Spring 2020

Developing a New Water-Soluble Porphyrin as a Potential Photodynamic Cancer Therapy Agent

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A Senior Thesis by Catherine L. Shirley

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Dedicated to Dr. Joseph Bradshaw and Dr. Tim Knight. Thank you for pouring into me during the last four years. You inspire me to reach every goal that I set!

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Abstract

Photodynamic cancer therapy (PDT) is a type of treatment involving the use of light in conjunction with a photosensitive agent- a chemical or series of chemicals designed for activation when exposed to light. This research project investigated the synthesis and identification of the novel photosensitive agent, H_2TPP-Pro-OH. To create the water-soluble porphyrin, (S)-(+)-prolinol was reacted with the tetra-carboxyl porphyrin, H_2TPPC, to form the final H_2TPP-Pro-OH product. This compound was then purified using syringe filtration and column chromatography, and subsequently characterized using infrared (IR), nuclear magnetic resonance (NMR), and Ultraviolet-visible (UV-vis) spectroscopies, as well as High Performance Liquid Chromatography (HPLC). Finally, the utility of the material as a PDT agent was determined by examining the cytotoxicity of the H_2TPP-Pro-OH product using an MTT assay on MDA-MB-231 triple negative breast cancer (TNBC) cells comparing dark and light exposure.

Background

What is Photodynamic Therapy?

Photodynamic Therapy (PDT) is a way to treat cancer using light and a photosensitive compound **(Figure 1)**. When the photosensitive agent is injected into the patient, it is "activated by light of a specific wavelength," which can cause three outcomes. Primarily, a reactive oxygen species, known as an oxygen free radical, can form which then destroys the surrounding tumorous cancer cells[2]. In addition to destroying the cancer cells via a reactive oxygen species, the activation of the

photosensitive agent can “destroy the blood vessels that feed the cancer cells,” and finally, the activation can “[alert] the immune system to attack the cancer”[3].

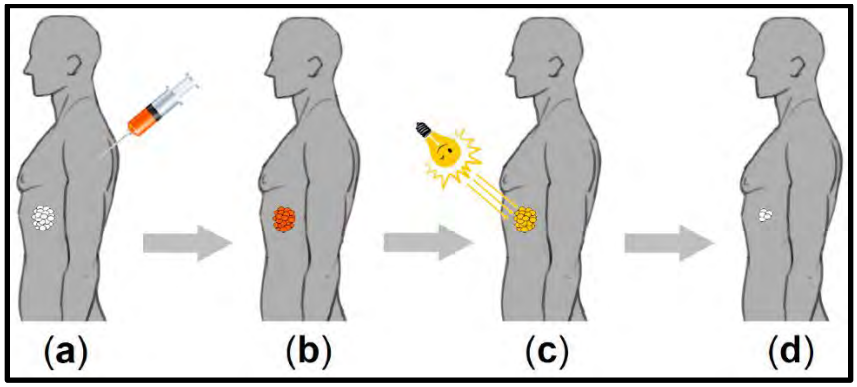

Figure 1. (a) A patient diagnosed with a tumor is injected with a photosensitizer. (b) Due to the nature of the photosensitizer, it collects in the tumor over time. (c) The tumor is exposed to light for a given amount of time at a given wavelength of light, activating the photosensitizer. (d) The tumor is selectively destroyed.[1]

Why PDT?

Normal breast cancer cells **(Figure 2)** have receptors for estrogen, progesterone, and HER2 hormones, but triple negative breast cancer (TNBC) cells **(Figure 3)** lack these receptors, so these cells are unable to be treated in the same way as normal

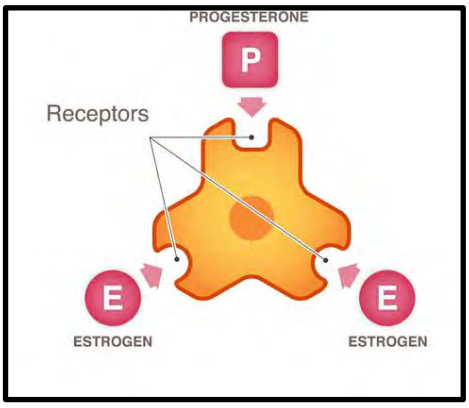

Figure 2. A type of normal breast cancer cells, containing receptors for estrogen and progesterone.[4]

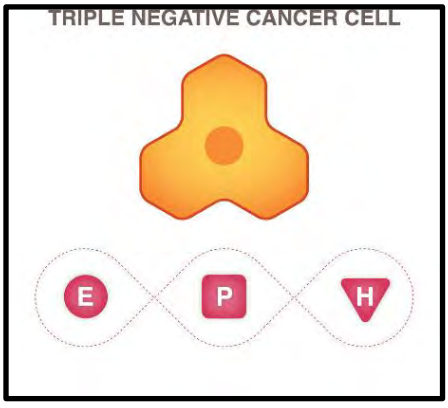

Figure 3. A triple negative breast cancer (TNBC) cell, lacking receptors for estrogen, progesterone and HER2.[5]

breast cancer cells due to the lack of receptors. TNBC cells are the cause for ten to twenty percent of breast cancer cases[6]. These cells are more likely to spread and recur, and they give the affected person an average survival rate of less than five years. Unfortunately, TNBC cells do not respond to chemotherapy or radiation in the same way that normal breast cancer cells respond. One method of treatment for TNBC that is being explored is Photodynamic Therapy (PDT). Through testing with PDT, the efficacy of the porphyrin compound H_2TPP-Pro-OH on the treatment TNBC cells can be concluded.

What are Porphyrins?

Porphyrin molecules are most known for their role in the active site of hemoglobin, the blood oxygen transporter of the body. An organic porphyrin ring is the basis for the formation of the heme group, the “non-protein active site within myoglobin and hemoglobin”[7]. Regarding PDT, however, porphyrin photosensitizers are one of the three main functional elements of the process. The other two main functional elements of the PDT process are ensuring an optimal wavelength of light, and ensuring the formation of singlet, molecular oxygen[8].

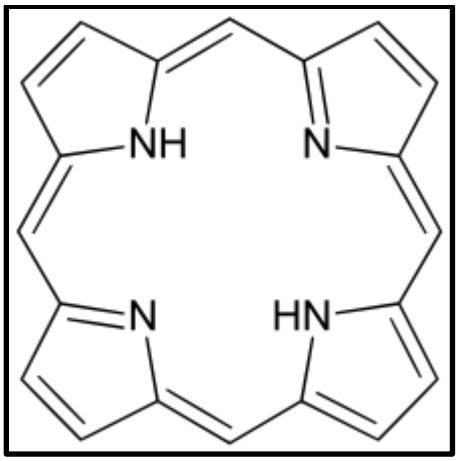

Figure 4. The structure of a porphyrin molecule. Chemical formula $C_{20}H_{14}N_4$.[9]

A porphyrin is a large ring molecule containing four pyrrole groups connected by single and double bonds **(Figure 4)**. The alternating single and double bonds give aromaticity and stabilization. Porphyrin molecules are ideal for PDT because of their likeliness to accumulate in tumor tissue. Most tumors contain “highly perfused areas” allowing for greater blood flow in the tumor than in an area with healthy, non-tumorous cells[10]. When the photosensitive porphyrin is injected into the body, it travels throughout the body via the blood, but because tumors have greater blood flow, the porphyrin molecules are more likely to gather in the tumor. Furthermore, when activated by light, porphyrin photosensitizers are able to create singlet, molecular oxygen, which results in cytotoxicity of the tumor tissue cells[11].

