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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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Contents

ABSTRACT

Triple negative breast cancer (TNBC) is a particularly aggressive form of breast cancer that lacks the three molecules typically targeted for treatment. Standard treatment methods leave much to be desired– the rates of metastasis and recurrence are high and the prognosis for most patients with TNBC is poor. One potential treatment for TNBC is photodynamic therapy (PDT), which uses compounds called photosensitizers that are taken up by all tissues in the body. The tumor is exposed to light, activating the photosensitizer and creating reactive oxygen species that cause cell death. This method is relatively pain-free, effective, and does not harm cells that are not exposed to light.

The goal of these experiments was to assess the effectiveness of porphyrin derivatives as PDT agents *in vitro* on MDA-MB231 triple-negative breast cancer cells. The polyhydroxy R-groups (e.g., H_2TPP-3NH) produced LD_{50}s of 40-50 μM and showed little to no dark toxicity.

For one compound, experiments were performed to determine the mechanism of cell death. A TUNEL assay was performed to measure DNA fragmentation, and the TUNEL-stained cells were co-stained with antibodies to help identify the method of cell death. Results indicated that the cells were dying primarily by caspase-mediated apoptosis.

BACKGROUND

Triple Negative Breast Cancer

Triple negative breast cancer, which makes up 15% of all breast cancers, is one of the most difficult cancers to treat. TNBC cells lack expression of estrogen receptors (ER)

and progesterone receptors (PR). There is also an absence of overexpression of human epidermal growth factor receptor-2 (HER2) in these cells that is commonly seen in other breast cancers [5].

There are many varieties of TNBC, and these different characteristics often overlap, making them difficult to categorize. Most TNBCs are ductal carcinomas with central necrotic regions within the tumor, most likely due to a lack of blood flow to these tissues. TNBCs have a very high proliferative rate. Immunohistochemical analyses show that TNBC is associated with high expression of proliferation marker Ki-67 and several other factors that favor cancer cell growth (mutated p53, cyclin E, P-cadherin, and BRCA1 mutations) [5].

Basal cells and luminal cells are the two distinct types of epithelial cells found in the human mammary gland. They can be distinguished by immunohistochemistry. Basal epithelial cells are characterized by keratin 5, keratin 17, integrin-β4 and laminin gene expression clusters, while luminal cells have gene expression cluster characteristics of transcription factors that include ER [6]. Other features shared by TNBC and basal-like breast cancers typically include a grade 3 tumor rating, high mitotic counts, and high apoptotic rates [2].

These basal-like characteristics seen in many TNBCs may be attributed to an epithelial-to-mesenchymal transition (EMT), or to the claudin-low phenotype. EMT is a transdifferentiation process marked by changes in protein expression. One of these changes is the upregulation of vimentin, a mesenchymal marker. High vimentin levels are associated with metastatic potential, increased aggressiveness, and a poor prognosis. The claudin-low subtype is ER, PR, and HER2 negative, but only reflects 5-10% of human

breast cancers. In addition to low expression of claudin, this subtype has EMT characteristics as well, such as upregulation of mesenchymal markers and other stem-cell signatures [2].

There seems to be an overlap with basal-type breast cancers and BRCA1-related breast cancers, however, some still express ER, PR, and HER2. Of basal-like breast cancers, 15-54% express at least one non-TNBC marker, and not all patients with TNBC express the markers for the basal-like phenotype. The BRCA1 protein plays a role in DNA repair and transcriptional regulation, so mutation of the BRCA1 gene could favor tumor growth. BRCA1-related breast cancers are very similar to triple negative breast cancers pathologically and histologically. Over 50% of BRCA1 carriers have triple negative breast cancer, but not all do [5].

There are several risk factors for TNBC. Those at risk are of a younger age (less than 50 years old) [2], have the BRCA1 mutation, and/or have a family history of breast cancer [5]. African Americans are the most affected ethnicity, with up to 47% of breast cancers being triple-negative in these patients. Their tumors have higher expression levels of cell-cycle and apoptotic-related proteins. They are also more likely to present with larger and higher-grade tumors [2].

There are some related risk factors for the basal-like phenotype, such as younger age at menarche, higher parity, higher BMI among postmenopausal women, and a shorter duration of breastfeeding [5]. Women with abdominal obesity, a risk factor for type II diabetes, present TNBCs and basal-like breast cancers more often, yet the antidiabetic drug metformin seems to induce apoptosis of TNBC cells. Metformin has also been observed to enhance chemotherapy treatment in breast cancer patients. In addition, there

is an increased risk of TNBC in women who are less than 40 years old and use oral contraceptives [2].

