Light Directed Targeted Photodynamic Therapy of Breast Cancer

Sara Shaut
sns7692@rit.edu

Follow this and additional works at: https://scholarworks.rit.edu/theses

Recommended Citation

This Thesis is brought to you for free and open access by RIT Scholar Works. It has been accepted for inclusion in Theses by an authorized administrator of RIT Scholar Works. For more information, please contact ritscholarworks@rit.edu.
Light Directed Targeted Photodynamic Therapy of Breast Cancer

Sara Shaut

A thesis submitted in partial fulfillment of the requirements for the degree

Master of Science in Chemistry

Supervised by
Dr. Hans Schmitthenner

School of Chemistry and Materials Science
College of Science
Rochester Institute of Technology

August 2022

Signature of the Author ______________________________

Accepted by _______________________________________

Director, M.S. Degree Program                  Date
The M.S. Degree Thesis of Sara Shaut has been examined and approved by the thesis committee as satisfactory for the thesis required for the M.S. Degree in Chemistry.

Dr. Hans Schmitthenner, Thesis Advisor

Dr. Maureen Ferran

Dr. Michael Heagy

Dr. Suzanne O’Handley

Date
Abstract

Breast cancer is the most prevalent cancer among women besides non-melanoma skin cancer. Even when caught in early stages, the treatment for breast cancer is often accompanied by harsh and debilitating side effects. For better quality of life, treatment options that have minimal side effects but maintain effectiveness are invaluable. If such a treatment option is a targeted form of therapy, it could selectively treat cancerous cells and not healthy cells. Photodynamic therapy (PDT) is a relatively new light-based therapy that utilizes inherent photochemical properties of certain dyes called photosensitizers (PS) in order to kill cancerous cells. It shows great promise as an alternative therapy for breast cancer with temporary photosensitivity as the only main side effect. The goal of this research was to make a molecularly targeted agent for photodynamic therapy of breast cancer by reliable and replicable methods. This was done through the use of a breast cancer targeting peptide called 18-4 discovered by Dr. Kamaljit Kaur, the synthesis of which was modified within our lab to be done on Sieber resin by solid phase peptide synthesis (SPPS). To this peptide, a tetra-pyrrole dye based on 2-[1-hexyloxyethyl]-2-devinyl pyropheophorbide-a (HPPH), was conjugated to form a molecularly targeted photosensitizer (MTPS). This was accomplished by two different approaches, the first by conjugation at the third residue of lysine on the 18-4 decapeptide. The second was by the addition of a puzzle piece based on a modular method developed earlier in our lab, which was the PS dye attached to side chain of a lysine residue. These two single modal compounds are the first reported MTPS agents that utilize 18-4 for the molecularly targeted photodynamic therapy of breast cancer and will be tested, along with a Cy5.5-3S analog of the MTPS agents, by our collaborators at the University of Rochester in the PDT of breast cancer models in mice.
Acknowledgements

This thesis and the research detailed within could not have been possible without the support of many. I would first like to thank Dr. Hans Schmitthenner, my research advisor, for his support and guidance throughout the whole process. I have gained both a wealth of knowledge and skills from working with him through this sometimes difficult and troublesome project.

I am also very grateful all the members of the Molecular Imaging Laboratory at RIT that I have had the pleasure of working with or alongside. There is much that I have learned from them and it is invaluable to have such support in the lab. I specifically would like to thank Basant Kaur for teaching me hands-on the methods and processes of solid phase peptide synthesis used in this project. I would also like to thank Matthew Law, who lent his knowledge and expertise in working with the breast cancer targeting peptide as well as helping to provide such peptide for me to work with. From previously detailed methods in the group, I also learned coupling techniques, that helped provide me with a foundation in an otherwise new research project.

I also would like to thank the RIT College of Science, specifically School of Chemistry and Material Science for providing me with financial and academic support during my graduate studies at RIT.

Last but certainly not least, I would like to thank my friends and family for their continual support during my journey at RIT. They were a great source of motivation and positivity through all the ups and downs that kept me sane and from burning myself out. It is moving to have people that were as excited about what I was working on as I was supporting me. I couldn’t have done this without all of them.
# Abbreviations

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>ACN</td>
<td>Acetonitrile</td>
</tr>
<tr>
<td>AmAc</td>
<td>Ammonium Acetate</td>
</tr>
<tr>
<td>BCS</td>
<td>Breast Conserving Surgery</td>
</tr>
<tr>
<td>BrCa</td>
<td>Breast Cancer</td>
</tr>
<tr>
<td>CFM</td>
<td>Confocal Fluorescence Microscopy</td>
</tr>
<tr>
<td>CT</td>
<td>Computerized Tomography</td>
</tr>
<tr>
<td>DCM</td>
<td>Dichloromethane</td>
</tr>
<tr>
<td>Dde</td>
<td>1-(4,4-dimethyl-2,6-dioxacyclohexidene)ethyl</td>
</tr>
<tr>
<td>DEA</td>
<td>Diethylamine</td>
</tr>
<tr>
<td>DIPEA</td>
<td>N,N-Diisopropylethylamine</td>
</tr>
<tr>
<td>DMSO</td>
<td>Dimethyl Sulfoxide</td>
</tr>
<tr>
<td>ERBB2</td>
<td>Human Epidermal Growth Factor Receptor 2</td>
</tr>
<tr>
<td>Fmoc</td>
<td>Fluorenylmethoxycarbonyl</td>
</tr>
<tr>
<td>HOMO</td>
<td>Highest Occupied Molecular Orbital</td>
</tr>
<tr>
<td>HPLC-MS</td>
<td>High Performance Liquid Chromatography-Mass Spectrometer</td>
</tr>
<tr>
<td>HPPH</td>
<td>2-[[1-hexyloxyethyl]-2-devinyl pyropheophorbide-a</td>
</tr>
<tr>
<td>HR</td>
<td>Hormone Receptor</td>
</tr>
<tr>
<td>HRMS</td>
<td>High Resolution Mass Spectroscopy</td>
</tr>
<tr>
<td>ICS</td>
<td>Intersystem Crossing</td>
</tr>
<tr>
<td>KRT1</td>
<td>Keratin 1</td>
</tr>
<tr>
<td>LDL</td>
<td>Low-Density Lipoprotein</td>
</tr>
<tr>
<td>LUMO</td>
<td>Lowest Unoccupied Molecular Orbital</td>
</tr>
<tr>
<td>MRI</td>
<td>Magnetic Resonance Imaging</td>
</tr>
<tr>
<td>MTPS</td>
<td>Molecularly Targeted Photosensitizer</td>
</tr>
<tr>
<td>Mtt</td>
<td>4-methyltrityl</td>
</tr>
<tr>
<td>NHS</td>
<td>N-Hydroxysuccinimide</td>
</tr>
<tr>
<td>NIR</td>
<td>Near Infrared</td>
</tr>
<tr>
<td>NMR</td>
<td>Nuclear Magnetic Resonance Spectroscopy</td>
</tr>
<tr>
<td>OMI</td>
<td>Optical Molecular Imaging</td>
</tr>
<tr>
<td>PDT</td>
<td>Photodynamic Therapy</td>
</tr>
<tr>
<td>PET</td>
<td>Positron Emission Tomography</td>
</tr>
<tr>
<td>PhiPr</td>
<td>2-phenylisopropyl ester</td>
</tr>
<tr>
<td>Prep-HPLC</td>
<td>Preparative High Performance Liquid Chromatography</td>
</tr>
<tr>
<td>PS</td>
<td>Photosensitizer</td>
</tr>
<tr>
<td>ROS</td>
<td>Reactive Oxygen Species</td>
</tr>
<tr>
<td>SERDS</td>
<td>Selective Estrogen Receptor Downregulators</td>
</tr>
<tr>
<td>SERMS</td>
<td>Selective Estrogen Receptor Modulators</td>
</tr>
<tr>
<td>SPE</td>
<td>Solid Phase Extraction</td>
</tr>
<tr>
<td>SPECT</td>
<td>Single-Photon Emission Computerized Tomography</td>
</tr>
<tr>
<td>SPPS</td>
<td>Solid Phase Peptide Synthesis</td>
</tr>
<tr>
<td>TFA</td>
<td>Tetrafluoroacetic Acid</td>
</tr>
<tr>
<td>THF</td>
<td>Tetrahydrofuran</td>
</tr>
<tr>
<td>TMIA</td>
<td>Targeted Molecular Imaging Agent</td>
</tr>
<tr>
<td>TSTU</td>
<td>N,N,N',N'-Tetramethyl-O-[(N-succinimidy]uronium tetrafluoroborate</td>
</tr>
<tr>
<td>UHPLC-MS</td>
<td>Ultra High Performance Liquid Chromatography-Mass Spectrometer</td>
</tr>
<tr>
<td>US</td>
<td>Ultrasound</td>
</tr>
</tbody>
</table>
# Abbreviations of Amino Acids in Peptide 18-4