Photodynamic Therapy Used in Oral Treatment

Malignant tumors that develop in “the oral cavity, the pharynx, the nasal cavity, and the larynx” **(Figure 5)** are categorized as head and neck cancer, or squamous cell carcinoma (SCC), and are seen to be caused by age, sunlight, alcohol abuse, and

smoking or other tobacco use[13]. Some symptoms associated with SCC include a white or red patch on the gums or tongue (oral cavity), trouble breathing or speaking (pharynx), pain when swallowing (larynx), or chronic sinus infections and nose bleeds (nasal cavity)[14].

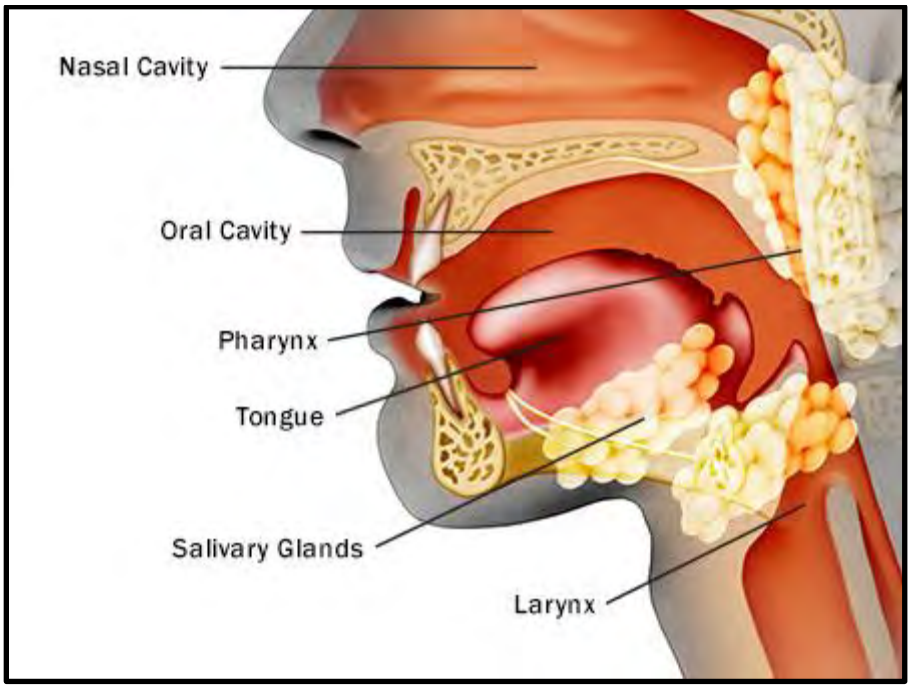

Figure 5. Oral Cavity Anatomy[12]

Photodynamic Therapy has recently begun an upward trend to its effectiveness in destroying head and neck cancer. The three photosensitizers currently being used in testing are Photofrin®, Foscan®, and 5-Aminolevulinic acid (ALA)[13].

Photofrin®

Photofrin® **(Figure 6)** is the most studied photosensitive agent and has "generally excellent" clinical results due to its reliability, easy activation, and non-toxic, pain-free administration, but it has shown to not be highly selective at 2 mg/kg (normal injection) and demonstrates "significant prolonged skin photosensitivity" for up to six

weeks after initial administration[13]. The prolonged skin sensitivity observed with Photofrin® is due to it being a first-generation PDT agent, meaning that it lacks long wavelength absorption and thus the activating light does not penetrate deeply into the tissues, and it is not easily cleared from the body[16].

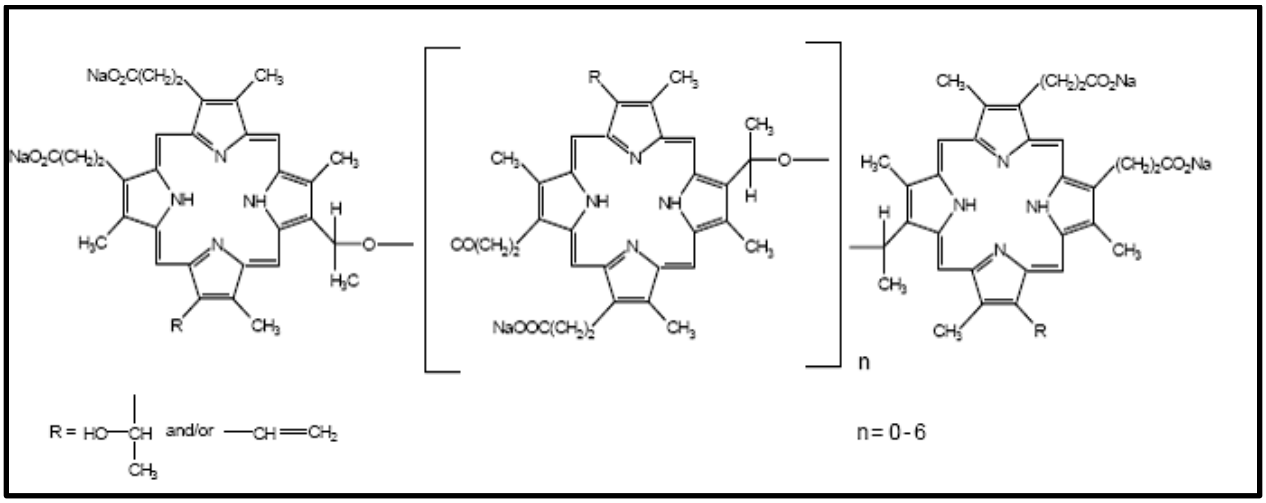

Figure 6. Photofrin® (Porfimer Sodium) Structure[15]

Foscan®

The second photosensitive agent being explored in the treatment of head and neck cancer is Foscan® **(Figure 7)**. Foscan® is a second-generation PDT agent that provides “greater tumor selectivity and deeper light penetration… with the use of longer wavelengths of activating light” when compared to first-generation photosensitizers such as Photofrin®[18]. Foscan® is injected at 0.15 mg/kg and is activated by laser lights at 652 nm[13]. While Foscan® has shown at least 93% efficacy in patients with oropharyngeal cancer, this photosensitizer has only been approved for use in Europe[13].

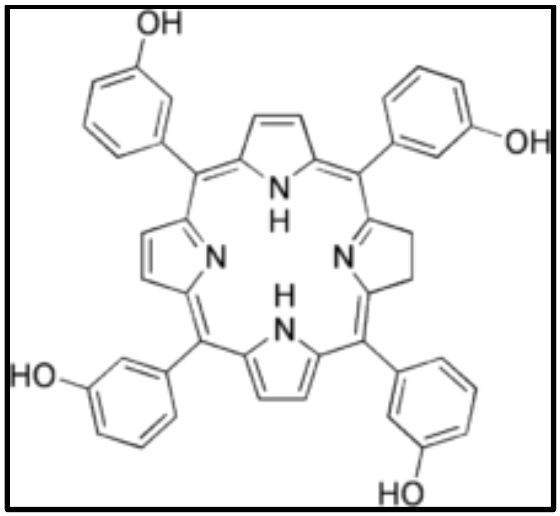

Figure 7. Foscan® Structure[17]

5-Aminolevulinic Acid

Though it has limited light penetration ability at 635 nm, and is restricted to superficial lesions, 5-Aminolevulinic acid (ALA) **(Figure 8)** is a promising photodynamic cancer therapy agent[13]. ALA administered topically and orally "has been used for the treatment of pre-malignant and malignant lesions in the oral cavity" and has been proven to "rapidly" clear from the body within 48 hours, with skin sensitivity lasting "less than 24 hrs"[13]. Among patients treated for oral dysplastic lesions, there was a 100% success rate in the "regression of the lesions to normal or less dysplastic"[13]. Unfortunately, due to its limited light penetration, ALA is not effective in the destruction of deep tumors.