The prognosis for patients with TNBC is poor compared to those with other kinds of breast cancers. Although TNBC tumors respond to typical chemotherapy treatments, they relapse more often than hormone receptor-positive, luminal subtypes. Studies actually suggest that improvements in chemotherapy may preferentially benefit TNBCs because of their defects in DNA repair mechanisms and rapid proliferation. However, high response rates become irrelevant due to the high relapse rate of TNBCs. The recurring tumors are much worse and have a lower survival in the basal-like breast cancers and HER2-enriched tumors [2].

Usually TNBCs are in advanced stages when found. This could partially be because TNBC is less likely to be detected by mammography than other breast cancers. There is also a lack of correlation between tumor size and lymph node positivity, possibly contributing to the advanced-stage tumors found when patients are first diagnosed with the cancer. Although it has similar responses to cytotoxic chemotherapy as other breast cancers, patients with TNBC still have a poorer outcome in the long run. There is a high risk of distant recurrence and metastasis in other tissues, even after treatment with chemotherapy [5].

Unlike breast cancers with estrogen receptors, progesterone receptors, or overexpression of HER2, TNBCs cannot be treated with hormone therapy or monoclonal antibody-based drugs such as Trastuzumab. Current treatment for patients with TNBC is limited to cytotoxic chemotherapy, which can eventually result in harmful side effects.

New treatment options are desperately needed to improve the prognosis of patients with TNBC [5].

Some of the newest treatments being designed to target TNBCs include poly (ADP-ribose) polymerase (PARP) inhibitors, angiogenesis inhibitors, EGFR- targeted agents, and src kinase inhibitors. PARP inhibitors block nuclear enzymes that are involved in the detection and repair of DNA damage. PARPs are upregulated in many cancers including TNBCs and BRCA1 tumors. Knocking out this defense allows chemotherapy and radiation to be more potent. Epidermal growth factor receptor (EGFR) is also upregulated in several cancers, so EGFR inhibitors have recently become a potential target for cancer therapy. Low efficacy has been seen in treatment of TNBCs with EGFR inhibitors. Angiogenesis inhibitors target proteins such as VEGF in an attempt to limit the proliferation of blood vessels that give nutrients to growing tumors. Increased motility and invasiveness are seen in tumors with upregulated expression of the tyrosine kinase c-src. Inhibitors of src-kinase seem to be effective in basal-like breast cancers [2]. Another possible alternative to standard treatment for TNBC is photodynamic therapy.

Photodynamic Therapy

Photodynamic therapy is a method of destroying tumor cells using a photosensitizing agent and light. The photosensitizer is introduced to the body intravenously and eventually is taken up by the tumor tissue. When exposed to a specific wavelength of light, the photosensitizer creates reactive oxygen species that kill the tumor cells. There are different types of photodynamic reactions, but the most important

one for photodynamic therapy is a type II photochemical reaction in which the photosensitizer interacts with oxygen to create a singlet oxygen that destroys the tumor tissue. Other effects of the photodynamic reaction can be the ablation of the vasculature that supplies the tumor with blood. Photodynamic therapy is especially attractive as a cancer treatment because unlike chemotherapy and radiation, it spares healthy tissue. If only the tumor cells containing the photosensitizer are exposed to light, they should be the only cells that are killed [1].

Photodynamic therapy was discovered accidentally by Oscar Raab, a medical student researching fluorescent dyes, in the late 1800s. He noticed that in the presence of strong light, the dyes killed the microorganisms he was working with. His professors, Jesionek and von Tappeiner, clarified the dyes' ablative ability. This photodynamic reaction began to be used in cancer treatment by the early 1900s. However, the therapy was soon forgotten until the 1950s, when it was rediscovered by Lipson and Schwarz. These studies reconfirmed the tumor ablation ability of photosensitizing compounds, as well as their ability to fluoresce. In the 1970s, Dougherty created a commercially suitable photosensitizing drug using porphyrins, as well as finding reliable light sources and conducting the appropriate clinical trials to prove the efficacy of photodynamic therapy as a cancer treatment. He is now known as the "Father of Photodynamic Therapy" [1].

Photosensitizers are natural or synthetic compounds that transfer light energy. An efficient and useful photosensitizer should have several characteristics. Ideally, a photosensitizer is nontoxic unless exposed to light and is eliminated relatively quickly from the body without forming any toxic degradation products. Clinically useful photosensitizers should be hydrophilic so that they can be applied systemically, be

activated by a practical light wavelength, and be pain free. Treatment can also be optimized by choosing the best wavelength of light and exposure time for the specific photosensitizer and tumor [1].