<table>
<thead>
<tr>
<th>Amino Acids</th>
<th>Three Letter Abbreviations</th>
<th>One Letter Abbreviations</th>
</tr>
</thead>
<tbody>
<tr>
<td>Alanine</td>
<td>Ala</td>
<td>A</td>
</tr>
<tr>
<td>Glutamic Acid</td>
<td>Glu</td>
<td>E</td>
</tr>
<tr>
<td>Glutamine</td>
<td>Gln</td>
<td>Q</td>
</tr>
<tr>
<td>Leucine</td>
<td>Leu</td>
<td>L</td>
</tr>
<tr>
<td>Lysine</td>
<td>Lys</td>
<td>K</td>
</tr>
<tr>
<td>Phenylalanine</td>
<td>Phe</td>
<td>F</td>
</tr>
<tr>
<td>Tryptophan</td>
<td>Try</td>
<td>W</td>
</tr>
<tr>
<td>Tyrosine</td>
<td>Tyr</td>
<td>Y</td>
</tr>
<tr>
<td>Norleucine</td>
<td>Nle</td>
<td>X</td>
</tr>
</tbody>
</table>
# Table of Contents

**Abstract** ...........................................................................................................................................ili

**Acknowledgements** .........................................................................................................................iv

**Abbreviations** .................................................................................................................................v

**Abbreviations of Amino Acids in Peptide 18-4** ...........................................................................vi

**List of Figures** .................................................................................................................................ix

1. **Introduction:** ...............................................................................................................................1
   
   1.1 Overview .....................................................................................................................................1
   
   1.2 Cancer ........................................................................................................................................2
   
   1.3 Treatment Options .......................................................................................................................4
   
   1.4 Photodynamic Therapy .............................................................................................................6
   
   1.5 Photosensitizers ..........................................................................................................................9
   
   1.6 HPPH .........................................................................................................................................13
   
   1.7 Targeting Breast Cancer ..........................................................................................................14
   
   1.8 Cancer Imaging ..........................................................................................................................16
   
   1.9 Modular Synthesis .......................................................................................................................20
   
   1.10 Targeted OMI and PDT Molecule ...........................................................................................22
   
   1.11 In Summation ...........................................................................................................................23

2. **Strategy for Targeted BrCa Imaging and Photodynamic Therapy** ........................................23
   
   2.1 Imaging dye for OMI ..................................................................................................................24
List of Figures

Figure 1. The singlet ground state, the singlet excited state, and triplet excited state which is fundamental to the photochemical mechanism of PDT. .................................................................7

Figure 2. The photochemical reactions a photosensitizer undergoes following light activation to initiate cell death in photodynamic therapy. Figure provided by Katherine Leising, illustrator, Personal Healthcare Technology (PHT 180), RIT, 2022. ................................................................................8

Figure 3. The three tetrapyrrole frameworks that many PS dyes derive from porphyrins (aromatic), chlorins (anti-aromatic), and bacteriochlorins (aromatic).............................................................12

Figure 4. The structure of chlorin-derived photosensitizer dye HPPH (2-[1-hexyloxyethyl]-2-devinyl pyropheophorbide-a)..........................................................................................13

Figure 5. The structure of breast cancer targeting decapeptide 18-4........................................................................16

Figure 6. Modular approach to adding imaging agents to a targeting molecule to create a TMIA. The approach uses “puzzle pieces” in which the dye is attached to the side chain of an Fmoc-protected lysine which can be brought into a peptide through a coupling reaction.39.....................21

Figure 7. CFM images of Triple negative MDA-MB-231 BrCa cells stained with M2 TMIA in collaboration with Dr. Irene Evans, GSOLS, RIT ..................................................................................24

Figure 8: (Left) Probe design with the wavelength of NIR dye (IRdye800cw, Abs 775/Em 800nm) and a HPPH type PS dye (Abs 633 nm). (Right) Probe design with the wavelength of NIR dye (Cy5.5, Abs 680/Em 710 nm) and PS dye padoporfin (Abs 750 nm) for dual-modal TMIA-MTPS agents. ..........................................................................................................................26

Figure 9: Solid phase synthesis (SPPS) of BrCa targeting peptide 18-4 on polymeric Sieber resin.13 ........................................................................................................................................27
Figure 10: Synthesis of a single-modal TMIA containing Cy5.5-3S on the 3rd lysine residue of 18-4.

Figure 11: Synthesis and structure of proposed chlorin conjugate, an MTPS for PDT of BrCa for irradiation at 660 nm.

Figure 12: Dual modal MTPS-TMIA with Cy5.5-3S at the third lysine residue and HPPH on an added 11th lysine residue of the 18-4 peptide.

Figure 13: Chlorin e6 trimethyl ester, the chlorin chosen as our starting material for chlorin-based photosensitizer synthesis.

Figure 14: The two-pot synthesis of HPPH as described in previous literature.

Figure 15: The structure of the methyl pyropheophorbide-a (1), the product obtained from the Dieckmann condensation followed by a thermal decarboxylation of chlorin e6 trimethyl ester.