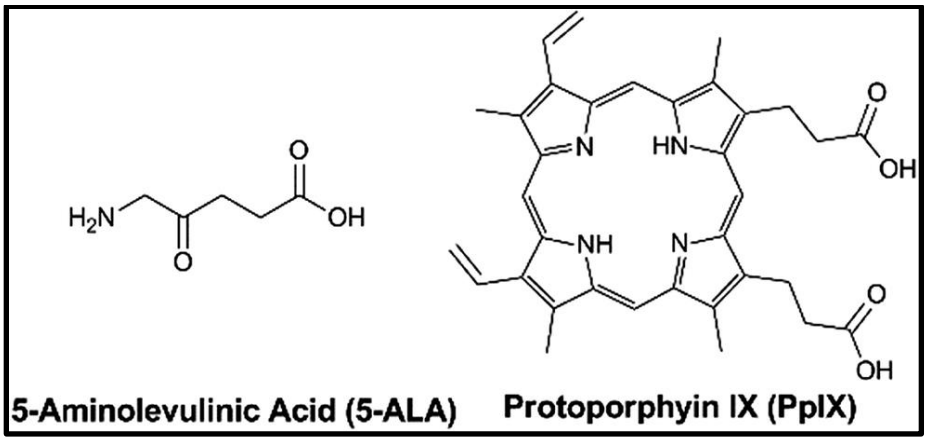

Figure 8. 5-Aminolevulinic acid (ALA); precursor to Protoporphyrin IX (PpIX)[19]

Other Clinical Applications of Photodynamic Therapy

Though PDT is actively being explored as a method of treatment to cure diseases of the oral cavity, its other clinical applications have made significant progress as well. The photosensitizer Photofrin® has been FDA approved for the treatment of obstructive esophageal cancer and early esophageal cancer. By using PDT, dysphagia was relieved in both types of cancer, and in early esophageal cancer, “72% of PDT patients showed complete elimination” of high-grade dysplasia[20]. Photofrin® has also shown positive results in the treatment of microinvasive non-small cell lung cancer. Treatment has resulted in a “79% complete response” of early lung cancer and an average of a 51% response rate of late stage lung cancer[20]. In addition to this, ALA and Photofrin® have been used in the treatment of skin cancer with the ALA showing a 64% complete response rate and “excellent cosmetic results” after three years of treatment[20]. The newest clinical trials being performed are aiming to see positive results in the

treatment of age-related macular degeneration and coronary heart disease. No clinical results of these trials have been published.

Introduction to Project

As shown above, photodynamic therapy is currently being explored as a method to treat different types of cancer, including breast cancer. This project aimed to create a novel, water-soluble photosensitive agent that could destroy triple negative breast cancer cells using PDT. This was achieved using a porphyrin molecule, the backbone of the final product, H_2TPP-Pro-OH.

Methods

Porphyrin Synthesis

The amine used to create the novel porphyrin was (S)-(+)-prolinol. Water solubility was a critical factor in the synthesis of the molecule because, when administered, PDT agents are injected into the bloodstream. The concentration of water in the blood is approximately 92%, meaning that it is vital for the photodynamic therapy agent to be water soluble.

Formation of H_2TPPC, (3)

To create the final water-soluble product, the starting material 5, 10, 15, 20-tetrakis(4-carboxyphenyl) porphyrin, or H_2TPPC **(3)**, was first synthesized **(Figure 9).** The material **(3)** was synthesized by adding 3.00 g of 4-carboxybenzaldehyde and 1.4 mL of pyrrole to a 500 mL round bottom flask containing approximately 200 mL of propionic acid and a stir bar. The flask was then completely wrapped in aluminum foil, to prevent any possible light sensitive reactions from producing extra side products, and

the mixture was allowed to reflux for an hour. Afterward, the heat was removed allowing the solution to cool. Once cooled, the flask was covered with parafilm and placed in a freezer overnight at -20°C.

Next, vacuum filtration was performed on the newly formed H_2TPPC by attaching a medium sintered glass funnel to a filter flask. Once the product had been filtered, CH_2Cl_2 was added to the round bottom flask to obtain any additional product. After all of the product had been vacuum filtered, the solid H_2TPPC **(3)** was scraped into a vial and dried overnight.

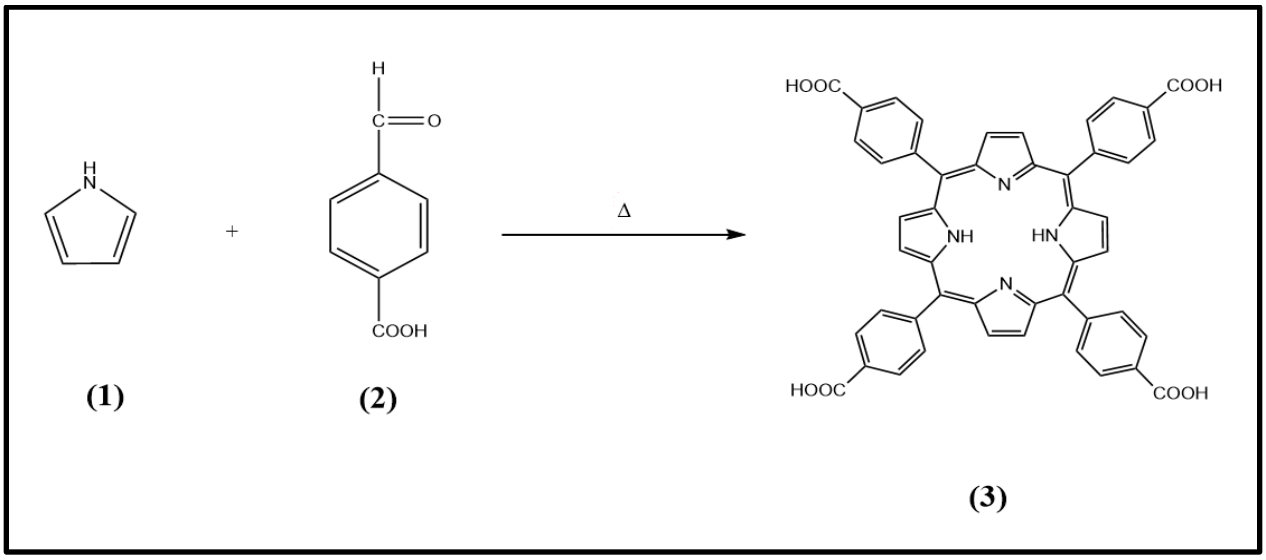

Figure 9. Reaction 1. 4-formylbenzoic acid **(2)** reacts with pyrrole **(1)** in propionic acid to form H_2TPPC **(3)**.