Porphyrins have been shown to be excellent photosensitizing agents. They are aromatic organic compounds containing four five-sided rings connected to one another (Fig. 1). Each ring has four carbons and one nitrogen and is connected to the next ring by a single hydrocarbon bridge. Porphyrins have a very similar structure to chlorin, the main constituent of the photosynthetic pigment, chlorophyll. Porphyrins are found in nature as well. They are abundant in hemoglobin, as heme is the molecule that binds to iron and allows for oxygen to be transported through the body [3]. The modified porphyrins created by Dr. Joe Bradshaw's lab at Ouachita Baptist University contain the hydrophobic core ring structure, but they vary by the different R-groups attached on the outside of the ring. These R-groups replace the hydrogen on the carbon bridge in the ring structure. Optimizing these side chains may make a difference in the porphyrins' ability to be taken up by cells or to create reactive oxygen species. The porphyrin derivatives tested in these experiments are H_2TPP-3NH, H_2TPP-2-Me-1,3, and H_2TPP-Tris (Fig. 1).

There are different combinations of characteristics in various porphyrins that affect their performance as photosensitizers. Photofrin (HPD) was the first commercially available photodynamic therapy agent. It is nontoxic and pain free. However, HPD stays in the body for 6-8 weeks post-administration. Because the photosensitizer will react in any tissue exposed to light, the patient must avoid direct sunlight for weeks after treatment. Aminolevulinic acid (ALA) is a precursor that is enzymatically converted to Proto-Porphyrin IX upon introduction to the body. It is a powerful photosensitizer that

has been shown to be effective in skin disease, as it can be topically administered with no systemic phototoxicity [1].

Another photosensitizer approved in the EU and in clinical trials in the U.S. is Foscan® (mTHPC) [8]. Foscan® is not a porphyrin, but rather a plant based chlorin derivative. It creates a very fast and powerful photodynamic reaction. It is so strong that patients must be under anesthesia during treatment and must stay in a dark room for at least 24 hours after treatment [1]. Foscan® has been shown to produce cell death through apoptotic, necrotic, and autophagic pathways [8]. The potency of Foscan® as a photodynamic therapy agent seems to be not only in its photochemical properties, but in its *in vivo* interactions as well. Pharmacokinetic studies describe extremely high concentrations of Foscan® in the liver, showing its role in the elimination of Foscan® from the body. It is eliminated from the body faster than many other photosensitizers. Studies also show that Foscan® has an additional antitumor effect early after drug administration due to its accumulation in blood plasma and interstitial spaces. It damages the vasculature in the tumor, depriving it of oxygen and nutrients, which ultimately leads to tumor cell death [4].

As previously noted, one aspect of photodynamic therapy is the way in which it induces cell death. Knowing this information can help optimize and personalize treatment based on the type of tumor [7]. Previous research has shown that PDT tumor destruction occurs primarily by apoptotic pathways or by necrotic pathways, although autophagy, parthanatos (regulated necrosis), and mitotic catastrophe have also been shown [9]. The ideal method of cell death is hard to determine. Necrosis could be helpful if the tumors have developed mutations that make them resistant to apoptosis. However, apoptosis is a

natural and much cleaner way to dispose of unnecessary or unhealthy cells. It seems that the high intensity light used in PDT results in necrosis, while low intensity light is more likely to induce apoptosis. It may be possible to take advantage of the different effects of light intensity in a clinical setting to favor either apoptotic or necrotic pathways [1].

MATERIALS AND METHODS

Cell Culture

MDA-MB231 cells were grown in MEM/10% FBS/2mM L-glutamine/pen-strep at 37°C. Cells for assays were plated in 96 well plates.

Porphyrin Derivatives

We tested novel porphyrin derivatives with polyhydroxy side chains: H_2TPP-3NH, H_2TPP-2-Me-1,3, and H_2TPP-Tris (Fig. 1 and 2).

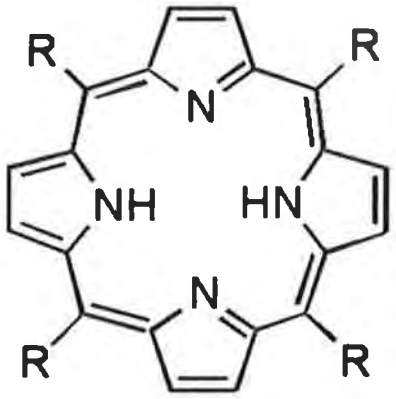
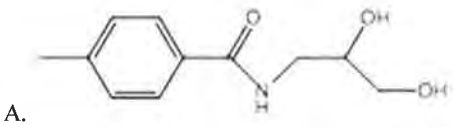


Figure 1. *Porphyrin Ring Structure*



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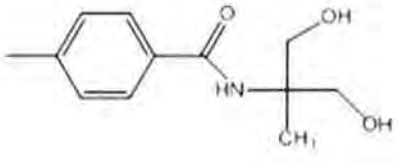

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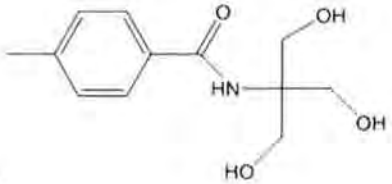

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Figure 2. *Polyhydroxy R-Groups. A. H_2TPP-3NH. B. H_2TPP-2-Me-1,3. C. H_2TPP-Tris.*

Exposure to Light

Porphyrins diluted to different concentrations in growth medium were added to duplicate 96-well plates when the cells were approximately 50% confluent. 8 wells were used for each condition. 18 hours later, the medium was replaced, and one plate was exposed to white light for 16 minutes (0.5 J/cm^2). MTT assays were performed 3 days later.