Figure 16: The optimized synthesis of methyl pyropheophorbide-a (1) from chlorin e6 trimethyl ester.

Figure 17: Pyropheophorbide-a, CAS No.: 24533-72-0, the pheophorbide with an acid functionality that was commercially obtained to continue photosensitizer synthesis.

Figure 18: The hydrobromination product resulting from the addition of HBr across the peripheral double bond of pyropheophorbide-a.

Figure 19: The methoxy substitution (a, left) and the hexyloxy substitution (b, right) of the hydrobromination product facilitated with methanol and hexanol respectively.

Figure 20: The reaction scheme for the hydrogenation of pyropheophorbide-a.

Figure 21: The structure of meso pyropheophorbide-a (2), resulting from the hydrogenation of pyropheophorbide-a.

Figure 22: The absorption spectra for mPPa (2), with maximums at 407 nm and 660 nm.

Figure 23: The fluorescence spectra for mPPa (2), with a maximum emission at 661 nm.
Figure 22. The NHS ester activated coupling of mPPa to 3-dLys of 18-4 targeting peptide to yield 6.

Figure 23. The NHS ester activated coupling of Cy5.5-3S to 3-dLys of 18-4 targeting peptide to yield 8.

Figure 24. a) The final MTPS 7 after Fmoc deprotection. b) The final TMIA 9 after Fmoc deprotection.

Figure 25. The NHS-ester activated mPPa puzzle piece for addition to the N-terminus of 18-4.

Figure 26. The Dde protected 18-4 peptide prepared for conjugation at the N-terminus.

Figure 27. The MTPS product resulting from the coupling of the activated mPPa puzzle piece and Dde protected 18-4.

The synthesis of the MTPS was halted at 12 without deprotection of the Fmoc or the Dde protecting groups due to the small amount of yielded product. However, in the experience of Matt Law in our lab that has worked more familiarly with Dde, its removal, which is facilitated by hydrazine, is not ideal in difficulty and byproducts.

Ultimately, it was determined that Dde is not a suitable protecting group for this purpose. The conditions for its removal caused degradation of the peptide, resulting in very low yields of deprotected product after purification. Thus, the value in testing this strategy was ultimately to spare future students who follow in similar synthesize and to steer them into a more viable strategy.

The advice for future researchers is that it would be wise to either make use of a protecting group that is more easily removed, or to add a dye at that position first in the synthesis of a dual-modal molecule in future syntheses, or to simply add a blocking group such as a acetyl or phenyl amide which should have no effect on binding.
1. Introduction:

1.1 Overview

The goal of the research undertaken herein is the synthesis of a targeted molecule consisting of a porphyrinoid-based photosensitizer (PS) coupled to the peptide 18-4 to enable the targeted treatment of breast cancer by photodynamic therapy (PDT). A further goal for this project is to create a multimodal molecule with the breast cancer (BrCa) targeting peptide 18-4, a PDT photosensitizer, and a near infrared (NIR) dye for optical molecular imaging for the purpose of visualizing breast cancer and directing not only surgical procedures such as lumpectomy but also the targeted PDT. If effective, such an agent would be a selective and targeted molecule for the therapy of breast cancer without the harsh side effects that are present in other types of cancer therapies.

Moreover, the combination of a PS dye combined with an imaging dye would avoid the use of ionizing radiation that is inherent in imaging techniques such as X-ray, and CT based mammography and PET imaging. In the broader scope, the process developed here would have the potential application to other cancers by utilizing other targeting modules as well as the use of other PS modules and when needed, combined into dual agents to include imaging agents for various modalities.

To understand the importance and function of this goal, cancer, and BrCa specifically, must be understood first, along with the viable treatment options and what the major drawbacks and limitations of these treatments are. We will then discuss photodynamic therapy as a cancer treatment option and solution as well as its mechanism of action following with the use of photosensitizers.
Secondly, the important class of photosensitizer (PS) dyes known as chlorins, which are related to the porphyrin family, will be discussed. In particular, the specific chlorin 2-[1-hexyloxyethyl]-2-devinyl pyropheophorbide-a (HPPH) is historically important in PDT research, and will form the basis of our synthesis.

Thirdly, the targeting strategy will be presented. For this purpose, a peptide discovered by Professor Kaur, “Peptide 18-4”, will be thoroughly examined as a tool for the specific targeting of breast cancer (BrCa).

Lastly, molecular imaging and various imaging modalities, including optical molecular imaging (OMI), will be discussed and then we will discuss the benefits and nature of modular synthesis as an approach for total synthesis.

1.2 Cancer

Cancer is a persistent disease that takes the lives of hundreds of thousands of individuals each year and affects millions. It is the second lead cause of death in the United States with cardiovascular disease as the number one.¹ At the beginning of 2022, the American Cancer Society estimated that there would be 1,918,030 new cancer cases and 609,360 cancer deaths for the year in the United States alone.¹

Cancer by definition is applied to various types of abnormal, unhealthy cells that grow at uncontrollable rates and usually form masses.² This often results in the loss of normal body organ function and cancer cells have the potential to metastasize, or spread, to other areas of the body where they continue to grow. It can metastasize to nearby organs or travel through the bloodstream or lymphatic system as well.² Cancers are typically classified by either the organ the cancer originates from or the type of cells that have become cancerous.
Early detection and treatment of cancerous tissue are crucial to the viability of those unfortunate enough to develop this disease.

Breast cancer in particular is the 2nd most common cancer in women, and nearly 12% of women in the United States will be diagnosed with breast cancer at some time in their lifetime.\(^3\) At the beginning of 2022, it was estimated that there would be 290,560 new cases of breast cancer in the United States and 43,780 deaths caused by breast cancer with 2,710 of those cases and 530 deaths being in men.\(^1\) Breast cancer can develop in several different locations and types of tissue of the breast, with cancer that begins to grow into the healthy tissue (either within or outside of the breast) being considered invasive.

There are three main subtypes of breast cancer based on the presence or lack of different receptors in the cancer cells. They either have the hormone receptors of estrogen and/or progesterone, human epidermal growth factor 2 receptors (ERBB2, formerly called HER2), or lack all three of these receptors. They are referred to as hormone receptor (HR) positive/ERBB2 negative (HR+/ERBB2-), ERBB2 positive (ERBB2+), and triple-negative respectively.\(^3\) HR+/ERBB2- is the most common type of these three, making up nearly 70% of breast cancer cases with ERBB2+ making up 15-20% and triple-negative, where the cancer has no response to hormone therapy, making up 15% of cases.\(^3\)

A staging system is used to identify the location, size, and extent of the spread of the cancer with five stages, ranging from stage zero through stage IV.\(^4\) Stage zero is considered non-invasive as the tumor has not spread to normal surrounding tissue, whereas stages I through IV are considered invasive breast cancer.\(^4,5\) Stage I is a very early stage where cancer has invaded a small limited area of normal tissue and is up to 20 mm in size.\(^5\) Stage II is a localized stage of further growth where the tumor has spread to the lymph nodes and is between 20-50 mm in size.\(^5\) Stage III is marked by regional spread where the tumor has either growth larger than 50 mm or has
spread further to a greater number of lymph nodes, the skin, or the chest wall. Stage IV is metastatic breast cancer, it is the most severe stage where the cancer has spread to other organs.\textsuperscript{4,5}

Early detection means that any potentially cancerous tumors can be evaluated and then appropriately treated at a lower severity (or stage) and prevalence (being contained to a single site) than if detection happened with a later stage cancerous tumor, which would have less viable and more severe treatment options.

