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Effectiveness and Mechanism of Action of Modified Porphyrins for Photodynamic Therapy of Triple Negative Breast Cancer Cells

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SENIOR THESIS APPROVAL

This Honors thesis entitled

**“Effectiveness and Mechanism of Action of Modified
Porphyrins for Photodynamic Therapy
of Triple Negative Breast Cancer Cells”**

written by

Alex Abbott

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

Dr. Tim Hayes, thesis director

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Date

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Abstract

Triple negative breast cancer is an aggressive family of cancers that are extremely difficult to treat. Therefore, the prognosis for most patients with TNBC is poor. The goal of this research is to determine if photodynamic therapy could be a possible therapy option for TNBC in the future using MDA-MB231 cells. MDA-MB231 cells were originally isolated from a patient with triple negative breast cancer and have been used for many studies, so they would work well for this study. Photodynamic therapy uses compounds called photosensitizing agents which are taken up by all tissues in the body and then activated by light. This creates a reactive oxygen species in the cell which is thought to cause cell death. To quantify cell death, an MTT assay was performed. The assay showed concentration-dependent cell death in the plates that were exposed to light. The plates that weren't exposed to light showed some dark toxicity at the highest concentrations. However, the cell death due to dark toxicity is small compared to cell death seen in the cells exposed to light. In addition to measuring cell death, experiments were performed to determine the mechanism of cell death. Antibodies were used to stain the cell for DNA fragmentation, which is a sign of apoptosis. The cells were also co-stained with four antibodies to test for the mechanism of cell death. The results from the antibody-staining assays suggested that the cells were dying mostly by caspase-mediated apoptosis. In addition, staining for oxidative damage and autophagy were also seen.

History of Photodynamic Therapy

Photodynamic therapy is a relatively new idea and practice. It was "accidentally" discovered over 100 years ago by a medical student by the name of Oscar Raab [1]. Raab was studying the effects of fluorescent dyes on infusoria, which are a class of microorganisms. He found that

when intense light was applied to the dyes, the microorganisms were rapidly destroyed. From there, Raab's professors took over and furthered studied this process. His professors, Jesionek and von Tappeiner, described and named this process, now known as photodynamic therapy, or PDT [1].

By the early 1900s, patients were successfully being treated for various cancers, particularly of the skin, by PDT. Although PDT had shown promising results, it was not able to build enough momentum and died out until about 50 years later. In the 1960s, PDT was rediscovered by Lipson and Schwartz through studies done at the Mayo Clinic [5]. These studies not only showed tumor ablation, but also showed the ability of photosensitizing agents to fluoresce and define the boundaries of tumors. Then, in the 1970s, a scientist by the name of Dougherty began working with porphyrin compounds for PDT. With this knowledge and experience, Dougherty was able to produce a commercially suitable photosensitizing drug, reliable light sources, and appropriate clinical trials that proved the value of PDT to the oncology community [1]. For this reason, Dougherty is known by many as the "The Father of PDT."

Background

Triple-negative breast cancer (TNBC) is an aggressive set of cancers that have few promising therapy options. Because of this, the prognosis for most cases of TNBC is very poor. Typically, TNBC is found as an advanced stage disease and patients have low survival rates compared to other types of breast cancer. The goal of our research is to determine whether photodynamic therapy is a viable option for treating TNBC in the future.

TNBC is described as any breast cancer that is lacking in expression of estrogen receptor (ER), progesterone receptor (PgR) and of HER2 overexpression. This cancer accounts for about 15% of all breast cancer [4]. TNBC is usually found as high-grade tumors, although there have been cases of low-grade tumors found. This is most likely due to the fact that TNBC tumors are typically very difficult to find through mammography [4]. There are many risk factors that are seen with TNBC's. This cancer occurs more frequently in patients younger than 50 years old. In addition, TNBC is seen 2 to 3 times more often in African Americans than other racial groups in the United States. Other important risk factors involved include a strong family history of breast cancer, young age at first birth, multiparity, and a high waist:hip ratio [3].