MTT Assay

An MTT assay was performed to quantitate cell death. MTT diluted in growth medium was added to the duplicate 96-well plates. After 3-4 hours, the medium was replaced with DMSO. If the mitochondria in the cell were still functioning (indicating cell viability), the MTT reacts to become Formazan, which produces a purple color. The absorbance of each well was read in a plate reader at 570 nM with a 630 nM correction. Averages and standard deviations were calculated for the 8 wells in each condition.

TUNEL Assay

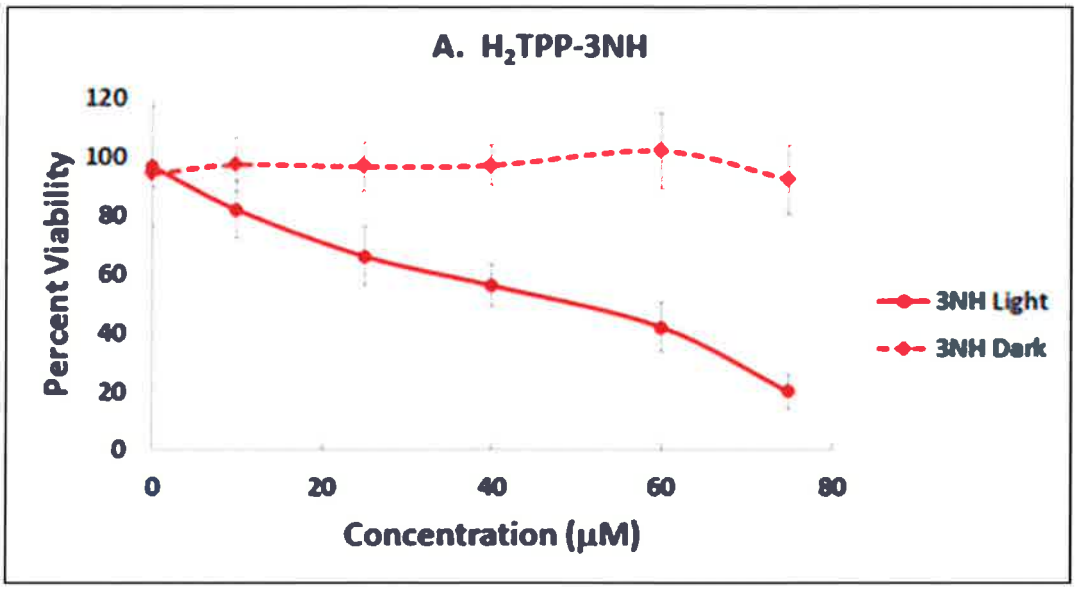
Cells were plated in 8-chamber slides and treated with the porphyrin H_2TPP-PipOH at the LD_{50} for 24 hours. Wells were left in the dark or exposed to light (0.5 J/cm^2) as indicated. 24 hours later, the cells were fixed for analysis.

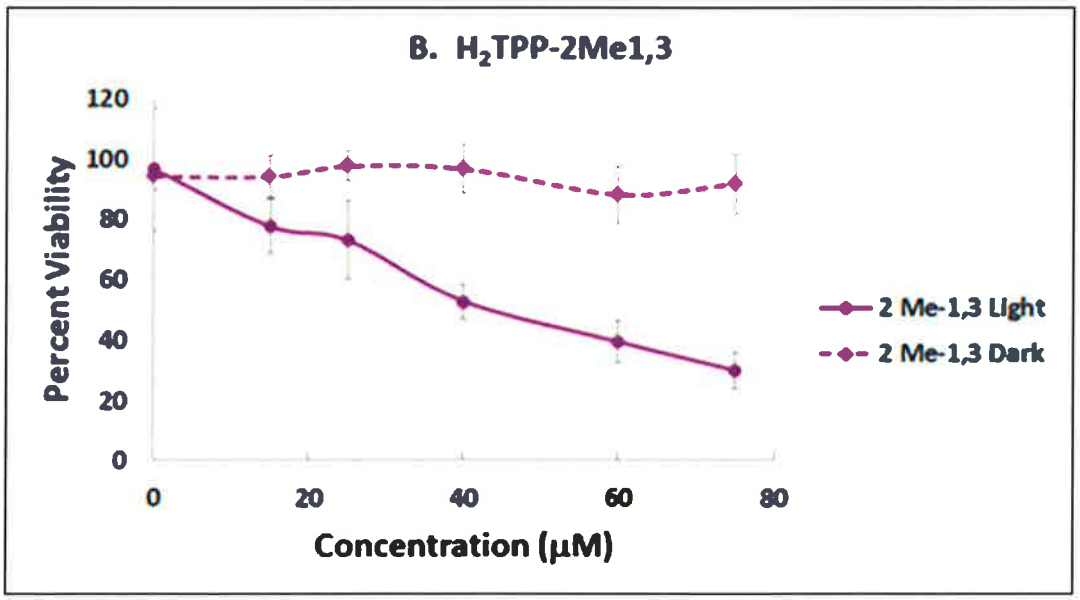
The cells were used for the TUNEL assay, which measures DNA fragmentation, and co-stained with DAPI and antibodies against caspase 3 (activated in apoptosis), EndoG (activated in caspase-independent apoptosis), OGG1 (a marker for autophagy), and HO1 (a marker for oxidative damage).

