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Treating Breast Cancer with Light: The Creation of Two Photodynamic Therapy Agents

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Dedicated to Dr. Joseph Bradshaw,
Dr. Tim Knight, and Dr. Angela Douglass
for inspiring me to reach higher. I wouldn't
have the love for science I do today without
you.

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ABSTRACT

Photodynamic therapy (PDT) is a treatment for a wide display of diseases, including cancer, that has become gradually widespread. The procedure requires the usage of photosynthesizing agents, which are activated in the presence of light. One quite successful photodynamic therapy agent is an aromatic structure made up of four pyrrole rings called a porphyrin. This research focused on producing the water-soluble porphyrins, H_2TPP-3-PEG-OH and H_2TPP-PiperMe-OH, through the attachment of the starting porphyrin, H_2TPPC, with 3-polyethyleneglycol and 3-piperidinemethanol, respectively. The novel, water-soluble agent was purified and characterized by infrared spectroscopy (IR), nuclear magnetic resonance spectroscopy (NMR), and UV-vis spectroscopy. Purity was determined using high performance liquid chromatography (HPLC). To find the cytotoxicity level of novel PDT porphyrins, H_2TPP-3-PEG-OG and H_2TPP-3-PiperMe-OH, the agents were conditionally tested in the presence and absence of light, using MTT assay on MDA-MB-231 triple negative breast cancer cells.

BACKGROUND

According to the National Cancer Institute, roughly 25% of the US population will develop cancer at some point within their lifespan. [4,21] Annually, billions of dollars across the globe are spent on cancer research, to cure or to improve treatment options for such a diagnosis. As of today, the primary treatment for cancer is typically surgical and is paired with radiation and/or chemotherapy. This course of action, however, especially when chemotherapy is the treatment option, has major and broad side effects for the patient. All rapidly dividing tissues are affected, which leads to a loss of hair, extreme loss of appetite, painful digestion, severe anemia, and skin sensitivity. Due to the side effects, the dosage that can be administered during treatment is limited and reduces the patient's quality of life substantially. Using chemotherapy, in combination with surgery and radiation, as a treatment for cancer, is dangerous. Sensing the need for a more target-specific treatment in relation to cancer, researchers began the development of Photodynamic Therapy (PDT).

How Photodynamic Therapy Works

In PDT, an agent, or photosensitizer, is used in combination with a light source to make tissue light-sensitive, thereby killing cancerous cells. [1] It functions by allowing a physician to inject a PDT agent, which is non-toxic until exposed to specific wavelengths of light, into the affected area. Both healthy and malignant cells throughout the body absorb the PDT agent. Compared to normal cells, malignant cells take in greater amounts of the agent. [3] After an incubation period, from minutes to a couple of days, the agent dissipates from healthy cells and

remains in malignant ones. [22] It is still currently being investigated, as to why the malignant cells retain the drug for a longer period.

The physician then directs light at the target tissue, via a laser, UV light, or other light source, which is now light-sensitive due to the PDT agent. Upon exposure to the chosen wavelength, the PDT agent produces a singlet, reactive oxygen species, or an oxygen free-radical, that can destroy cancer cells. The product is believed to treat the cancer by damaging the tumor's blood cells, aiding the body's own immune system in attacking the malignant cells, and killing the cancer cells itself. [4,23] FIGURE 1, below, lays out each step of PDT.

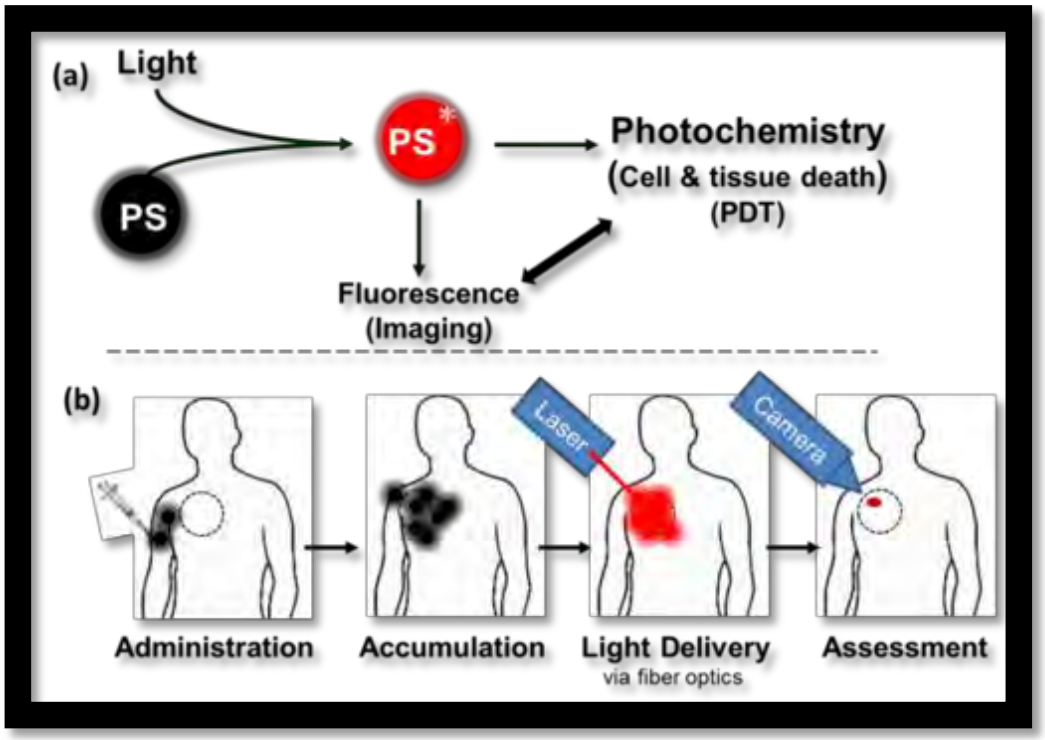


FIGURE 1:
In part a, light and a photosensitizer is being combined to produce a photochemical effect, which triggers tumor necrosis. In part b, the PDT agent is administered, and an incubation period is allotted to allow for accumulation of the drug within the malignant cells. Then, the affected area is treated with a light source and the physician uses a camera to assess the tumor and determine if the treatment was effective.
(http://sites.dartmouth.edu/pdt/)

How PDT Came to Be

PDT, in a modern sense, is a new, evolving science, but the idea of it is almost ancient. After careful examination of ancient German, Danish, and French texts, one sees light used for medicinal purposes that can be traced from antiquity to present day. [26] Light first began as a therapeutic agent in the Egyptian, Indian, and Chinese peoples. Their cultures used light to treat vitiligo, rickets, psoriasis, skin cancer, and psychosis. [24,25] 3000 years ago, the Greeks spoke highly of heliotherapy, or total body exposure to the sun. Herodotus, a renowned physician in 2nd century BC and the father of heliotherapy, stressed the importance of the sun in the restoration of one's health. These practices and beliefs were forgotten though and were eventually regarded as pagan practices, as Christianity became the primary religion throughout the world.

However, in 1815, the thought of using light as a medicinal tool was rediscovered. A physician by the name of Cauvin re-established our perception of the sun's benefits, as he noticed it was an effective therapy for rickets. From this point, research in phototherapy took off and it developed into a science. Niels Finsen, a Danish physician, popularized phototherapy when his work in the field won a Nobel prize in 1903. [26]

Light research also looked at the combination of light and photosensitizers through the field of photochemotherapy. Individuals used exogeneous sensitizers to absorb photons with the intention of a reaction for a therapeutic effect. [26] The photosensitizers, or porsalens, were used in India as early as 1400 BC and by Egyptians around 12th century AD. These peoples used porsalens obtained from various plants combined with natural ultraviolet light to treat skin conditions, such as vitiligo and psoriasis. [2] At the beginning of the 20th century, Oscar Raab, a medical student, examined photosensitized reactions in specific ways and ultimately introduced

the subject to western medicine. [27] He hypothesized that there was a transfer of light energy to chemical energy, but his explanation of the science was incomplete due to the limited understanding of fluorescence at the time. In order to address this issue, his teacher, Professor Herman von Tappeiner and Dermatologist Jesionek, took over Raab's research and developed a clinical study. [28] They set out to determine if eosin could be used as a photosensitizer in the treatment of skin cancer, lesions of the skin, and chondylomata of female genitalia. In 1904, they reported that oxygen was required for an affected area to become photosensitized and three years later, von Tappeiner compiled the experiments into a book, coining the term 'Photodynamic Therapy'. [29] His predictions for the applications of photosensitizers and his push for the use of phototherapy for tumors, make von Tappeiner one of the most important early pioneers for the field of PDT.

Are there Advantages or Limitations to Using PDT as a Treatment?

With more and more findings in the field of photodynamic therapy, many physicians are suggesting PDT as a viable treatment option that could one day work as well as standard treatments, like chemotherapy or surgery. [16,19] Of course, both physician and patient must keep in mind that medicine is never a "one size fits all" situation and some treatments might work better for one individual, while not at all for the next. [30]

Physicians promote PDT because of its benefits for the patient. Due to this treatment option, physicians have more control and may eventually be able to selectively destroy malignant cells, sparing all healthy tissues. [20]

There are, however, advantages and limitations to PDT. One benefit to PDT is, while every procedure has some risk, the risks associated with PDT are quite minimal and much less dangerous than surgery. The therapy can also be applied in regions of the body where surgery may not be an option and may be repeated many times in the affected area if the first treatment is unsuccessful, unlike radiation therapy. [22] PDT is also a much safer option due to its minimally invasive nature, especially for patients considered too high a risk to undergo chemotherapy, radiation, or surgery. These patients consist of the elderly, those vulnerable to surgery, those with immunological disorders, etc.[20] PDT has fewer adverse effects, shorter treatment time, and is usable in outpatient settings, which make it a prime treatment option for patients who are considered high risk or for those who wish to make the treatment experience as good as possible without reduction of quality of life. [19] PDT also leaves little to no scarring post healing process and typically has a lower cost than other treatment options. [12, 18]

While, physicians say that while PDT is a good option, patients should still understand that there are side effects, like every therapy. [10] After treatment, it is common for patients to experience photosensitivity, or sensitivity to light due to the medication given. These symptoms may include unexpected sunburns or dermatitis on skin exposed to the sun. The medication can also cause onycholysis (FIGURE 2), or the lifting of the nail plate from the nail bed. [6,7] To help decrease side effects, physicians recommend keeping shades and curtains on windows, wearing clothing to cover as much skin as possible, or walking with a darker umbrella, and making sure to not rely on sunscreen for at least one-month post-treatment, as it will not prevent any reactions the one might have. [18]