TNBC's, in addition to lacking ER, PgR, and HER2 expression, show many other pathological features. TNBC comprises a heterogeneous subgroup of tumors including basal-like and claudin-low subtypes. In addition, these tumors can be of ductal type, metaplastic, medullary, or adenoid cystic type. TNBC's frequently express basal cytokeratins and the epidermal growth factor receptor (EGFR) HER1 which are features that are usually associated with a poor prognosis in breast cancer. TNBC also shows overlap with basal-like and BRCA-1 cancers. The BRCA-1 protein plays a role in DNA repair and transcriptional regulation [4]. This may promote genetic instability and favor tumor growth, and BRCA-1 tumors account for about 5% of all breast cancers [4]. Both BRCA1 and TNBC tumors have been shown through immunohistochemical (IHC) analyses to be associated with high Ki-67 expression, p53 mutation, and basal-like expression. In addition, greater than 50% of BRCA-1 carriers have TNBC [4]. Therefore, people with a BRCA1 mutation are at higher risk of developing TNBC.

Perhaps one of the most disappointing things about TNBC is the fact that there are currently no specific treatment options. In other cases of breast cancer, hormonal therapy is a

viable option. However, since TNBC's lack the receptors that are the usual targets in hormonal therapy, this will not work. TNBC is also not a candidate for agents such as trastuzumab, which is a HER-2 directed agent [4]. As of right now, the only viable option for treating TNBC is chemotherapy, which is not a very effective or friendly treatment option. Because of the lack of treatment options, the survival rates for TNBC's are generally lower than other types of cancer. In a study done by the California Cancer Registry, TNBC's were present at a more advanced stage disease and had lower 5-year survival rates (77% vs. 93%) than non-TNBC cancers [4]. TNBC's also tend to show a characteristic pattern of early relapse and shorter overall survival in patients [3]. For these reasons, it is important to find a viable treatment option for triple negative breast cancer.

One therapy option that may be a viable treatment for TNBC is photodynamic therapy (PDT). PDT is a method of destroying cells through the use of molecules called photosensitizers (PS) and light. Cells take up the PS, and, when exposed to light of a specific wavelength, create a photodynamic reaction (PDR) which causes the cell to die [1]. Although it is not known for sure how the cell dies during the PDR, studies have determined a basic idea of how PDT works in the cell. Once light is applied to the cell, the energy from the light alters the PS via energy transfer from the photon to the PS [1]. There are a few pathways in which the PS can lose energy, however, the most important pathway for clinical PDT is the generation of a type II photochemical reaction [1]. In this pathway, the PS interacts with oxygen to generate singlet oxygen, which then destroys the cell through oxidative damage. In addition, the tumor could die through ablation of the cells of the vasculature that supplies the tumor or could initiate a immune response to the tumor cells.

To make sure that PDT gives the best possible treatment results for cancer, there are many characteristics PS agents need to be successful. Some of the most important characteristics of PS agents are: nontoxic until activated by light, hydrophilic properties for easy application, activated by a useful wavelength of light, and a reliable generator of the PDR [1]. Successful PS agents should also concentrate in the tumor cells only and not healthy cells, should leave the patient's body fairly quickly, should not create any toxic by-products, and should provide a pain-free therapy [1]. This is a long list of characteristics of PS agents, and finding one that checks all the boxes is difficult. Currently, there are several PS agents on the market such as Hematoporphyrin derivative (HPD), Foscan, Mono-L-aspartyl chlorin e6, aminolevulinic acid, and Fotosens [1]. Although all of these are able to create a PDR to kill cells, each of them have their drawbacks. For example, HPD is non-toxic and does not cause pain, but, it stays in the body for 6 to 8 weeks after introduction. During this time, patients must stay out of a strong light source, like intense sunlight, to avoid severe burns [1]. This is not the most ideal situation for the patient after cancer treatment. Another PS agent that is very similar to the agents used in this paper is Foscan. Foscan is a plant based chlorin derivative that produces a rapid and significant PDR [1]. The drug is so active that patients must stay in a dark room for 24 hours after treatment to avoid severe burns to the body. PS agents that are used today are also not cancer cell-specific, which means that the PS agents are taken up by all cells. In the clinic, this can be compensated for by only shining light on the tumor itself. However, if the PS agent stays in the body for a long period after treatment, the patient has to avoid other strong sources of light. The treatment using Foscan is also very painful, so patients must undergo anesthesia during treatment [1]. However, Foscan is still a popular drug of choice for PDT because of its effectiveness in killing tumor cells.