Formation of the Acid-Chloride Porphyrin, (4)

Once the H_2TPPC had been synthesized, the acid-chloride intermediate **(4)** was able to be synthesized **(Figure 10).** This reaction began by dissolving 0.25 g of H_2TPPC **(3)** in approximately 20 mL of dry dimethylformamide (DMF) in a clean, oven dried, 100 mL round bottom flask. While stirring, the flask was put under the constant flow of

nitrogen. When the flask had been effectively flooded with nitrogen, a 1 mL syringe was used to retrieve 0.25 mL of $SOCl_2$ (thionyl chloride) which was quickly added to the 100 mL round bottom flask via a rubber septum.

After the reaction had been stirred under the flow of nitrogen for an hour, the DMF was removed by evaporation and the flask placed under vacuum overnight.

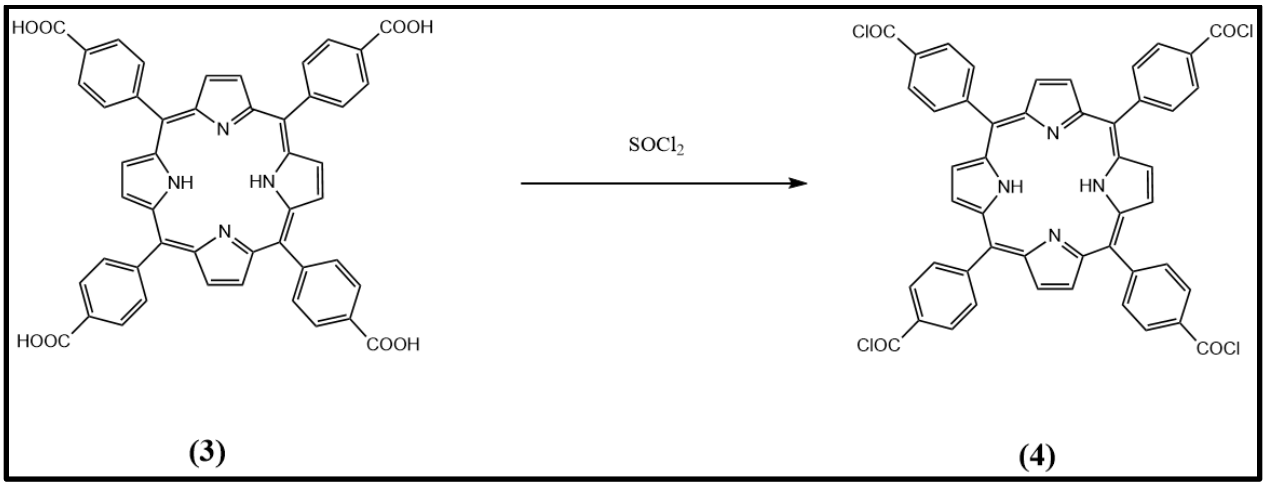

Figure 10. Reaction 2. H_2TPPC (3) reacts with thionyl chloride ($SOCl_2$) in dimethylformamide (DMF) to form the acid chloride porphyrin, H_2TPPCl (4).

Formation of the Final Product, H_2TPP-Pro-OH, (5)

In order to form the final H_2TPP-Pro-OH product **(5) (Figure 11)**, 0.30g (S)-(+)-prolinol was added to an oven dried vial and then dissolved in freshly distilled methanol. This solution was mixed and then transferred into the round bottom flask containing the acid-chloride porphyrin **(4)** intermediate. The flask was again wrapped in aluminum foil to eliminate any light dependent reactions and placed under the constant flow of nitrogen. After stirring for one hour the methanol was removed under reduced pressure.

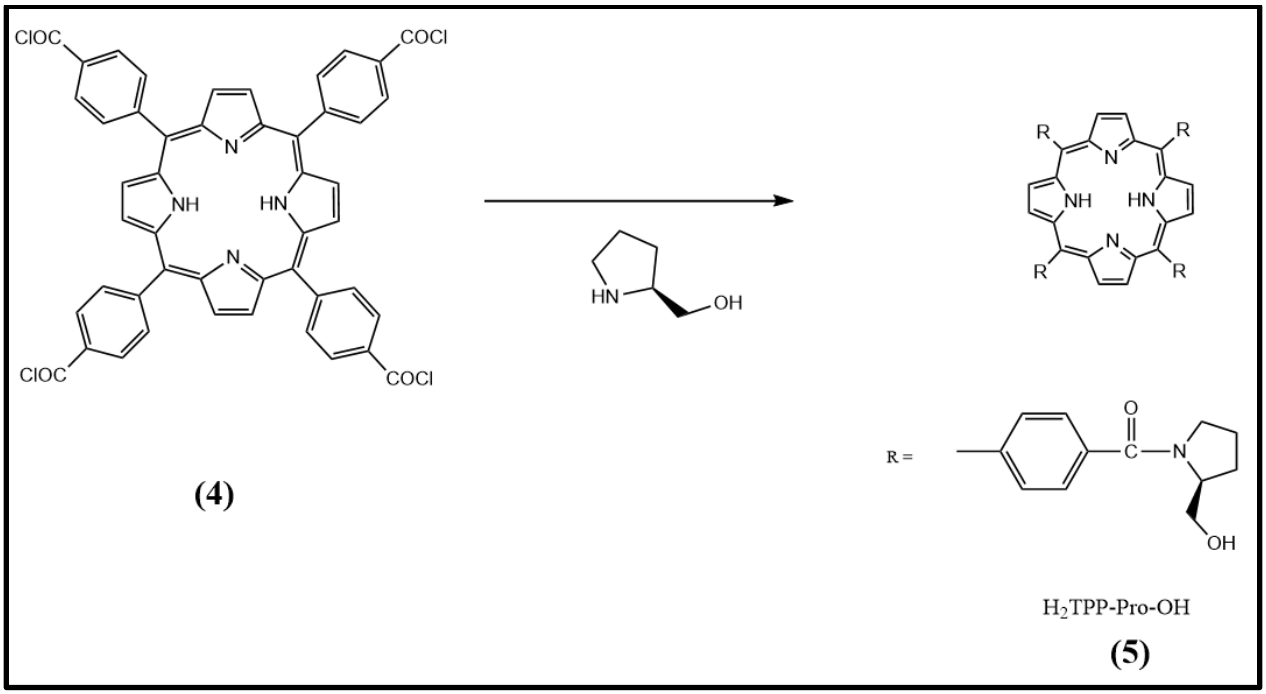

Figure 11. Reaction 3. The acid chloride porphyrin (4) is reacted with the amine, (S)-(+)-prolinol, in distilled methanol to form the final product, H_2TPP-Pro-OH.

Purification

To purify the H_2TPP-Pro-OH **(5)**, the process began by adding a small amount of 50/50 methanol/water to the round bottom flask containing the water-soluble porphyrin until it dissolved. Next, a syringe filter was used to filter the product into a 100 mL beaker. Using a Pasteur pipette, half of this product was added in a swirling motion to a chromatography column packed with Sephadex LH-20, then eluted with 50/50 methanol/water in the chromatography column **(Figure 12)**. Chromatography removes impurities from the desired product. After all of the desired porphyrin-colored product had been collected from the LH-20 chromatography column, the product was rotavapped and then placed under vacuum to dry overnight.

Figure 12. Liquid chromatography column.

In order to ensure purification, the material was run through a second Sephadex G-50 chromatography column using Milli-Q water as the eluent. First, Milli-Q water was added to the round bottom flask containing the product that had previously been purified through the Sephadex LH-20 column and the product dissolved. A Pasteur pipette was used to add half of the product into the column in a swirling motion, which was then eluted with Milli-Q water and allowed to pass through the column. Once the impurities had passed through the column, the purified product **(5)** was collected, rotavapped, and this final product was evaporated under reduced pressure until dry.