1.3 Treatment Options

The options for treatment are often limited depending on many factors including the patient’s financial status, physician specialty, existing health conditions, stage of the cancer, location and prevalence, treatment availability, and patient preference.\textsuperscript{6} In the case of breast cancer, treatment can also depend on the subtype of the cancer. Many treatments also come with a laundry list of adverse side effects and consist of surgery, chemotherapy, hormonal therapies, radiation therapies, or some combination thereof.\textsuperscript{6} Each has their own risks and benefits as treatment options as well as some intrinsic limitations. The most common treatment for early-stage breast cancer (stage I or II) in women is breast-conserving surgery with adjuvant radiation therapy. Whereas, the majority of patients with stage III breast cancer undergo mastectomy, many of whom also undergo adjuvant chemotherapy.\textsuperscript{7} In metastatic breast cancer (stage IV) most women undergo radiation and/or chemotherapy for treatment.

Surgery to rid the body of cancer is largely dependent on the imaging available to know the size and location of a cancerous tissue for how effective it is. Surgery is most effective in the case of solid tumors that are contained to only one site and haven’t yet metastasized. The two main types of surgery for breast cancer are breast-conserving surgery (BCS) also called lumpectomy, the
removal of the cancerous area and a small amount of surrounding tissue, and mastectomy, the removal of the entire breast.

In BCS, there is a potential that some of the cancerous tissue or precancerous tissue could be left behind or that the cancer could reoccur after surgery depending on the accuracy of the procedure. Thus, it is common for BCS or mastectomy to be followed by either radiation, hormonal therapy, or chemotherapy as an adjuvant therapy to kill any remaining cancer cells left in the body and reduce the chance of it coming back.

Chemotherapy is the use of drugs to kill cancer cells and prevent them from growing. Chemotherapy drugs can be grouped in the following classes based on their mechanism of action: alkylating agents, anti-metabolites, anti-tumor antibiotics, topoisomerase inhibitors, and tubulin-binding drugs.\(^8\) Chemotherapy in particular provides a well-established increase in the probability of survival, but due to their mechanisms of action various chemotherapy agents have different types of toxicity which presents dangers to other parts of the body and major side effects like nausea, vomiting, arrhythmia, anorexia, opportunistic infections, alopecia, and edema that are lessened by using a lower dosage.\(^9\) This is due to the function of chemotherapy agents to kill rapidly growing cells and though cancer cells are the most effected, healthy cells that are fast-growing can be damaged as well.

Hormonal therapy is a viable option for treatment in cancers that use hormones to grow. Hormonal therapy works by either blocking the body’s ability to make these hormones or alter how these hormones behave. Typical classes of drugs for hormonal therapy are selective estrogen receptor modulators (SERMs), selective estrogen receptor downregulators (SERDs), aromatase inhibitors, and luteinizing hormone releasing hormone agonists.\(^10\) However, some cases of breast cancer are resistant to hormonal therapy and many of the current hormonal therapies have side effects like bone loss, arthralgia, hot flashes, fatigue, and increased risk of endometrial cancer and
thromboembolism.\textsuperscript{10,11} Hormonal therapy also cannot be used for triple negative breast cancer as it lacking the hormone receptors necessary for the treatment to be beneficial.

In radiation therapy, high energy (x-ray, proton, or electron) rays are targeted at cancerous tissue where the radiation forms ions. These ions deposit energy in the tissue cells they pass through, which can kill cancer cells or cause damage to genetic material that can result in cancer cell death.\textsuperscript{12} Radiation therapies, however, are not able to be done for all types and stages of cancers. It also isn’t always effective on its own and requires combination with other modalities in order to garnish the desired effect on the cancer in question.\textsuperscript{12} Radiation therapies are also prone to side effects due to damage to healthy cells including long-term fatigue (with some causes being severe or intolerable), skin reactions (including swelling, blisters, and burning), pain, damage to healthy tissue, and sleeping difficulties.\textsuperscript{13,14}

Therefore, treatments with reduced side effects are needed for better quality of life of cancer patients. However, these treatments still need to maintain their effectiveness. In order to achieve such a favorable combination, the treatment should be a targeted therapeutic approach to selectively affect only the cancerous cells over healthy cells to the greatest extent possible.

\subsection*{1.4 Photodynamic Therapy}

Photodynamic therapy (PDT) is a relatively new treatment modality that functions through the administration of a photosensitizer, a light sensitive molecule. This molecule undergoes a series of photochemical reactions after exposure to light that results in an oxygen species that kills the cells that uptake the photosensitizer.\textsuperscript{15} The photochemical reactions involved occur between the photosensitizer and dissolved oxygen present within the cancerous cells. The photosensitizer absorbs the light, whose wavelength coincides with the photosensitizer’s absorption spectrum, and
is excited from its singlet ground state to its singlet excited state. Some of the generated singlets relax through fluorescence or vibrational relaxation, but the rest undergo intersystem crossing to the triplet state with the corresponding reversal of the spin state, as seen in Figure 1.16

![Diagram of singlet ground state, singlet excited state, and triplet excited state.]

**Figure 1.** The singlet ground state, the singlet excited state, and triplet excited state which is fundamental to the photochemical mechanism of PDT.

The process of intersystem crossing (ICS) is a spin-forbidden transition as it violates the spin conservation rule due to the transition from different states of multiplicity, in this case singlet to triplet state. However, in the case of photodynamic therapy it is a competitive process in relation to the other forms of decay of the excited singlet state due to spin-orbit coupling. Furthermore, the rate at which the photosensitizer undergoes intersystem crossing over other forms of decay is dependent on the structure of the photosensitizer and the presence of “heavy” atoms.17