For this study, PS agents known as porphyrins were tested. Porphyrins, as mentioned earlier, are very similar to Foscan in structure. They consist of a hydrophobic ring structure, as shown in Figure 1.

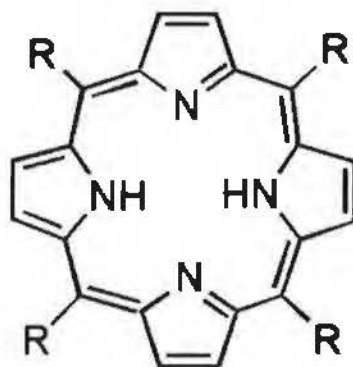


Figure 1 - Porphyrin ring structure

This very hydrophobic ring is thought to help the molecule enter the cell. The four R groups attached to the ring structure are what differentiates porphyrins from one another. These R groups are usually hydrophilic and polar to allow for easier travel through the blood. Foscan, a chlorin, differs from porphyrins by having one less double bond in the ring structure. The R group for Foscan is shown Figure 2.

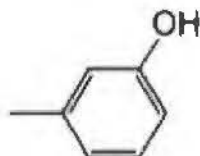


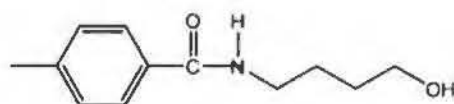
Figure 2 - R group in Foscan

In this paper, Foscan was used as a comparison for the porphyrins that were tested in the lab. Two important criteria that were analyzed were the LD_{50} and the dark toxicity of the porphyrins.

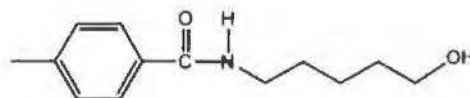
TNBC is a very aggressive type of cancer that has few viable treatment options. The prognosis is generally poor for patients with TNBC and patients have lower survival rates compared to patients with other types of breast cancer. For these reasons, it is important to find better treatment options. This study looks at photodynamic therapy using porphyrins as a potential treatment option.

Materials and Methods

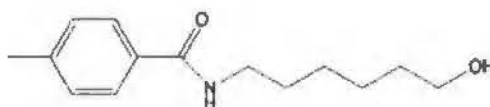
For this study, MDA-MB231 triple negative breast cancer cells were grown in MEM/10% FBS/2mM L-glutamine/pen-strep at 37°C. These cells were used in the experiments to test porphyrin effectiveness. For the assays, the cells were plated in 96-well plates. The porphyrin derivatives tested in this study were synthesized by Dr. Joe Bradshaw's lab at Ouachita Baptist University. These porphyrins were H₂TPP-A4OH, H₂TPP-A5OH, and H₂TPP-A6OH. The structures of their R groups are shown in Figure 3.



H₂TPP-A4OH



H₂TPP-A5OH



H₂TPP-A6OH

Figure 3 - R groups of porphyrins tested

For the photodynamic treatments of the cells, the porphyrins were diluted in 2 ml of growth medium to the desired concentration. These concentrations were added to the cells in the 96-well plates once the cells were about 25% confluent. Eight wells were used for each condition. Twenty-four hours after adding the porphyrins to the cells, the medium was replaced with fresh medium to remove any porphyrins not in the cells. One plate was then exposed to white light (0.5 J/cm^2) for 16 mins and then was moved to the incubator in the dark. The second plate was placed in the incubator in the dark immediately after replacing the medium. MTT assays were then done three days later.