Characterization and Results

Infrared Spectroscopy

Infrared (IR) spectroscopy is a tool used by chemists to determine molecular structure based on the fact that functional groups absorb infrared light at different wavelengths in accordance to their structure[21]. As a functional group absorbs different energy, it stretches or bends and this wavenumber and transmittance is recorded graphically. Based on the percent transmittance shown in the figures below, the amine, (S)-(+)-prolinol, **(Figure 13)** and the final porphyrin product **(5) (Figure 14)** both have a broad -OH stretch at *ca.* a wavenumber of 3300 cm^{-1} and a C=O double bond stretch at *ca.* a wavenumber of 1700 cm^{-1}, while the final porphyrin product, additionally, contains an -NH stretch. These

signals consist of amide I, amide II, and amide III bands, at 1580 cm^{-1}, 1533 cm^{-1}, 1376 cm^{-1}, respectively, which indicate the attachment of the amine to the porphyrin core.

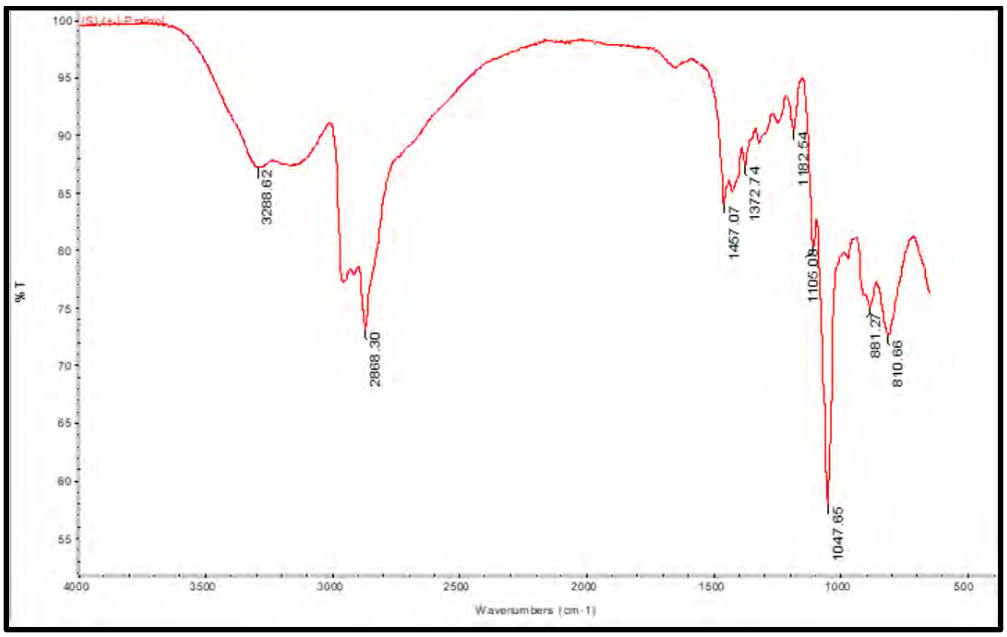

Figure 13. Infrared results for (S)-(+)-prolinol.

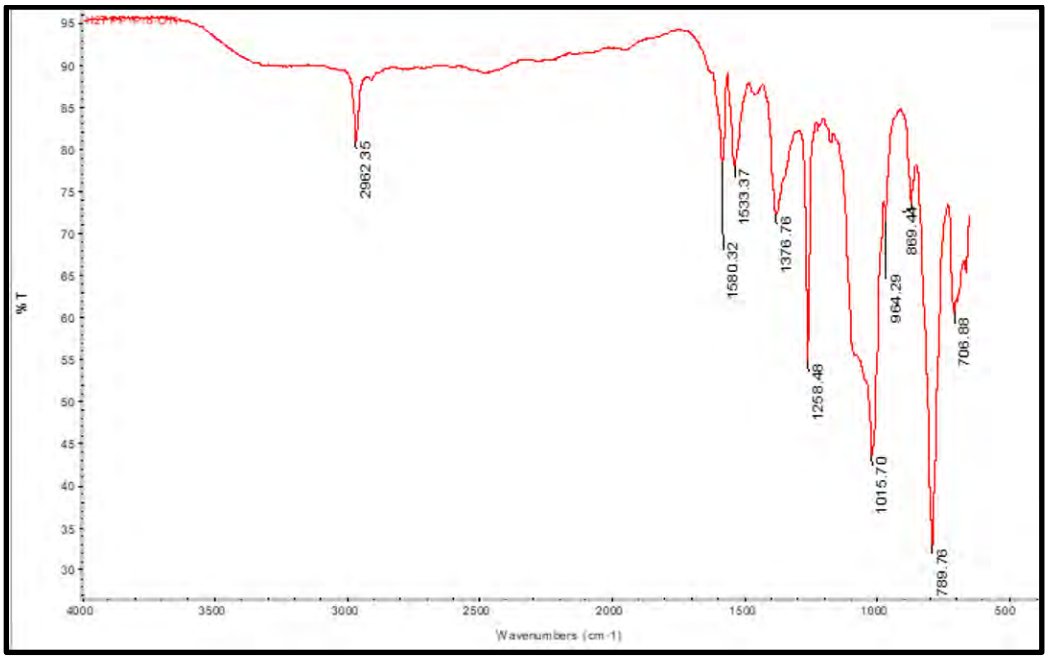

Figure 14. Infrared results for H_2TPP-Pro-OH.

Ultraviolet-Visible Spectroscopy

Ultraviolet-Visible (UV-Vis) Spectroscopy is a tool used in chemistry to determine the absorbance of a particular sample. The absorbance, or reflectance, of a particular molecule is indicative of the perceived color of the molecule either in the ultraviolet or visible spectra. Molecules containing "extensively conjugated pi-electrons" or non-bonding electrons are able to absorb wavelengths of ultraviolet or visible light, and the energies associated with these wavelengths excite the electrons to "higher energy orbitals"[22]. If the electrons are more easily excited, they are able to absorb a longer wavelength of light. To perform UV-Vis spectroscopy, a certain wavelength of light passes through a solution, which absorbs this radiation **(Figure 15**).

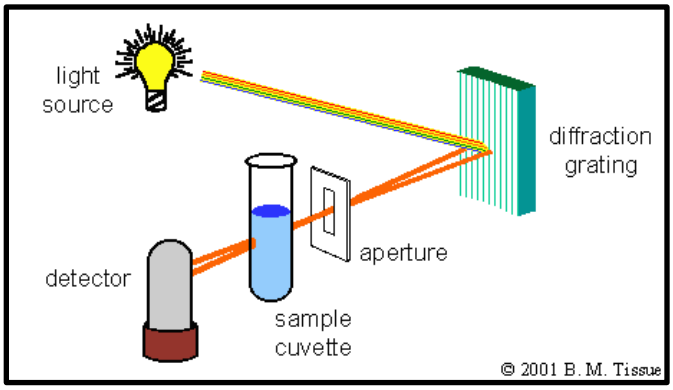

Figure 15. UV-Vis Spectroscopy diagram showing how light is transmitted[23].

