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The Development of a New Water-Soluble Zinc Porphyrin, ZnTPP--5AP, for Photodynamic Therapy

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The Development of a New Water-Soluble Zinc Porphyrin, ZnTPP-5AP, as a Photodynamic Therapy Agent

Senior Thesis by Kaylee Johnson

Ouachita Baptist University

Dedicated to my grandparents Rick and Debbie Johnson. My grandpa, Rick, died due to non-small lung cancer and my Mamaw, Debbie, died due to breast cancer. You both were taken from this Earth too soon. I am inspired daily by you both to work on this research project in your honor.

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ABSTRACT

This research focuses on synthesizing a new water-soluble porphyrin with a zinc core that can be used as a photosensitizing agent in photodynamic therapy. Photodynamic therapy is a process in which light is used to activate a photosensitizer to cause cell death. For this study, the porphyrin was synthesized by adding 5-amino-1-pentanol to the ZnTPPC. Once the porphyrin was synthesized it was purified using column chromatography through Sephadex LH-20 and G-50. The structure of the purified ZnTPP-5AP was then analyzed using nuclear magnetic resonance spectroscopy (NMR), infrared spectroscopy (IR), and ultraviolet-visible spectroscopy (UV-vis). Purity of the ZnTPP-5AP was tested using high performance liquid chromatography (HPLC). The ZnTPP-5AP was then tested as a photosensitizing agent on the cancer cell line A549 using a MTT assay in the presence of red and white light. Red light was used with the zinc porphyrin to test whether its cytotoxicity was similar to white light and whether it provided deeper penetration for tumors than previous methods. For further testing of the porphyrin's cytotoxicity under light, an MTT assay was run on the A549 cell line using a smaller range of porphyrin concentrations. Additionally, since a tumor in the body has low oxygen levels, the A549 cells were exposed to hypoxic conditions to mimic these *in vivo* conditions. To model this hypoxic environment, the A549 cells were kept in a hypoxic chamber prior to exposing the A549 cells to the porphyrin.

BACKGROUND

Lung Cancer Disparities

The leading cause of cancer-related deaths in the world is lung cancer. Around 85% of lung cancer cases in the United States are specifically non-small lung cancer, NSLC. The prevalence of lung cancer is highly linked to the elevated rates of cigarette smokers. Unless significant changes are made to decrease smoking globally, lung cancer is likely to persist as a leading cause of death.[1] The global epidemic of lung cancer is why the cell line A549 NSLC was chosen for this specific project. Once diagnosed with lung cancer, the five-year survival rate is between 10-20%. Typically, the treatment for local lung cancer tumors is surgery followed by chemotherapy.[2] However, looking at statistics, the outcome after diagnosis and treatment is not positive. Therefore, for this research project, NSLC was analyzed using a new method of treatment that could potentially provide a less invasive way to battle lung cancer.

Photodynamic Therapy Background

Cancer is defined as the "uncontrolled growth and spread of abnormal cells." When these abnormal cells begin to spread, the effects can be detrimental, with death as a likely outcome. The diagnosis of cancer often involves finding a malignant tumor through imaging techniques, biopsies, etc.When this malignant tumor expands and affects more regions of the body, it has metastasized. The goal in treating cancer is to kill these malignant cells before they can spread to other regions of the body. Photodynamic therapy (PDT) is a new approach that uses light, oxygen, and a photosensitizer to kill these rapidly growing cells.[3]

The process of photodynamic therapy involves a photosensitizer that is activated by a light source to cause a specific response. In photodynamic therapy, the photosensitizing agent, the porphyrin, is administered first to the patient. The porphyrin will then selectively concentrate at the site of the tumor. The photosensitizing agent is then exposed to light and absorbs a photon of energy at a specific wavelength. The energy absorbed is then transferred to oxygen where an excited singlet oxygen is produced. This singlet oxygen is unstable due to the oxygen containing two electrons of opposing spin that occupy a higher energy orbital than its ground state. This singlet oxygen is what is toxic to the cancerous cell, therefore, leading to cell death (**Figure 1**). This process is short lived and cell death during photodynamic therapy can only occur in the presence of oxygen.[3] Therefore, photodynamic therapy provides a way to selectively target specific cancerous tissues within the body without harming surrounding areas.

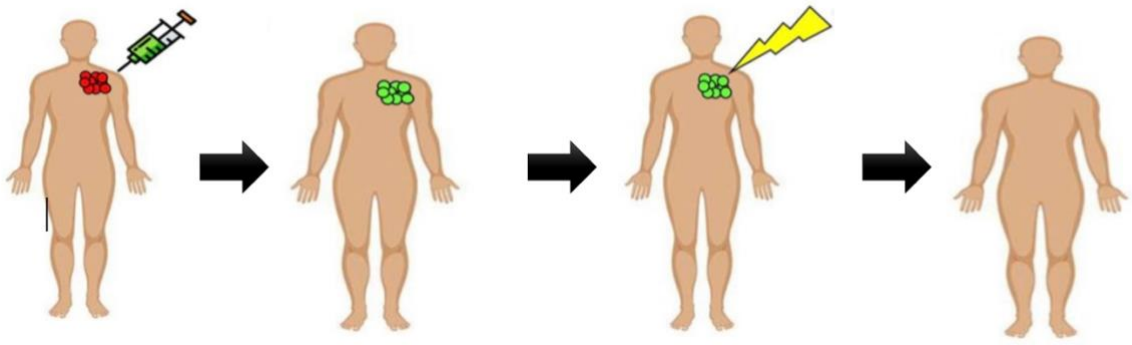


Figure 1: Photodynamic Therapy

Porphyrin Background

This research specifically utilizes and analyzes the light sensitivity of porphyrins, a photosensitizer, and how they respond by resulting in cell death of lung cancer cells.

Porphyrins are tetrapyrrolic molecules that contain four pyrrolic subunits linked through methine (CH) bridges. The resulting structure then can be substituted at the *meso*-position with a non-hydrogen atom, creating a porphyrin (**Figure 2**). The Greek word *porphura* (purple) is the origin of the word "porphyrin." These porphyrins are a group of intensely colored compounds that naturally occur.[4]

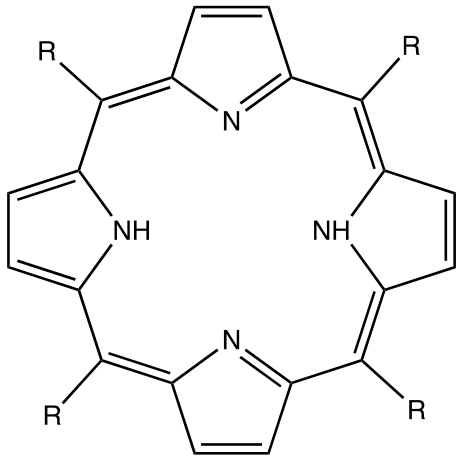


Figure 2: Standard Porphyrin Core Structure (unsubstitued)

The porphyrin's conjugated structure (**Figure 2**) allows it to absorb specific wavelengths which allows it to be an ideal photosensitive agent. The ideal photosensitizer for cancer treatment should be pure, have a known composition, have minimal dark toxicity and only be cytotoxic in the presence of light, be specific to the target tissue, be rapidly excreted from the body, have a high singlet oxygen yield, and have an absorption of around 600-800 nm.[5] Beyond PDT, porphyrins are found throughout nature and within the human body. Some examples of porphyrins are heme in the blood, pigments, chlorophyll, and bacteriochlorophylls.[3] Porphyrins are used for several processes within the body including gene regulation, drug and iron metabolism, oxygen transport, and as an electron transport medium.[4]

INTRODUCTION TO RESEARCH PROJECT

The purpose of this research project was to synthesize a new water-soluble porphyrin that could be used for photodynamic therapy. The goal for this project was to synthesize a porphyrin that is water soluble, has a metalated core, is pure, and of a low lethal dosage (LD_{50}). This project was accomplished by first synthesizing the new water-soluble porphyrin. Afterward, the porphyrin was purified; it was then characterized to ensure the porphyrin had the correct chemical structure. Finally, the new water-soluble porphyrin was applied to A549 NSLC cells in hypoxic and nonhypoxic conditions to test the drug's efficacy. After drug treatment, the cells were exposed to red light and white light. The new porphyrin was tested using a MTT assay on 1549 cells. The MTT assay measures cellular metabolic activity to test for cell viability. The intended outcome of the project was to achieve a low LD_{50} for potential photodynamic therapy.