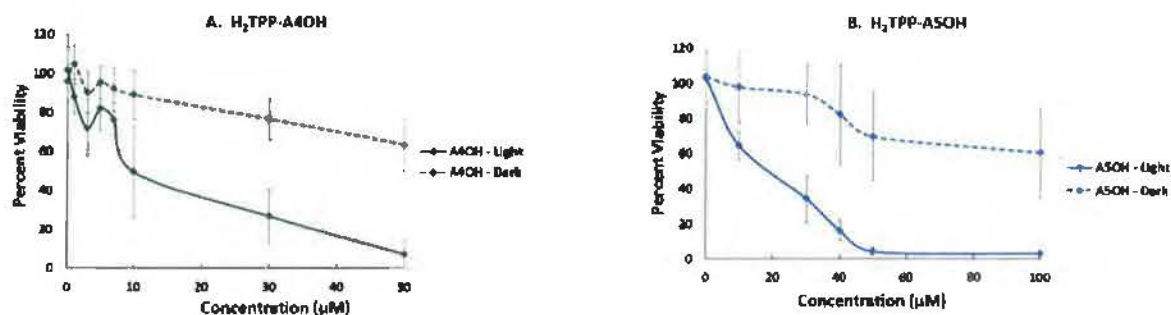
To test for cell viability, MTT assays were performed for each plate. The MTT assays test the viability of the cells by using a yellow chemical called MTT. The cells will reduce the yellow MTT to a purple formazan, which can be dissolved in DMSO. The more purple the solution in the wells, the more viable the cells. The absorbance of each plate was measured in a plate reader at 570 nm with a 630 nm correction. The averages and standard deviations for each condition were then calculated and graphed.

To test for the mechanism of cell death, a TUNEL assay was performed at the University of Arkansas for Medical Sciences (UAMS). The cells were plated in 8-well chamber slides and were treated with $\text{H}_2\text{TPP-PipOH}$ at the LD_{50} for 24 hours. PipOH had been previously tested and had shown effectiveness as a PS agent, so it was used in this assay instead of the other compounds that were used in this study. The cells were then exposed to light in the same way as the 96-well plates. Twenty-four hours after exposure, the cells were washed using PBS (phosphate buffered saline) and fixed with a 4% PFA-based buffered solution. Paraformaldehyde (PFA) is converted into formaldehyde by PBS in solution. Formaldehyde then terminates all

ongoing biochemical reactions in the cells and increase the cells' stability. This is called "fixing" cells. A TUNEL assay stains the cells for DNA fragmentation. In addition, the cells were co-stained with DAPI and four antibodies: Caspase-3, EndoG, OGG1, and HO1. These antibodies stain for cell death mechanisms such as apoptosis, autophagy, and oxidative damage. Caspase-3 is a marker for many apoptotic pathways, so if it is seen the cell most likely died due to apoptosis. Endo-G is activated in caspase-independent apoptosis. OGG1 stains for nuclear oxidative damage, which occurs when the nucleus is damaged by reactive oxygen species, while HO1 stains for oxidative damage. Since the mechanism of PDT involves reactive oxygen species, these antibodies are expected to stain in the cells exposed to light.

Results

The porphyrins H₂TPP-A4OH, H₂TPP-A5OH, and H₂TPP-A6OH were tested for their cell toxicity in this study. After three separate experiments of the porphyrins on cells, the cell viability values were averaged and graphed together. The standard deviations of these values were also found. Figure 4 shows the graphs of each porphyrin and an overlay of the three porphyrins together.