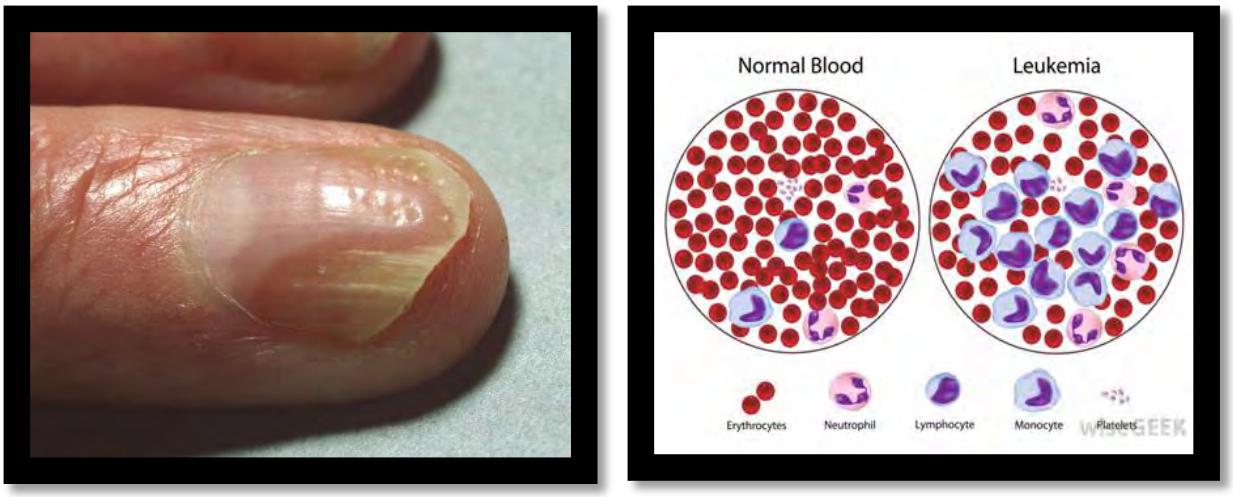


FIGURE 2:
On the left is a photo of onycholysis, or the lifting of the nail plate from the nail bed. The image to the right shows what leukemia, a blood cancer, does to the body. There is a rapid increase of immature blood cells and due to the overcrowding, healthy blood cells cannot be produced.
(http://biyaninursingcollege.com/what-is-leukemia-its-type-etiology-classificationsign-symptomdiagnostic-findings-treatment/ (right); https://www.researchgate.net/figure/Onycholysis-semilunaris-A-is-characterized-by-its-half-moon-shape-and-clear-border_fig19_221924704 (left))

As it is a new treatment modality and still in development, PDT also has limitations. The treatment's effectiveness largely depends upon how accurately light is being delivered to the tumor or affected site. Tumors that are too deep for visible light to penetrate have proven to be difficult to treat, as they are not accessible without surgical intervention. For now, PDT only has the ability to target cells within 1/3 of an inch of the light source. [18] Oxygenation of the tumor and surrounding tissue is also important for PDT. A patient who has a tumor surrounded by necrotic tissue will have a limited photodynamic therapy treatment. Without oxygen, free-radicals, which kill the cancerous cells, are not produced. PDT is also limited to specific sites; therefore, the PDT effect only takes place at an irradiated site. With the current available technology, treating metastases or performing whole body radiation with PDT is not available. [19] Since it functions by targeting tumors, PDT cannot treat cancer that have spread throughout the body, nor can it treat diseases, such as leukemia (FIGURE 2). [16]

What Types of Treatments is PDT Used For?

However, despite its limitations, physicians still developed ways to use the treatment. As PDT has grown, researchers have increased their support its use for uses aside from just dermatological ones, such as acne, skin lesions, and skin cancers, as you can see in FIGURE 3.

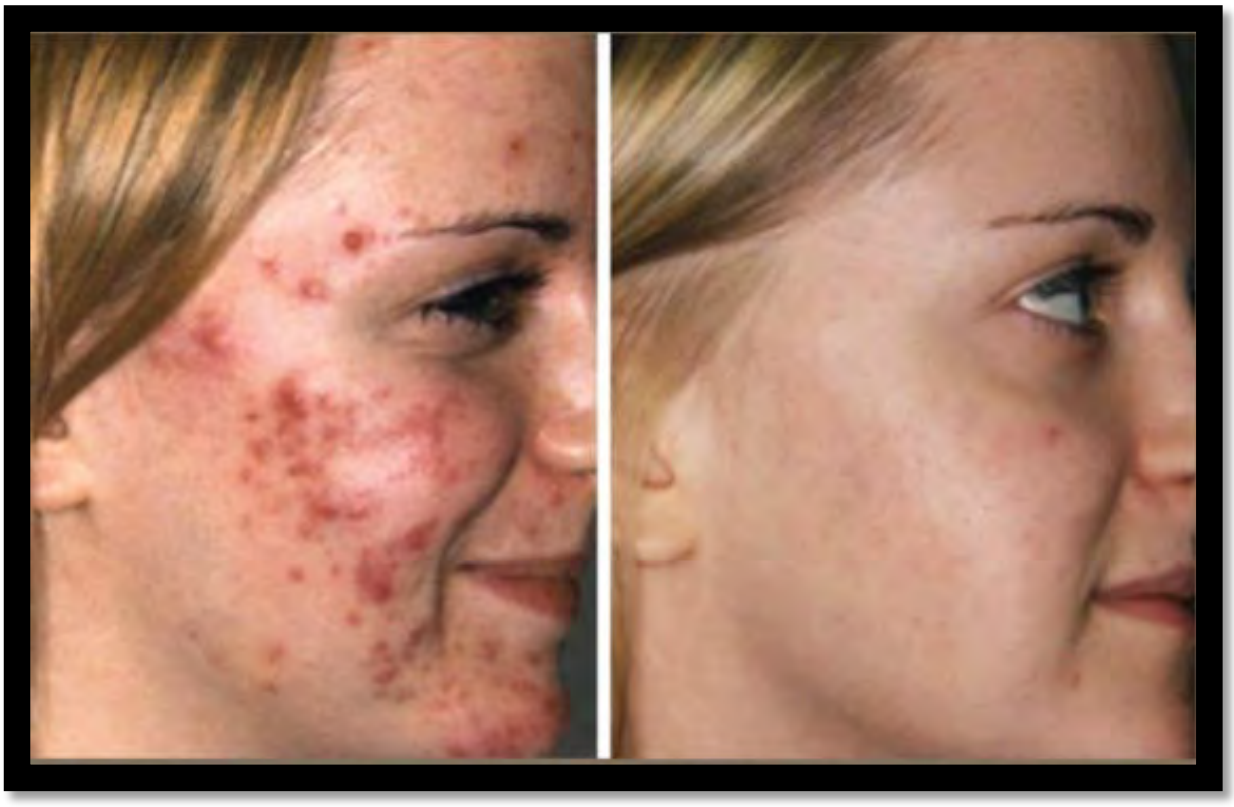


FIGURE 3:
Jane underwent PDT for her acne. This is the result after 3 treatments.
(https://www.skinmdandbeyond.com/faq-face-photodynamic-therapy/)

Katie, a 21-year-old who suffered from anaplastic ependymoma, or an aggressive form of brain cancer, says that she had seven brain surgeries before Dr. Harry Whelan, a neurologist, suggested PDT. [5] After three aggressive treatments, the tumors have not rematerialized on Katie's brain. PDT is also being used for esophageal cancers, which affects around 12,000 Americans every year. [14] To save Francis' life, the doctor would have had to remove a large

portion of the esophagus, leaving Francis to eat through a feeding tube for the remainder of his life. After his PDT treatment, he reported to Current Science Magazine, "I can eat anything I want." [5] For now, especially in the US, PDT is only approved for patients that have no other alternatives and are diagnosed with late-stage esophageal cancer or early-stage lung cancer. [16]

A new field of PDT is also being explored: photoangioplasty. This treatment uses the photosensitizer to break up the plaque build up in blood vessels, so the patients' arteries are no longer clogged. This process is illustrated in FIGURE 4 and FIGURE 5 below. Doctors are hoping that these studies will one day offer new options to patients suffering from ischemia, or a disease causing an inadequate blood supply to an organ or other part of the body, especially the heart muscles. [17]

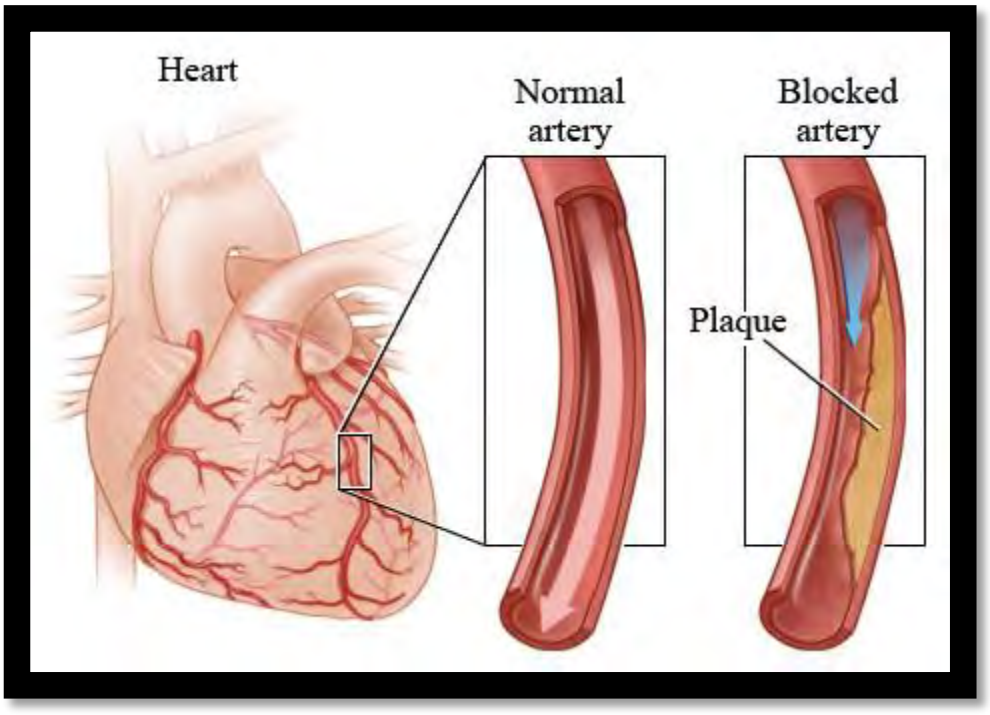


FIGURE 4:
An Illustrated example of a blocked artery due to plaque buildup, that would typically be solved using more invasive means outside of PDT. Now, the issue can be solved with a photoangioplasty.
(https://metrohealth.net/healthwise/coronary-angioplasty/)

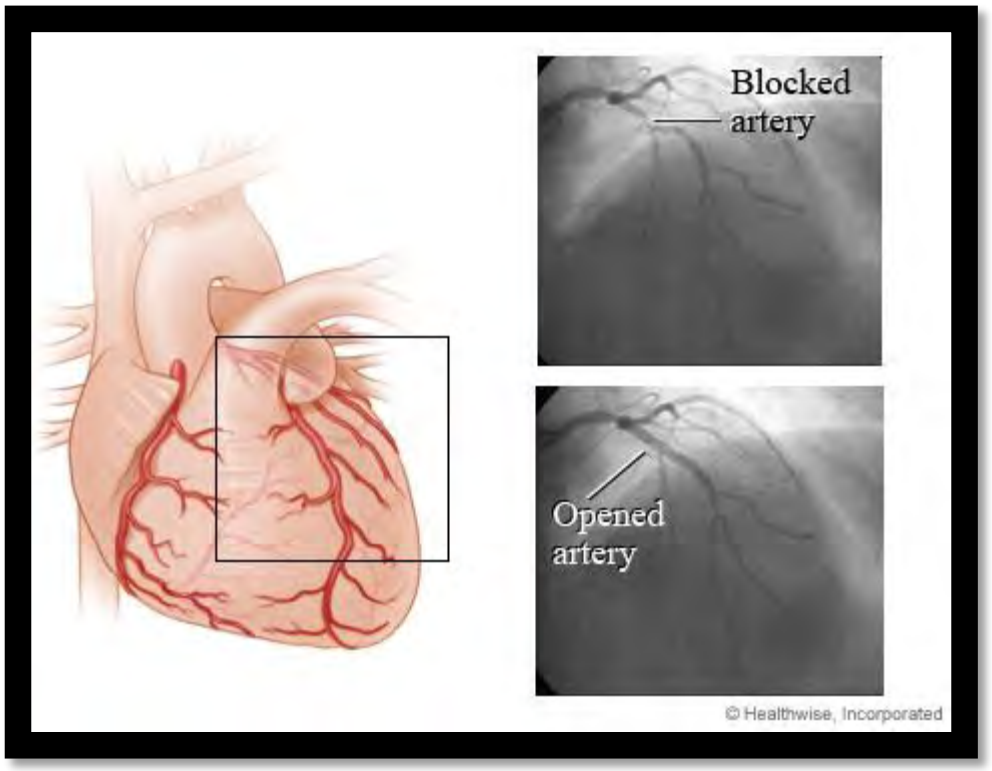


FIGURE 5:
An imaging, or real-life, example of a blocked artery due to plaque buildup, that would typically be solved using more invasive means outside of PDT. Now, the issue can be solved with a photoangioplasty.
(https://metrohealth.net/healthwise/coronary-angioplasty/)

Currently, verteporfin, a photosensitizing drug licensed for PDT, is used in the treatment of age-related macular degeneration (AMD) and pathological myopia. [2] These diseases result in damage to the macula, a central area of the retina. AMD also occurs when blood vessels in the eyes begin to leak, causing scar tissue build-up in the light sensitive portion of the eye. While PDT cannot remove scar tissue already present, it does stop the disease from progressing. [14] FIGURE 6 shows how PDT functions within the eye and FIGURE 7 shows the treatment illustrated and being used on a woman diagnosed with AMD.