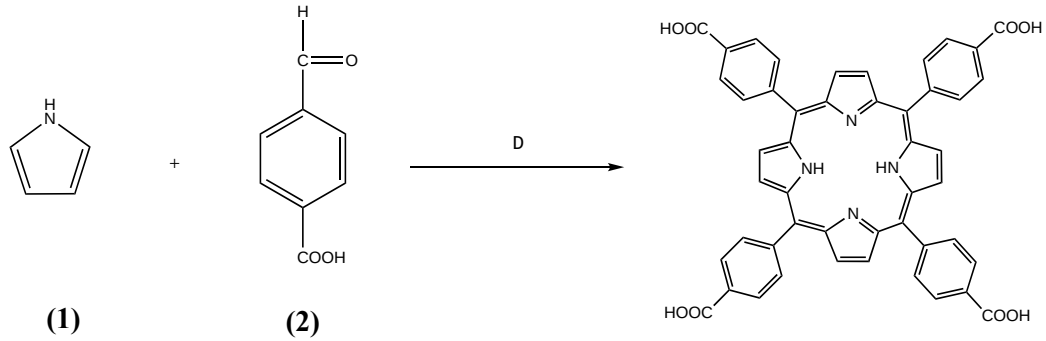
METHODS

Synthesis of the Porphyrin Compound, ZnTPP-5AP

Formation of H_2TPPC

To begin the synthesis of ZnTPP-5AP, the first goal was to synthesize H_2TPPC **(3)** as the starting material. The porphyrin was synthesized by adding 3.0 g of 4-carboxybenzaldehyde **(2)** and a stir bar to a 500-mL round bottom flask in a heating mantle. The round bottom flask was filled halfway with propionic acid to serve as the solvent. Pyrrole, 1.5-mL, **(1)** was added into the round bottom flask using a syringe. The reaction was then covered with aluminum foil to minimize light and refluxed for 1 hr. Afterward, the reaction was allowed to cool to room temperature, the round bottom flask was covered with parafilm and stored in the freezer and put in the freezer at -20°C overnight.

The H_2TPPC **(3)** was then vacuum filtered using a medium fritted filter to collect the desired product. The round bottom flask was washed with deionized water, 10-mL, twice and filtered. Dichloromethane, 10-mL, was then used to wash the product. The H_2TPPC was then air-dried and collected.



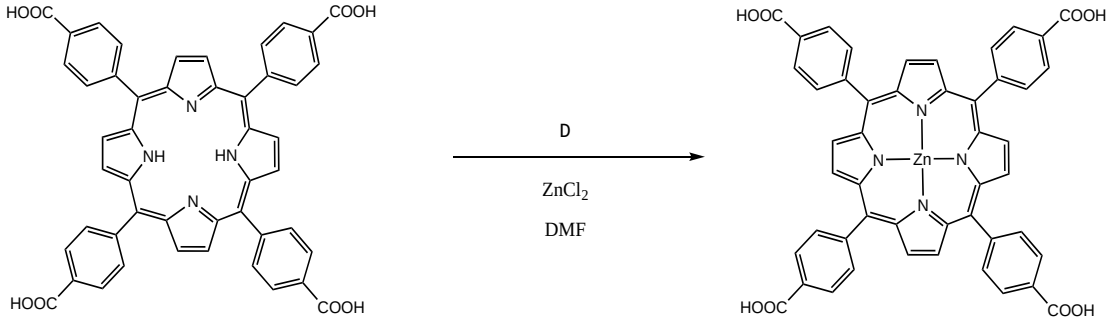
(1) **(2)**

Figure 3: Reaction 1. 4-carboxybenzaldehyde **(2)** reacted with pyrrole **(1)** in propionic acid as a solvent to form H_2TPPC **(3).**

Formation of ZnTPPC

The next step in the synthesis of the final product was to add zinc to the porphyrin core of the H_2TPPC **(3**). In order to accomplish this goal, 1.0 gram of H_2TPPC and zinc chloride ($ZnCl_2$), 0.365 grams, were added to a 100-mL round bottom flask with a stir bar. The round bottom flask was then filled halfway with dried dimethylformamide (DMF), a condensing column connected to water was added to the round bottom flask, the round bottom was covered in aluminum, and the reaction was then heated to reflux. This reaction was then refluxed for three hours. Afterward, the round bottom flask was connected to the rotovap to evaporate the DMF.

Next, the crude ZnTPPC was dissolved in methanol and filtered using vacuum filtration. The product was filtered for purification by removing any excess Zn from the desired product. The filtrate collected was evaporated to dryness by rotary evaporation. The product, ZnTPPC, was a purple residue in the round bottom flask. The ZnTPPC was scraped out of the round bottom flask into a vial and dried overnight. The following day, the ZnTPPC product **(4)** (**Figure 4**) was put in an oven at 110°C and heated for thirty minutes, and then cooled in a desiccator to room temperature.



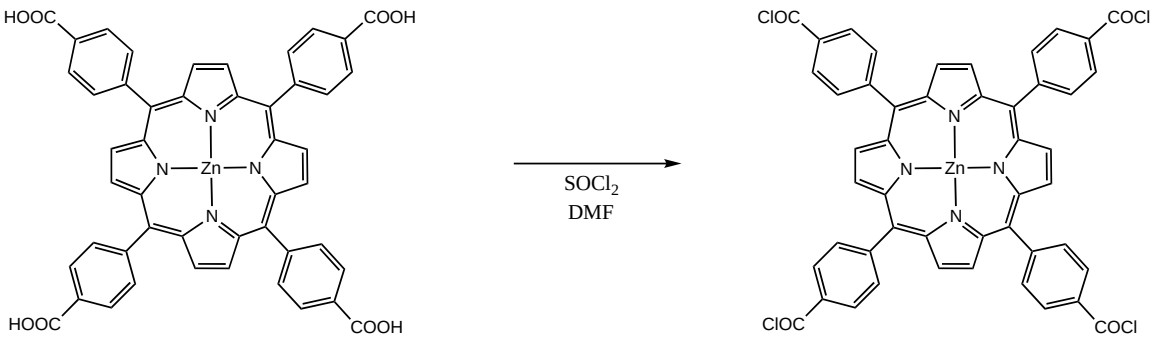
(3) **(4)**

Figure 4: Reaction 2. H_2TPPC (**3**) reacted with $ZnCl_2$ in DMF to form ZnTPPC (**4**).

Formation of the Acid Chloride Porphyrin Intermediate

The next step in the synthesis of the desired product was to form an acid chloride intermediate. This was done by replacing the carboxylic acid side chains -OH group with a chloride. The acid chloride was used because chlorine is a better leaving group than hydroxide; therefore, for reaction 4 (**Figure 6**) the 5-aminopentanol can easily undergo a substitution reaction and replace the chlorine leaving group. For reaction 3 (**Figure 5**) to be successful, the environment must be dry to avoid water interacting and forming the carboxylic acid group again.

To begin reaction 3 the round bottom flask, with a stir bar, was dried in an oven at 110°C to remove any moisture and then placed in a desiccator to cool. Once the round bottom flask was cooled, the flask was stoppered immediately to avoid exposure to water. ZnTPPC **(4)**, 0.152 grams, was then added to the dried round bottom flask. Dried DMF, 25-mL, was then added and then capped. The flask was kept under a nitrogen atmosphere while stirring. Using a syringe 0.15-mL of thionyl chloride, $SOCl_2$, was then added to the round bottom flask. The reaction stirred for one hour, and the reaction resulted in a green color. The DMF was then evaporated off using the rotovap, and the dried product appeared blue. The round bottom flask was immediately placed under vacuum overnight to remove any excess $SOCl_2$. The blue product was the acid chloride intermediate **(5)** seen in **Figure 5**.