Normally, UV light has wavelengths ranging from 200-400 nm, and visible light has wavelengths ranging from 400-800 nm. Characteristically porphyrins have a strong absorbance at 415 nm, called the "Söret" band, and a series of satellite absorptions from 600 to 800 nm, called the the "fingerprint region." For this particular porphyrin molecule, the Söret band is seen at 414 nm **(Figure 16)**, and the approximate ε values for the final compound, including the fingerprint region, are shown in the table below **(Table 1)**.

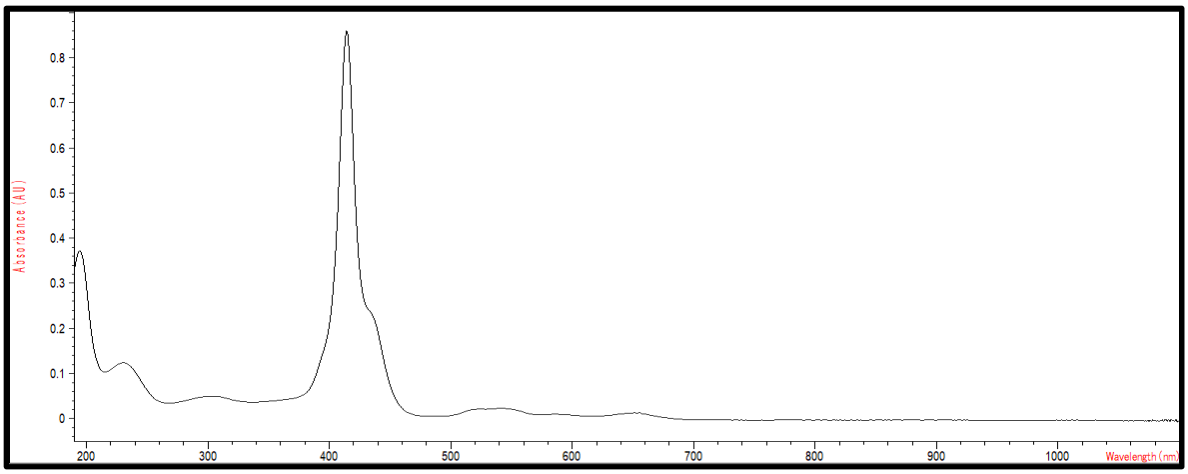

Figure 16. UV-Vis results for H_2TPP-Pro-OH.

Peaks (nm)	Molar Absorptivity Coefficient, ε (cm^{-1} mM^{-1})
414	361
518	10.3
555	6.32
581	5.66
637	5.03

Table 1. Peaks (nm) and epsilon (ε) values for the UV-vis spectroscopy of H_2TPP-Pro-OH. Epsilon values calculated using Beer's Law: $A = \varepsilon c l$.

^{1}H Nuclear Magnetic Resonance Spectroscopy

Nuclear Magnetic Resonance Spectroscopy (^{1}H NMR) is a technique used for the characterization of organic molecules that contain hydrogen atoms. The sample is prepared in an NMR tube and subsequently placed in a magnetic field. Compounds are characterized by observing the chemical shifts of the hydrogens in the magnetic field[24]. Based on the location of the signals when comparing the free amine **(Figure 17)** to H_2TPP-Pro-OH, it is concluded that the final product contained hydrogen atoms characteristic of aromatic rings, hydrogen atoms on the pyrrole ring of the porphyrin, and hydrogens associated with (S)-(+)-prolinol **(Figure 18)**.

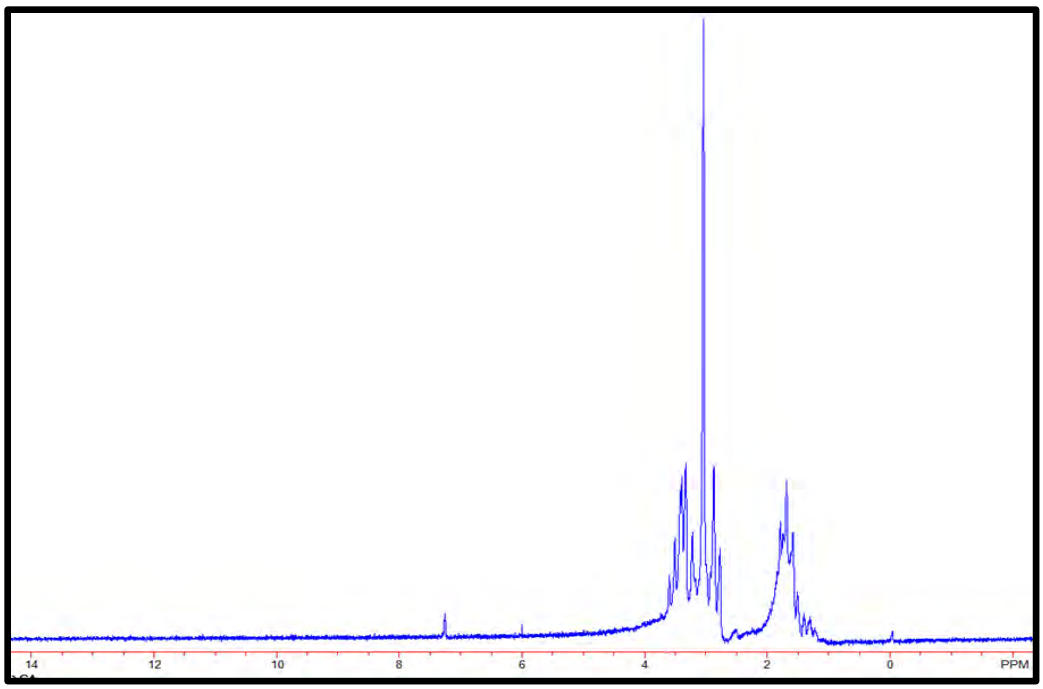

Figure 17. Results from ^{1}H NMR spectroscopy of the free amine, (S)-(+)-prolinol, in $CDCl_3$.

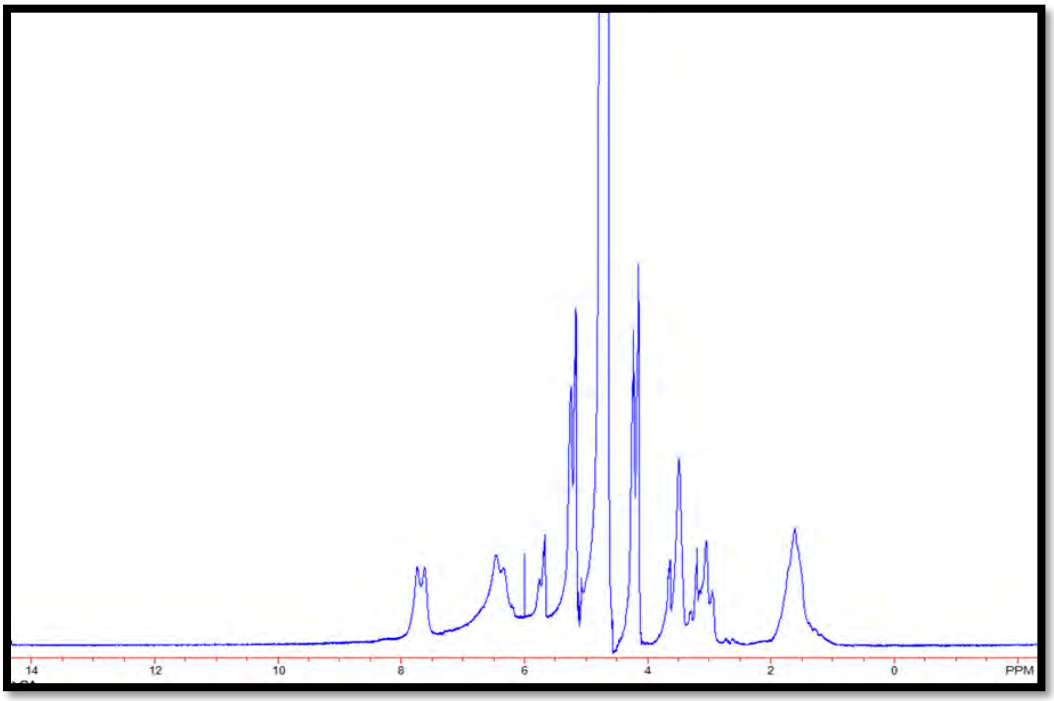

Figure 18. Results from ^{1}H NMR spectroscopy of final product, H_2TPP-Pro-OH, in D_2O.