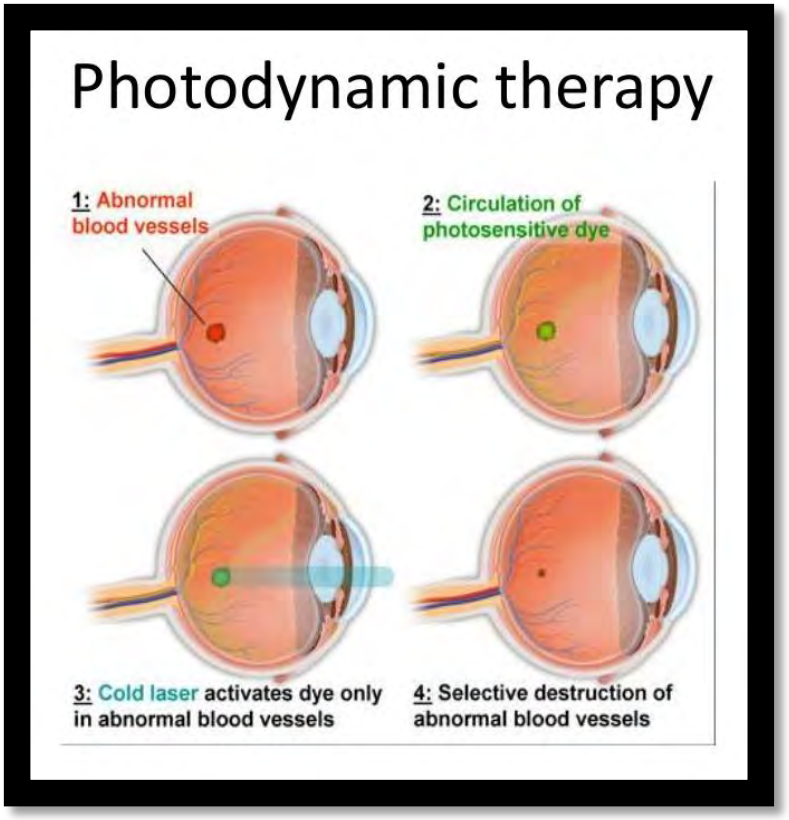


FIGURE 6:
PDT can treat AMD by stopping the leakage of abnormal retinal tissue in the back of the eye. The procedure calls for a photosensitizing dye to be injected into the patient's arm, which is then absorbed by structures in the eye. The photosensitizer is then activated by the laser. Abnormal blood vessels are selectively treated without damaging any normal tissues.
(https://www.slideshare.net/JaheedKhan/age-related-macular-degeneration-a-glimpse-into-the-future-by-jaheed-khan)

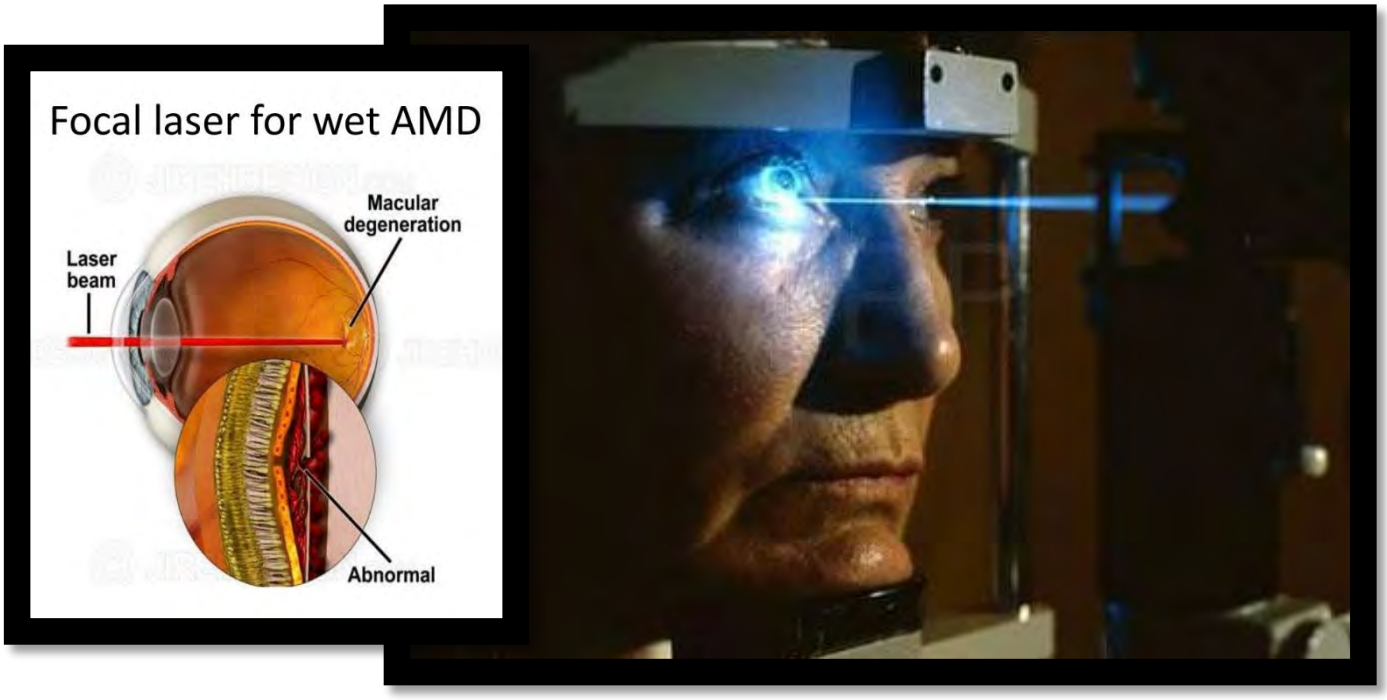


FIGURE 7:
The image on the left, is an illustrated example of how the laser penetrates the eye during an AMD PDT treatment. The photo on the right is of a woman undergoing the treatment.
(https://retinamaculainstitute.com/photodynamic-therapy/)

How is Optimal PDT Achieved?

To help combat the limitations of PDT and aid in new research, scientists from all over the world have been working to improve light dosimetry and develop new photosensitizers, both key components in PDT.

As the depth of the tissue penetration is affected by the light's wavelength, scientists began their research by looking into the science behind light dosimetry. The most frequent light source used today is a laser, but other sources include, intense pulsed light, light-emitting diodes (LEDs), and light of various colors, like blue, red, and natural sunlight. Researchers found that skin treatments typically require the light source to be directly shown on the skin for an appropriate amount of time and light for internal cancers, in the bladder, lungs, stomach or esophagus, can be delivered by means of small, fiber-optic cables. [18] By using wavelengths of 600 to 800 nm, researchers have found that light delivery can be enhanced. [8,9,19]

To have an ideal photosensitizer, it must be able to produce the singlet oxygen necessary for the degradation of tumors. [13] It should have a highly absorbent coefficient, or the ability to absorb light at a particular wavelength. [20] During testing, the photosensitizer should not accrue toxicity in the dark and should selectively congregate in the cancerous cells. There should only be cytotoxicity in the presence of light. [13] Also, in a clinical trial, the photosensitizer must be chemically pure, stable, and produce minimal skin toxicity. [20] With these characteristics in mind, scientists began to develop photosensitizers with the end goal that they would one day be used to help cure cancer.

The Development of Porphyrins as PDT Agents

After the development and FDA approval of Photofrin, a hematophorphyrin derivative and the first photosensitizer for PDT, the therapy option has been accepted "for treating various forms of cancer in many countries…and is also being tested as a possible therapeutic agent against abdominal and thoracic cancers, brain, breast, and skin." [11,13,30] Other porphyrin derivatives have also been approved for clinical use: Visudyne, Temoporfin, and Talaporfin. These FDA approvals have led to significant PDT research using both natural and synthetic porphyrins.

Recently, researchers have concentrated on using porphyrins as the photosensitizer, due to its unique properties of a multiring structure, its presence in biological systems, and its photosensitizing abilities. After its detection in 1897, the molecule has been applied in various fashions, such as gene regulation, hormone synthesis, and as a solar cell.

In the words of Igor Stojiljkovic, *et. al,* [15] "Prophyrins are the most colorful and probably most widespread enzyme co-factors in nature." The porphyrin molecule is a heterocyclic macrocycle containing four pyrrole subunits, which is shown in FIGURE 8.

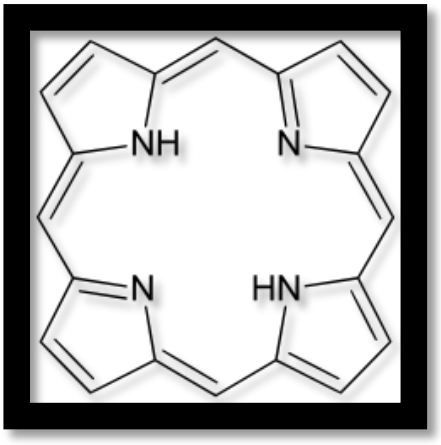


FIGURE 8:
The structure of a porphyrin molecule, with molecular formula $C_{20}H_{14}N_4$.
(https://en.wikipedia.org/wiki/Porphine)

The most recognizable structure that contains a porphyrin macrocycle is hemoglobin in red blood cells. Characteristics of a porphyrin include a dark purple, photosensitive pigment with specific absorption readings when characterizing the created porphyrin with UV-vis spectroscopy.

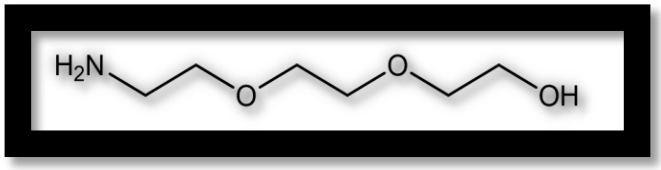
RESEARCH

MATERIALS & METHODS

Porphyrin Synthesis

This research utilizes the light absorbing characteristic of porphyrins, which allow the molecule to be activated by photons and results in a desired reaction with the targeted cells. The goal was to create two, novel PDT agents that were water-soluble and contain typical porphyrin characteristics. The two amines used to create the porphyrins in this investigation are amino-PEG-3-alcohol and 3-piperidinemethanol.