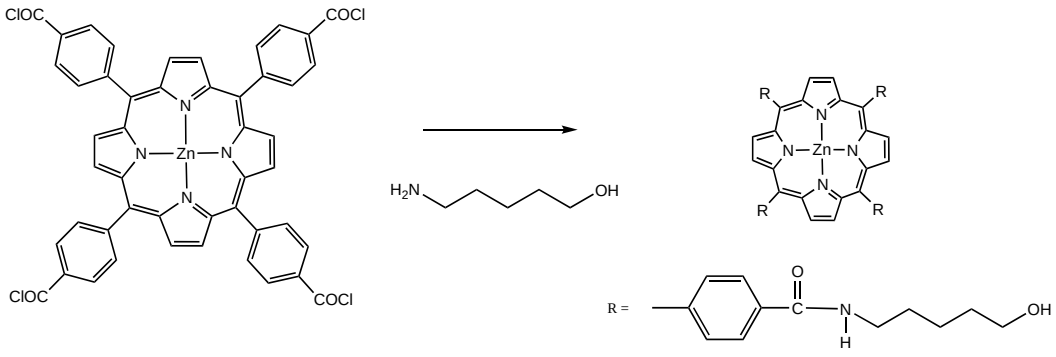


(4) **(5)**

Figure 5: Reaction 3. Formation of the Acid Chloride Intermediate by reacting ZnTPPC with $SOCl_2$ in dried DMF.

Formation of ZnTPP-5AP

For the final step in the synthesis of ZnTPP-5AP, the chloride group in the acid chloride porphyrin intermediate **(5)** was substituted using 5-aminopentanol to form the final porphyrin product, ZnTPP-5AP **(6)**. In a vial that had been dried in the oven, 0.363 grams of 5-aminopentanol was added. Freshly distilled methanol was added to the vial, and this solution was added to the round bottom flask containing the acid chloride porphyrin intermediate **(5)** while under nitrogen flow. The reaction was then allowed to stir for one hour. After the stirring was complete, the methanol was rotovaped off leaving behind a dark purple oil, which was the final product, ZnTPP-5AP **(6) (Figure 6).** This synthetic scheme was repeated four times.



(5) ZnTPP-5A **(6)**

Figure 6: Reaction 4. Acid chloride porphyrin intermediate (5) reacted with 5-aminopentanol to form ZnTPP-5AP **(6)**.

Purification of ZnTPP-5AP

Once the desired porphyrin, ZnTPP-5AP, had been synthesized, the porphyrin needed to be purified using chromatography to remove any byproducts or impurities from the synthesis. A Sephadex LH-20 column and a Sephadex GH-50 column were both run to separate impurities from the desired ZnTPP-5AP. The Sephadex LH-20 is lipophilic, fat-loving, and it separated the porphyrin and any impurities based off lipophilicity. The Sephadex GH-50 column was used after the LH-20 to then separate the porphyrin's impurities based on molecular size. The setup for the columns is shown in **Figure 7**, and in **Figure 8** the range of separation by color can be seen in the Erlenmeyer flasks. In **Figure 8** the purple product is the final purified product that was isolated.

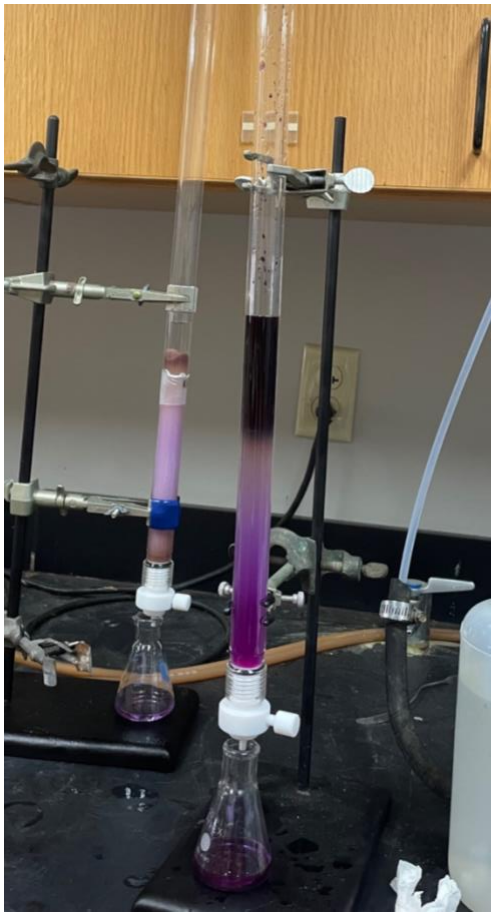


Figure 7: Sephadex LH-20 and G-50 Column Chromatography set up

Figure 8: The product collected through the column. The Erlenmeyer flask containing a purple product was the final product that was kept, and the rest was discarded because it was the unwanted impurities.

To begin the purification, the Sephadex LH-20 column was first set up by putting six scoops of Sephadex LH-20 in a beaker, pouring 50/50 MeOH/H_2O in the beaker, parafilming the beaker, and letting the LH-20 hydrate overnight. The parts used to build the column were soaked in 50/50 MeOH/H_2O the night before, as well, to avoid any change in condition. The following day, the column was set up as seen in **Figure 7** by pouring the hydrated Sephadex LH-20 down the column to pack it. Enough 50/50 MeOH/H_2O was then added to the ZnTPP-5AP to dissolve the product. Before the porphyrin was run through the column, syringe filtration using a 0.45-micron nylon syringe filter was used initially to filter the product. Once filtered, the ZnTPP-5AP was run through the LH-20 column and the purple product was collected (**Figure 8**). Once all of the product had been collected, the solvent was rotovaped off leaving behind the purified ZnTPP-5AP.

The purified ZnTPP-5AP from the Sephadex LH-20 column was then run through the Sephadex G-50 column. The Sephadex G-50 was prepared and set up as the LH-20

column, except the solvent used was Milli-Q H_2O, instead of the 50/50 MeOH/H_2O. The dissolved ZnTPP-5AP was then run through the Sephadex G-50 column, and the purple product was collected. The water was then rotovaped off leaving behind the final purified ZnTPP-5AP product.

Characterization of ZnTPP-5AP

High-Performance Liquid Chromatography, HPLC

High-Performance Liquid Chromatography, HPLC, was run on the final product, ZnTPP-5AP, to test its purity. HPLC uses high pressure to push the liquid of interest, and a mobile phase, through a packed column, stationary phase, to separate a mixture.[6] The mixture separates into its individual components based on the component's interaction with the stationary phase that affects retention time.[6]

By performing HPLC the purity of the ZnTPP-5AP could be determined. The porphyrin, ZnTPP-5AP, was dissolved Milli-Q H_2O with 100% acetonitrile as the solvent for HPLC. The dissolved porphyrin was then run through a Hamilton PRP-1 5µm column 4 x 150 mm, with 100% acetonitrile as the mobile phase, at a flow rate of 1.00-mL per minute. The purity for ZnTPP-5AP was found to be greater than 99%. As seen in **Figure 9,** the HPLC resulted in a single large peak that indicated there were no other components in the final porphyrin.

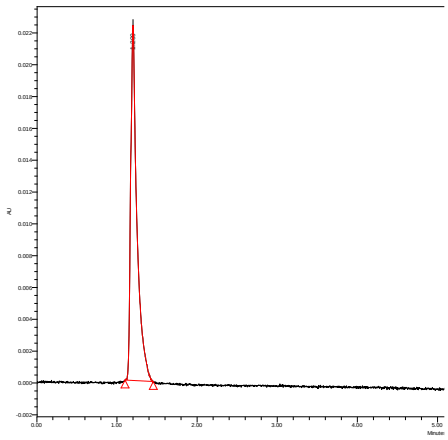


Figure 9: HPLC of ZnTPP-5AP

Infrared (IR) Spectroscopy

Infrared (IR) spectroscopy is a way to analyze the structure of unknown molecules by using infrared radiation. Infrared radiation is absorbed by chemical bonds in molecules, and IR spectroscopy measures this absorption. IR specifically measures each functional group in the same frequency range regardless of the overall structure. These peaks are measured using wavenumbers, cm^{-1}, and specific wavenumbers correlate with specific functional groups. This allows an unknown compound to structurally be analyzed using IR.[7]

Before the amine functional group was added to the porphyrin, IR was run on 5-amino-1-pentanol for comparative purposes. The IR spectrum of 5-amino-1-pentanol **(Figure 10)** showed a large peak around 3300 cm^{-1} that was the N-H stretch of the primary amine. The broad stretch around 3000 cm^{-1} seen in figure 10 was the -OH stretch from the alcohol group.