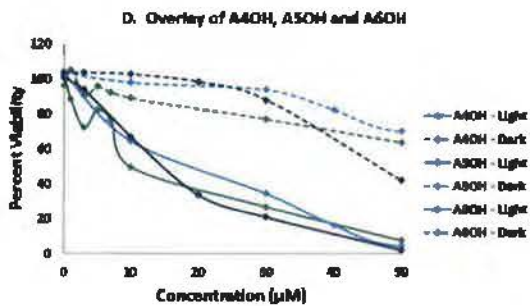
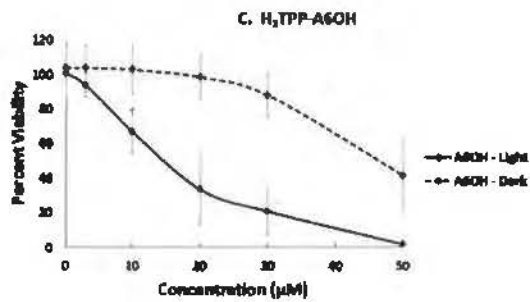


Figure 4 - Graph A shows the cell viability for A4OH; Graph B shows the cell viability for A5OH; Graph C shows the cell viability for A6OH; Graph D shows the three porphyrins together.

The results indicate the porphyrins showed concentration-dependent cell death when exposed to light. The LD₅₀ was in the 10-20 µM range for each of them. One thing to note is that at concentrations needed to kill >80% of the cells, significant dark toxicity was seen.

The TUNEL assay done at UAMS showed some insight into how the cells treated with these porphyrin derivatives were dying. Figure 5 shows a graph of the mean intensity of OGG1 staining in cells treated with porphyrin and light.

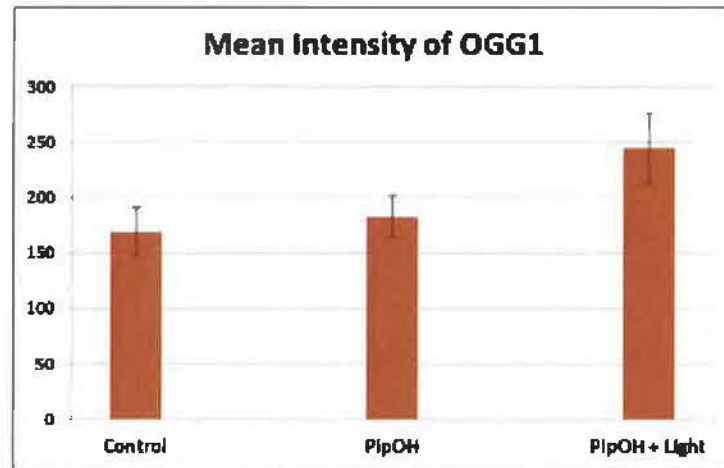


Figure 5

These results show that there was a significant increase in the amount of OGG1 staining, which shows oxidative damage, in cells that were both exposed to PipOH and light as compared to cells that had no porphyrin and cells that were not exposed to light.

Figure 6 shows the percentage of TUNEL positive cells with Caspase-3 colocalization.

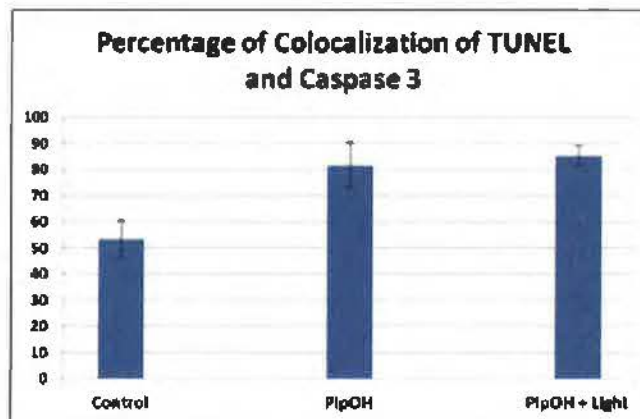


Figure 6

This graph indicates that a higher percentage of cells that were exposed to PipOH and light showed both higher amounts of DNA fragmentation and higher amounts of Caspase-3. This could indicate that the cells are dying through apoptosis.

Figure 7 shows the merged staining picture of the cells that were exposed to PipOH and light. The cells in this figure were stained with TUNEL and co-stained with DAPI and Caspase-3. This figure shows that the cells expressed Caspase-3 and were TUNEL-positive due to the blue and bright orange stains, respectively.