FIGURE 9:
The structure of *Amino-PEG-3-Alcohol.*
(1.67 X 10^{-3} mol, MW = 149.2)



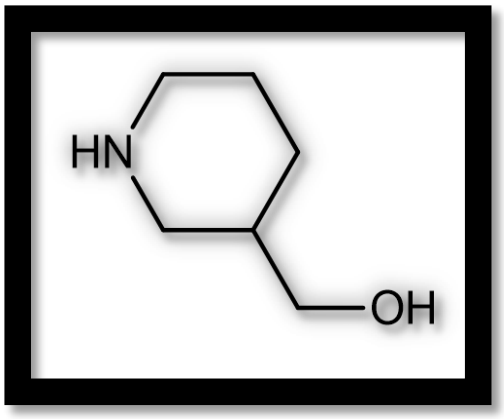


FIGURE 10:
The structure of *3-Piperidinemethanol*
(3.79 X 10^{-3} mol, MW = 115.18)

Water solubility was a critical factor in this project as the main goal was to make a porphyrin with the intention that it could one day be used *in vivo*, meaning within a living organism. A physician administers PDT agents to a patient through skin application or injection to the affected area. As the body consists of roughly of 60% water and the blood has a concentration of water approximately 92%, it is important that the agent is water-soluble. This ensures successful entry into the cells and allows the drug to accumulate where it is needed.

Synthesis of H_2TPPC

With the goal of attaching the amines to the porphyrin structure and making the new agent water-soluble, first H_2TPPC, the starting material, was synthesized. The experiment began by adding 3 g of 4-formylbenzoic acid and a stir bar to a clean, dry 500 ml round bottom flask. The flask was then placed on a heating mantle and 250 ml of propionic acid was added. Then, Gloves were appropriated for protection against the harsh chemicals when adding 1.5 ml of pyrrole via syringe. The solution was refluxed for 1 hour and the entire apparatus was wrapped in aluminum foil to protect it from light. After an hour, the flask and its contents were left to cool. Then, the flask was wrapped in aluminum foil, parafilm was placed across the top, and the flask was labeled. The flask was then placed in a freezer overnight.

The product should then be filtered using a medium sintered glass filter and washed with roughly 30 ml of dichloromethane. Washing was repeated until no product remained in the flask. The M glass filter with the final product (it should be a purple/black color) was left to dry overnight.

Synthesis of H_2TPP-3-PEG-OH

Once the starting material was synthesized, the acid chloride had to made, so the amino-PEG-3-alcohol could be attached to the porphyrin structure itself. All required glassware was dried in the oven overnight. The reaction is air sensitive, and its environment must stay dry at all times. The reaction began by weighting out 0.13 g of H_2TPPC, adding it to a 50ml round bottom flask, and placing the flask under a nitrogen sparge. Then, 10 ml of Dimethylformamide (DMF) and a stir bar was added to the flask. 0.15 ml of thionyl chloride ($SOCl_2$) was then added via syringe. The reaction was allowed to sit and stir for 1 hour. The product, H_2TPPCl, was green.

After an hour, the flask was disconnected from the nitrogen sparge and connected to the rotary evaporator (rotovap), as seen in FIGURE 11, using KECK clips. The Rotovap's purpose was to eradicate chemicals that contain lower boiling points from a mixture, while isolating the desired product. The mixture was evaporated to dryness. After evaporation of the DMF, the dried product was be a shimmery blue-green color. The product was kept under vacuum overnight.

FIGURE 11:
This is a photograph of a Rotovap. Glassware is connected to the machine using KECK clips (green adapters). All unwanted solvents are removed via vacuum, are trapped in the condenser, and are collected in the larger flask for later disposal. It utilizes a hot water bath to prevent the mixture from freezing during evaporation.

Next, methanol was distilled so it could be used in the final step of the porphyrin synthesis. The flask was removed from vacuum and placed under a nitrogen sparge. After retrieving a small, clean glass vial, 0.25 g of amino-PEG-3-alcohol was added and then dissolved in 25ml of freshly distilled methanol. The reaction was allowed to stir under nitrogen for 1 hour. After an hour, the solution was rotovapped to dryness, evaporating all traces of methanol. The

final product was be a deep purple color, as seen in FIGURE 12. This product was named H_2TPPC-3-PEG-OH. Next, the product was purified using column chromatography.

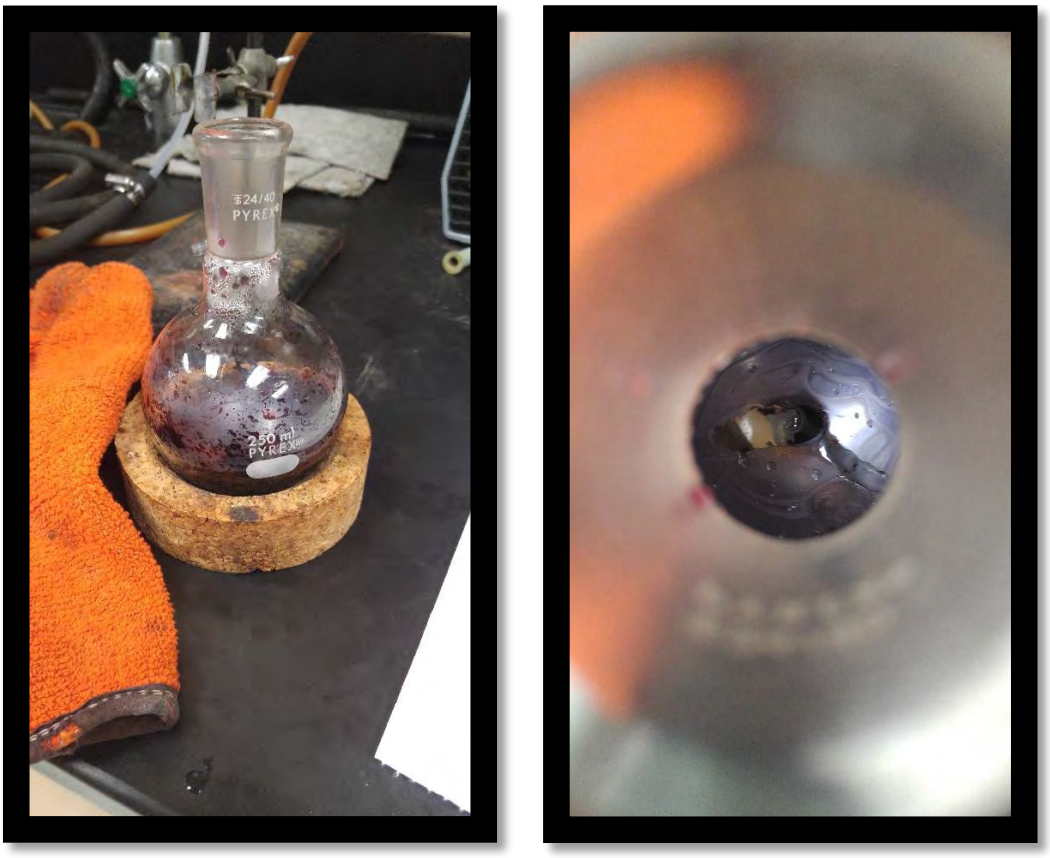


FIGURE 12:
These are photographs of the final porphyrin product. Notice the color is purple, a characteristic of a porphyrin.

Synthesis of H_2TPP-3-PiperMe-OH

The porphyrin synthesis was repeated using a second amine, 3-piperidinemethanol. However, the acid chloride reaction began with 0.25 g of H_2TPPC and 0.25ml of thionyl chloride. The remaining synthesis was identical to that of H_2TPP-3-PEG-OH, with the substitution of 0.437 g of 3-piperidinemethanl for animo-PEG-3-alcohol in the final reaction. This product was named H_2TPPC-3-PiperMe-OH. Next, the product was purified using column chromatography.

Summary of Porphyrin Synthesis

So, in summary, the porphyrins were synthesized through a three-step reaction process:

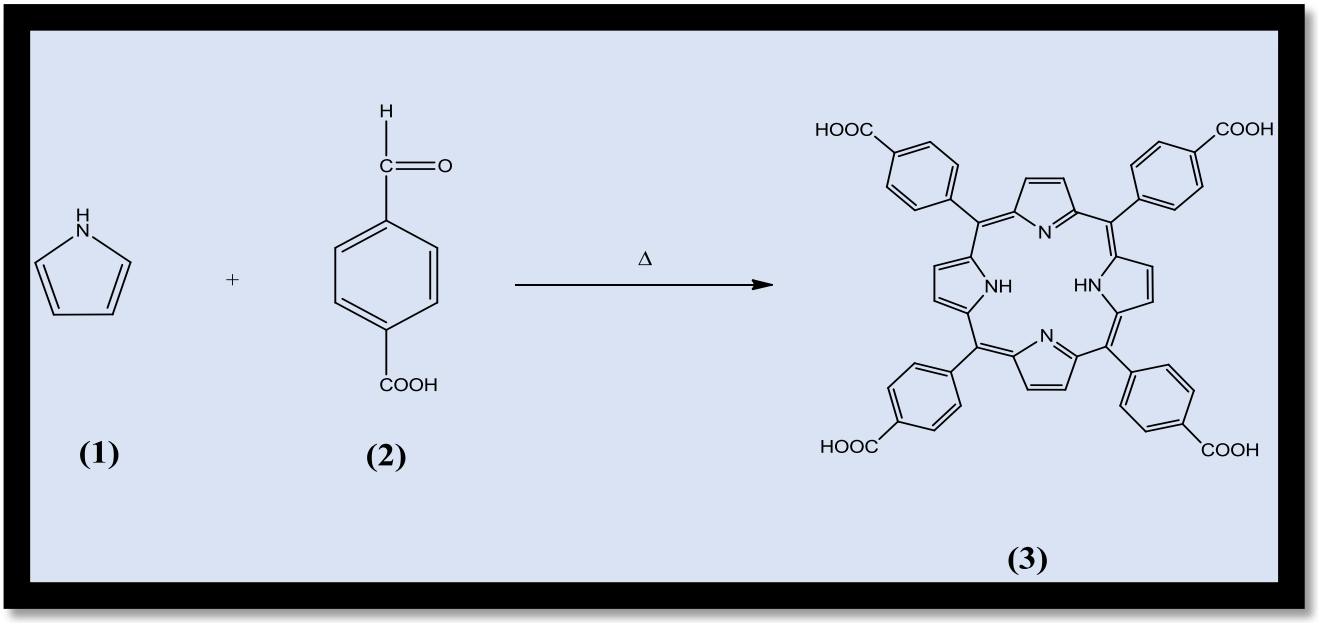


FIGURE 13:
In step 1, 4-formylbenzoic acid (2) was reacted with pyrrole (1) in propionic acid forming H_2TPPC (3)

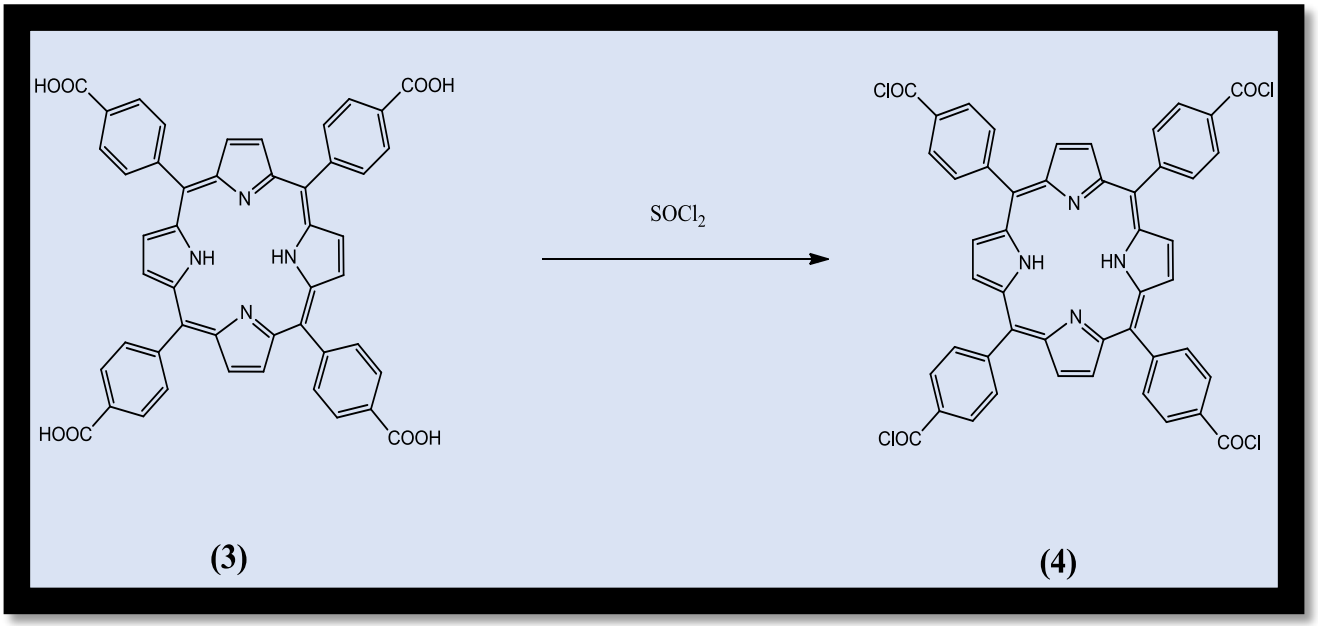


FIGURE 14:
In step 2, H_2TPPC (3) was reacted with $SOCl_2$ in DMF to form Acid Chloride (4), or H_2TPPCl.