The final porphyrin product, ZnTPP-5AP, was also run on IR to ensure that the amine functional group was correctly attached to the porphyrin. The broad peak at ~3300 cm^{-1} was the -OH group from the attached 5-amino-1-pentanol. Three amide bands were observed. The amide peaks around 1400 cm^{-1}, 1550 cm^{-1}, and 1600 cm^{-1} **(Figure 11)** were the three amide bands from the stretching of C=O, N-H, and N-H deformation functional groups. This confirms that the amine functional group was added to the porphyrin core during synthesis to form ZnTPP-5AP.

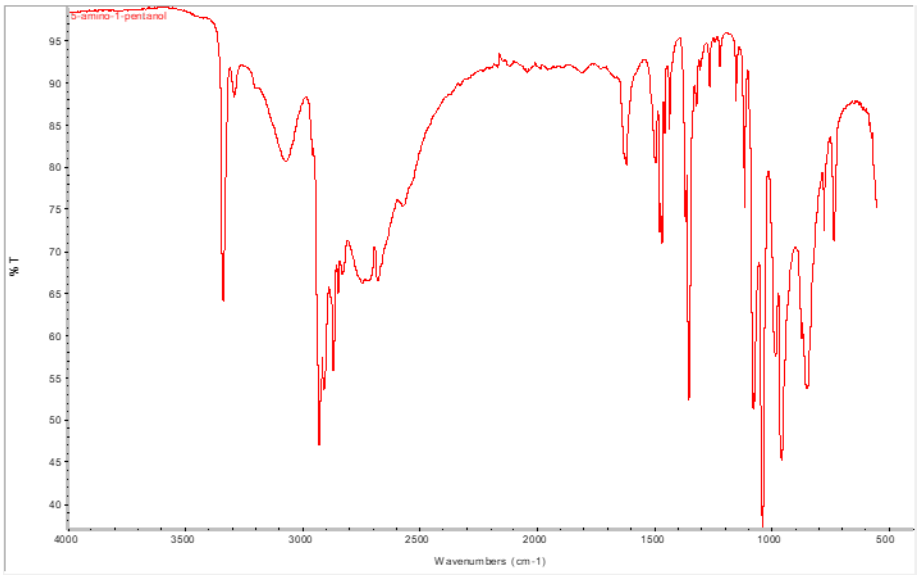


Figure 10: IR of 5-amino-1-pentanol

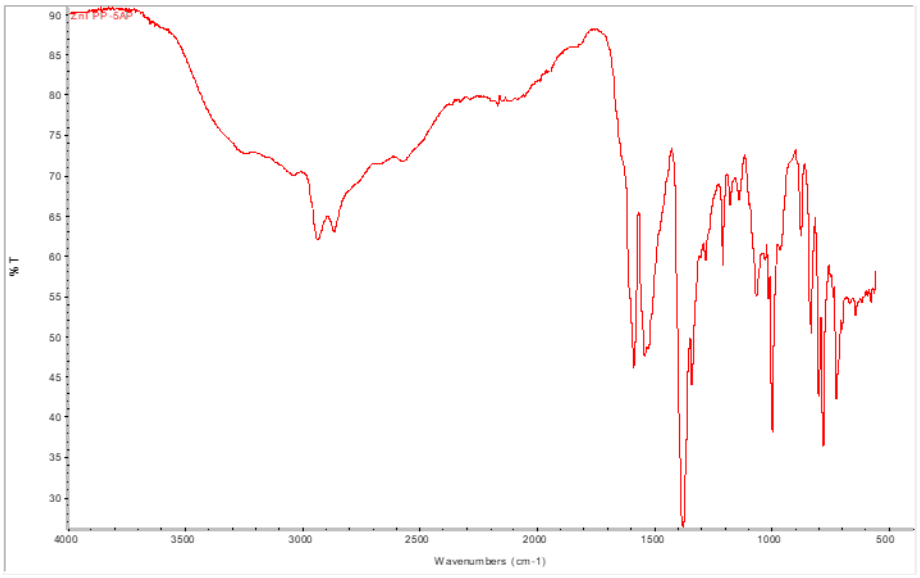


Figure 11: IR of ZnTPP-5AP

Nuclear Magnetic Resonance, NMR, Spectroscopy

Nuclear Magnetic Resonance, NMR, is a spectroscopic technique that characterizes molecules structures when nuclei have specific spin states and are exposed to an external magnetic field. For an atom to be observed using NMR, it must have non-zero nuclear spin to interact with the magnetic field to be detectable. NMR detects transitions between spin states of nuclei that are specific to the nucleus in question and its chemical environment. NMR has high specificity and can differentiate between isotopes because each specific nucleus absorbs at a highly specific frequency. Electrons surrounding a nucleus can result in nuclear shielding, which causes there to be chemical shifts in NMR. Therefore, due to the chemical environment surrounding a nucleus, the level of shielding around each nucleus differs, resulting in a peak for each individual environment. These properties of NMR allow it to be highly specific and accurate in determining a molecule's structure.[8]

In **Figure 12** ^{1}H, NMR was run on 5-amino-1-pentanol to analyze the structure of the starting material. The peak at 0 is TMS, which is an internal reference. There are three aliphatic signals at 1.5 ppm that are the CH_2 groups in the center of the amine structure. The peak at 7.24 ppm is due to $CHCl_3$ that was used as a solvent for NMR. The peak around 3.5 ppm is the -CH_2 group that is closest to the -OH group. The peak around 2.7 ppm is the -CH group beside the amine group.

^{1}H NMR of the final product ZnTPP-5AP is shown in **Figure 13**. The large peak at 4.2 ppm is the D_2O that was used as the solvent for NMR. A water suppression program called WEFT was used to suppress the large D_2O peak. The three peaks between 6.5 and 8 ppm indicate aromatic protons, H atoms, that are the pyrrole and benzene ring

protons. The three peaks that represent the aliphatic groups of the amine are seen in figure 13 between 1.4 and 3.0 ppm. This again helps to confirm that the amine group was successfully added to the porphyrin.