From there two types of reactions can occur with oxygen. In a Type I reaction, the triplet excited state photosensitizer transfers either an electron or a hydrogen to a substrate of the cell (the cell membrane or nearby molecules) to form radical ions. These radical ions interact with ground state oxygen to produce reactive oxygen species (ROS), usually in the form of superoxide anion radicals ($O_2^\cdot-$).16 While superoxide anions are not very reactive in biological systems, they react further to generate other ROS that are very reactive and perpetuate this reaction cascade that can lead to oxidative stress and ultimately cause cell death.16,18 This photochemical process is the basis of PDT and is shown in Figure 2.
In the case of our MTPS agents, the main driver is likely the Type II reaction, as it is believed to contribute more towards the efficiency of PDT in our porphyrinoid PS dyes. In the Type II reaction, a direct energy transfer occurs between the triplet excited state photosensitizer and ground triplet state oxygen. This is possible due to the photosensitizer and the oxygen having the same triplet state spins, and the chlorin (porphyrinoid) type of PS dyes are well suited for this.\textsuperscript{16} The ground state triplet oxygen is excited into its singlet state, which is highly reactive and though short-lived, is able to cause damage that cascades toward both programmed (apoptosis) and non-programmed (necrosis) cell death pathways as well as autophagy depending on the cell type, photosensitizer used, and its intracellular location.\textsuperscript{19,20}

\textbf{Figure 2.} The photochemical reactions a photosensitizer undergoes following light activation to initiate cell death in photodynamic therapy. Figure provided by Katherine Leising, illustrator, Personal Healthcare Technology (PHT 180), RIT, 2022.
While the properties of the photosensitizer used affects the pathway by which PDT induces cell death, there is no singular cell death pathway that is shown to lead to cell death after PDT treatment.\textsuperscript{21} Which cell death pathway is induced and how it is induced is most dependent on where the chosen PS is localized in the cell as that is where the singlet oxygen or other ROS will cause oxidative damage in the cell. Apoptosis is observed when the PS localizes at the mitochondria due to Bcl-2 damage, an apoptosis regulator protein, and cytochrome c release or at the cytoplasm due to nuclear factor kappa B, NF\textsubscript{k}B, damage which hinders anti-apoptotic gene stimulation.\textsuperscript{20,21} However, photodynamic therapy can also induce necrosis though it is harder to study the factors that cause cellular necrosis. It is accepted that higher doses of either or both the PS dye or the light dose administered for PDT favors necrosis over apoptosis.\textsuperscript{20} However, more lipophilic photosensitizers are also considered to cause necrosis as they localize in membranes and cell membrane disintegration and loss of plasma membrane integrity are evident signs of necrosis.\textsuperscript{20–22}

\subsection*{1.5 Photosensitizers}

Some photosensitizers can accumulate in cancerous and precancerous tissue at a greater rate than in healthy cells. Thus, PDT shows great promise as an alternative therapy as well as being synergistic and able to be implemented along with another treatment option.\textsuperscript{23} PDT is a USFDA (United States Food and Drug Administration) approved treatment for endo-bronchial and endo-esophageal cancer as well as premalignant and early malignant lesions that occur at the skin, stomach, oral cavity, bladder, and breast.\textsuperscript{20} PDT has even been considered as a treatment for those who have undergone radiation for breast cancer but experienced chest wall recurrence that the radiation could not salvage with gratifying results.\textsuperscript{24}
A good photosensitizer for cancer is pure and of known composition, has limited toxicity in dark conditions and only exhibits cytotoxic nature in the presence of light. It is preferentially retained in the target tissue, leaves the body quickly with low systematic toxicity, displays a high yield for the photochemical event (the generation of singlet oxygen), and exhibits a strong absorbance with a high extinction coefficient in the 600-800 nm range such that the light is energetic enough to produce singlet oxygen as well as exhibit maximum tissue penetration of light.25

As current PS dyes used in PDT are not targeted, their efficacy is based on their preferential accumulation in cancerous cells (as well as precancerous cells) which is thought to be due to lower intracellular pH, poor lymphatic drainage, leaky microvasculature, greater amounts of collagen, and elevated levels of low-density lipoprotein (LDL) receptors.26 They can be localized in different organelles of cancerous cells, including mitochondria, lysosomes, endoplasmic reticulum, Golgi apparatus, and plasma membranes.20 The localization and distribution of the photosensitizer is affected by its solubility and charge. Hydrophobic species bind to LDL while hydrophilic compounds bind to both globulins and albumin, whereas cationic species collect in the mitochondria and anionic species are often found in the lysosomes.26

Photosensitizers are categorized into three generations. First generation photosensitizers are the oldest and first used photosensitizers starting in the 1970s, including Photofrin®, which still remains as the most commonly used photosensitizer.16 Photofrin®, or porfimer sodium, is a mixture of oligomers with porphyrin units connected by ether or ester linkages.27 However, first generation photosensitizers had low chemical purity, poor tissue penetration, and long half-lives that caused hypersensitivity of the skin to light for weeks after treatment.

Second generation photosensitizers aimed to offset these downfalls, bringing forth hundreds of new molecules with higher chemical sensitivity, better tissue penetration, and greater singlet
oxygen formation. They also demonstrated fewer side effects as a result of higher selectivity and faster removal from the body. Surprisingly, few of these were used in clinical trials and even fewer were officially approved for clinical use in anti-cancer PDT.

Third generation photosensitizers synthesized with a focus of developing substances with high affinity for tumor tissue and new drug delivery systems. These include nano-structures and targeted agents such as those we are developing, but this field is still in the infant stage.

Current photosensitizers that are in clinical or preclinical research include hematoporphyrin derivatives, core-modified porphyrins, phthalocyanines, chlorin and bacteriochlorin derivatives, texaphyrins, cationic dyes Rhodamine 123 and AA1, triaryl-methane cations, chalcogenoxanthylum dyes, Photofrin® (hematoporphyrin derivative), Levulan® and Metvix® (5-aminolevulinic acid derivatives), as well as Foscan® (m-THPC). Many of these are either cationic dyes or porphyrin-related compounds.

Cationic dyes show a great affinity for mitochondria that make them desirable. Their disruption of mitochondrial function increases the specificity of PDT. Their positive charge also likely increases the selectivity for cancer cells as they have higher metabolism and membrane potential. However, some cationic dyes aren’t very good photosensitizers. They inhibit mitochondrial processes, but the evidence of current cationic dyes of Rhodamine 123 and AA1 reflect that they inhibit further growth of cancer cells rather than actually initiating cell death. Thus, more recent cationic dyes require modification with heavy atoms like sulfur, selenium, or tellurium to replace the oxygen and nitrogen in these structures in order to increase singlet oxygen production and effectively initiate cell death.

Porphyrians are heterocyclic compounds composed of four pyrrole rings interconnected at their α carbons by methine bridges that are aromatic and absorb strongly in the visible light
spectrum. These porphyrin-related molecules are very efficient producers of singlet oxygen, which makes them very attractive considerations for PDT photosensitizers.\textsuperscript{28}

Chlorins are partially hydrogenated porphyrins and thus hold a similar core tetrapyrrole structure. Chlorins, however, absorb longer wavelengths over porphyrins while still remaining efficient singlet oxygen generators. This is a notable characteristic because short wavelengths of light cannot penetrate tissue as optimally.\textsuperscript{28} The increased light penetration of longer wavelength dyes is paramount to the process of PDT as shorter wavelength dyes cannot reach adequate depth of tissue penetration.