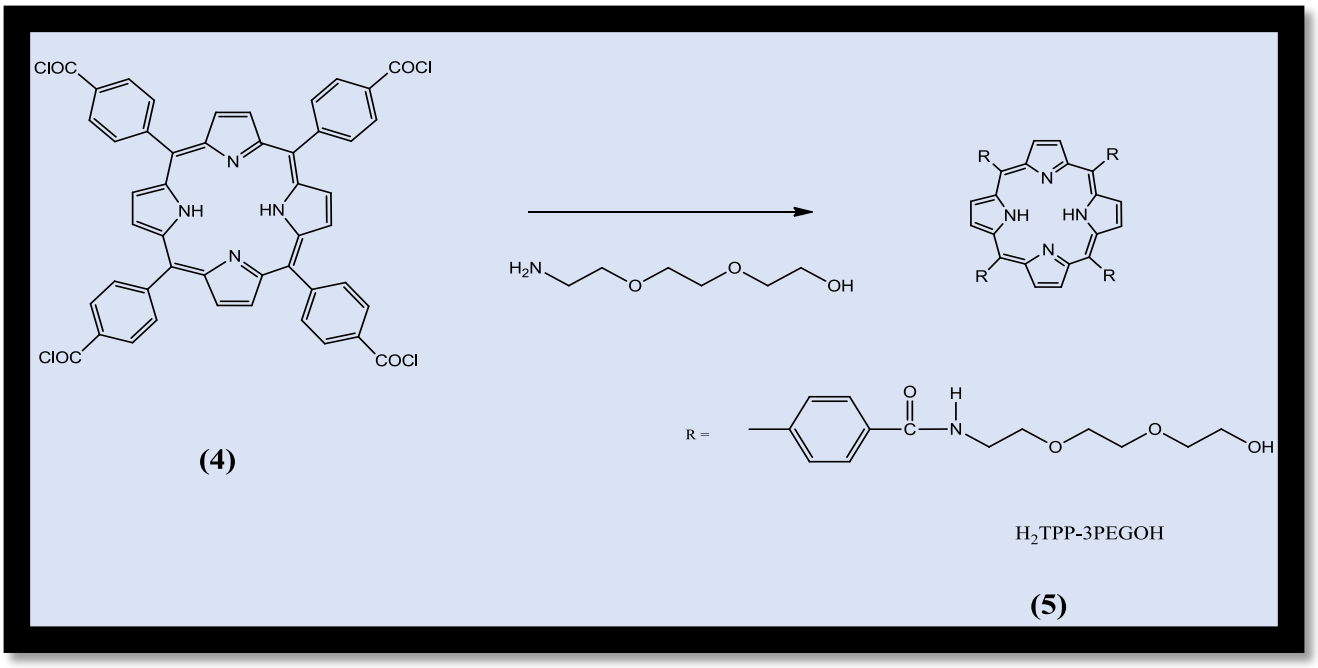


FIGURE 15A:
Finally, in step 3 A, the acid chloride (4), or H_2TPPCl, was reacted with the amine, amino-PEG-3-alcohol, in methanol to form the final product, H_2TPP-3-PEG-OH.

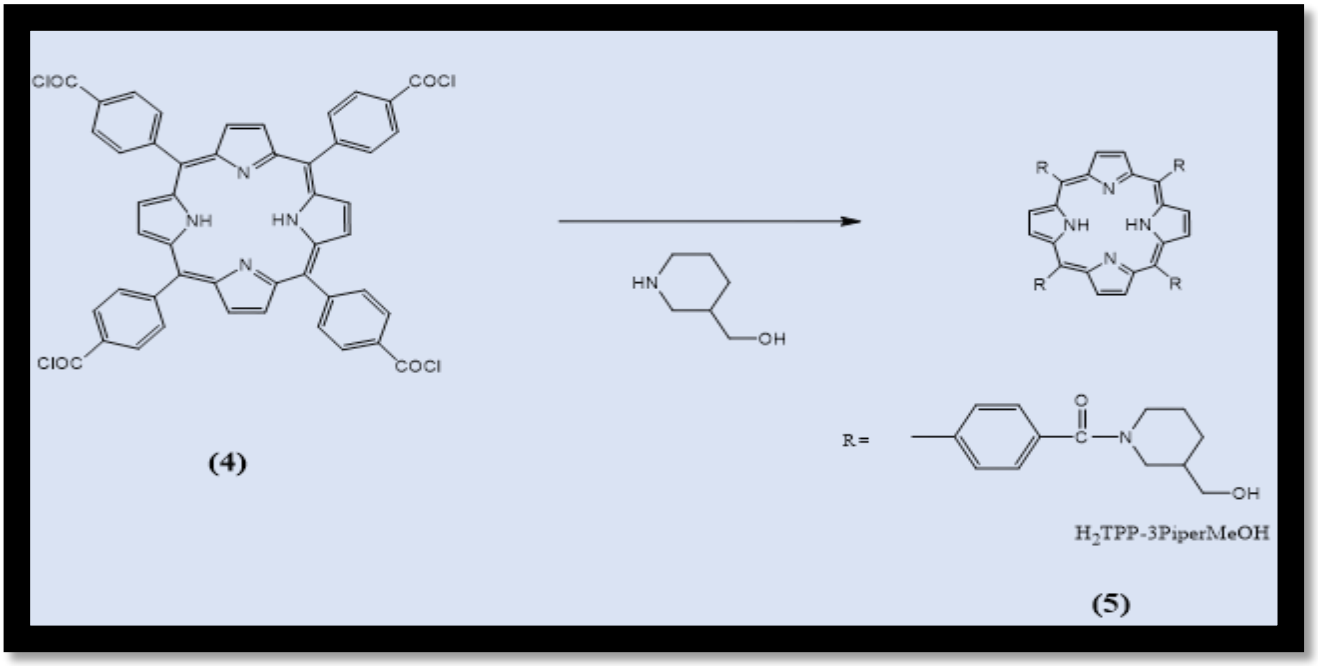


FIGURE 15B:
Finally, in step 3 B, the acid chloride (4), or H_2TPPCl, was reacted with the amine, 3-piperidinemethanol, in methanol to form the final product, H_2TPP-3-PiperMe-OH.

Purification of the Porphyrin Products

Low pressure liquid column chromatography was used for the porphyrin purification. This type of chromatography was used to separate proteins, nucleic acids, or small molecules in complex mixtures. It uses a liquid phase, which is mobile, and a solid phase, which is stationary. The desired molecules in the mobile phase are separated based on their differing physiochemical interactions with both the stationary and mobile phases. [32]

Low pressure liquid column chromatography was prepared by setting up a glass column with pressurized air at the top of the apparatus, as seen in FIGURE 16. The column material, or slurry, was prepared 24-hours in advance by mixing LH-20 with 50:50 MeOH- H_2O, or G-50 with Milli-Q H_2O, so that the material could hydrate under the added eluent.

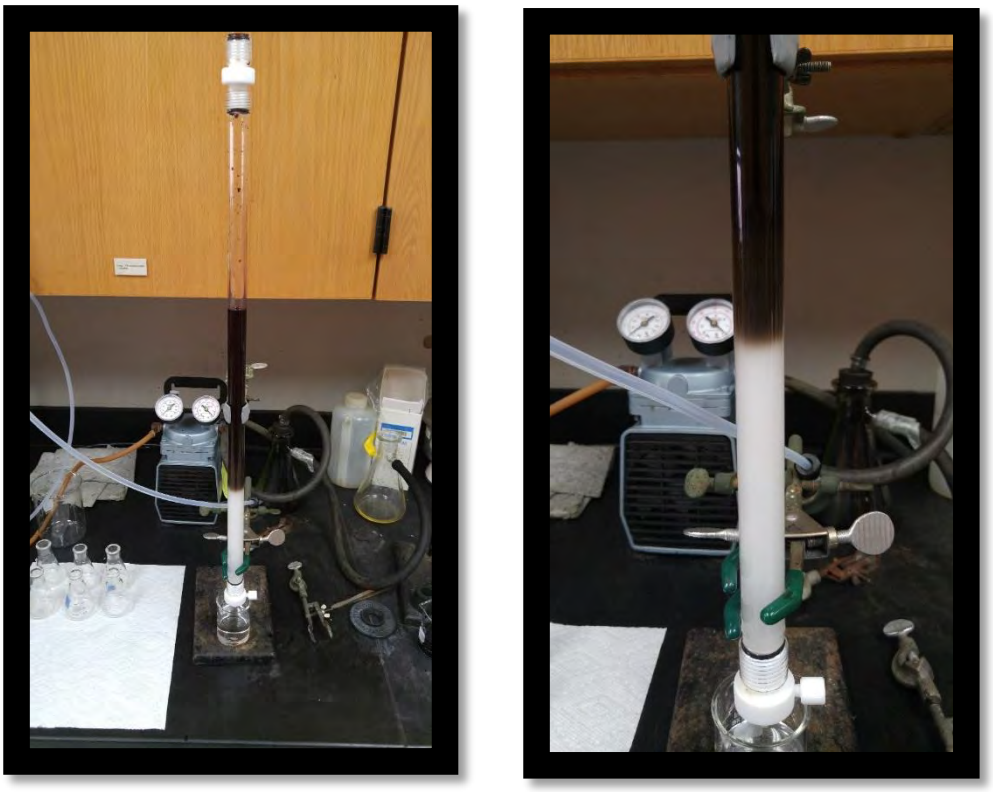


FIGURE 16:
The photo above shows an example of low pressure liquid column chromatography. Notice the air apparatus at the top of the glass column (seen in photo on left), the product being filtered through the LH-20 column material, and the collection of the product at the bottom in an Erlenmeyer flask.

Each porphyrin was purified separately by first passing it through 0.45mm nylon syringe filters and then through Sephadex LH-20 using a 50:50 methanol/water mixture as the eluent. The material was subsequently purified by passing it through 0.45mm nylon syringe filters and then through Sephadex G-50 using Milli-Q H_2O. The purified material was then passed through 0.45mm nylon syringe filters and a final column of Sephadex G-50 using Milli-Q H_2O to ensure purity. Between each column, the material was rotovapped. During collection of the material, close attention was paid to the column to ensure only the dark purple/pink materials, which is the porphyrin, were collected. After purification, the material was tested for purity using HPLC and characterized by UV-vis and ^{1}H-NMR spectroscopy.