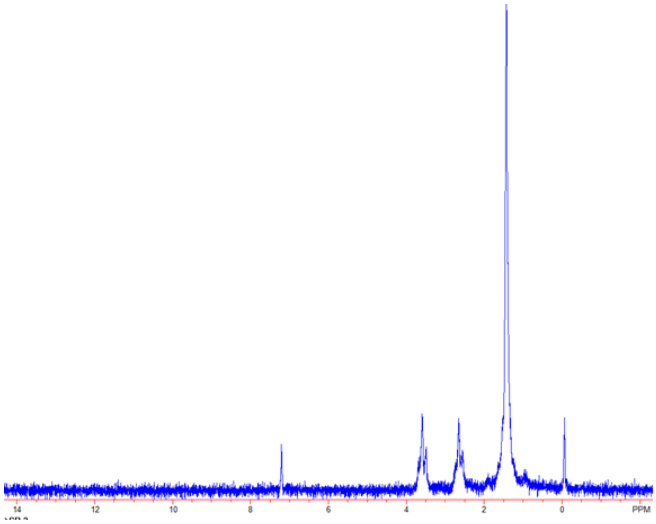


Figure 12: NMR of 5-amino-1-pentanol

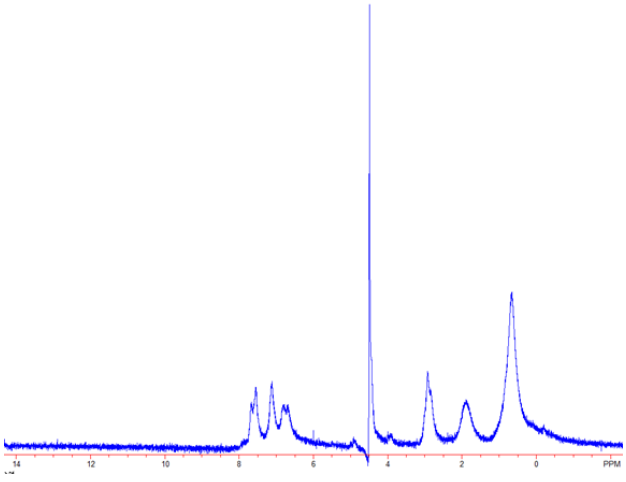


Figure 13: NMR of ZnTPP-5AP

UV-Vis Spectroscopy

UV-Vis spectroscopy was used to measure the amount of light absorbed across a range of wavelengths in ZnTPP-5AP. One purpose of doing UV-Vis on the porphyrin was to determine the wavelength at which the porphyrin absorbed light for photodynamic therapy. In **Figure 14** UV-vis was run, and the Soret band was found at a wavelength of 423 nm. Two more peaks were found at 557 and 597 nm (fingerprint region). The results were used to calculate the molar absorption coefficients, the epsilon value, using Beer's law. The product from reaction 1 H_2TPPC would have its Soret band at 415 nm; however, when the porphyrin core is metallated with Zn the Soret band shifts to 423 nm. The peaks in the fingerprint region are the outside structures of the conjugated core. To determine the epsilon values for the Soret band, a diluted solution of the porphyrin was used, and to determine the epsilon values in the fingerprint region, a more concentrated solution was used. These solutions were prepared using DI water. The UV-Vis results in **Figure 14** confirm that the porphyrin was successfully metalated. The molar absorptivity values for ZnTPP-5AP are shown in **Table 1**.

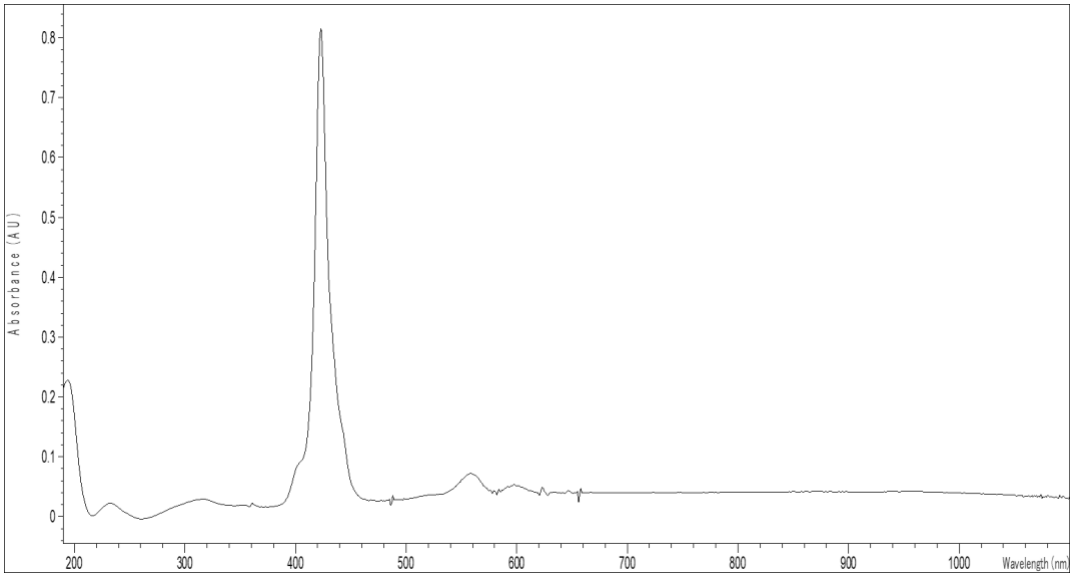


Figure 14: UV-Vis of ZnTPP-5AP.

Peaks (nm)	Molar Absorptivity Coefficient, e ($mM^{-1}cm^{-1}$)
423	**497**
557	**15.5**
597	**8.67**

Table 1: Table of the Molar Absorptivity Coefficient of ZnTPP-5AP.

Cytotoxicity Testing

Background

An MTT assay was chosen to test if the new water-soluble porphyrin, ZnTPP-5AP, is toxic to cancer cells when exposed to light. The MTT reagent that was used was a monotetrazolium salt, and when reduced it results in a violet-blue molecule called formazan. The MTT reagent is positively charged, so it can pass through the cell membrane and mitochondrial membrane of living cells. The MTT reagent gets reduced to formazan by metabolically active cells. Therefore, MTT assays are a colorimetric-based measurement of cell viability. The more viable cells, the stronger the violet-blue color, whereas less color present means more cell death.[9] An MTT assay was chosen for this experiment because it was able to provide data on the survival of the A549 NSLC cells undergoing photodynamic therapy. The MTT assay allowed analysis of the porphyrins efficacy of disrupting metabolism and resulting cell death to be measured. Visually, the difference in cell death among treatment groups could be seen, **figure 15**, but doing the MTT assay allowed the results to be evaluated visually and numerically. Porphyrins with greater efficacy when exposed to light should have decreased the metabolic activity and resulted in less formazan being generated; Whereas, the cells containing the porphyrin kept in the dark should have had no change in metabolic activity because the porphyrin should be non-toxic in the dark. Therefore, to test the porphyrin's cytotoxicity, an MTT assay was chosen.

MTT Assay Methodology

Cell culture growth media and trypsin were prepared as follows. In an empty vial 150-mL of base medium, 2-mL of 200mM L-glutamine, 2-mL 200mM penicillin/streptomycin, and 20-mL of fetal bovine serum (FBS) were added. The base medium used was that of F12-K, which contains glucose, amino acids, and other nutrients the cells need for survival. The FBS was added to help the cells proliferate. The penicillin/streptomycin was added to minimize bacteria infecting the cell cultures. Trypsin was used to detach the cells from the flask so that the cells could be split or plated. The trypsin was tenfold concentrated, so the trypsin was diluted in 9-mL of PBS with 1-mL of trypsin. This gave a 1x solution of trypsin that could be used on the cells. The growth media and diluted trypsin were then stored in the refrigerator for future use.

A549 non-small lung cancer cells were used. When cells had proliferated, the cells were split into three vials. To split the cells, the old media was removed from the flask. The cells were then washed with 10-mL of PBS to clean any dead cells remaining. Once the PBS was removed, 1.5-mL of trypsin was gently added to the wall of the flask containing the cells. The trypsin was gently swirled to help release any cells sticking and then placed in the incubator for 3-4 minutes. The flask of cells was then placed under a microscope to ensure that the cells were moving and no longer adhered to the flask. To prevent the trypsin from damaging the cells, 2-mL of growth media was then added to the flask. Depending on the concentration of cells compared to the area of the flask, calculations were done each time cells were split to adjust the volume of the cells suspended in trypsin that were added to a new flask. Once cells were added to a new flask, enough growth media was added to have a total volume of *ca.* 20-mL covering the

bottom of the flask. This same procedure was followed each time the cells were to be split or prepared for plating for an MTT assay.

For the experiments run using the A549 non-small lung cancer cells with ZnTPP-5AP, 96-well plates were used. The cells were added to the well plates using the same procedure that was followed for splitting the cells. When the cells were suspended in trypsin instead of adding them to a new flask, 10 microliters of cell suspension and 5 microliters of trypan blue were added to a centrifuge tube. The cell suspension with trypan blue was then added to a microscope slide, and using a grid, the cells were counted. Counting the cells allowed the total cell count in the flask to be estimated so that the cells could be plated in the 96 well plates evenly and at the correct concentration. To begin seeding the cells in the 96 well plates, the A549 non-small lung cancer cells were used and 1,000 cells per well were calculated. In a sterile tube, the calculated volume of cell suspension and growth media were added together and mixed. Using a sterile tray and an 8 well pipette, all three 96-well plates used for the experiment were plates with 100 microliters. The 96-well plates were then placed in the incubator for three days to let the cells adhere to the wells.