Many chlorins can be derived from natural sources like chlorophyll, but synthetic chlorins also seem to display potent phototoxic abilities. Bacteriochlorins are partially hydrogenated chlorins that possess one pi-bond less than chlorins and two pi-bonds less than porphyrins. They are naturally derived from bacteriochlorophyll that, as aptly named, is produced in bacteria. Bacteriochlorins absorb at even longer wavelengths than both chlorins and porphyrins.

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{tetrapyrrole frameworks.jpg}
\caption{The three tetrapyrrole frameworks that many PS dyes derive from porphyrins (aromatic), chlorins (anti-aromatic), and bacteriochlorins (aromatic).}
\end{figure}

It is important to note that with 22 pi electrons, porphyrins are an aromatic system as derived from the Huckel’s 4n+2 rule, whereas chlorins are anti-aromatic for having 20 or 4n pi electrons. Bacteriochlorins, with 18 pi electrons, are also aromatic. This has an impact on their
maximum absorption wavelengths, their bands most applicable for PDT being 630 nm, 650-700 nm, and 700-800 respectively depending on exact structure.\textsuperscript{30} Based upon theoretical molecular orbital calculations, his red shift is thought to be the consequence of the increase in the HOMO energies due to their destabilization with each subsequent reduction from 22 to 20 to 18 pi electrons.\textsuperscript{17} Meanwhile, the LUMO energies are not as sensitive to the number of pi electrons. Thus, this leads to a smaller HOMO-LUMO gap from porphyrin to chlorin to bacteriochlorin.

1.6 HPPH

HPPH (2-[1-hexyloxyethyl]-2-devinyl pyropheophorbide-a) also known as Photochlor\textsuperscript{®} is a chlorin derivative that has been investigated in clinical trials for esophageal cancer, non-small cell as well as early and late stage lung cancer, and basal cell carcinoma.\textsuperscript{29} The structure of HPPH can be seen in Figure 3.

![Figure 3. The structure of chlorin-derived photosensitizer dye HPPH (2-[1-hexyloxyethyl]-2-devinyl pyropheophorbide-a).](image)

More specifically, HPPH is a second-generation photosensitizer that strongly absorbs at 665 nm, allowing for deeper light penetration into tissue, and a pheophorbide.\textsuperscript{15,26} It is known to
selectively couple to the cytoplasm of cancer or precancerous cells and result in more mild photosensitivity after treatment compared to first generation photosensitizers that declines rapidly after a few days.\textsuperscript{31}

HPPH is a member of a series of ethers that originally derived from pyropheophorbide-a and the preparation of such derivatives traditionally involved tedious extractions that are simply unfeasible at a large scale.\textsuperscript{32} Thus, alternative synthetic routes have been explored to allow for HPPH to be synthesized at larger scales for clinical and commercial use. Instead of the tedious isolation of methyl pheophorbide-a from algae, this compound could be synthesized from chlorin e\textsubscript{6} trimethyl ester which is significantly easier to obtain.\textsuperscript{32} It is reported to be a two-step process with pyropheophorbide-a as an intermediate.

A critical aspect of the synthesis of targeted molecular agents for the PDT of cancer is availability of starting materials and replicable or trustworthy synthetic methods. In our experience, a number of published procedures, including the conversion from the trimethyl ester above, failed to yield the reported results and these included the synthesis of HPPH and its related precursors. Therefore, one of our goals became to develop and provide the research community with more reliable methods to synthesize useful PS dyes for PDT. A secondary goal was to synthesize such dyes that also contained a carboxylic acid functional group for the purpose of conjugating the PS dye to various targeting agents.

1.7 Targeting Breast Cancer

While PS dyes can accumulate in cancer cells, there is often non-specific targeting in the body as well. This means that light induced therapy can have considerable side effects off-target
and damage healthy cells. As we gain a better understanding of cancer, groups have been able to develop molecules that target receptors and proteins that are unique to cancer cells, thereby increasing specificity towards cancer cells over healthy cells, which will spare the healthy tissue from collateral damage.

For breast cancer targeting, the dodecapeptide, p160 (VPWMEPAYQRFL), a cancer specific peptide ligand that displays large preferential binding and internalization by breast cancer cells, was discovered early on.\textsuperscript{33-35} Of the numerous known specific types of breast cancer cells, it was found that this 12-mer peptide, p160, targets several of them including MCF-7 and MDA-MB-435.\textsuperscript{34,35}

Using p160 as a lead peptide, Dr. Kamaljit Kaur embarked on a quest to develop a smaller and more proteolytic stable peptide. In her early research, a decapeptide peptide, named 18 was identified and upon screening was shown to possess 3-fold greater binding affinity to MDA-MB-435 and MCF-7 breast cancer cell lines than p160.\textsuperscript{36} Peptide 18 also displayed negligible affinity for control noncancerous cells. However, peptide 18 is an $\alpha$-peptide much like p160 and is susceptible to \textit{in vivo} instability to proteases which hinders their bioavailability in both tissues and organs.\textsuperscript{36} Thus, analogues of peptide 18 were explored as potential solutions to this proteolytic susceptibility while still maintaining high affinity for cancerous cells over noncancerous cells.

The decapeptide 18-4 (WxEAAYQRFL), was one such analogue of peptide 18, with two L- to D-amino acid replacements, exceptionally high proteolytic stability in biological fluids, and even higher uptake by breast cancer cell lines (MDA-MB-435, MDA-MB-231, and MCF-7) when compared to peptide 18.\textsuperscript{33,36} 18-4, shown in Figure 4, binds to the surface receptors of breast cancer cells and is then rapidly internalized, usually through endocytosis. Endocytosis is the process
by which the cell membrane surrounds and engulfs external material then buds off inside the cell to form a vesicle.

It was later discovered that 18-4 is found to bind, along with p160, to the keratin 1 (KRT1) receptor on breast cancer cells. KRT1 is minimally expressed in normal mammary cells but is largely overexpressed in breast cancer cells. Thus, using 18-4 as a targeting molecule would significantly improve more selective uptake of any therapies tethered to it.

It is our hypothesis, that if 18-4 is attached to a photosensitizer, it would be selective for breast cancer cells, and therefore would have greater uptake of the given photosensitizer dye and greater efficacy in inducing cancer cell death.

![Figure 5. The structure of breast cancer targeting decapeptide 18-4.](image)

### 1.8 Cancer Imaging

As previously mentioned, early detection is crucial to one’s well-being as potentially cancerous tumors can be evaluated and diagnosed at an earlier stage. Earlier stage tumors have less severe treatment options and better chance of survival as it hasn’t spread or grown nearly as far. Imaging is also an important part of the process in the diagnosis and treatment of cancer as it aids in the visualization and understanding of the cancerous tissue including how severe it is, if it
has spread elsewhere in the body, how it has progressed, if there has been any recurrence, and the effectiveness of a treatment regime.