RESULTS

High Performance Liquid Chromatography

High Performance Liquid Chromatography (HPLC) is a tool used in analytical chemistry to determine the purity of a material. It is able to separate, identify, and quantify each component in a mixture. Each component within the sample will interact slightly different with the stationary phase, causing different elution rates for each component and leading to the separation of materials as they elute out of the column. The HPLC instrument uses a pump to pass a pressurized liquid and the sample through a column filled with a stationary phase. This leads to the separation of components in a mixture. FIGURE 17 and FIGURE 18 show the HPLC results for the novel porphyrins.

FIGURE 17:
HPLC results for porphyrin H_2TPP-3-PEG-OH. The sample indicated a 98.8% purity.

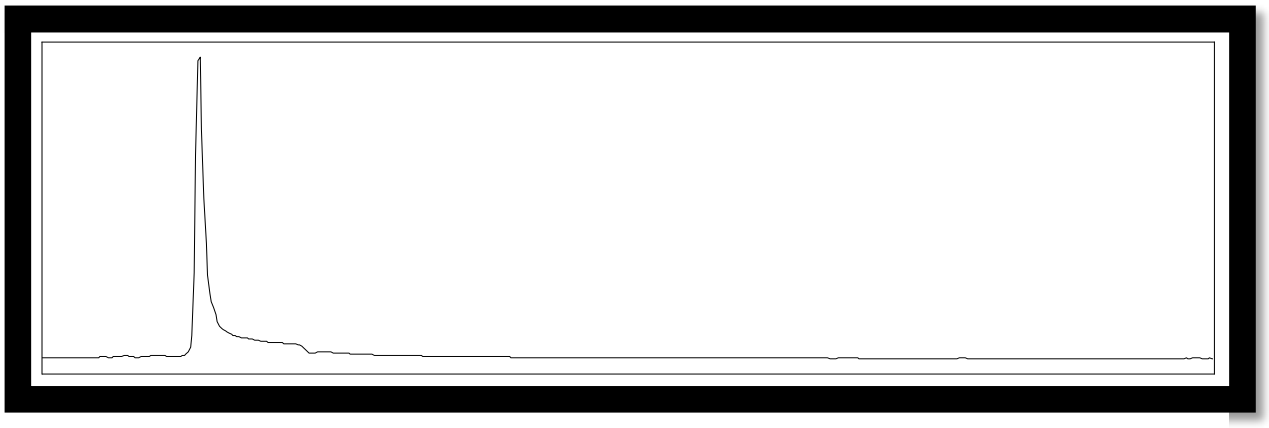


FIGURE 18:
HPLC results for porphyrin H_2TPP-3-PiperMe-OH. The sample indicated a 99.3% purity.

Prepared samples were dissolved in Milli-Q H_2O and analyzed. H_2TPPC-3-PEG-OH indicated a 98.8% purity and H_2TPPC-3-PiperMe-OH indicated a 99.3% purity. The HPLC analysis was completed using a Waters Nova-Pak C18, 3.9 x 150 mm column, with 100% acetonitrile at a flow rate of 1.00 mL/min.

The purity of a material is tested because the development of a pure photosensitizer allows higher dosages to be given to patients and decreases the amount of side effects a patient may have, such as skin photosensitivity. [26]

Characterization of the Porphyrin: UV-Vis Spectroscopy

Ultraviolet-Visible Spectroscopy (UV-Vis) a tool used in analytical chemistry to determine the absorbance a particular sample has. "Molecules containing π-electrons or non-bonding electrons can absorb energy in the form of ultraviolet or visible light to excite these electrons to higher anti-bonding molecular orbitals." [31] The more easily excited the electrons, the longer the wavelength of light it is able to absorb. The instrument functions by allowing a beam of light from a visible and/or UV light source, to separate into its component wavelengths by a prism or diffraction grating (monochromator). One beam passes through a small transparent container, called a cuvette, that contains the sample. The beam's intensity is measured by electronic detectors and are compared to a control sample. The UV region is typically 200 to 350 nm, while the visible portion is from 350 to 800 nm. Characteristically porphyrins have a strong reading at 400 nm, called the "Söret" band, and a series of satellite absorptions from 600 to 800 nm, called the Q-bands or the "fingerprint region". [20]

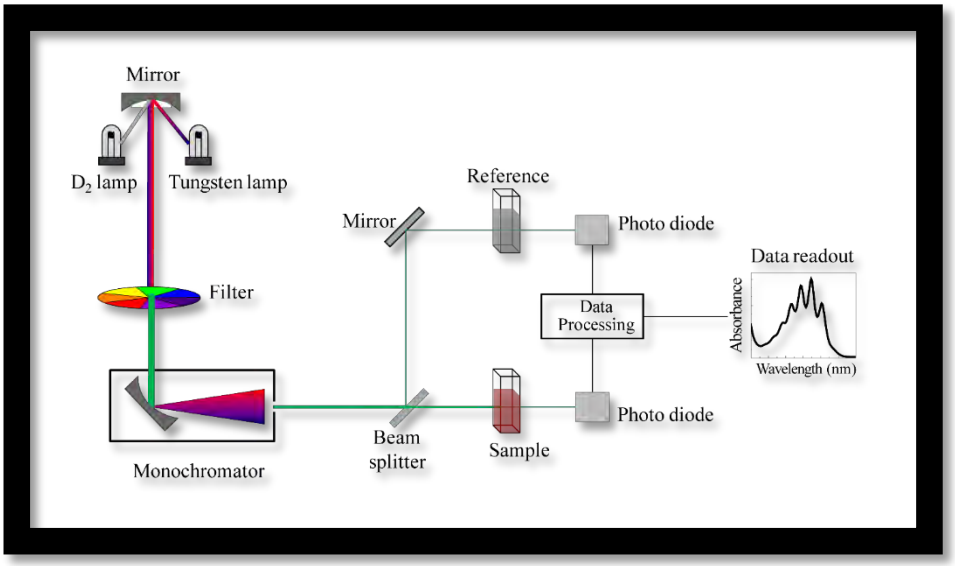


FIGURE 19:
The UV-vis spectroscopy diagram above shows how light is transmitted through a color filter and a monochromator, hits the sample, and data is processed in absorbant amount and wavelengths. (https://commons.wikimedia.org/wiki/File:Schematic_of_UV-_visible_spectrophotometer.png)

The UV-Vis of both H_2TPP-3-PEG-OH and H_2TPP-3-PiperMe-OH were obtained. In the table below, each porphyrin demonstrates a Söret band at approximately 414 nm, which corresponds to a wavelength of maximum absorption, and contained a satellite region with appropriate Ɛ values, as shown in FIGURE 20 and FIGURE 21. Accurate sample masses were diluted to stock solutions of 25 or 50 ml.

Peak (nM)	Ɛ ($mM^{-1}cM^{-1}$)
409	424.55
522	38.19
558	20.15
593	15.89
653	14.41

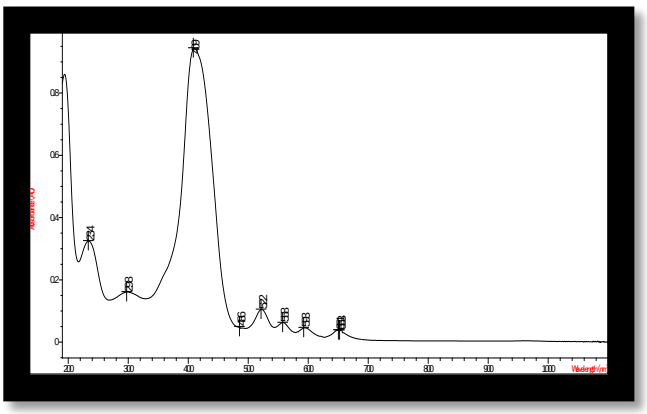


FIGURE 20:
UV-vis results for porphyrin H_2TPP-3-PEG-OH. The epsilon values were calculated by Beer's Law: $A = \varepsilon Cl$

Peak (nM)	Ɛ ($mM^{-1}cM^{-1}$)
414	363.85
520	8.02
541	8.10
581	4.22
640	3.79

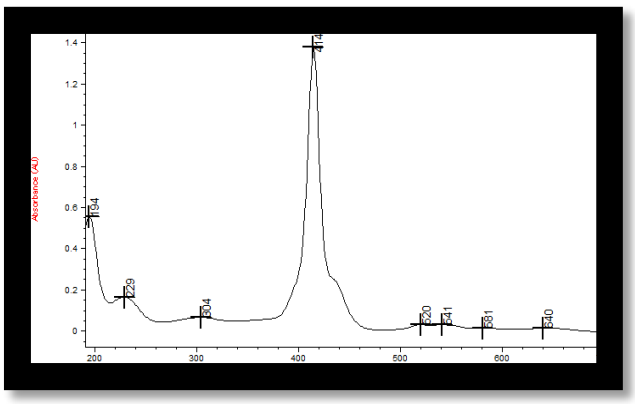


FIGURE 21:
UV-vis results for porphyrin H_2TPP-3-PiperMe-OH. The epsilon values were calculated by Beer's Law: $A = \varepsilon Cl$

Characterization of the Porphyrin: NMR Spectroscopy

Nuclear Magnetic Resonance Spectroscopy (NMR) is a technique used for the characterization of organic molecules that contain hydrogen atoms. Every NMR sample is prepared in a thin, glass tube (FIGURE 22), or NMR tube. When ready, the sample is placed into a spinning holder inside of a very strong magnet. Then, the results are placed in a graph.

FIGURE 22:
The photo above is of a glass tube used for ^{1}H-NMR.

After comparison of the ^{1}H-NMR of each anime and the final porphyrin product, the anime successfully attached to the R group on the porphyrin core structure, as seen in FIGURE 23 and FIGURE 24. These results confirmed that the intended porphyrin was produced and could proceed to the next step in the research.

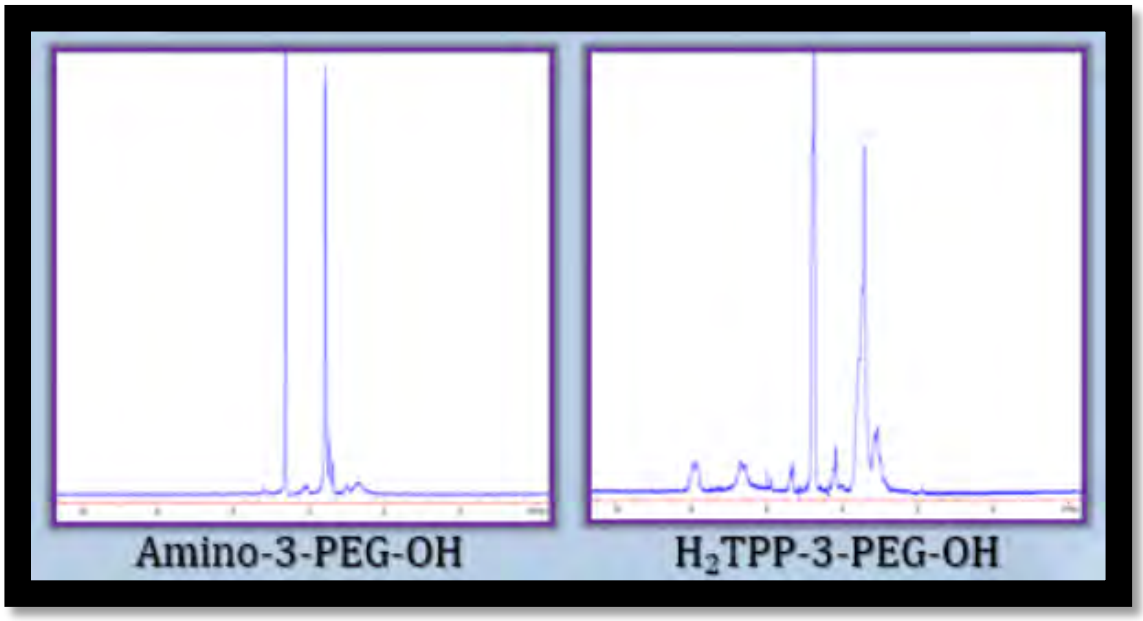


FIGURE 23:
The results from the ^{1}H-NMR show that that H_2TPPC combined with the anime to produce the final product, H_2TPP-3-PEG-OH. The ^{1}H-NMR for amino-PEG-3-alcohol is on the left, while the ^{1}H-NMR for the final porphyrin is on the right. Notice how the peaks are added together to make the final product.

