxo-MeOH. Once the porphyrin was formed, it was then characterized using infrared spectroscopy (IR), UV-vis spectroscopy, and nuclear magnetic resonance (NMR). Its photocytotoxicity or its effect on cell death in triple-negative breast cancer cells, specifically MDA-MB-231, was tested using an MTT Assay with two conditions, dark and light.

Introduction

Cancer is something that is relatively common and will affect approximately 38.4% of people at some point in their life[2]. Since its discovery, researchers have spent years distinguishing its forms and developing new and more effective diagnostic techniques as well as treatments. The rate of cancer survival has only increased as scientific research in the area has progressed. Since it is a disease that affects so many people, there are thousands of fundraising events and billions of dollars are donated to continue the research. Cancer therapy and treatment is an area that is constantly being researched and improved in many ways. The most common form of cancer treatments are chemotherapy or radiation. These treatments not only lack the ability to target certain cells but also may cause many severe side effects. They can also lead to many complications that can actually hinder a patient's healing and decreases their quality of life[3]. A newer form of therapy known as photodynamic therapy lacks the side effects that chemotherapy has while also being able to target specific cells within the body.

Background

Triple Negative Breast Cancer

Triple- Negative Breast Cancer (TNBC) is named as such because this type of cancer does not have the markers for estrogen (ER), progesterone (PR), and HER2. Simply put, TNBC does not have those hormone receptors present and therefore does not respond to typical anti-hormonal treatments. In the beginning, researchers thought that all breast cancers were the same[4]. With advances in science came new ways to test cancer and read

genes. Because of these advances, researchers learned that not all breast cancer is the same and have been developing new ways to treat each type of breast cancer. While anyone can develop breast cancer, it has been found that young women, African American women, and *BRCA1* mutation carriers are at a greater risk for developing TNBC than other groups of people[5,6]. Around 70%–90% of cases of TNBC have a basal-like genetic pattern. Simply put, this means that the cancer cells look similar to the cells that line the breast ducts. They typically over express genes that encourage the growth of these cancer cells[4].

Treatments

In order to treat Triple Negative Breast Cancer, there are five different options. The first is a lumpectomy. As its name suggests, a surgeon will simply remove the lump from the breast and check to make sure cancer has not spread. A mastectomy is similar to the first but it removes the entire breast rather than a part. The next two treatments, chemotherapy and radiation tend to have similar impacts on the body[7]. Chemotherapy utilizes different drugs to target any rapidly growing cell. Due to its lack of specificity, it also targets rapidly growing healthy cells such as hair cells and the lining of the intestinal tract[3]. Radiation is similar to chemotherapy but rather than a drug, it uses high-energy radiation and is site-specific. It has very similar side effects to chemotherapy[7]. A more recently developed treatment for cancer is that of photodynamic therapy (PDT). Unlike other types of treatment, it is minimally invasive, targeted, and limited in its side effects.

Development of Photodynamic Therapy

The idea of photodynamic therapy has been around since ancient Greece but was lost until 1841 with the discovery of hematoporphyrin. Phototoxicity, which is the death of cells

after exposure to a chemical and then light, was discovered in a study done from 1861-1871. Further studies were done and in 1903 N.R. Finsen received the Nobel Prize for Phototherapy[9]. Porphyrins, which are photosensitizing agents used in PDT, were examined in 1925 by H. Fischer[9]. The basis for the modern version of PDT was the hematoporphyrin derivative (HpD) study done in 1960 by Lipson, et al[9]. Photodynamic therapy has three functional elements that are necessary to its effectiveness. The first is a photosensitizer. This can be a drug, such as a porphyrin, or another small molecule that is used to target cancer cells and is activated by light. The next element is an optical wavelength of light. This is the trigger for the photosensitizer to interact with oxygen and release the chemical necessary for cell death. Wavelength can vary depending on what drug is used and what researchers are trying to investigate. The last element is molecular oxygen. This element is tied to the photosynthesizer and is required to create an oxygen radical species needed to initiate cell death[10].

PDT in Practice

Pros and Cons of PDT

When debating photodynamic therapy as a treatment, it becomes necessary to examine both the positives and negative of the treatment. The biggest advantage of PDT is that it has no lasting side effects. Unlike chemotherapy and radiation, PDT is relatively easy on the body as a whole. It also less invasive and is a quicker outpatient option. Many treatments, such as radiation or surgery, require long stays in the hospital that can be very draining on the patient. PDT's use of light and light sensitive agents allows it to target certain areas in the body. Because it is easier on the body, it can be repeated many times in the same area. One of the biggest pros for PDT is its affordability. Financial burden is

something that many people who have cancer struggle with. The cost for the more common cancer treatments and hospital stays is more than most people can afford.

While Photodynamic Therapy has a long list of benefits in its favor, it also has its drawbacks. Due to its use of photosensitive agents, some people can be affected by the sun after treatment. Therefore, extra precaution must be taken to remain out of direct sunlight until the agents have left the patient's system. PDT is also limited in areas that it can reach within the body. It can only treat tumors that can be reached with a light source. It is also not able to treat cancers that have spread to many areas within the body. Lastly is the fact that individuals with certain diseases cannot receive the agents used in PDT. One such case is those with porphyria. Porphyria is a rare blood disease that affects the skin and is a result of the build up of naturally produced porphyrins. In extrememly rare cases, people can be allergic to the porphyrins used in the treatment[10].

How PDT Works

Photodynamic Therapy is a treatment that utilizes light and photosensitizing agents to target and kill cancer cells. The agent or drug can be administered one of two ways, through an injection or topically. How it is administered is dependent on the location of the cancer cells. Once the cancer cells absorb the agent, it can be activated by light. After being activated, it interacts with oxygen to create a singlet oxygen species that kills cancer cells. Depending on the drug used, the drug-to-light interval or the time between when the patient receives that drug and when the light can be applied ranges from two hours to two days[9,10].

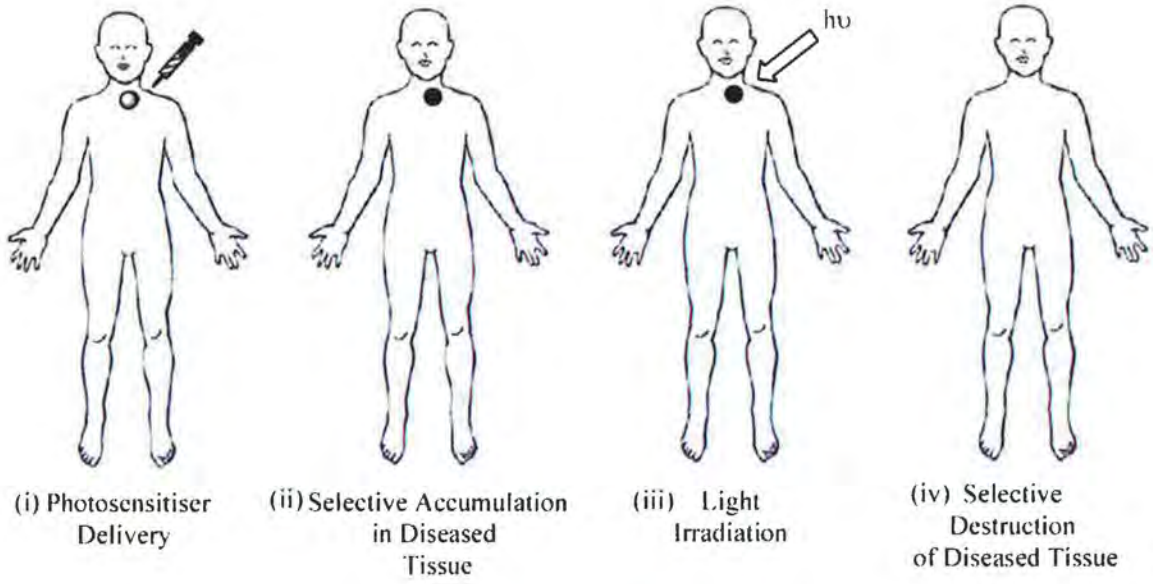

FIGURE: 1
The procedure used in PDT[11]
(https://www.ncbi.nlm.nih.gov/pmc/articles/PMC3475217/)