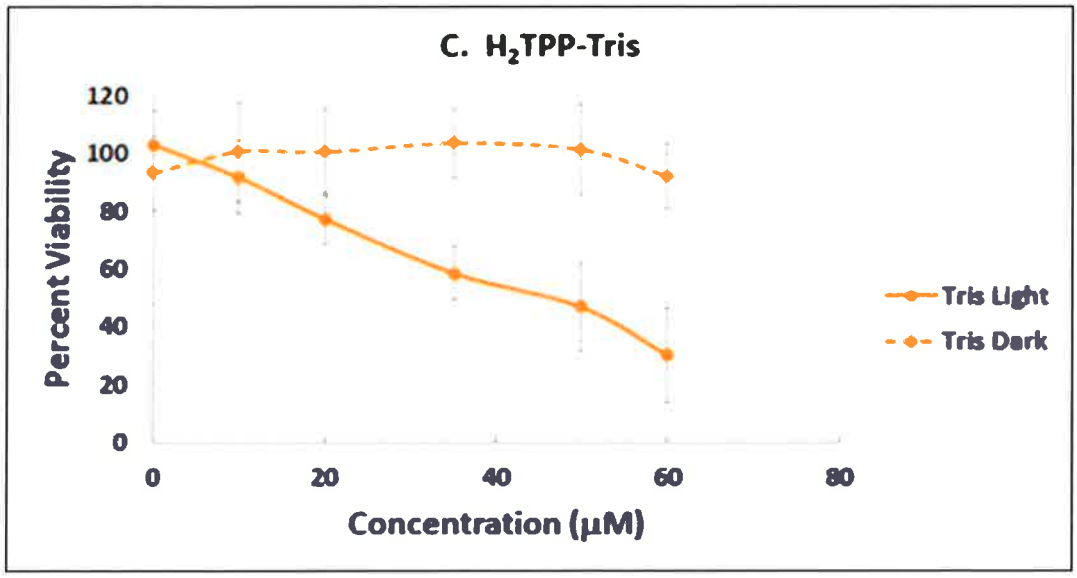
RESULTS

Toxicity of Polyhydroxy Side Chains

Each of the related porphyrin derivatives showed little to no toxicity in the dark but concentration-dependent toxicity when exposed to light. The phototoxicity for each was very similar. The LD_{50} for each is in the range of 40-50 μM (Fig. 3).