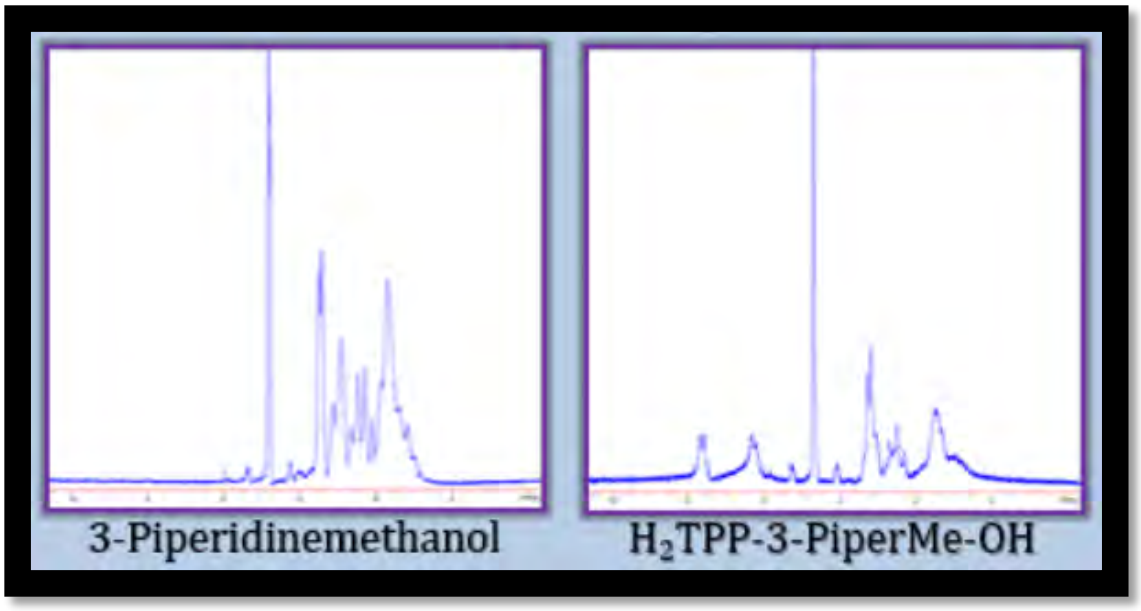


FIGURE 24:
The results from the ^{1}H-NMR show that that H_2TPPC combined with the anime to produce the final product, H_2TPP-3-PiperMe-OH. The ^{1}H-NMR for 3-piperidinemethanol is on the left, while the ^{1}H-NMR for the final porphyrin is on the right. Notice how the peaks are added together to make the final product.

MTT ASSAY OF TNBC CELLS

In many cancer diagnoses, PDT is given to the patient as a treatment option, not as a primary choice, but because it is typically the patient's only option. This is the case for individuals diagnosed with MDA-MD-231 triple-negative breast cancer (TNBC). In the United States, 1 in 8 women are diagnosed with breast cancer. Those diagnosed with TNBC account for nearly 10-15% of all breast cancer cases. Treatment options for TNBC are restricted due to the cancer cells not expressing genes for estrogen (ER), progesterone (PR), or human epidermal growth factor (HER2) receptors. Cancers with the receptors can be treated with standard cancer treatment options, such as chemotherapy. The chemotherapy drug sends a signal to the cancer cell to "stop growing". However, due to the lack of receptors on TNBC cells, a message to "stop growing" cannot be transmitted. Individuals diagnosed with TNBC have a high likelihood of reoccurrence and an increased risk of dying within five years of diagnosis. PDT is highly recommended in cases of TNBC, due to standard cancer treatments having limited effectiveness.

After characterization, the porphyrins were tested against TNBC cells to determine how effective they would be as a treatment option for PDT. To begin, MDA-MD-231 TNBC cells were cultured and then plated in 2, 96 well-plates. Approximately 72-hours after plating, H_2TPP-3-PEG-OH and H_2TPP-3-PiperMe-OH were tested in multiple concentrations against the TNBC cells. Each treatment was added to 8 replicate wells within the 96 well-plate, with one plate designated "dark" and the other "light". Each plate was wrapped in aluminum foil to protect against any light.

After 18-24 hours of incubation, the cells in the "light" plate were exposed to white light (0.5 J/cm^2) for approximately 16 minutes. Then, the plate was wrapped in aluminum foil and returned to the incubator. The parallel "dark" plate remained in the incubator. 72 hours post light

treatment, cytotoxicity of the porphyrin was measured using an MTT assay and was quantitated spectrophotometrically.

MTT Assay Results

MTT assays are widely used to assess cell viability. [33] The drug MTT-formazan is catalyzed by mitochondrial succinate dehydrogenase, meaning the assay is dependent upon mitochondrial respiration. This also suggests that it indirectly assesses the cellular energy capacity of a cell. The MTT assay indicated that cell viability is greater in cells on the "dark" plate compared to those in the "light" plate, which were exposed to light. The cytotoxicity for each porphyrin is concentration dependent for both cell environments. The purple formazan color indicates living cells.

One is able to discern from the 96 well-plates in FIGURE 25 and FIGURE 26 below, there are two controls and two sections of porphyrin testing. The first row is a positive control, containing only TNBC cells and media. The next row is also a positive control, containing TNBC cells plus DMSO and media. Then, there are five rows, each with a different concentration of the porphyrin, tested in 8 replicate wells. The more viable the cells, the more purple the well is. If the well is clear, that indicates no mitochondrial activity, meaning cell death. The "dark" plate itself is also a control, as you need to confirm the porphyrin is only causing cell cytotoxicity when exposed light and the cells are not dying while in the dark.

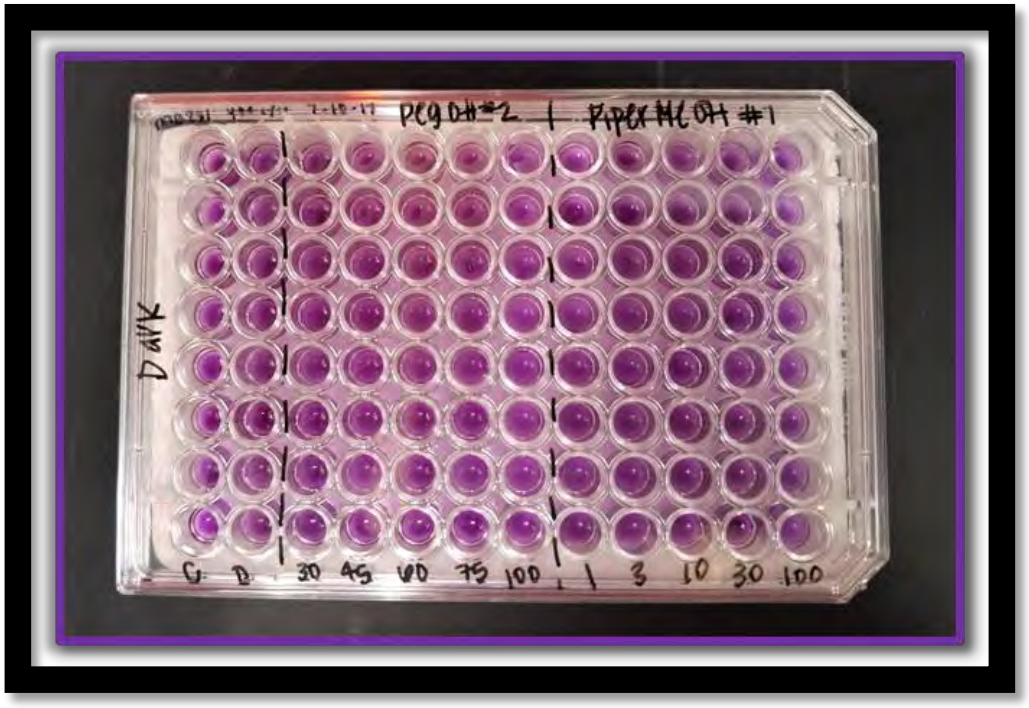


FIGURE 25:

Above is a photo the "dark" plate in the MTT assay of TNBC cells for the first porphyrin, H_2TPP-3-PEG-OH. Notice there are two controls and 5 rows of various treatment concentrations, with each concentration in 8 replicate wells.

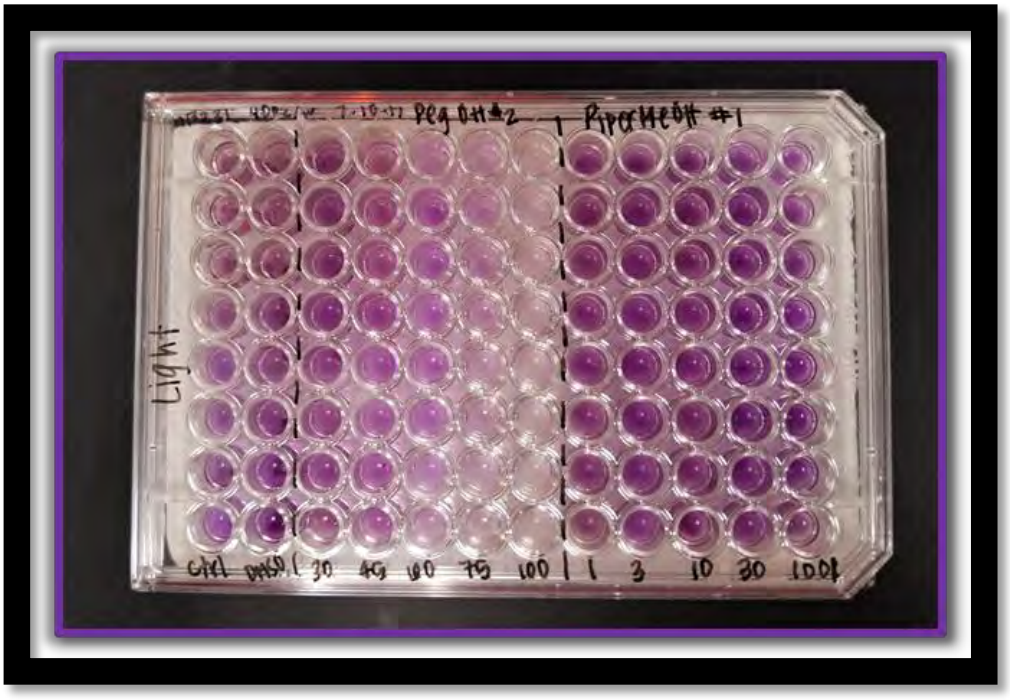


FIGURE 26:

Above is a photo the "light" plate in the MTT assay of TNBC cells for the first porphyrin, H_2TPP-3-PEG-OH. Notice there are two controls and 5 rows of various treatment concentrations, with each concentration in 8 replicate wells. At concentrations higher than 45 µMol, there is significant cell death.