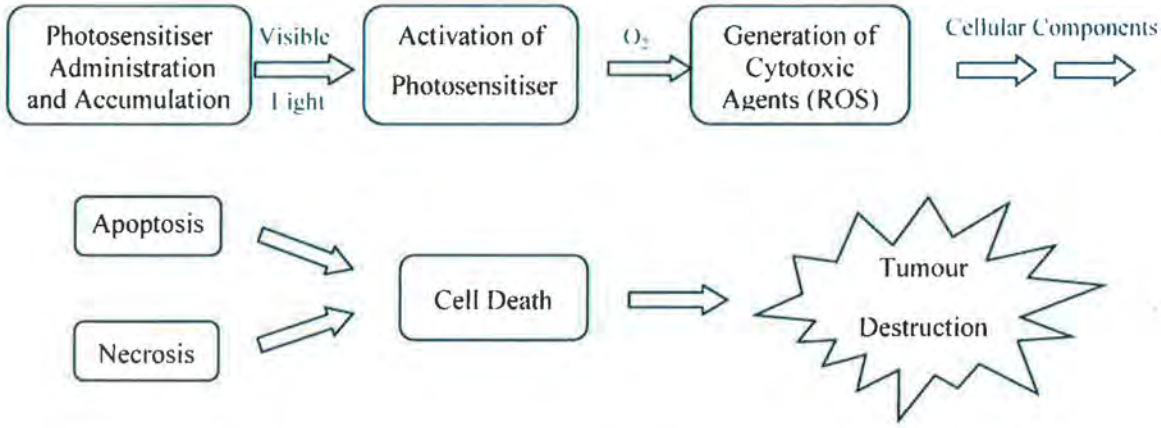

FIGURE: 2
Process of cell death after activation of the Photosensitizer[11]
(https://www.ncbi.nlm.nih.gov/pmc/articles/PMC3475217/)

Health Problems Treated with PDT

While cancer is the main issue that is treated with Photodynamic Therapy, it is also used to treat different skin problems. These issues include sun damage, acne, signs of aging and many more. The basic process of PDT is the same no matter what is being treated; the

types of drugs and light wavelength change. When dealing with skin issues, Levulan and blue light are used. Levulan contains the active ingredient 5-aminolevulinic acid hydrochloride which acts as the photosensitizing agent[12].

FIGURE: 3
Example of PDT using blue light[12]
(https://www.medicinenet.com/photodynamic_therapy/article.htm)

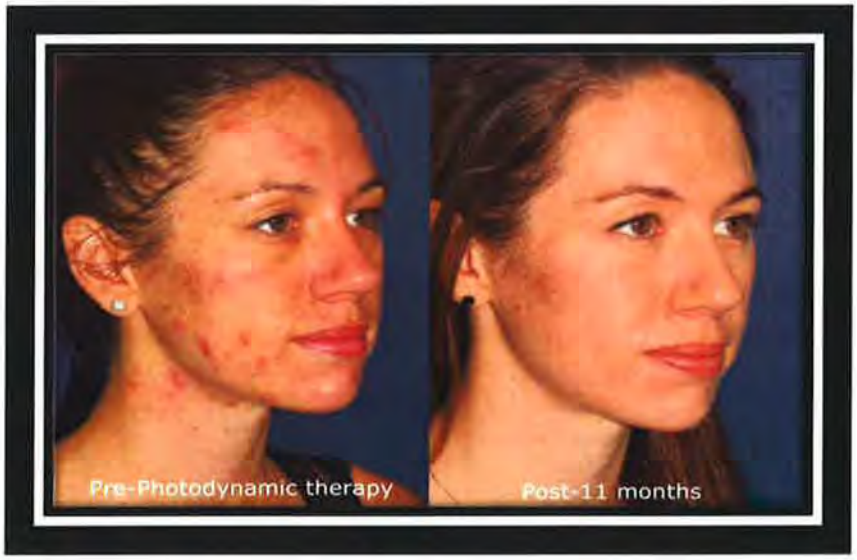

FIGURE: 4
Before and After PDT [13]
(https://clderm.com/procedure/photodynamic-therapy/)

Future of PDT

As the research in the area of Photodynamic therapy continues, more health problems may be able to receive this treatment and it will become more effective as time goes on. Some doctors even use PDT to treat issues such as actinic keratosis and certain eye conditions such as macular degeneration. While this treatment has not been approved by the FDA to treat all types of cancer, it has been approved for some and has also been approved for different medical trials. Another area of improvement for PDT is the development of novel photodynamic agents. These future drugs may be able to target tumors that are even deeper in the body as well as become more directed in the targeting of cancer cells as opposed to healthy cells. The next area of change would occur in the light source itself. This specifically looks at the use of light in differing doses in order to limit the side effects. The final area that researchers are looking at is the combination of PDT and other existing treatments in order to make it more effective. If research into PDT continues, it potentially may become the primary cancer treatment in many cases, replacing the existing versions of chemotherapy and radiation as treatments.

Materials and Methods

Research Overview

In this research, a novel light-absorbing compound was created. This compound, known as a porphyrin, can be activated by light photons and be used to target specific cells. The two most important factors in this research were that the new PDT agent created was water-soluble and had a negative impact on the growth of TNBC cells when exposed to light. The goal of this project was to create a porphyrin using the amine, 3-amino-oxetane-3-

yl methanol that would accomplish the two requirements listed above.

Porphyrin Synthesis

The synthesis of the porphyrin compounds can be described in three steps. In the first step, 4-carboxybenzaldehyde reacts with pyrrole in propionic acid to form H_2TPPC **(3)**. The H_2TPPC from step one was then reacted with thionyl chloride ($SOCl_2$) in dimethylformamide (DMF) to form H_2TPPCl **(4)** in step two. In the final reaction, the acid chloride was reacted with 3-amino-oxetane-3-yl methanol in methanol to create the final porphyrin product H_2TPP-Oxo-MeOH **(5)** which was used in the rest of the study.