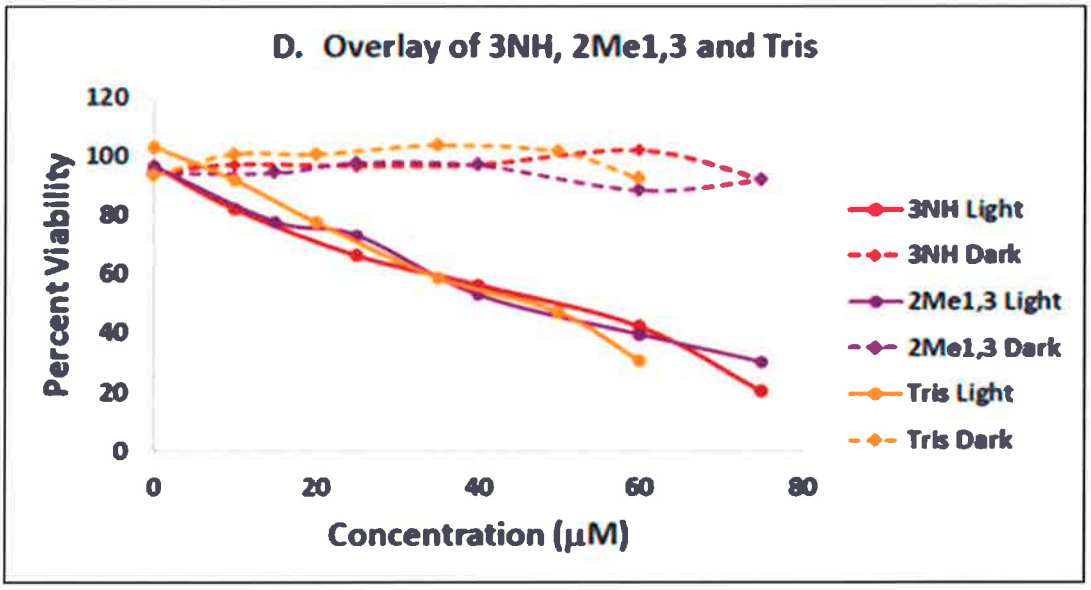


Figure 3. *MTT assays of cell viability of 3 polyhydroxy side chains. Each graph shows the average and standard deviation of at least three independent experiments. A- H_2TPP-3NH. B- H_2TPP-2Me1,3. C- H_2TPP-Tris. D- Overlay of the individual graphs; error bars are omitted for clarity.*

Mechanism of Cell Death

We treated our cells with the porphyrin derivative PipOH in these experiments because its phototoxicity had been previously studied. In our experiments, PipOH had an LD_{50} of

20 μM. The fraction of the cells that were TUNEL-positive after treatment with PipOH was quantitated, as was the intensity of the staining for each antibody. A few cells treated with PipOH and light show DNA fragmentation and activated caspase 3. Most cells treated with PipOH and light show oxidative damage (Fig. 4).

A. B.

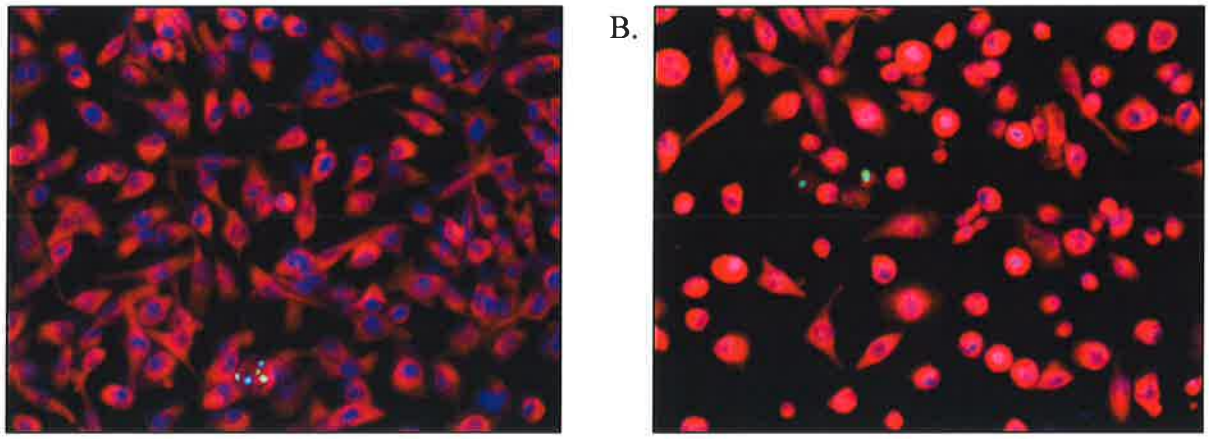
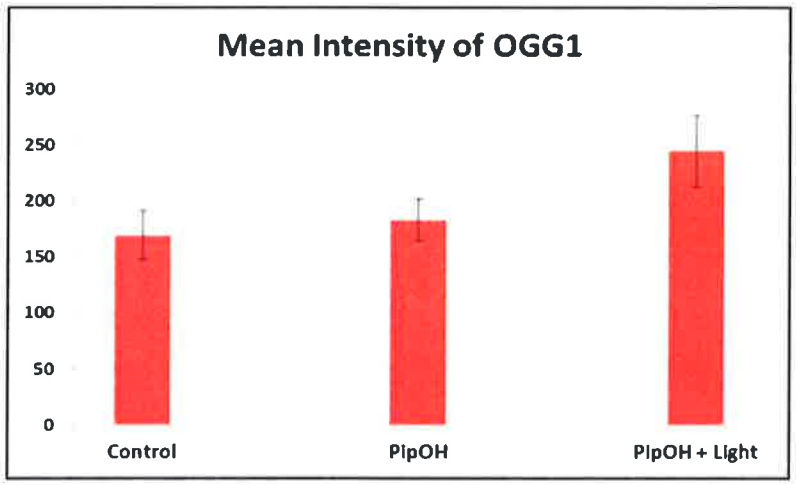