Imaging, in particular, aids a physician in choosing the appropriate treatment option as it visualizes cellular function and molecular processes and can reveal molecular properties of the tumor. There are five modalities of molecular imaging including ultrasound (US), X-ray computed tomography (CT), radionuclide imaging, magnetic resonance imaging (MRI), and optical molecular imaging (OMI). Most of them operate upon the principle of administering molecules into a living subject to target specific molecular processes and produce a signal that can be observed to provide insight about these processes.

Ultrasound is an imaging technique that utilizes the nature of how sound waves of high frequency travel through tissue. In particular, the probe sends out a series of ultrasound waves that enter the body with some of them being reflected back and detected by the probe. The detected waves are converted into electrical signals that are processed and displayed as an image based upon the distances and intensity of the reflections. Though often used without them, emerging contrast agents, in the form of lipid or biopolymer-coated gas-filled microbubbles, can be administered to boost the signal-to-noise ratio for reflections for blood in ultrasound. It also ultimately improves the sensitivity of the technique.

CT is an X-ray imaging technique that relies on the differing attenuation, loss of intensity, of X-rays by the tissues in the body in order to produce an image of internal anatomy. The scans appear as black and white with tissues that are poor at absorbing X-rays appearing as black and those that are strong absorbers of X-rays, like bone, appearing as white. CT differs from traditional X-ray scans through the use of tomography, imaging by sections, to generate a three-dimensional image. Oftentimes, a contrast agent is treated with iodine and administered to enhance spatial resolution and provide better contrast for soft tissues.
Radionuclide imaging includes positron emission tomography (PET) and single-photon emission computerized tomography (SPECT) techniques and makes use of radioactive isotopes to produce a signal. Both techniques can be used to evaluate biochemical changes or processes and have limitless penetration, meaning that no tissue or organ is too deep in the body to be targeted and imaged. The radioisotope selected for a PET or SPECT scan is specific and selective for the area of interest in the body.

PET is an extensively used imaging modality in the clinical setting and creates tomographic images from the signal that results from the radioactive decay of neutron-deficient radioisotopes that have been injected into the bloodstream. PET specifically operates using the property of radioactive isotopes that decay via positron emission and the nuclei of these isotopes convert a proton into a neutron, a positron, and a neutrino in order to stabilize itself from having an excess of protons over neutrons. The positron is ejected from the nucleus, losing kinetic energy as it interacts with electrons from the surrounding tissue, until it collides with an electron in a process called annihilation. The collided electron and positron convert into two photons of gamma-ray levels on the electromagnetic spectrum that travel out in opposite directions to be detected by the rings of the PET instrument.

SPECT operates similarly to PET but uses different radioisotopes that decay through the emission of single gamma-rays of varying energies. It is a common nuclear medicine modality that is used despite its lower sensitivity relative to PET and the inability of most SPECT imaging agents to provide specific biochemical information. This is in part due to the origin of a photon being unable to be traced from SPECT’s position detection. However, an advantage that SPECT has is the ability to image multiple biochemical targets simultaneously due to the nature of different nuclides producing gamma rays of differing energies. The radioisotopes used in SPECT also stay in the bloodstream (as opposed to being absorbed by surrounding tissue for PET radioisotopes).
and though this limits imaging to areas where blood flows, it also makes it valuable in applications where visualizing blood flow to tissues and organs is of importance—such as seizures, brain studies, stress fractures, tumors, and strokes.\textsuperscript{40}

MRI is an imaging modality that makes use of a strong magnetic field and radiofrequency energy to allow visualization of internal morphology and atomic nuclei by similar principles to nuclear magnetic resonance (NMR).\textsuperscript{38} Protons and neutrons within the nucleus are always in rotational motion, and if there is an unequal number of protons and neutrons this creates angular momentum and sometimes a small magnetic field if the atom has appropriate mass and charge. These nuclei when inside of an external magnetic field, such as in an MRI machine, act as magnetic dipoles that either align in parallel or anti-parallel with the magnetic field.\textsuperscript{38} The contrast between different tissues in MRI is due to the dipoles in these tissues having different relaxation times after a radiofrequency pulse.\textsuperscript{38} Clinical MRI most often uses the $^1$H nucleus as all $^1$H is magnetically active and is able to be detected from the water in tissues. MRI, however, can make use of contrast agents to help improve its relatively low sensitivity to improve the visualization of one type of relaxation over another.\textsuperscript{37,38}

Optical Molecular Imaging (OMI) is performed by using light to visualize cells and tissues with the most widely used modalities being bioluminescence imaging and fluorescence imaging. Similar to that of PDT, the utilization of low-energy photons limits the depth of penetration of these techniques. Bioluminescence imaging, unlike PDT and fluorescence imaging, relies on the passage of light out from the body, not into it. Instead, it takes advantage of luciferase enzymes and the light that is produced when they undergo an oxidation reaction with their substrate.\textsuperscript{38} Since tissues don’t naturally exhibit bioluminescence within the body, this technique possesses high sensitivities and signal-to-background ratios. Fluorescence imaging makes use of fluorescent
proteins, dyes, or other materials that exhibit fluorescent properties when under the appropriate wavelength to excite to fluorescent molecule.\textsuperscript{38}

The most significant challenge for this type of imaging is loss of intensity as the light passes through tissue and light scattering. This can be overcome to an extent by employing fluorescent imaging agents with emission in the near-infrared (NIR) with wavelengths in the range of 650 and 900 nm, where absorption of light by hemoglobin, water, and cytochromes within the body is minimal.\textsuperscript{38} Such absorption significantly reduces the fluorescent signal so minimizing it is advantageous. Choosing fluorescent imaging agents in this emission region also lends to increased sensitivity and the capacity to image deeper parts of the body.\textsuperscript{38}

\textbf{1.9 Modular Synthesis}

In creating this molecularly targeted photosensitizer (MTPS), the order of synthesis and how the targeting molecule will be linked to the PS is of great importance. Some linkages may have ineffective yields, reduce the effectiveness of the therapy or the targeting peptide, and may require several steps to achieve.

Modularity for total synthesis involves synthesis of an integrated system into small separate building blocks or “modules” that have the potential to be interchanged and can allow for a diverse array of structures that can be attained by varying the modules.\textsuperscript{18} It is an established approach to total synthesis in organic chemistry. Not only does it allow for potential interchangeability, it also reduces the number of steps especially in regards to protecting and deprotecting functional groups. A total synthesis without modularity may involve some steps for reactions of one part of the molecule that interfere and need protection to not react unnecessarily with another part of the larger molecule. Within a modular synthesis, the synthesis steps from one module are entirely independent and not affected by the synthesis steps of another.
One particular approach for modular synthesis for targeted applications has been reported by our research group for targeted molecular imaging agents (TMIAs) for the targeted use of detection and imaging of prostate and breast cancer.\textsuperscript{39,41} In this approach a synthesized imaging agent for a molecular imaging technique was attached to a targeting molecule with lysine as a linker as shown in Figure 6.