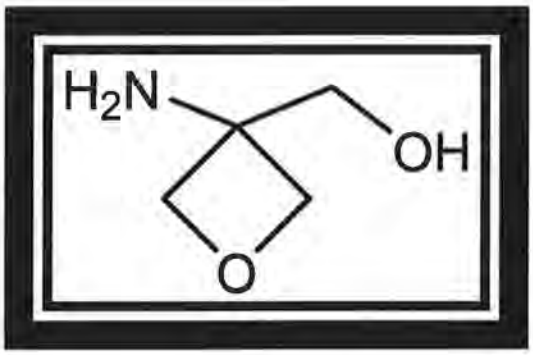

FIGURE: 5
3-amino-oxetane-3-yl-methanol

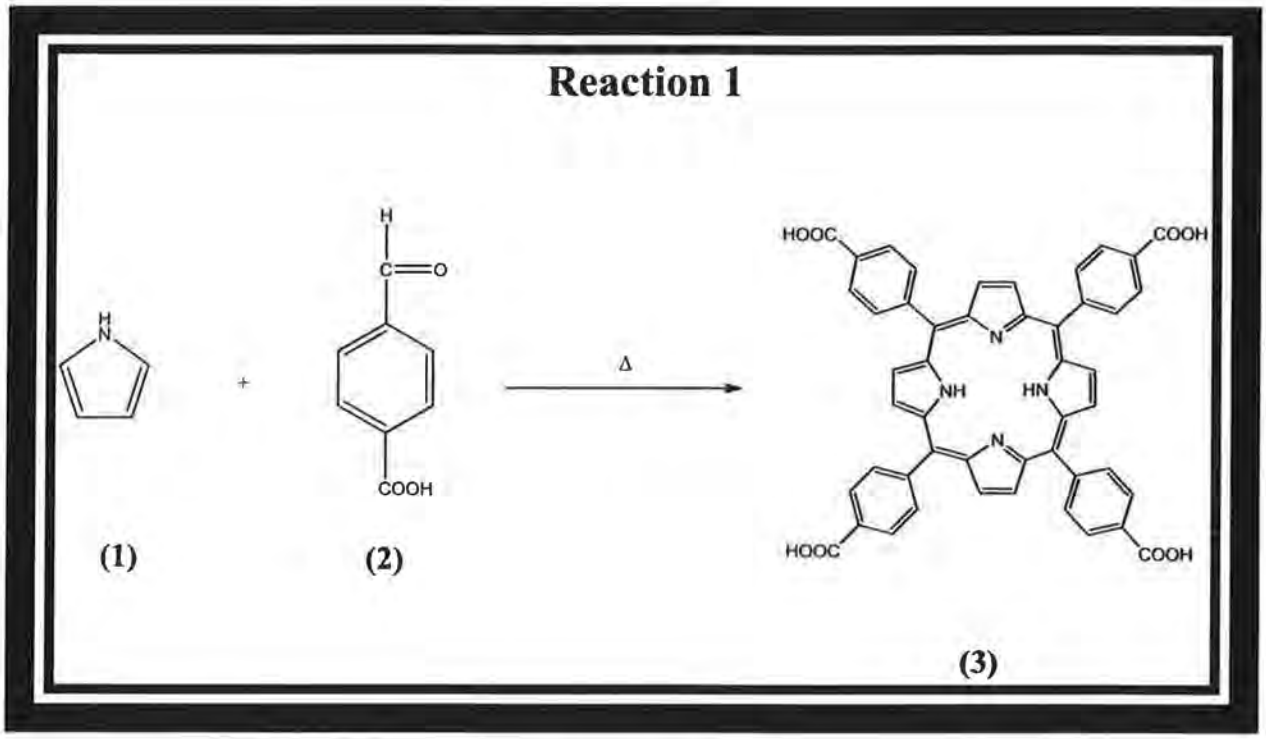

FIGURE: 6
Pyrrole **(1)** reacts with 4-carboxybenzaldehyde **(2)** in propionic acid to form H_2TPPC **(3)**

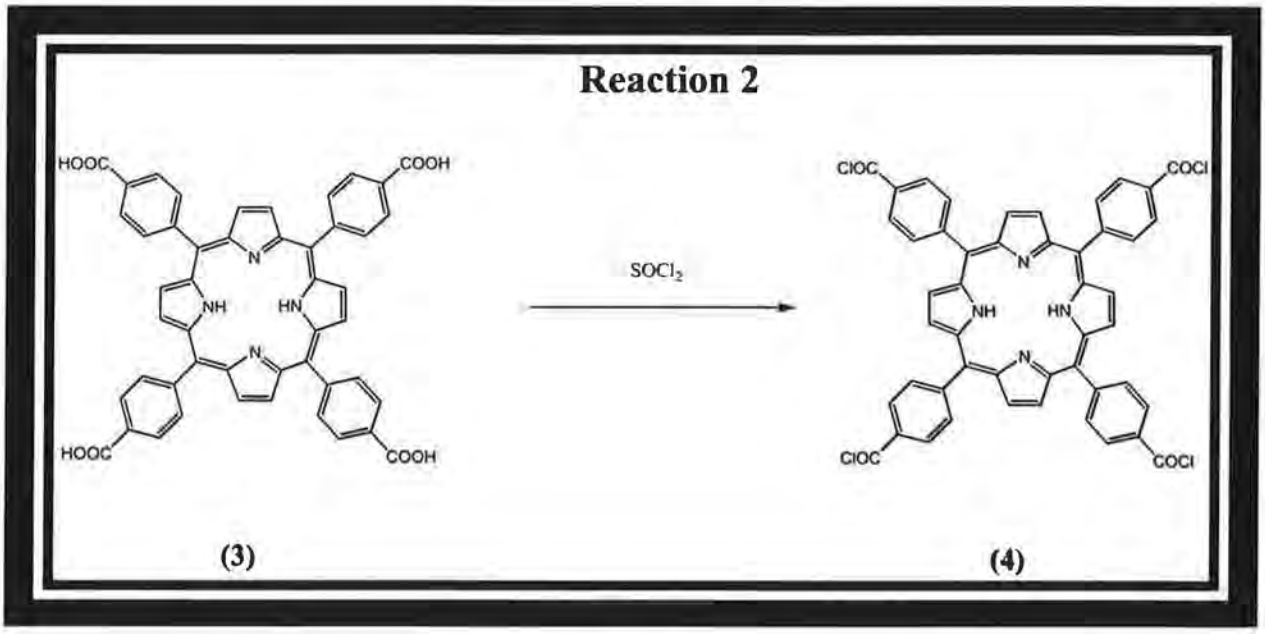

FIGURE: 7
H_2TPPC **(3)** from step 1 reacts with $SOCl_2$ in DMF to create H_2TPPCl **(4)**

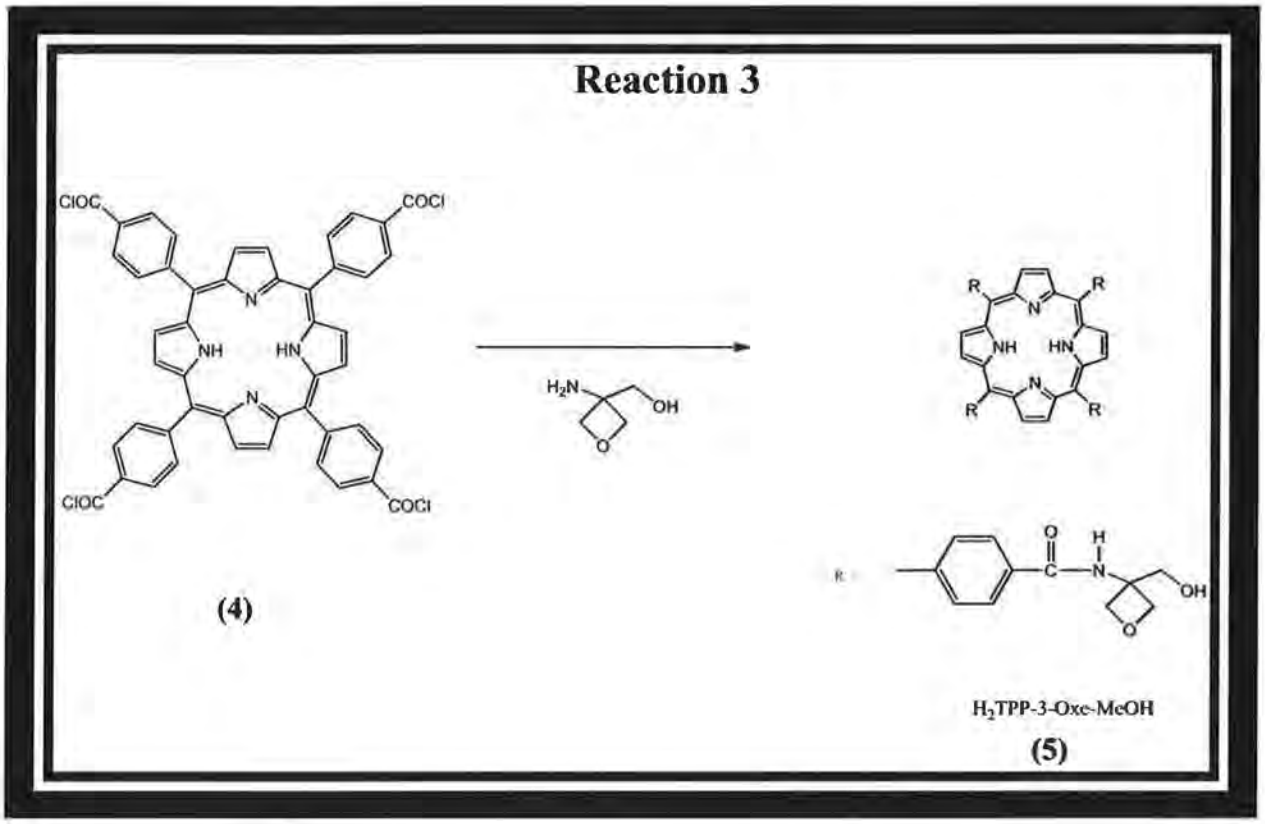

FIGURE: 8
The acid chloride intermediate **(4)** reacts with 3-amino-oxetane-3-yl-methanol in methanol to form H_2TPP-Oxo-MeOH **(5)**

H_2TPPC Synthesis

In order to create a water-soluble compound using the amine (3-amino-oxetane-3-yl-methanol) the starting material H_2TPPC first had to be synthesized. The first step in this process was to add 3.00g of 4-carboxybenzaldehyde ($2.0x10^{-2}$ mol) to a clean, dry 500 mL round-bottom flask with a stir bar. After the flask was placed on the heating mantle, 200 mL of propionic acid was added. With the aid of a syringe, approximately 1.4mL of pyrrole ($4.8x10^{-2}$ mol) was then added to the mixture. After all three compounds were in the flask, the flask was wrapped in aluminum foil and allowed to reflux for 1 hour. The aluminum foil was necessary to protect the reaction from light. After an hour, the reaction was removed from the heat and left to cool. After the flask and its contents cooled completely, parafilm

was placed over the opening and it was placed in the freezer overnight to crystalize.