While the well plates with the A549 non-small lung cancer cells were under incubation, calculations were made to determine the correct concentration of porphyrin, ZnTPP-5AP, to add to the cells. A 50 mM DMSO stock solution of the porphyrin was made. After it had been made, UV-Vis was done on the stock solution to determine the concentration. The absorption in theory should have been 1, but the actual absorption was found to be 1.1. Using Beer's Law, the actual concentration of the stock solution made was found to be 56.675 mM. Then using the stock solution, calculations were done to

make vials of 1 μM, 3 μM, 10 μM, 30 μM, and 100 μM stock solutions with growth media to be put on the cells in the well plates.

Once the three-day incubation period of the three 96-well plates ended, it was time to treat the cells with ZnTPP-5AP. The 96-well plates were removed from the incubator, and the old media was removed. Each well plate was then given the same treatments: media, media and DMSO, and increasing ZnTPP-5AP treatment concentrations. The prepared vials of stock solution with 1-100 μM concentrations were used on the cells for the ZnTPP-5AP treatment. Once the cells had been treated with the porphyrin solution, they were given 24 hours to treat the cells in the incubator. To limit light exposure during this time, all three well plates were covered in aluminum foil.

Following the 24-hour time period, the porphyrin was removed from the cells in the well plates to begin the photodynamic therapy. One plate was kept in the dark to test that the porphyrin itself not toxic to cancer cells. The second plate was exposed to white light for 26.5 minutes, and the third plate was exposed to red light for 56 minutes. After the period of light exposure, the media was removed from the well plates, and a mixture of MTT and growth media was added to the well plates. The three well plates were then incubated for four hours with the MTT on. DMSO was then added, shaken for thirty minutes, and the well plates were placed in a microplate reader to obtain the results of cell viability. The data was then analyzed to compare no light, white light, and red-light conditions on cell death of A549 non-small lung cancer cells with ZnTPP-5AP as a treatment.

For the second experiment with the A549 non-small lung cancer cell line and the porphyrin, ZnTPP-5AP hypoxic conditions were tested. To try to replicate *in vivo* conditions of a tumor, a hypoxic chamber was used to remove oxygen from the cells in the well plate. Four well plates were plated with the A549 non-small lung cancer cells using the same method as previously described. After the three-day incubation period allowing the cancer cells to adhere to the well plate, three 96-well plates were placed in the hypoxic chamber. Nitrogen gas was run through the hypoxic chamber for four minutes to remove any oxygen. After four minutes of nitrogen flow, the hypoxic chamber was sealed to trap the nitrogen in and prevent oxygen from entering. The hypoxic chamber was then placed in the incubator for four hours. The concentration range of the porphyrin treatment was decreased to 1 μM, 2.5 μM, 5 μM, 7.5 μM, and 10 μM ZnTPP-5AP concentrations. The concentration range was decreased because in the previous experiment, LD_{50} was seen at lower porphyrin concentrations. After the hypoxia treatment was completed, cells on all four well plates were treated the same with media, DMSO and media, and increasing porphyrin concentrations. The porphyrin concentrations remained on the cancer cells for 24 hours. After 24 hours, the porphyrin was removed, new media added, and photodynamic therapy begun. The well plate that received oxygen was exposed to white light for 26.5 minutes. One hypoxic well plate was exposed to white light for 26.5 minutes, a second hypoxic well plate was exposed to red light for 56 minutes, and a third hypoxic well plate was kept in the dark. The cells were then prepared with MTT as before and placed in a microplate reader for results. The results were then analyzed to compare hypoxic and nonhypoxic A549 non-small lung cancer cells under white and red light with ZnTPP-5AP.

MTT Assay Results

The first MTT assay experiment done was to test the cytotoxicity of ZnTPP-5AP under red and white light. The porphyrin ZnTPP-5AP was added to the A549 non-small lung cancer cells in increasing concentrations of 0 μM, 1 μM, 3 μM, 10 μM, 30 μM, and 100 μM. The top plate that was kept in the dark showed the most metabolic activity as seen by its strong violet color, **Figure 15**. The bottom well plates both revealed a color change as the porphyrin concentration would increase from 0 μM to 100 μM, **Figure 15**. Through observation of the plates, it is evident that as ZnTPP-5AP was added in increasing concentrations across the well plate cell viability decreased (cell death increased).

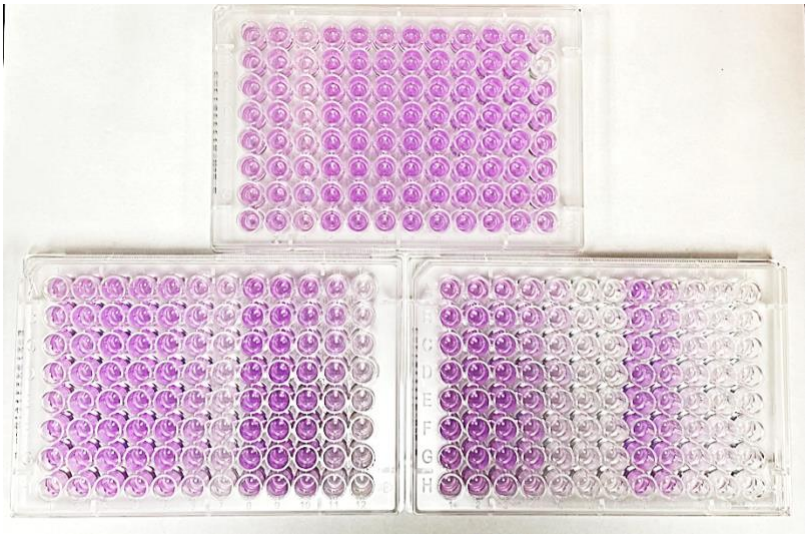


Figure 15: MTT Assay Results (experiment 1). The top 96-well plate was kept in the dark, the bottom left well plate was exposed to red light, and the bottom right well plate was exposed to white light.

After the MTT reagent had been added to the well plates, the well plates were then placed in a microplate reader that used software to calculate the number of viable

cells within each well. Using the raw data, a graph was created to visualize the MTT assay results better. In **Figure 16,** one can observe the A549 non-small lung cancer cells with ZnTPP-5AP had a high survival rate when kept in the dark. This high cell survival rate is what was expected because there was no light exposure to activate the porphyrin. Under white light ZnTPP-5AP had a LD_{50} of around 5 µM. Photodynamic therapy with increasing concentrations of ZnTPP-5AP under red light had a LD_{50} of around 50 µM.

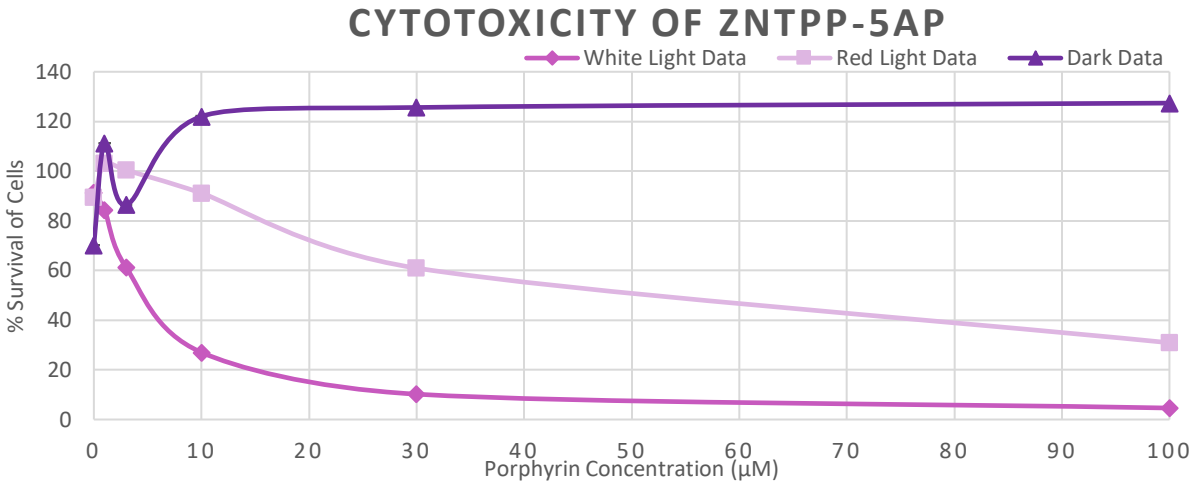


Figure 16: Graph of experiment 1 showing the results of photodynamic therapy with red vs. white light and increasing porphyrin concentrations (µM).