Figure 4- *Cells stained with DAPI (blue), TUNEL (green), and HO1 (red). A. Control. B. PipOH + Light.*

Stained slides were analyzed for signal intensity and the number of positive cells. Quantitation of the staining revealed evidence of oxidative damage and caspase 3 dependent apoptosis (Fig. 5). Cells treated with PipOH + light showed a significant increase ($p<0.05$) in staining for OGG1. TUNEL staining of control cells showed low background (~0.1% of cells positive). Treatment with PipOH alone caused a 0.6-fold increase in the fraction of TUNEL-positive cells, while treatment with PipOH + light caused a 3-fold increase. A significant majority of the cells treated with PipOH, either with or without light, that were TUNEL-positive were also positive for activated caspase-3 ($p<0.05$).

A.



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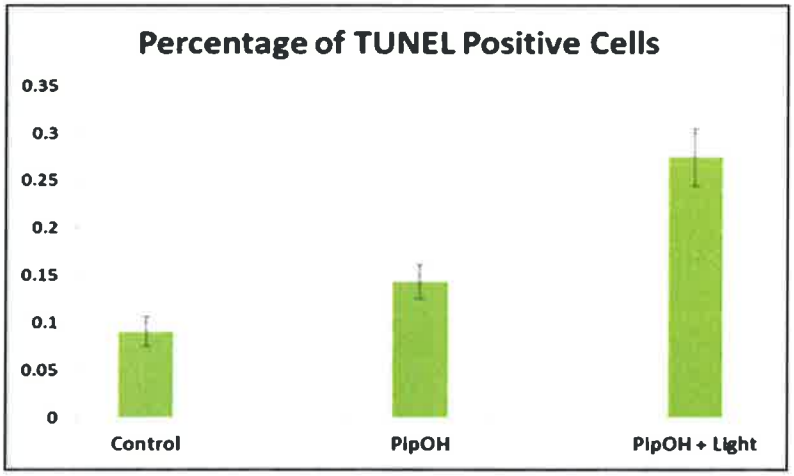

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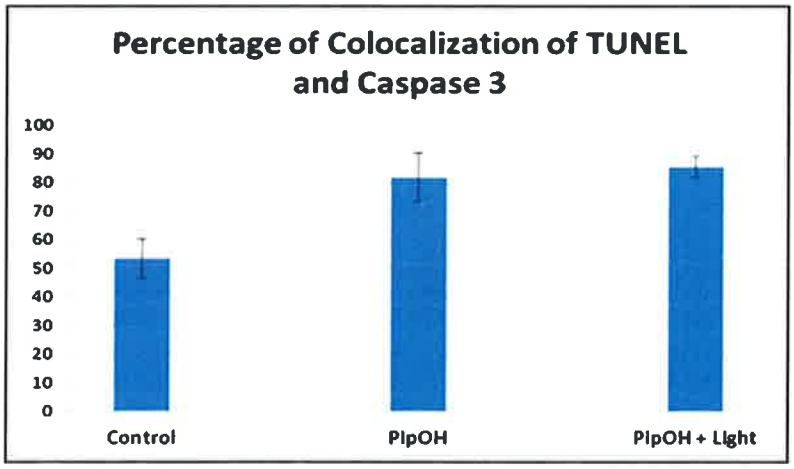

Figure 5- A. Intensity of staining with OGG1. Quantitation of staining for B. TUNEL and C. TUNEL and caspase-3.

DISCUSSION

The polyhydroxy porphyrin derivatives show promise as photosensitizers in photodynamic therapy. They effectively killed the cells when treated with white light of moderate intensity. When the cells were not exposed to light, there was only dark toxicity in cells treated with high concentrations of porphyrin. This is good for clinical applications because room light ideally should not be able to activate the photosensitizer. Unwanted exposure to light could cause cell death all over the body since the photosensitizer does not accumulate only in the tumor. The LD_{50} of 40-50 μM is a good concentration for clinical use because it is not so toxic that it reacts under minimal light, but it is effective enough to use only a small amount. This is beneficial because only the minimal amount of drug necessary for treatment should be used.

Novel porphyrin derivatives such as the polyhydroxy family of porphyrins are being synthesized to maximize ideal characteristics of photodynamic therapy agents. The porphyrin ring is the same for all porphyrins. It is hydrophobic, which may help the compound be able to get into the cell. There are four identical and hydrophilic R-groups that have more than one hydroxyl group in the side chain. This may increase the molecule's polarity, making it easier to travel through the bloodstream.

It seems that the cells treated with photodynamic therapy in our experiments are dying through caspase 3-dependent apoptosis. As expected, we saw evidence of oxidative damage in our experiments. We would like to repeat these experiments using flow cytometry so that we can include in the staining the cells that detached from the dish during the experiment to better assay what fraction of the cells die via apoptosis, autophagy, necrosis, or another mechanism.