The following day, the compound was filtered using a medium sintered glass filter and dichloromethane was used to wash the product, H_2TPPC **(3)**. The filter and the compound were left to air-dry overnight. At this point, the compound was purple/black in color.

H_2TPP-Oxo-MeOH Synthesis

In order for the amine to attach to the porphyrin, an acid chloride intermediate needed to be created. This reaction is not only air sensitive but water sensitive as well. In order to limit any possible interference, all the glassware was placed in an oven to dry overnight. To start this reaction, 0.13g of H_2TPPC ($1.64x10^{-4}$ mol) was measured and placed in a 50 mL round bottom flask. The flask was then placed under a nitrogen sparge in order to keep the environment dry. A stir bar and 10 mL of DMF (dimethylformamide) were added to the flask to dissolve the H_2TPPC. The mixture was stirred under N_2 sparge for 1 hour after using a syringe to add 0.15 mL of $SOCl_2$ (thionyl chloride, $2.06x10^{-3}$ mol). A green product (H_2TPPCl) was formed.

Once the reaction had stirred for an hour, it was removed from the nitrogen sparge and placed on a rotary evaporator or rotovap with the help of KECK clips. The purpose of this step was to remove the DMF solvent and to leave the desired product behind. This process was done by heating the mixture under vacuum. The flask and its contents remained on the rotovap until it was completely dry. The flask with the dried acid chloride intermediate **(4)** was then kept under vacuum overnight. The product in the flask was dark and shimmery green color.

For the final reaction, fresh methanol was distilled. The flask was moved from the

vacuum and placed back under a nitrogen sparge. 0.25g of the amine 3-amino-oxetane-3-yl-methanol ($2.42x10^{-3}$ mol, Figure 5), and 25 mL of the methanol were added to a clean, glass vial. The mixture was transferred to the flask containing the acid chloride intermediate and was then stirred for an hour. Once the time was finished, the methanol was removed under vacuum. The final product H_2TPP-Oxo-MeOH **(5)** was dark purple in color.

This process was repeated three times in order to obtain a sufficient amount of product. Once these three syntheses were achieved, the final productwas purified using column chromatography.

Purification of the Porphyrin

In order to purify the porphyrin a process known as column chromatography was used. Chromatography is used to separate various components of mixtures. The column is broken down into two specific areas. The top is liquid (mobile phase) and is used to push the compound through the solid (stationary phase) bottom half. The mixture is separated into different components based on how it interacts with the stationary phase (Figure 9). The stationary phase is placed in the column to separate the impure product[14].

The first column was prepared by filling a glass column with Sephadex LH-20 and using 50:50 methanol/water MeOH-H_2O as the column material. The second column was prepared in the same way but used Sephadex G-50 and Milli-Q H_2O as the column material.

Before the impure porphyrin product was placed in the first column, it was first filtered through a 0.45 mm nylon syringe filter to purify it. The desired porphyrin product was a dark purple/pink color and paying close attention to the columns was necessary in order to collect only the desired material. After passing through the column, the desired fractions, recognized by their color, were rotovaped. After the purification process was

complete, UV-vis and ^{1}H-NMR spectroscopy were used to characterize the purified porphyrin **(5)**.

FIGURE: 9
The LH-20 column

FIGURE: 10
The G-50 column

Results

UV-vis Spectroscopy

To determine the absorbance of the product, Ultraviolet-Visible Spectroscopy (UV-vis) was used. The wavelength of light recorded is determined by the excitation of the electrons in the sample. Excitation occurs when molecules absorb the energy in the form of light. Through the use of a prism, this device is able to separate the wavelengths after a beam of light is emitted. The intensity is then measured by electronic detectors and compared to the control sample. The light spectrum consists of visible light, which ranges from 350 to 800 nm and ultraviolet light which ranged from 200 to 350 nm. A typical porphyrin will have a "Söret" band at a wavelength of 415 nm. It will generally have "finger

print" absorptions in the 500 to 750 nm range. These are sometimes referred to as the Q Bands[15,16]. In the synthesized porphyrin, the Söret band appeared at 415 nm. The sample was later diluted to a 30mM solution and eventually a 10 mM and 1 mM stock solutions for the use in an MTT Assay.

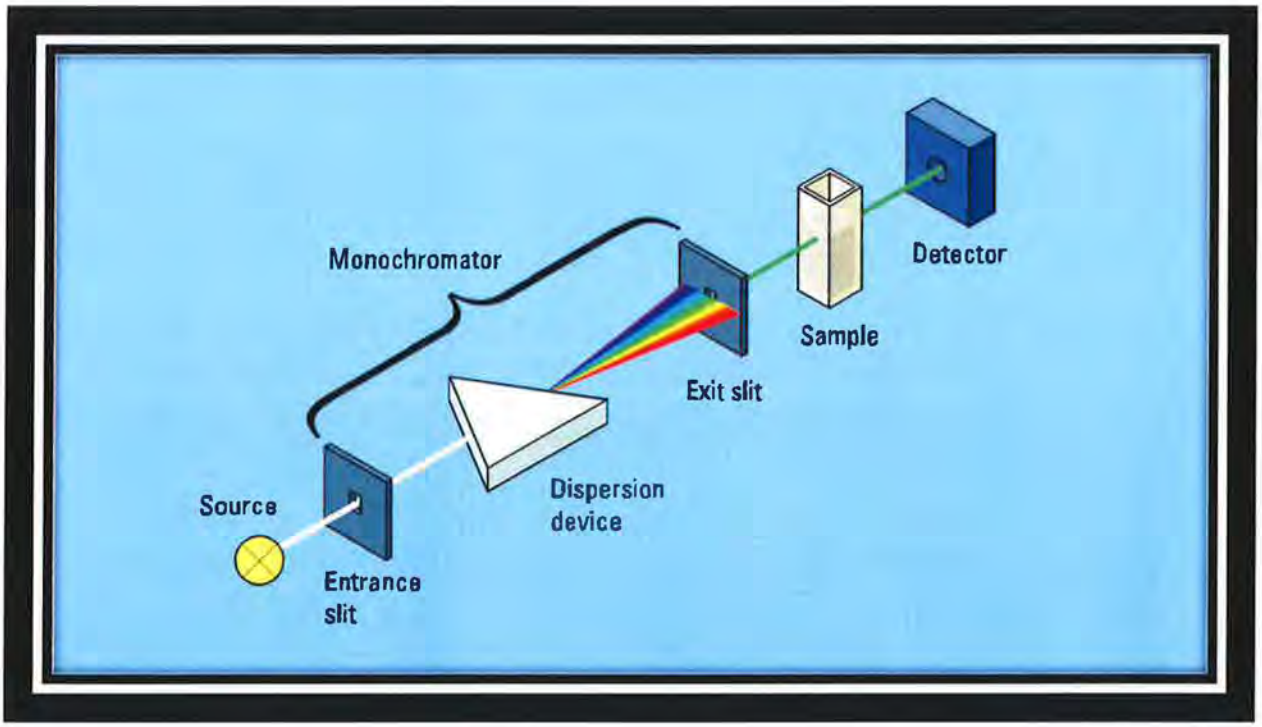

FIGURE: 11

This above diagram is an example of UV-vis spectroscopy and how light would pass through the monochromator, the sample and be processed by the detector. [17]

Peak (nM)	Ɛ ($mM^{-1}cM^{-1}$)
415	98.30
518	3.54
555	2.95
582	2.18
638	2.29

FIGURE: 12

UV-vis spectra results for porphyrin H_2TPP-Oxo-MeOH. The epsilon values were calculated with Beer's Law: $A = \varepsilon Cl$

Infrared Spectroscopy

The use of infrared light in the reading of absorption, and emission of a molecule is known as Infrared Spectroscopy (IR). This is done through the reading of the vibrations that occur by the atoms in the molecule[18]. The IR results (Figure 14) show that the H_2TPP-Oxo-MeOH contains the amide bands (1577 cm^{-1}, 1534 cm^{-1}, and 1378 cm^{-1}), indicating that the amine has been attached to the porphyrin.