For the second experiment with ZnTPP-5AP hypoxic conditions were employed to mimic *in vivo* tumor conditions. The hypoxic cells that underwent porphyrin treatments but never had light exposure experienced a decrease in survival rate. This could be due to the hypoxic conditions causing stress on the cancer cells and contributing to cell death. The hypoxic cells exposed to red light did not have a much different survival rate than the control cells. This trend is probably because treatment with red light has a higher LD_{50}, **figure 16**, and in experiment 2 the concentration range is only 0-10

µM. For cells kept in normal oxygen with white light conditions, the LD_{50} was around 9 µM for ZnTPP-5AP. The hypoxic cells exposed to white light had the lowest LD_{50} of 6 µM for ZnTPP-5AP. In both experiments, the LD_{50} was lower when white light was used over red light. The addition of hypoxia conditions in experiment 2 lowered the LD_{50} of white light exposure. To determine if hypoxic conditions are lowering cancer cells $LD_{50,}$ more trials would need to be run.

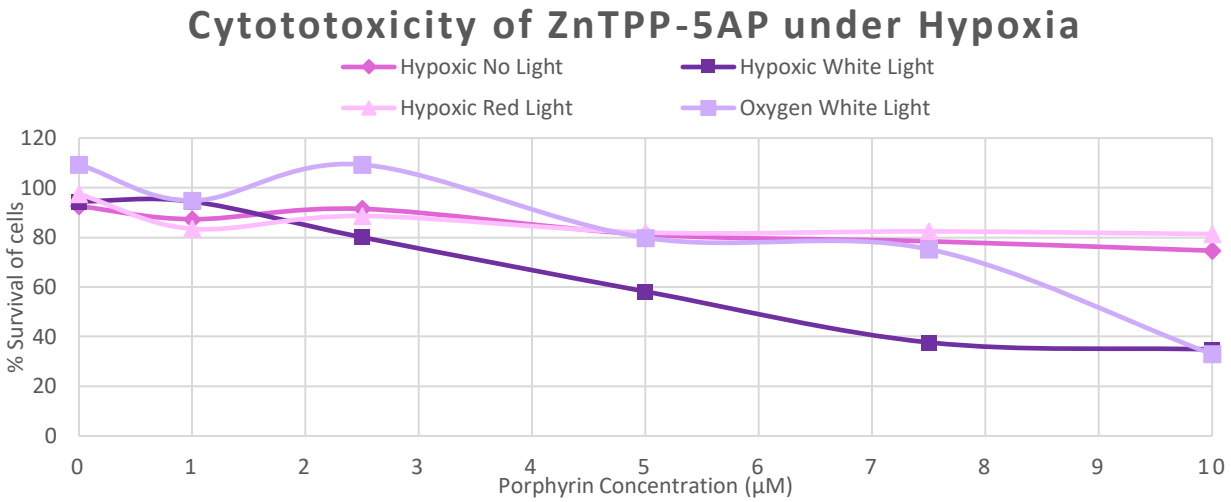


Figure 17: Graph of Experiment 2 with cells undergoing photodynamic therapy treatment with ZnTPP-5AP under hypoxic and nonhypoxic conditions.

Conclusion

A new water-soluble zinc porphyrin, ZnTPP-5AP, was able to successfully be synthesized for potential use in photodynamic therapy. The hydro amine group, 5-amino-1-pentanol, was successfully able to be attached to the porphyrin core. The final porphyrin was purified using Sephadex LH-20 and G-50, and ZnTPP-5AP was found to have a purity of greater than 99% by HPLC. After synthesis, the porphyrin structure was characterized using UV-Vis, IR, and NMR spectroscopies. Characterization of ZnTPP-5AP indicates the correct final porphyrin structure.

The new porphyrin was then tested as a possible photodynamic therapy agent using A549 non-small lung cancer cells. To test the cytotoxicity of the porphyrin an MTT assay was run on the cells. In the first experiment cancer cells were kept in the dark, exposed to white light, and exposed to red light after having undergone exposure to differing concentrations of porphyrin. It was found that when A549 non-small lung cancer cells were kept in the dark, there was very limited cytotoxicity. After exposure to red or white light, as the concentration of ZnTPP-5AP was increased, cytotoxicity increased in A549 cells. Cells exposed to red light had a LD_{50} of 50 µM ZnTPP-5AP, and cells exposed to white light had a LD_{50} of 5 µM.

The second experiment was able to successfully mimic hypoxic conditions before the A549 cancer cells underwent photodynamic therapy. MTT assay of the cells kept under hypoxia showed an LD_{50} at a porphyrin concentration of 6 µM after exposure to white light. However, cells exposed to white light with oxygen had an LD_{50} of around 9µM. Under hypoxia, the cells in dark and red light showed no difference in cell death with the concentration of porphyrin ranging from 0 to 10 µM, and a higher concentration

range needed to be used. These results indicate that ZnTPP-5AP is effective as a photosensitizer for photodynamic therapy for A549 non-small cell lung cancer cells at low concentrations (5µM).

Future Work

First, ZnTPP-5AP as a photosensitizer should be tested using different cancer cell lines. Testing the cytotoxicity of ZnTPP-5AP with other cancer cell lines would allow conclusions to be made as to whether the porphyrin is able to work as a general photosensitizer for cancer treatment.

Second, the porphyrin ZnTPP-5AP could undergo *in vivo* testing in mice on a specific tumor type. Because ZnTPP-5AP has a lower LD_{50}, it could serve as a potential photosensitizer at low dosage concentrations *in vivo*.

Finally, the porphyrin ZnTPP-5AP could be synthesized differently in the future to test unmetalated versus metalated porphyrins. This would result in a new porphyrin H_2TPP-5AP to be synthesized. After the synthesis of this porphyrin, cytotoxicity could be run to see if removing the metalated core changes the cytotoxicity of the porphyrin in photodynamic therapy.

Photodynamic Therapy in Pediatric Age Patients:

Photodynamic therapy is a treatment option that would specifically be beneficial to pediatric patients. Some benefits of PDT are that it is non-invasive, an outpatient treatment, easy to repeat, and can be applied to wide areas of affected skin. PDT treatment is also great for fragile patients that are immunosuppressed or cannot undergo surgery. Although PDT has already been analyzed as a safe treatment for adults, this form of treatment is not well standardized in the pediatric population.[10] Increasing research in this field provides an opportunity to develop effective methods to treat children with a non-surgical option and limit harmful treatments, such as chemotherapy and radiation therapy. Not only is this form of treatment safer for children, but patients receiving this treatment have seen increased survival rates.[11]

Photodynamic therapy has currently been utilized in dermatological and dental diseases, respiratory diseases, eye diseases, and in brain tumors.[11] In these separate studies using PDT treatment, several different photosensitizers were administered to patients with specific tissue targets. A case study using PDT for malignant brain tumors used talaporfin sodium (TS) as a photosensitive agent. Using this photosensitizer, the results indicated that TS was safely utilized in children with dosages of 40 mg/m^2.[12] Another photosensitizer used to treat pediatric brain tumors is protoporphyrin IX (PPIX) with the metabolite five-aminolevulinic acid (5-ALA). PPIX showed strong fluorescence and was very selective in tumor cells. This porphyrin and metabolite have been approved in Europe and by the FDA. Using this specific photosensitizer, PDT selective cell death was demonstrated above 5-ALA concentrations of 50 µg/mL.[13] Both of these photosensitizers share the same basic heme structure but differ in their side chains and

unmetalated core. The ability for photosensitizers to have differing side chains and metal cores allows them to be specific to tumor cells. Having this capability to change the fluorescence, water solubility, and toxicity of the photosensitizer allows it to be an ideal drug for pediatric cancer patients. Depending on the depth, location, or type of tumor, certain photosensitizers could potentially be developed that are ideal for that specific treatment.