This experiment revealed that at lower concentrations the cells exposed to light have less cell viability than those kept in the dark. Shown above, in FIGURE 25 and FIGURE 26, is the second MTT Assay trial results for the porphyrin product, H_2TPP-3-PEG-OH. It is easy to notice that in concentrations higher than 45 µmol, the light treated places show significant cell death. Treatment concentrations for H_2TPP-3-PEG-OH in the first MTT assay were 1, 3, 10, 30, 100 µmol and the results are graphed in FIGURE 27. In the second MTT assay, the concentrations were changed to 30, 45, 60, 75, 100 µmol to better determine the LD-50 for the porphyrin against the TNBC cells. The results are graphed in FIGURE 28.

The second amine, H_2TPP-3-PiperMe-OH is still in testing stages and its results will be reported in future work by Dr. Joseph E. Bradshaw.

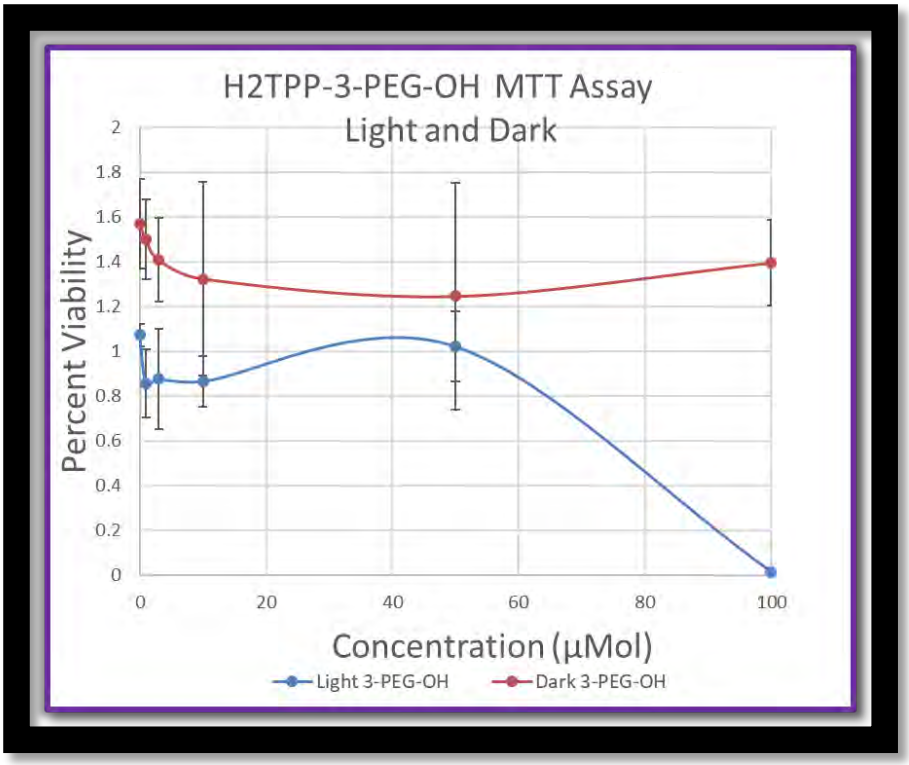


FIGURE 27:
This graph displays the first spectrophotometric MTT assay results for the "light" and "dark" plate of porphyrin H_2TPP-3-PEG-OH. The concentrations of porphyrin tested were 1, 3, 10, 30, and 100 µMol.

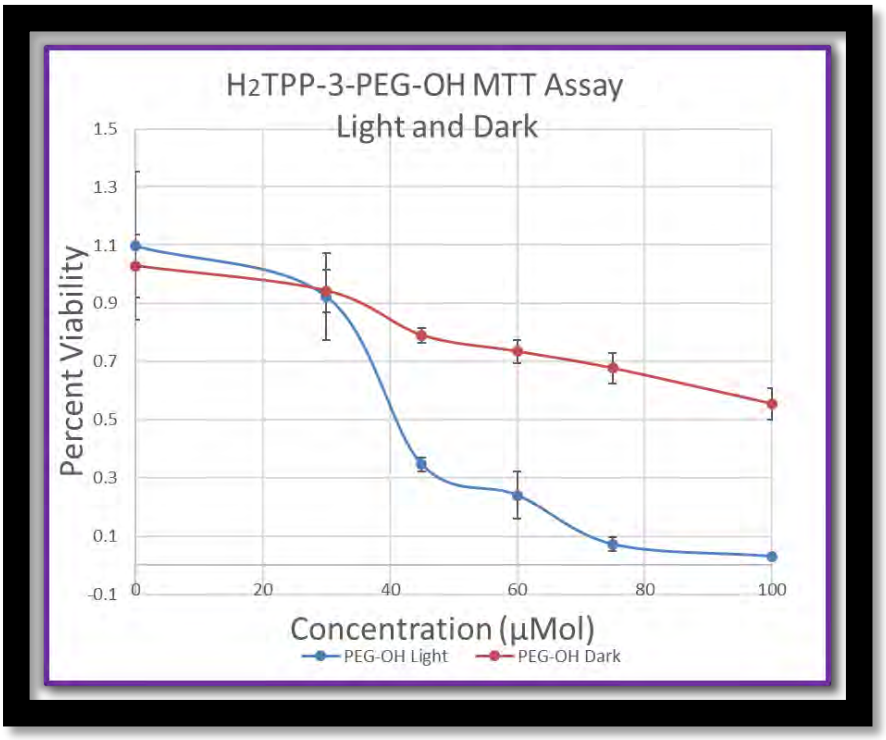


FIGURE 27:
This graph displays the second spectrophotometric MTT assay results for the "light" and "dark" plate of porphyrin H_2TPP-3-PEG-OH. The concentrations of porphyrin tested. The concentrations of porphyrin tested were 30, 45, 60, 75, 100 µMol.

With every MTT assay, the goal is to narrow down the LD-50, or the amount of a toxic agent (the porphyrin) that is sufficient to kill 50% of a population of animals (TNBC cells) usually within a certain time (3 days). The results shown in FIGURE 27 and FIGURE 28 were provided by a spectrophotometer, or an instrument that provides a measure of the amount of material in the solution absorbing the light. The LD-50 of porphyrin H_2TPP-3-PEG-OH was slowly being narrowed down, ranging from 25 µmol to 60 µmol. The lower the LD-50 is the more toxic the material. This means a lower dosage can be used to treat the patient and therefore, decreases the chances of having toxic side-effects.

CONCLUSION

In conclusion, this research was successful in the creation of two novel, water-soluble porphyrins. Each structure, H_2TPP-3-PEG-OH and H_2TPP-3-PiperMe-OH, was confirmed by ^{1}H-NMR and HPLC indicated each with high purities of 98.8% and 99.3%, respectively. Both also showed characteristics of porphyrins by having the pink or purple coloring in the final product and in UV-vis spectroscopy by having a strong Söret band around 414 nm and Q-bands from 600 to 800 nm.

Although results are still pending on H_2TPP-3-PiperMe-OH, the H_2TPP-3-PEG-OH porphyrin appears to kill the TNBC cells when exposed to light in the concentration range between 25 µmol to 60 µmol. Under dark conditions, normal growth patterns were seen until the porphyrins treatments grew higher in concentration (about 35 µmol). This data indicates that the novel porphyrin, H_2TPP-3-PEG-OH, may be a viable PDT agent and a possible candidate for the treatment against the TNBC cells in mid-to-high concentration ranges. More experimentation and trials, however, must be completed to receive more conclusive data.

FUTURE WORK

The synthesized novel porphyrins appear to have potential to become PDT agents. In the future, further testing will be conducted in an attempt to find each porphyrin's LD-50, or the amount of a toxic agent that is sufficient to kill 50% of a population of animals usually within a certain time (3 days), and to establish a trendline for the H_2TPP-3-PEG-OH and H_2TPP-3-PiperMe-OH treated cells.

Additionally, the porphyrins will be tested to determine if they are viable PDT candidates and if the porphyrins have more desirable properties in regard to killing MDA-MD-231 TNBC cells.

WHERE IS PDT HEADING?

Despite the good the therapy is doing, many areas in PDT pose problems. To realize the full potential of PDT, basic physics and engineering issues must be solved. Clinical results could be improved remarkably if a direct measurement of light fluorescence distribution during PDT was developed and if penetration depth was expanded. Additionally, there could also be advancement in light dosimetry, scientists would then have an enhanced understanding of tissues' optical properties, and one could possibly develop a more effective delivery mechanism to the treatment site. There are countless opportunities for PDT improvement and many avenues in which it might take, including more clinical trials or developing a way to irradiate the entire body. [12]

ACKNOWLEDGEMENTS

I would like to take this opportunity to extend my deepest gratitude to Dr. Joseph E. Bradshaw for being the main instructor for this research. He walked me though each step of the project, while also giving me much needed instruction in the laboratory. Without his support, this research would not have been as successful or as fun. I would also like to thank Dr. Timothy E. Hayes for the use of his laboratory and for giving each of us an insurmountable amount of information. He changed my outlook on the field of scientific research and for that I will be forever grateful. I am also appreciative of Alex Abbott, Hannah Brandon, Callie Clement, Savanna Harris, and Sally Owens. While we each had individual projects, we aided one another in our day-to-day tasks. It was a joy to work alongside them. They are an inspiration for the future scientific fields they will one day choose. I would also love to give my thanks to Ouachita Baptist University, and more specifically the Patterson School of Natural Sciences, for providing the resources, equipment, and laboratories necessary to complete this research. Finally, I would like to extend my greatest appreciation to the J.D. Patterson Summer Research Program. It gave me the chance to take part in such an incredibly inspiring project. It granted me the opportunity to work side-by-side with Dr. Bradshaw in a lab for an extensive period and enabled me to complete my own project. This research was a small step in time, but a giant step in my growth as both a student and an individual. I am eternally in debt to the J.D. Patterson Summer Research Program for funding the amazing opportunity I was able to partake in. Without the program's contributions, I would not have been given the knowledge and experience that will continue to aid me throughout the rest of my career.

KEY WORDS

Anaplastic Ependymoma – an aggressive form of brain cancer

Cancer – refers to any one of a large number of diseases characterized by the development of abnormal cells that divide uncontrollably and have the ability to infiltrate and destroy normal body tissue. Cancer often has the ability to spread throughout your body.

Condyloma – (Pl. condylomata) a raised growth on the skin resembling a wart, typically in the genital region, caused by viral infection or syphilis and transmissible by contact.

Ischemia – A clogging of the arteries that can lead to pain, inability to walk, and, in some cases, amputation of the leg; an inadequate blood supply to an organ or part of the body, especially the heart muscles.

Leukemia – a malignant progressive disease in which the bone marrow and other blood-forming organs produce increased numbers of immature or abnormal leukocytes. These suppress the production of normal blood cells, leading to anemia and other symptoms.

Metastases – the development of secondary malignant growths at a distance from a primary site of cancer.

Onycholysis – a common medical condition characterized by the painless detachment of the nail from the nail bed, usually starting at the tip and/or sides.

Porphyria – a family of metabolic diseases that produce chemicals, called porphyrins, which absorb sunlight in the skin and thereby cause damage

Psoriasis – a common skin condition that speeds up the life cycle of skin cells. It causes cells to build up rapidly on the surface of the skin. The extra skin cells form scales and red patches that are itchy and sometimes painful.

Psychosis – a severe mental disorder in which thought, and emotions are so impaired that contact is lost with external reality.

Rickets - is the softening and weakening of bones in children, usually because of an extreme and prolonged vitamin D deficiency.

Slurry – a thin sloppy mud or cement or, in extended use, any fluid mixture of a pulverized solid with a liquid (usually water), often used as a convenient way of handling solids in bulk. Slurries behave in some ways like thick fluids, flowing under gravity and are also capable of being pumped if not too thick.

Vitiligo – is a disease that causes the loss of skin color in blotches. It occurs when the cells that produce melanin die or stop functioning.

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