High Performance Liquid Chromatography

High Performance Liquid Chromatography (HPLC) is a chemical technique used to “separate, and identify compounds that are present” in a sample mixture[25]. The material is passed through a column containing an absorbent material using a liquid solvent to elute each component of a mixture that interacts differently with the materials in the column, thus leading to different elution rates of the components as they flow out of the column, separating the designed product from any impurities.

The sample was dissolved in Milli-Q H_2O and analyzed by HPLC. The HPLC analysis was completed using a Waters Nova-Pak C18, 3.9 x 150 mm column, using 100% acetonitrile as the eluent at a flow rate of 1.00 mL/min. H_2TPP-Pro-OH indicated a 98% purity **(Figure 19)**.

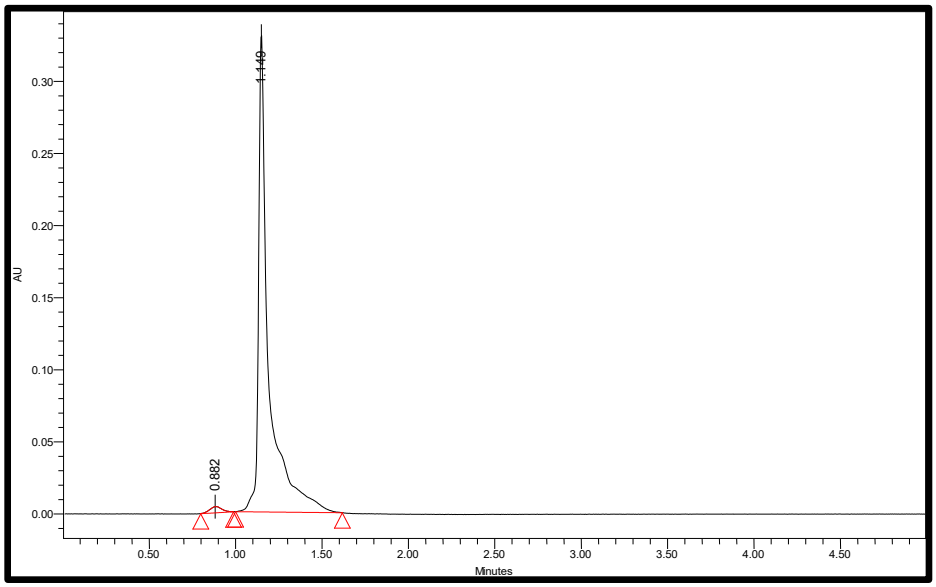

Figure 19. HPLC results for H_2TPP-Pro-OH indicating 98% purity.

Testing

MTT Assay Procedure

An MTT assay is a method of measuring cellular metabolic activity, and thus determining cell viability[26]. The viable cells contain an enzyme that reduces the MTT reagent to a crystalline product called formazan- the formazan crystals have a vivid purple color, which is seen in **Figure 20.** In this particular MTT assay, MDA-MB-231 TNBC cells were plated onto two 96-well plates and allowed to culture for approximately 72 hours. After 72 hours, various concentrations of H_2TPP-Pro-OH were added to the wells containing the TNBC cells and then placed under different conditions- “dark” and “light”. Both plates were wrapped in aluminum foil and set in the incubator, but after approximately 24 hours of incubation, the cells in the “light” plate were exposed to white light (0.5 J/cm^2) for approximately 17.5 minutes, then rewrapped in aluminum foil and returned to the incubator for 72 hours. After the final 72 hours of incubation, the formazan product was dissolved in DMSO and the plates were scanned via a spectrophotometer to quantify the results and determine the cytotoxicity of the H_2TPP-Pro-OH.

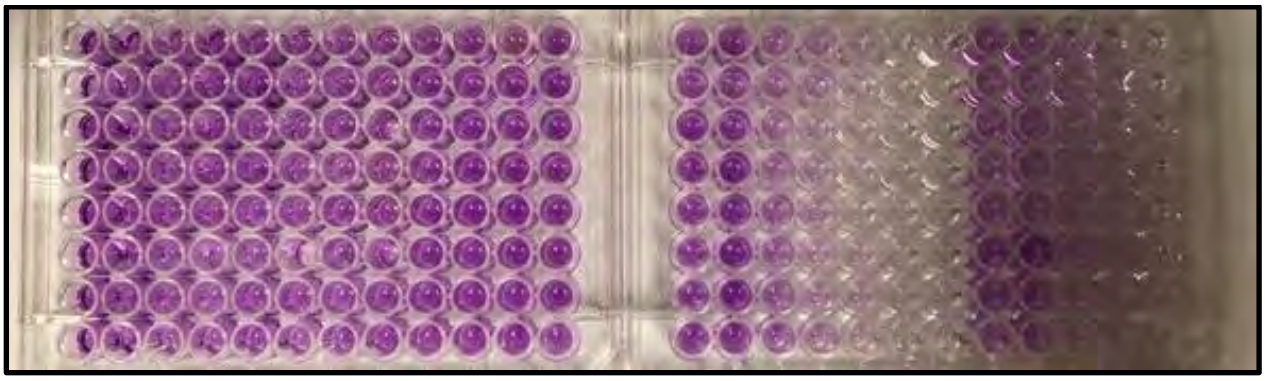

Figure 20. MTT assay results after full incubation period. The left plate was entirely in dark conditions, while the right plate was exposed to light. The purple color indicates living cells.