In the future, animal studies could be done to determine the pharmacokinetics of the polyhydroxy porphyrin derivatives. These experiments would show where these porphyrins tend to accumulate in the body and how long they stay before they are eliminated. It would also reveal if any toxic products are made from these photosensitizers while in the body, and what the best drug-light interval for these porphyrins would be.

Because the porphyrins accumulate in all tissues, there is a risk that healthy tissue could be unintentionally killed if exposed to light. Careful precautions must be taken to ensure that only tumor cells are targeted for treatment. Another complication is that surgery would be required to be able to access most tumors. It may be difficult to treat the tumor if it is quite large because the light may not be able to penetrate deep enough to react with the photosensitizer. One option would be to try to remove as much as the tumor as possible, and then treat the remaining tumor tissue with photodynamic therapy.

An underutilized aspect of photodynamic therapy is the ability of some photosensitizers to fluoresce. If our polyhydroxy porphyrin derivatives fluoresce, perhaps they could be used to detect where the tumor begins and ends, as well as to kill the tumor by creating reactive oxygen species. Future experiments might involve testing to see whether the polyhydroxy porphyrin derivatives fluoresce at a specific wavelength of light.

CONCLUSION

Photodynamic therapy would be a great alternative to today's treatment options for triple negative breast cancer. Unlike targeted treatments for other breast cancers, no receptors on the cancer cells are required to use photodynamic therapy. Since the photosensitizer accumulates in all cells, only the cells purposely exposed to light are marked for death. A better treatment for triple negative breast cancer is needed because it is generally more advanced at diagnosis than other breast cancers and has shorter survival times. Even when treated with chemotherapy, patients with triple negative breast cancer have poorer outcomes.

Our experiments have shown the effectiveness of polyhydroxy porphyrin derivatives in photodynamic therapy on triple negative breast cancer cells. The polyhydroxy family showed concentration-dependent toxicity when exposed to light, with LD_{50}s in the range of 40 –50 μM. Our data shows the presence of oxidative damage after light exposure and that some of the cancer cells are dying through caspase 3-dependent apoptosis.

There are still more experiments that would need to be performed before the polyhydroxy porphyrins could be used in a clinical setting. Further experiments using flow cytometry are needed to confirm the mechanism of cell death. Pharmacokinetic studies would show how effective these compounds would be *in vivo* and would provide further information about the best drug-light interval for these porphyrins.

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REFERENCES

[1] Allison, Ron R., and Keyvan Moghissi. "Photodynamic Therapy (PDT): PDT Mechanisms." *Clinical Endoscopy*, vol. 46, no. 1, 2013, p. 24., doi:10.5946/ce.2013.46.1.24

[2] Carey, Lisa, et al. "Triple-Negative Breast Cancer: Disease Entity or Title of Convenience?" *Nature Reviews Clinical Oncology*, vol. 7, no. 12, 2010, pp. 683-692., doi:10.1038/nrclinonc.2010.154

[3] Clegg, Brian. "Porphyrins." *Chemistry World*, 8 Feb. 2017, www.chemistryworld.com/podcasts/porphyrins/2500381.article.

[4] Jones, H J et al. "Photodynamic Therapy Effect of m-THPC (Foscan®®) *In vivo*: Correlation with Pharmacokinetics." *British journal of cancer* vol. 89,2 (2003): 398-404. doi:10.1038/sj.bjc.6601101

[5] Pal, Sumanta Kumar, et al. "Triple Negative Breast Cancer: Unmet Medical Needs." *Breast Cancer Research and Treatment*, vol. 125, no. 3, 2010, pp. 627-636., doi:10.1007/s10549-010-1293-1.

[6] Perou, CM et al. "Molecular Portraits of Human Breast Tumours." *Nature*, 2000; 406:747–752, doi:10.1038/35021093

[7] Rello-Varona, Santiago, et al. "'(Not) All (Dead) Things Share the Same Breath': Identification of Cell Death Mechanisms in Anticancer Therapy." *Cancer Res*, 15 Mar. 2015, (75) (6) 913-917; DOI: 10.1158/0008-5472.CAN-14-3494

[8] Senge M.O., Brandt J.C. Temoporfin (Foscan®®, 5,10,15,20-Tetra(m-hydroxyphenyl)chlorin)—A second-generation photosensitizer. *Photochem. Photobiol.*, 2011;87:1240–1296. doi: 10.1111/j.1751-1097.2011.00986.x

[9] Soriano, J et al. "Cell Death Mechanisms in Tumoral and Non-Tumoral Human Cell Lines Triggered by Photodynamic Treatments: Apoptosis, Necrosis and Parthanatos." *Scientific reports* vol. 7 41340. 23 Jan. 2017, doi:10.1038/srep41340