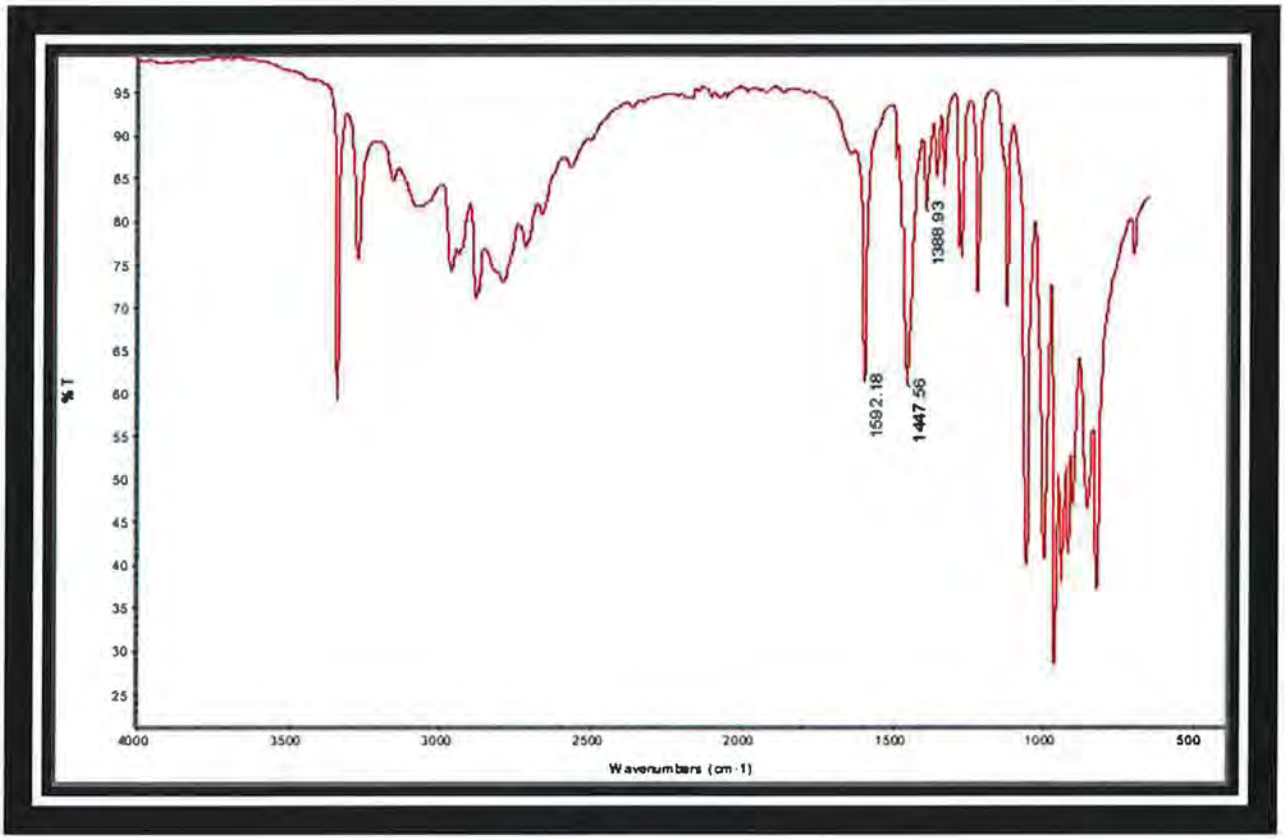

FIGURE: 13
3-amino-oxetane-3-yl methanol IR spectra results

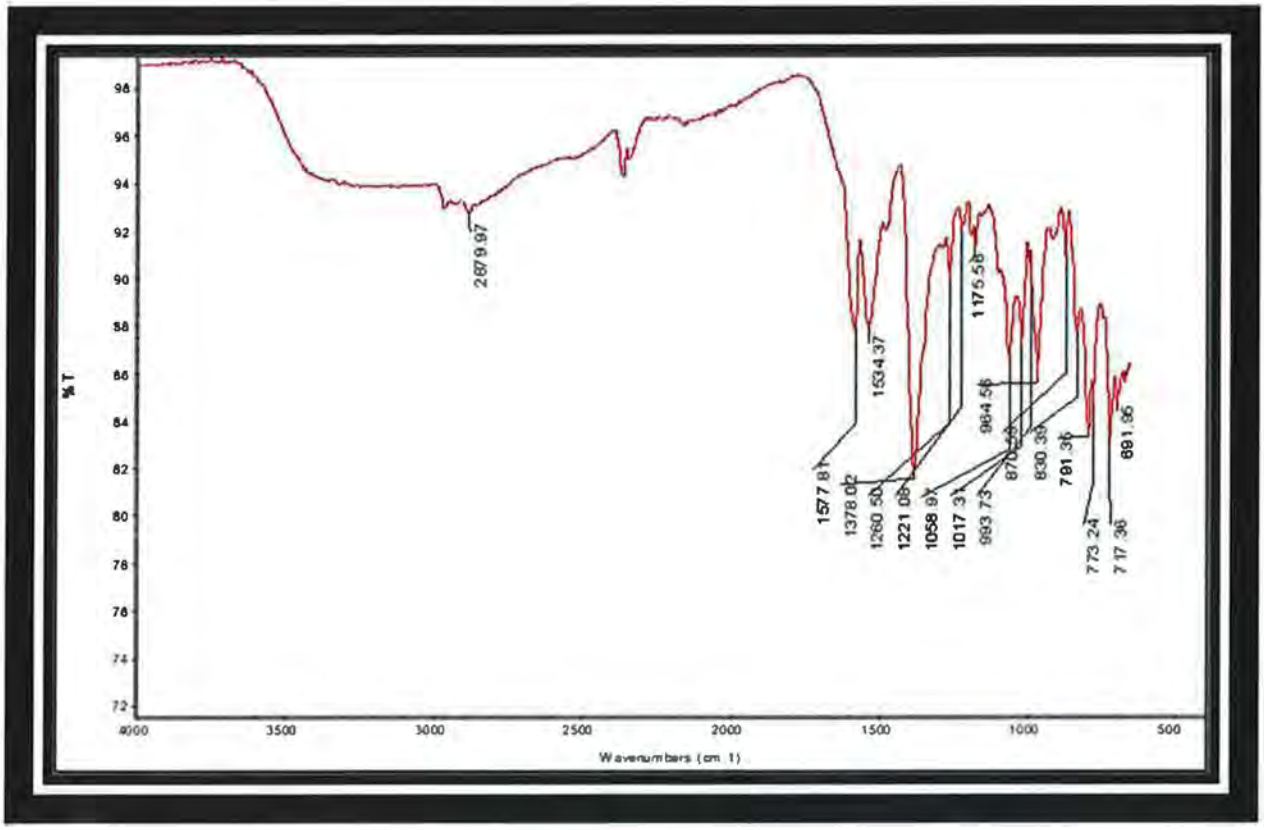

FIGURE: 14
H_2TPP-Oxo-MeOH IR spectra results

NMR Spectroscopy

NMR or Nuclear Magnetic Resonance Spectroscopy looks at organic molecules that contain hydrogen atoms and helps to characterize them. The sample is tested in an NMR tube, which is simply a thin, glass tube. The sample is dissolved in an appropriate solvent and placed in the ^{1}H NMR tube. The sample is then placed in a spinning holder that is surrounded by a magnet[19]. The ^{1}H NMR results are shown in Figure 15 and 16 for the amine and the desired porphyrin. The NMR (Figure 16) results for the porphyrin created in this research show that the desired porphyrin was produced by identifying the aromatic region of the spectra, 6.5-8.0 ppm.