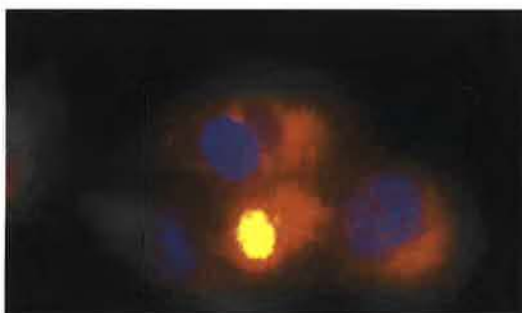


Figure 7 - The red staining shows Caspase-3, the blue staining shows DAPI, and the bright orange stain is for TUNEL.

Discussion

This study showed that the porphyrins used were effective in killing triple negative breast cancer cells when exposed to light. The graphs in Figure 4 show concentration-dependent cell death with a LD_{50} in the 10-20 μM range. These porphyrins contained monohydroxy side chains, which means that they had only one hydroxyl (-OH) group. These results were compared to results obtained from testing the effectiveness of polyhydroxy side chain porphyrins (more than one -OH group) (data not shown). The polyhydroxy side chains differed from the monohydroxy side chains shown here in that the LD_{50} was much greater (40-50 μM). Another difference

between the two families was that the polyhydroxy side chains showed little to no dark toxicity at any concentration, whereas the monohydroxy side chains showed dark toxicity at the higher concentrations.

From the TUNEL assay, the results indicate that the cells exposed to the porphyrin PipOH were dying mainly through caspase-mediated apoptosis, as well as oxidative damage. Since PDT is known to create reactive oxygen species within the cell, oxidative damage was expected to be seen. Figure 5 shows the staining of OGG1, which is a marker seen in cells that have gone through oxidative damage. As expected, the graph shows a higher amount of staining in the cells that were exposed to both the porphyrin and light as opposed to cells that were not exposed to the porphyrin and cells that were not exposed to light. Figures 6 and 7 suggest that the cells died by caspase-mediated apoptosis. The graph in Figure 6 shows a greater staining of TUNEL positive cells and Caspase-3 in cells exposed to PipOH and light. One interesting note about Figure 6 is that there is a greater percentage of cells showing TUNEL and Caspase-3 that were exposed to the porphyrin but no light. Through the cell viability assays, it is known that these cells did not die, however. This could indicate the cells may have been “preparing” for death by increasing the expression of Caspase-3. However, since there was no light given to the cells, they did not go through apoptosis.

The porphyrins tested here show great promise in being potential options for treating triple negative breast cancer in the future. However, more testing needs to be done in order to fully know the effect of these drugs. Since porphyrins are taken up by all cells and not just cancer cells, research on the pharmacokinetics needs to be done. This research would show how the drugs interact with the human body and how long they stay in the human body. This would also show any by-products the porphyrins degrade into; these products cannot be toxic to the

human body for these porphyrins to be successful PS agents. The TUNEL assays were extremely enlightening as to how these drugs kill the cells. It is important to know how the cells are dying to best formulate an effective treatment plan for patient use.

Conclusions

Triple negative breast cancer is an extremely aggressive form of cancer with few viable treatment options. One potential option explored in this study was photodynamic therapy. Results showed that the porphyrins tested have promise in becoming drugs for cancer treatment. The drugs killed cells that were exposed to light at low concentrations; the LD₅₀ was in the 10-20 μM range. These porphyrins also showed no dark toxicity in lower concentrations, however some dark toxicity was seen in cells that were given higher concentrations. TUNEL assays were also performed to determine the mechanism of cell death. Results showed that the cells were likely dying through caspase-mediated apoptosis. In the future, we would like to repeat the TUNEL experiments using flow cytometry to receive a better picture of the staining of the cells that detached from the surface of the plate. This would give a better representation of the fraction of cells that died due to apoptosis, necrosis, or another mechanism.