The administration of PDT to pediatric patients would follow a different course than other traditional treatments such as surgery, chemotherapy, or radiation therapy. In the study using TS as a photosensitizer, the TS drug was administered 22-27 hours before laser irradiation. Immediately after TS had been administered to the patient, light shielding was begun and continued until day 14, when the photosensitivity test was negative. To protect surrounding blood vessels, aluminum foil sheets were used to cover surrounding areas.[12] Pain levels were low to moderate for most pediatric patients, but in sensitive areas, an analgesic was given thirty minutes before PDT to help reduce any discomfort. In studies looking at dermatological diseases, children were able to topically apply the photosensitizer and then play in the sun. Using PDT for more topical diseases is ideal for pediatrics psychologically, because it prevents children from being trapped in the walls of a hospital.[10] For cases where stronger light sources are needed, children would have to avoid the sun and follow similar guidelines as used with TS. Although having pediatric patients undergo shielding would be difficult, pediatrics would benefit from the ability to have a quick recovery from treatments because the side effects are so minimal.

Photodynamic therapy used to treat diseases and cancer in children has low side effects, but still there are concerns that must be explored. A study analyzed the photosensitizer, Curcumin, in pediatric epithelial liver tumor cells and found that under visible light, the Curcumin was degraded. The Curcumin was not capable of reducing cell viability before light therapy began.[14] When evaluating which photosensitizer to use on pediatric tumors, cell viability and specificity are an issue. There are several photosensitizers that have positive results with only a specific tissue type, while being poor photosensitizers for PDT in other tissue types. One study shows that 5-ALA as a photosensitizer was "quite variable in different tumor cell types," and this could indicate a differing sensitivity to PDT.[13] Another limitation to PDT when selecting the correct photosensitizer is the selection of a non-toxic photosensitizer. The amount of photosensitizer administered to the patient must be minimized for efficient elimination of the tumor cells. Because the photosensitizer is administered prior to PDT, there is a long waiting period where the child could have a reaction.[11] If the tumor is in deep locations, the photosensitizer is administered intravenously, which can be painful for pediatric patients.[11]

Overall, the side effects associated with pediatrics PDT cases are similar to adults and can be categorized into early and late onset effects. A few early side effects of PDT include pain and local skin reactions. The pain can differ depending on the area of treatment in the child. Other side effects associated with PDT are erosive pustular dermatitis on the scalp, hair loss, onychodystrophy, and urticarial reactions.[10] One psychological side effect for children undergoing PDT is the light shielding. There are strict rules on using devices or being exposed to light during the light-shielding period of

PDT. Children experience stress due to these regulations, especially as children are becoming more addicted to electronics.[12] The main concern for children is PDT-induced skin cancer, but currently there is no evidence to suggest that PDT stimulates skin carcinogenesis in children.[10] Careful follow up of pediatric patients would be needed to confirm this hypothesis and help prevent any development of skin cancer after treatment.

Despite the occurrence of minor adverse effects, photodynamic therapy provides numerous advantages for the pediatric population. In all clinical pediatric cases in which PDT was used, dermatological, dental diseases, respiratory diseases, eye diseases, and brain tumors , results were improved. PDT had an overall 78-100% effectiveness in pediatric cases, further revealing its importance for treatment in children.[11]

Hepatoblastoma (HB) is a common pediatric liver tumor and Hepatocellular carcinoma (HCC) is a rare disease in pediatrics. These two types of liver cancers both have poor outcomes in pediatric patients, and the cancer cells do not respond to radiation. The main method for survival currently is complete removal of the tumor through surgery; therefore, PDT would provide a second line of defense to ensure that all cancer cells are removed in pediatric patients. Intraoperative PDT is able to be transferred to the OR so that PDT therapy can be done simultaneously with surgery.[15] PDT could also provide a second-line treatment in pediatric patients who are antibiotic or retinoid resistant, or help to prevent children from building up this resistance with time.[10] Photodynamic therapy offers a safer, non-invasive, and easier method of treating diseases such as cancer within pediatric patients.

References:

1. Molina, Julian R., et al. “Non-Small Cell Lung Cancer: Epidemiology, Risk Factors, Treatment, and Survivorship.” *Mayo Clinic Proceedings*, vol. 83, no. 5, Elsevier, May 2008, www.sciencedirect.com/science/article/abs/pii/S0025619611607350. Accessed 07 Feb. 2024.
2. “Lung Cancer - Small Cell - Statistics.” *Cancer.Net*, American Society of Clinical Oncology, 20 Mar. 2023, www.cancer.net/cancer-types/lung-cancer-small-cell/statistics. Accessed 07 Feb. 2024.
3. Miller, Jennifer. “Photodynamic Therapy: The Sensitization of Cancer Cells to Light.” *Journal of Chemical Education*, vol. 76, no. 5, May 1999, doi.org/10.1021/ed076p592. Accessed 07 Feb. 2024.
4. 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, 4 Oct. 2012, doi:10.7150/thno.4571. Accessed 07 Feb. 2024.
5. Detty, Michael R. “Current Clinical and Preclinical Photosensitizers for Use in Photodynamic Therapy.” *Journal of Medicinal Chemistry*, vol. 47, no. 16, American Chemical Society, 29 July 2004, doi.org/10.1021/jm040074b. Accessed 07 Feb. 2024.
6. Ali, Abdu Hussen. "High-Performance Liquid Chromatography (HPLC): A review." *Annals of Advance in Chemistry*, 2022, Ali AH, doi.org/10.29328/journal.aac.1001026. Accessed 07 Feb. 2024.
7. Ng, Lily M., and Reiko Simmons. “Infrared Spectroscopy.” *Analytical Chemistry*, vol. 71, no. 12, 1999, American Chemistry Society, doi.org/10.1021/a1999908r. Accessed 07 Feb. 2024.
8. Reusch, William. “Nuclear Magnetic Resonance Spectroscopy.” *NMR Spectroscopy*, www2.chemistry.msu.edu/faculty/reusch/virttxtjml/spectrpy/nmr/nmr1.htm. Accessed 07 Feb. 2024.
9. Ghasemi, Mahshid et al. “The MTT Assay: Utility, Limitations, Pitfalls, and Interpretation in Bulk and Single-Cell Analysis.” *InternationalJournal of Molecular Sciences* vol. 22,23 12827. 26 Nov. 2021, doi:10.3390/ijms222312827.
10. Di Bartolomeo, Luca et al. “Photodynamic Therapy in Pediatric Age: Current applications and future trends.” *Frontiers in pharmacology* vol. 13 879380. 16 Aug. 2022, doi:10.3389/fphar.2022.879380.
11. Mazur, Anna et al. “Photodynamic Therapy for Treatment of Disease in Children-A Review of the Literature.” *Children (Basel, Switzerland)* vol. 9,5 695. 10 May. 2022, doi:10.3390/children9050695.
12. Chiba, Kentaro et al. “Photodynamic Therapy for Malignant Brain Tumors in Children and Young Adolescents.” *Frontiers in Oncology* vol. 12 957267. 24 Nov. 2022, doi:10.3389/fonc.2022.957267.
13. Schwake, Michael et al. “In-Vitro Use of 5-ALA for Photodynamic Therapy in Pediatric Brain Tumors.” *Neurosurgery* vol. 83,6 (2018): 1328-1337. doi:10.1093/neuros/nyy054.

14. Ellerkamp, Verena et al. “Photodynamic Therapy Potentiates the Effects of Curcumin on Pediatric Epithelial Liver Tumor Cells.” *Antiancer Research* vol. 36,7 (2016): 3363-72.