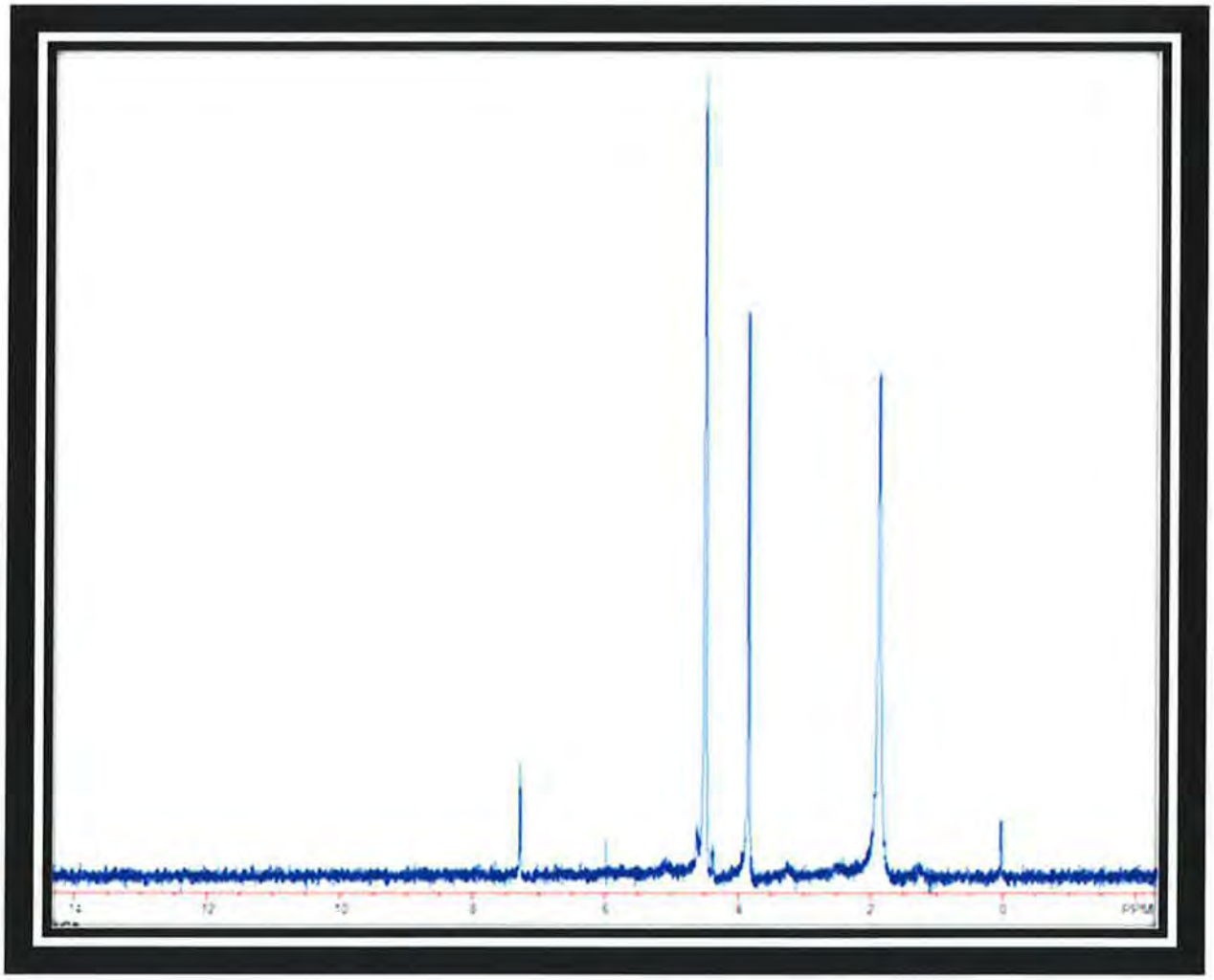

FIGURE: 15

3-amino-oxetane-3-yl-methanol ^{1}H-NMR in $CDCl_3$ results

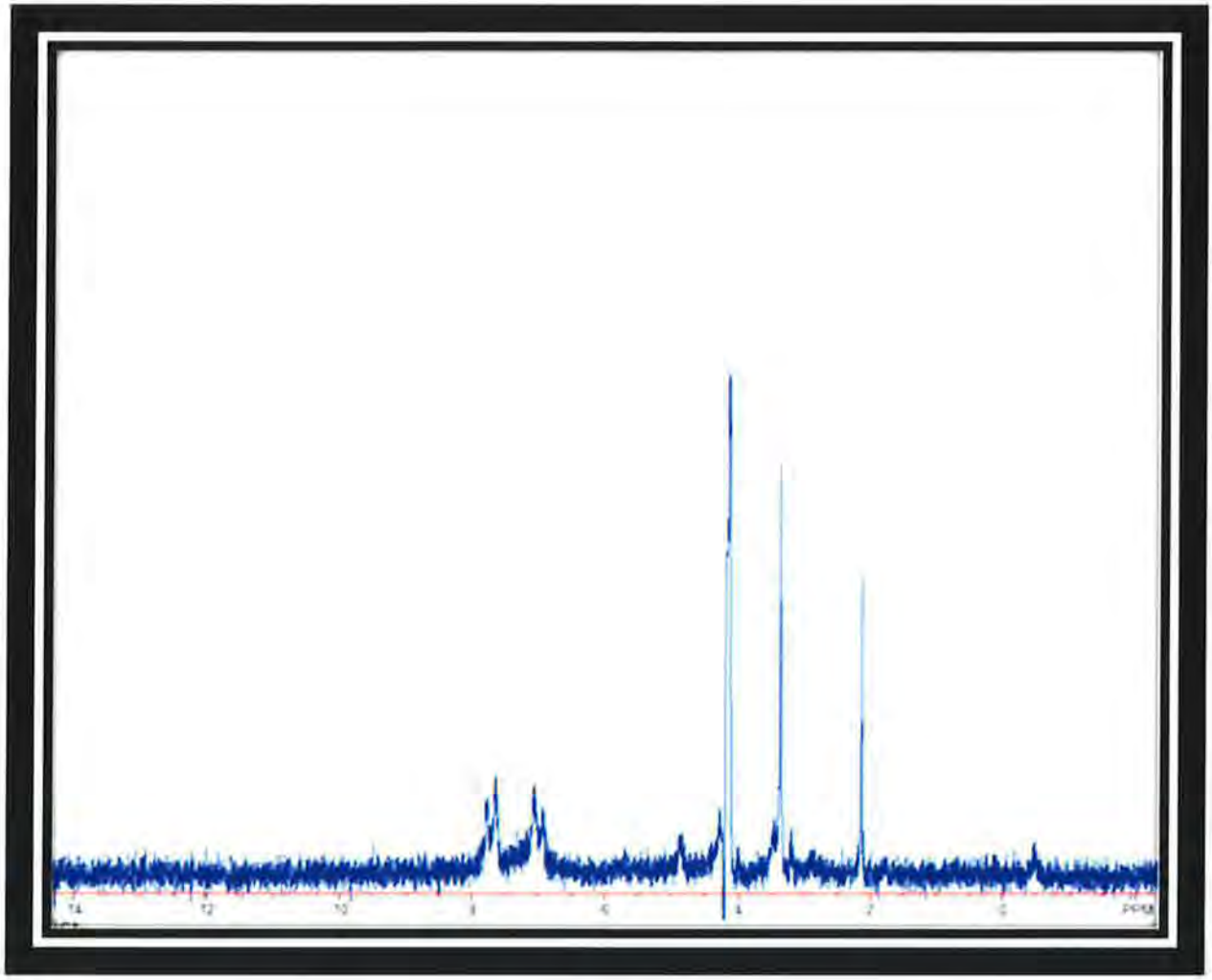

FIGURE:16
H_2TPP-Oxo-MeOH ^1H-NMR in D_2O results

MTT Assay of TNBC

While PDT is gaining popularity as a cancer treatment, it is not the primary treatment used. It is used when there are no better options available. As mentioned earlier, Triple Negative Breast Cancer lacks the three most common receptors that are the targets of some cancer treatments. This is why PDT could potentially be used for treating those with TNBC.