Other Uses of Photodynamic Therapy

Although the main focus of this paper is using PDT on breast cancer, PDT has been used to treat other ailments as well. Using PDT to treat severe acne is something that researchers are looking into currently [2]. Although it is only in clinical trials, researchers believe that PDT shows promise in being able to treat severe acne in the future. In addition, PDT is used to treat other types of cancer, such as skin cancer, esophageal cancer, and gastric cancer. Skin cancer is an especially popular choice for PDT treatment because of the easy access to the tumor.

Illuminating a tumor within the body with light is not as simple as a tumor on the skin.

Therefore, PDT has shown major success in treating skin cancers. One specific drug used to treat skin cancer is called aminolevulinic acid (ALA). This photosensitizer is enzymatically converted into Proto-Porphyrin IX, which is a powerful photosensitizing agent [1]. This means that the ALA can be applied to the skin topically, and then the body itself produces the drug from the ALA. Once this happens, the tumor can be illuminated and thus killed very easily. For this reason of ease of access, skin cancer is one of the more promising options for PDT treatment going forward.

Perhaps one of the most promising directions that PDT is headed is photoimmunotherapy. Photoimmunotherapy is a response to PDT's biggest weakness as a cancer treatment, which is its non-specificity. Photosensitizers used in PDT are not specific to cancer cells. This means that the drug is taken up by all the cells of the body, not just the cancer cells. This makes treatment very tricky; if healthy cells are illuminated by enough light, then those cells will die. The desired outcome of PDT is for only cancer cells to be killed by the treatment, however there is always a good chance that the desired outcome is not what happens.

Photoimmunotherapy utilizes a monoclonal antibody that it is specific to targets on the cancer cell membrane [2]. Scientists have also been able to add drugs to these antibodies to form complexes that not only find and bind to cancer cells, but also kill the cells. One study created a specific dye that was attached to a monoclonal antibody. Once the complex was bound to the cancer cell, a light of a wavelength of 690 nm was used to illuminate the cancer tissue. The light activated the dye portion of the complex, which changed the molecular makeup of the dye. This caused the cancer cell membrane to break apart, thus killing the cell [2].

This treatment, unlike PDT, is very new to the medical community. However, this process shows immense promise in treating cancer in patients much more efficiently. Although it is not exactly PDT, photoimmunotherapy is extremely similar and uses PDT mechanisms.

Future of PDT

As mentioned earlier, PDT has shown major promise as a treatment for cancer and other diseases in the future. PDT as a cancer treatment is one of the most interesting avenues it can take because of its comparison to other treatment options. For many cancers, including triple negative breast cancer, there are no specific treatments. The only way to treat these cancers is through radiation and chemotherapy. These processes are extremely harmful to the body as a whole and affect the patient greatly. Although PDT has its flaws, many believe it is and will be a better option for cancer treatment in the future.

A major aspect of PDT that scientists can improve on for the future is finding a great photosensitizing agent. Many problems with PDT arise from the drugs used not fitting all of the criteria of a “perfect” PS agent, such as toxicity, specificity, and light absorption. This is what the main focus of this study was. If better and better PS agents can be synthesized and used in PDT, this therapy option will continue to grow in popularity in treating cancer and other diseases.

Acknowledgements

First, I would like to thank Dr. Tim Hayes for allowing me to work with him this past summer. I learned so much from participating in this research and I truly believe that this experience will help me in my future career. I would also like to thank my fellow research student this past summer, Hannah Brandon. Hannah and I spent many hours together over the summer writing

notes and treating cells. Without her help, this summer would have been very difficult. I would also like to thank the J.D. Patterson Summer Research Program here at Ouachita Baptist University. This program is great for undergraduate students because it provides a research experience that very few undergraduates receive in college. This program allowed me to gain extremely valuable experience that will I will benefit from for my entire career. Finally, I would like to thank the Carl Goodson Honors Program for providing this great platform for student research. Conducting the research and writing this thesis taught me so much that I will use and appreciate my entire life.

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