MTT Assay Results

As previously stated, MTT assays are used to determine cell viability, and cell viability is visibly shown by the vivid purple color on the 96-well plates **(Figure 20).** An MTT assay of H_2TPP-Pro-OH on TNBC cells was run twice. The first trial used porphyrin concentrations of 1, 3, 10, 30, and 100 μM, and at these concentrations the lethal dose to kill 50% of the cells (LD_{50}) was at approximately 60 μM **(Figure 21).** For the second trial, an MTT assay was run again at more narrowed concentrations of 30, 50, 70, 90, and 110 μM, and the LD_{50} was seen to be approximately 30 μM **(Figure 22).**

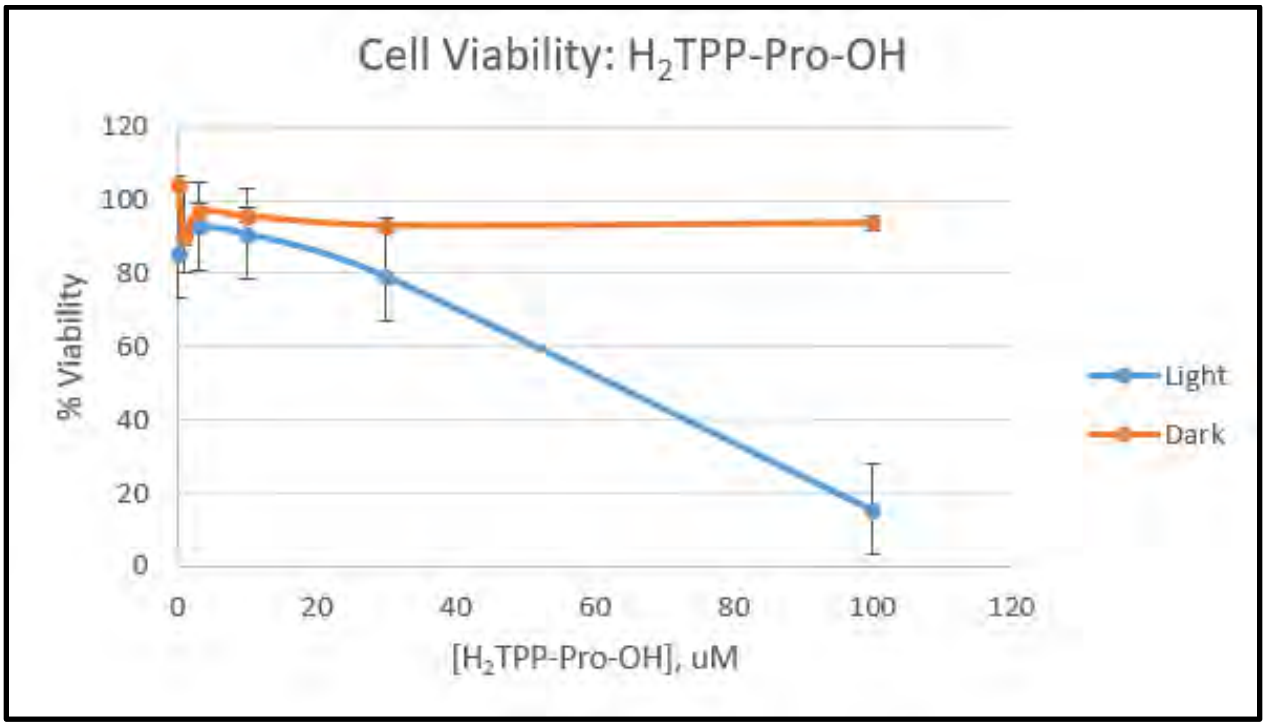

Figure 21. Spectrophotometric MTT assay results for trial one of the light and dark 96-well plates. The porphyrin concentrations used were 1, 3, 10, 30, and 100 μM

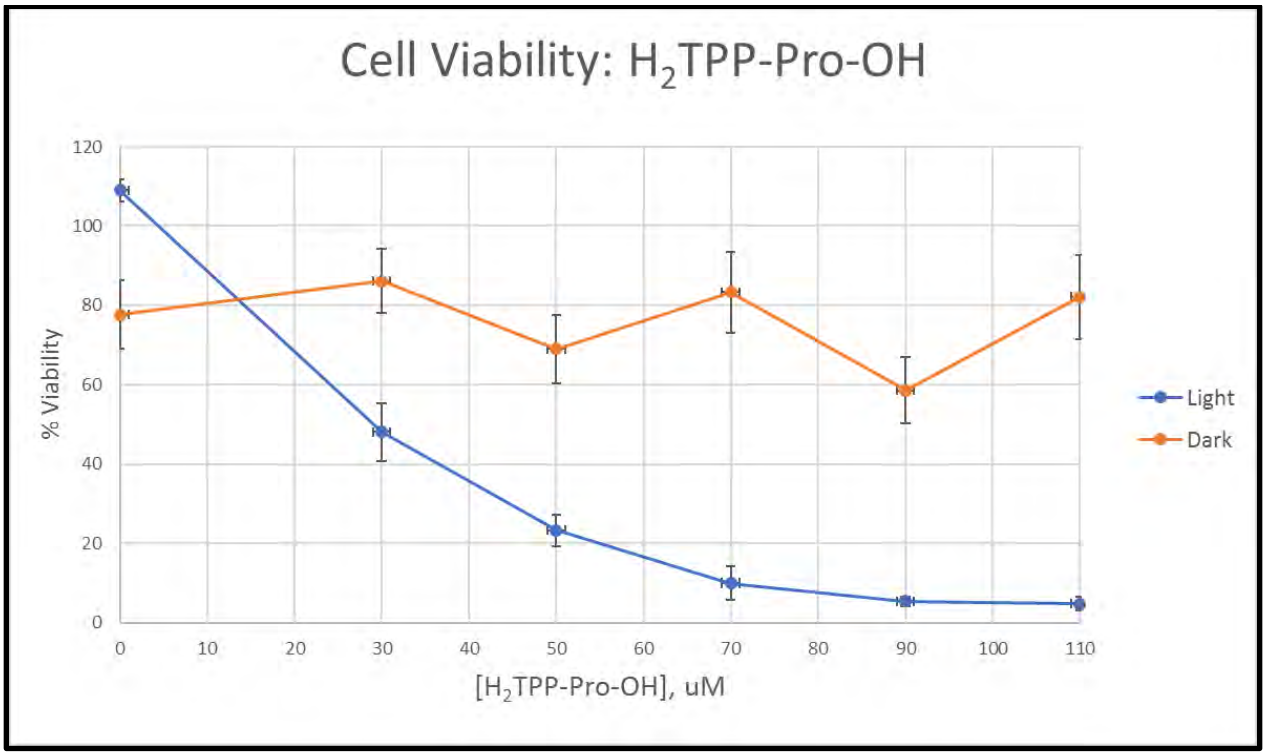

Figure 22. Spectrophotometric MTT assay results for trial two of the light and dark 96-well plates. The porphyrin concentrations used were 30, 50, 70, 90, and 110 μM

Conclusion

In conclusion, the novel water-soluble porphyrin, H_2TPP-Pro-OH, was successfully synthesized and its structure further confirmed by various characterizations. IR spectroscopy showed the proper -OH stretch, C=O double bond stretch, -NH stretch, and the amide I, II, and III bands. The Söret band seen at approximately 414 nm on the UV-vis spectroscopy indicated the presence of a porphyrin core. ^{1}H NMR spectroscopy showed that the hydrogen atoms were attached at the correct locations, while HPLC proved a 98% purity of the final porphyrin product.

When exposed to light, the H_2TPP-Pro-OH porphyrin killed at least 50% of the TNBC cells (LD_{50}) at a porphyrin concentration of 30 μM. When remaining in dark conditions, the TNBC cells grew at a normal rate. This data indicates that the H_2TPP-

Pro-OH porphyrin is more effective in killing TNBC cells when exposed to light than other porphyrin molecules, possibly making it a viable PDT agent. To conclude this data, further experimentation and trials should be performed.

Future Work

In the future, a new trial of the novel porphyrin will be tested against TNBC cells in an MTT assay using the same concentrations of H_2TPP-Pro-OH. Data will be collected to determine if these well plates exposed to light have the same photocytotoxicity as previously determined in the second trial. Other novel water-soluble porphyrin derivatives will also be synthesized to examine their cytotoxicity and to determine whether a different porphyrin derivative is more desirable in killing MDA-MB-231 Triple Negative Breast Cancer cells.

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