Once the porphyrin was synthesized, purified, and characterized it was tested with TNBC cells to determine its phototoxicity effectiveness. This was done to see if H_2TPP-

Oxo-MeOH could be used in photodynamic therapy. The specific cell type used in this project was MD-MBA 231 TNBC. The cells were grown and then plated onto two 96 well-plates. One plate was labeled "dark" (Figure 18) and the other was labeled "light" (Figure 17). The cells were allowed to grow for 72 hours in the wells. At the end of 72 hours, the H_2TPP-Oxo-MeOH was added in varying concentrations to each of the wells containing MD-231 cells. Eight wells were treated with each concentration. Exposure to light was minimized by covering the well-plate with aluminum foil. The cells were then incubated overnight. The next day, the plate titled "light" received a media change and was exposed to white light for 20 minutes (1/2 J/cm^2). The plate was placed back in the incubator under aluminum foil. The dark plate never received light and therefore only received a media before being placed back in the incubator under aluminum foil. The plates incubated for another 72 hours. At the end of this time, the phototoxicity was determined using MTT assay and the amount of purple in the cells was quantified through spectrophotometrics.

MTT Assay Results

Cell viability is commonly tested through MTT assays. The assay occurs in several steps. The yellow MTT turns a purple color when living cells are present since mitochondrial succinate dehydrogenzase catalyzes the formation of the purple MTT-formazan. So, the darker the color, the greater the number of living cells. Since there was some color difference between the "light" (Figure 17) and "dark" (Figure 18) plates, this indicated that there was more cell death in those well-plates exposed to light.

Normally, a difference in color would be able to be seen amongst the different concentrations due to the increase in cell death but that is not accurate in this case. The percent of cell death was so little that there was no clear color change. When looking at

figures (Figures 17 and 18), there are seven vertical columns that are important. The first is a negative control. The wells in this column contain only TNBC cells and media. The next column is another control but it contains DMSO in order to exclude it as a reason for cell death. Columns 3 through 7 contain varying concentrations of **(5)**. These rows contain 1, 3, 10, 30, and 74 μmolar concentrations. The remaining rows contain more negative controls. In order to make sure that the porphyrin is being triggered by light, the "dark" plate is used as another control to compare to the amount of cell death on the "light" plate.

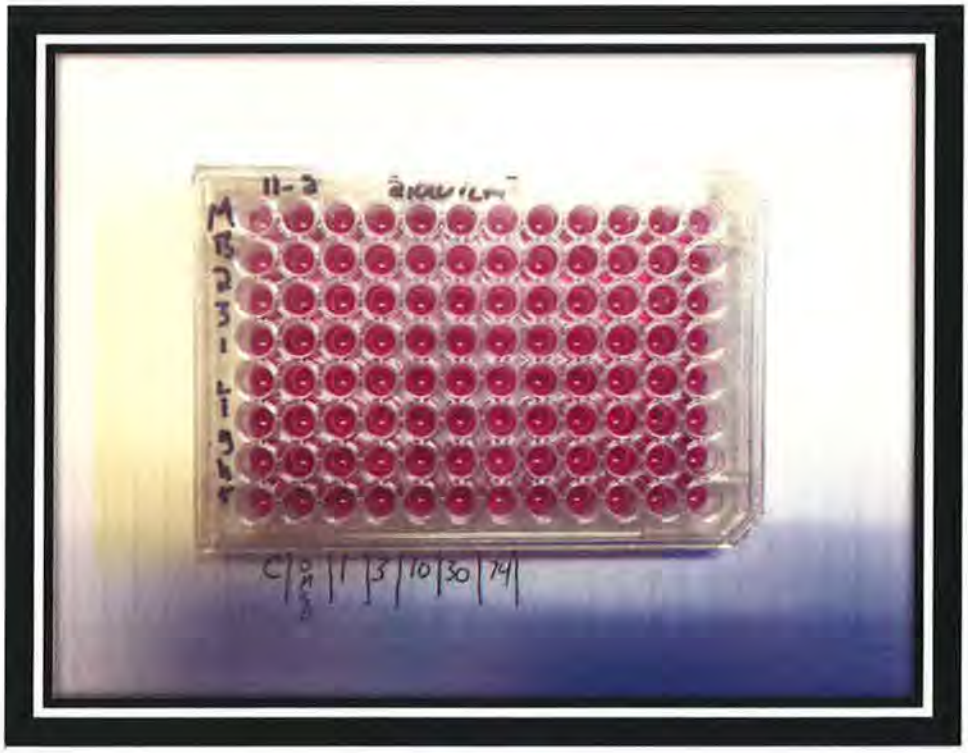

FIGURE: 17
The photo above is of the "light" plate with its varying rows marked. Color difference among the column signals cell death.

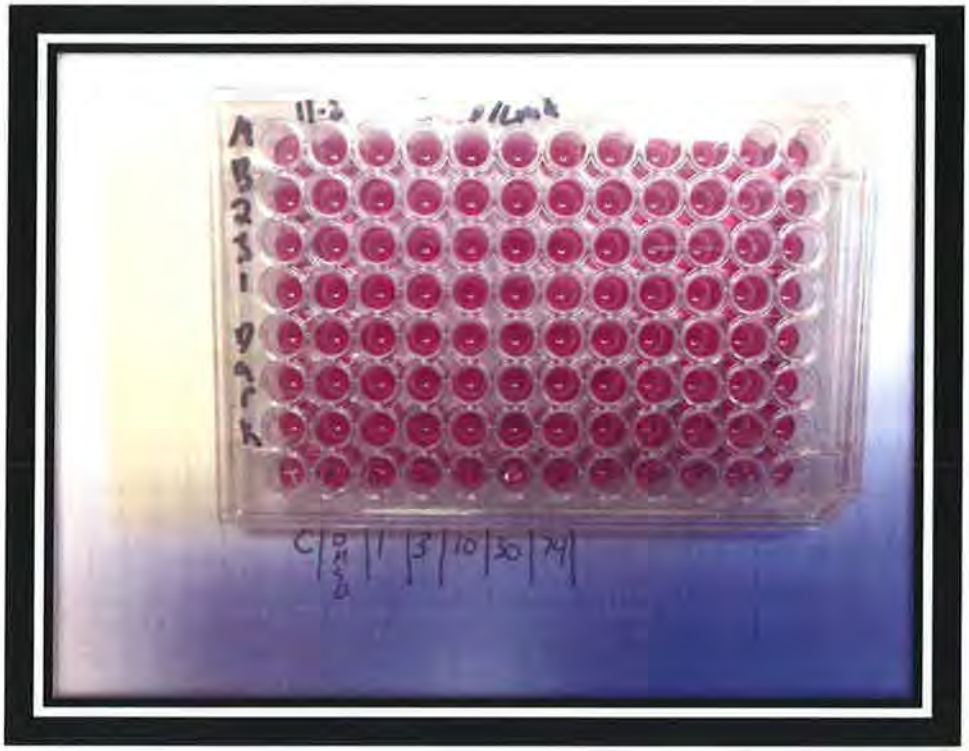

FIGURE: 18

The photo above is of the "dark" plate with the rows marked.

The MTT assay was run three different times in order to confirm the results. This experiment showed that among the lower concentrations, there was little variation between the two plates. The only condition to really see a slight difference was the 74 μmol concentration. While the light plate showed more cell death, it never reached the 50% cell death mark that was anticipated.

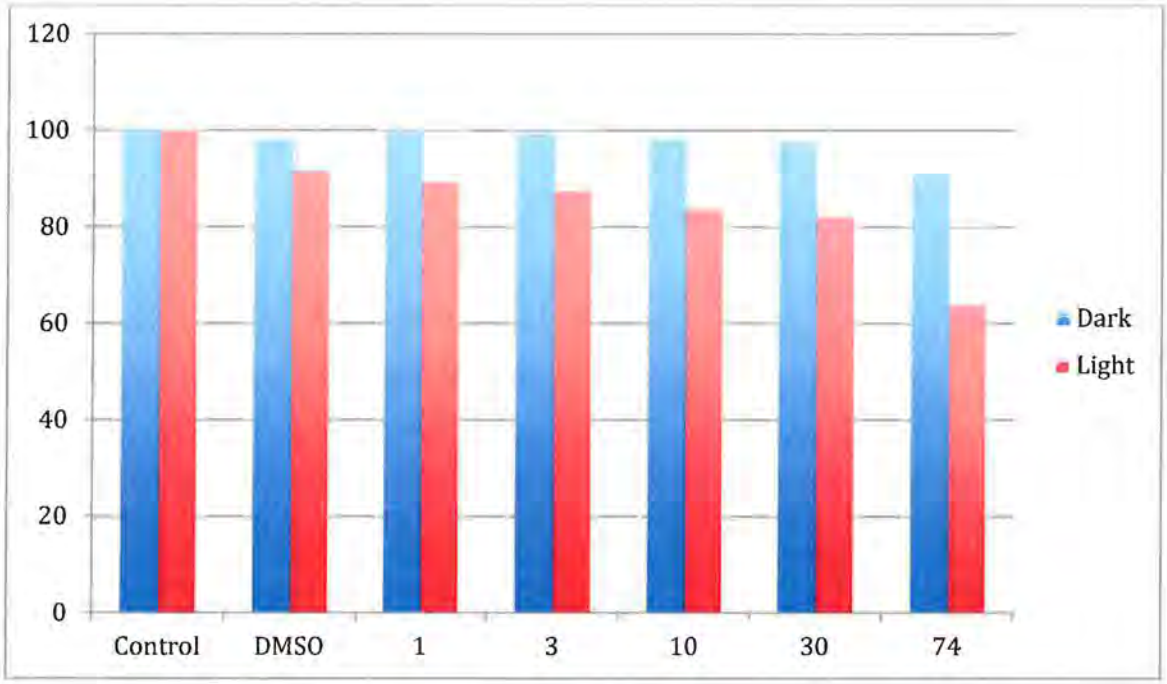

FIGURE: 19

Results of the second MTT assay. The y-axis is the percent viability of the cells and the x-axis is the varying concentrations of **(5)**.

Conclusion

The synthesis and characterization of a novel water-soluble porphyrin was accomplished. The porphyrin contained the spectroscopic characteristics that were expected. While it had the predicted porphyrin characteristics, it did not have the desired results as a potential PDT agent. It was expected that there would be a significant difference between the light and dark conditions as well as an increase in cell death among the varing concentrations of porphyrin. It is unclear if it is the structure of the amine on the porphyrin periphery that caused the porphyrin to be less effective or if other factors may play a role. Future research studies should repeat these MTT assay experiments to confirm the results or synthesize a more effective compound.

Acknowledgments

I would like to express my deepest gratitude to Dr. Joseph E. Bradshaw for not only helping me with my research but also, the completion of this thesis. Without him, this would not have been possible. He walked me through every step taken in the lab and provided extra instructions when I needed it. Dr. Bradshaw made this entire process fun and less stressful. I would also like to thank Dr. Timothy Hayes for the use of his lab and his support. He was always available for questions and even agreed to be my second reader. Finally, I would like to thank Ouachita as a whole and specifically the J.D. Patterson School of Natural Science for their continuous support and encouragement during the past four years. This campus and especially the science building became my home. They have taught me valuable lessons and provided memories that I will have with me for the rest of my life. It is hard to say goodbye but I know that I am a better person from my time as a Biology major and my time at Ouachita.

References

1. Gomes, Ana T.P.C., Neves, Maria G.P.M.S., and Cavaleiro, Jose A.S. "Cancer, Photodynamic Therapy and Porphyrin-Type Derivatives" *Annals of the Brazilian Academy of Sciences.* (2018) 90(1 Suppl. 2): 993-1026

2. "Cancer Statistics." *National Cancer Institute*, 27 Apr. 2018, www.cancer.gov/about-cancer/understanding/statistics.

3. "How Is Chemotherapy Used to Treat Cancer?" *American Cancer Society*, 2016, www.cancer.org/treatment/treatments-and-side-effects/treatment-types/chemotherapy/how-is-chemotherapy-used-to-treat-cancer.html.

4. "What Is Triple Negative Breast Cancer?" *Triple Negative Breast Cancer Foundation*, tnbcfoundation.org/what-is-tnbc.

5. "Triple Negative Breast Cancer." *Patient Resource Publishing: Immunotherapy Immune System*, www.patientresource.com/TNBC_Overview.aspx.

6. Anders, Carey and Lisa A Carey. "Understanding and treating triple-negative breast cancer" *Oncology (Williston Park, N.Y.)* vol. 22,11 (2008): 1233-9; discussion 1239-40, 1243.

7. "CDC - Triple-Negative Breast Cancer." *Centers for Disease Control and Prevention*, Centers for Disease Control and Prevention, 19 Mar. 2018, www.cdc.gov/cancer/breast/triple-negative.htm.

8. Daniell, M. D. and Hill, J. S. (1991), "A Hisotry of Photodynamic Therapy". *Australian and New Zealand Journal of Surgery*, 61: 340-348. doi:10.1111/j.1445-2197.1991.tb00230.x

9. Kou, Jiayuan et al. "Porphyrin photosensitizers in photodynamic therapy and its applications" *Oncotarget* vol. 8,46 81591-81603. 11 Aug. 2017, doi:10.18632/oncotarget.20189

10. "Photodynamic Therapy." *American Cancer Society*, 18 Mar. 2015, www.cancer.org/treatment/treatments-and-side-effects/treatment-types/photodynamic-therapy.html.

11. Josefsen, Leanne B and Ross W Boyle. "Unique diagnostic and therapeutic roles of porphyrins and phthalocyanines in photodynamic therapy, imaging and theranostics" *Theranostics* vol. 2,9 (2012): 916-66.

12. Cole, Gary W. "Photodynamic Therapy (PDT) Costs, Side Effects & Recovery." *MedicineNet*, 8 Feb. 2018, www.medicinenet.com/photodynamic_therapy/article.htm.

13. "Photodynamic Therapy PDT San Diego, CA | Cosmetic Laser Dermatology." *Cosmetic Laser Dermatology Skin Specialists in San Diego*, clderm.com/procedure/photodynamic-therapy/.

14. Libretexts. "Liquid Chromatography." *Chemistry LibreTexts*, National Science Foundation, 19 Dec. 2018, chem.libretexts.org/Bookshelves/Analytical_Chemistry/Supplemental_Modules_(Analytical_Chemistry)/Instrumental_Analysis/Chromatography/Liquid_Chromatography.

15. Rita Giovannetti (2012). The Use of Spectrophotometry UV-Vis for the Study of Porphyrins, Macro To Nano Spectroscopy, Dr. Jamal Uddin (Ed.), from:http://www.intechopen.com/books/macro-to-nano-spectroscopy/the-use-of-spectrophotometry-uv-vis-for-the-study-of-porphyrins

16. Royal Chemistry Society "Ultra-Violet Visible Spectroscopy". *Advancing the Chemical Sciences,* http://www.rsc.org/learn-chemistry/content/filerepository/CMP/00/001/304/UV-Vis_Student%20resource%20pack_ENGLISH.pdf

17. http://faculty.sdmiramar.edu/fgarces/LabMatters/Instruments/UV_Vis/Cary50.htm

18. Libretexts. "Infrared Spectroscopy." *Chemistry LibreTexts*, National Science Foundation, 26 Nov. 2018, chem.libretexts.org/Bookshelves/Physical_and_Theoretical_Chemistry_Textbook_Maps/Supplemental_Modules_(Physical_and_Theoretical_Chemistry)/Spectroscopy/Vibrational_Spectroscopy/Infrared_Spectroscopy.

19. Reusch, William. "Nuclear Magnetic Resonance Spectroscopy." *UV-Visible Spectroscopy*, 5 May 2013, www2.chemistry.msu.edu/faculty/reusch/virttxtjml/spectrpy/nmr/nmr1.htm.