![Figure 6. Modular approach to adding imaging agents to a targeting molecule to create a TMIA. The approach uses “puzzle pieces” in which the dye is attached to the side chain of an Fmoc-protected lysine which can be brought into a peptide through a coupling reaction.\textsuperscript{39}](image)

One of the approaches we utilized follows a similar process. A photosensitizer module, in this case a HPPH-like chlorin, and the targeting module, \textbf{18-4} for the targeting of breast cancer, would be synthesized separately. An Fmoc-protected lysine-based module or “puzzle piece” containing the PS dye on the side chain is coupled through activation of the carboxylic acid to the N-terminus of the \textbf{18-4} peptide. To build a multi-modal agent for both imaging and treatment, an optical molecular imaging module can be further added to this molecule in a similar manner.
1.10 Targeted OMI and PDT Molecule

In order to design a dual modal agent for imaging and therapy there are two strategies that were contemplated. In the first strategy, the imaging dye can be attached to the lysine on the third lysine residue on 18-4, and the PS dye can be attached to the N-terminus of the tenth tryptophan residue via a modular approach. In this approach the two dyes could be switched as well, depending on synthetic ease. There is evidence from Dr. Kaur, that dyes in either position do not affect binding adversely, but in discussions with her, it was determined that dyes in BOTH positions have not been tested. So this will be a preliminary goal.

The second strategy would be to use two puzzle pieces in an entirely modular approach where the imaging dye could first be added as part of a lysine module followed by conjugation of a lysine module containing the PS dye. Thus, the OMI module and a PDT module could be attached in series to the same targeting peptide which would allow for one targeted molecule to be utilized for both treatment and detection.

PDT and OMI being both light-based techniques provide some unique and interesting applications that can be undertaken due to their similar modalities and properties. If a fluorescent imaging agent and a photosensitizer of different absorption properties are utilized then one wavelength of light can be administered for fluorescent imaging and then another for the activation of the photosensitizer for PDT. If the desired activation order is reversed then bioluminescence imaging could preferably be used since the main side effect of PDT is photosensitivity of the skin and bioluminescence imaging requires no external light source. In either case, it opens the doors to several different light-based therapy and visualization combinations.
1.11 In Summation

Understanding and finding new ways to treat and image cancer are significant steps in helping individuals against this persistent and all too prevalent disease. The research undertaken and described utilizes synthetic organic chemistry as an avenue to help steer advancements of current techniques toward the design of a multifunctional targeted treatment guided by imaging. In particular, this is being done with a HPPH-type photosensitizer and targeting peptide 18-4 for treatment of breast cancer by photodynamic therapy with the addition of an optical molecular imaging module.

Now that a necessary background in the areas of cancer, breast cancer, treatment options, photodynamic therapy and photosensitizers, targeting peptides, molecular imaging, and modular synthesis has been discussed, the goal and function of this research is put in perspective and its importance evident. Thus, the specific synthetic route and research itself can be discussed along with the results and drawn conclusions.

2. Strategy for Targeted BrCa Imaging and Photodynamic Therapy

The ultimate goal in our research group, which is supported by a recently awarded NIH grant as well as a research award from the Foundation for Women’s Wellness, is to position two dyes on a targeting peptide, 18-4. One of these dyes will be for fluorescent imaging and the other dye will be for photodynamic therapy (PDT).

While the longer-term goal is a compound with two dyes, it is important to first make each as a single dye targeted agent separately, and then to test both the binding to BrCa cells and the fluorescence and PDT properties. According to Dr. Kaur, there are two positions where dyes can be added and not interfere with binding to the BrCa keratin 1 receptor. These are the lysine, the
third amino acid (from right to left), and the N-terminus after the tenth amino acid of tryptophan. The first goal was to test adding the dyes on the lysine in the third position.

For this initial goal, it was important to first synthesize the 18-4 peptide with only one fluorescent dye on the third amino acid residue, and a second agent with only one PS dye on that same position. These goals became the foundation of the work presented here.

2.1 Imaging dye for OMI

In previous work, a non-sulfonated Cy 5.5 dye was attached to the 18-4 peptide on the 10th tryptophan residue. In preliminary results, this was shown to bind to MDA-MB-231 BrCa cells by confocal fluorescence microscopy (CFM) as shown in Figure 7. However, the water solubility was insufficient for optimal results *in vitro* and would not have been suitable for *in vivo* testing.

*Figure 7. CFM images of Triple negative MDA-MB-231 BrCa cells stained with M2 TMLA in collaboration with Dr. Irene Evans, GSOLS, RIT*

To solve this problem, we chose a dye that was prepared and published by our lab which contained three sulfonates and was compatible with 630 nm, which is the highest wavelength laser
available in our CFM instrument. The peak absorption of Cy5.5-3S is 680 nm with fluorescence emission (Em) at 710 nm as shown in Figure 8, with adequate separation from the absorption of hemoglobin (Figure 8) to facilitate fluorescence or “light directed therapy” and to enhance monitoring the progress of PDT in vivo.

The ultimate three-year goal of the NIH project is to incorporate higher wavelength imaging dyes such as IR800cw, along with the PS dyes such as HPPH as shown in Figure 8 a. However, Cy5.5-3S is still important as an alternative strategy of using a lower wavelength imaging dye such as Cy5.5 and a higher wavelength PS dye such as padeliporfin (WST-11, or TOOKAD) as shown in Figure 8b.

However, the initial goal will focus on single dye molecules. The first will be a TMIA containing Cy5.5 to study the binding by CFM, and the second will be a MTPS containing an HPPH type dye which was to be determined by synthetic studies presented here.

### 2.2 PS dye for PDT

Porphyrins have been useful in PDT due to their remarkable propensity to create reactive oxygen species (ROS). As described in the introduction, the PS dye class we will begin with is the chlorin type represented by HPPH. With peak absorption at 660 nm, this dye could be used in tandem with the imaging dye IRdye800cw as the higher wavelength dye (Abs 775/Em 800nm) as shown in Figure 10B.
While the synthesis of Cy5.5-3S was thoroughly optimized and published by our group, the field of porphyrin dyes was previously unexplored in our group. In addition, there were no commercially available clinical PS dyes that could be obtained for purchase on synthesis scales as well as no PS dyes that were available that also contained a mono-acid functionality necessary for coupling. Moreover, we found that literature methods for preparation of these were scarce and we found the methods that were reported were often brief and lacking details, and as described below often non-reproducible.

Thus, our first goal was to achieve the synthesis of a chlorin type porphyrin with the same properties as HPPH, but that contained an acid functional group that would enable conjugation to targeting groups such as the peptide 18-4.

2.3 Synthetic Methodology: Strategy to Synthesize MTPS for PDT of BrCa

The design and synthesis of 18-4 based MTPS probes will be analogous to the synthesis of the TMIA M2, which is meticulously described in a M.S. thesis by Xinyu Xu, assisted by an
The solid phase peptide synthesis (SPPS) of decapeptide 18-4 was modified from the Kaur synthesis to enable the use of Sieber resin.

In our preliminary research, the Sieber resin allowed for cleavage of the peptide from the resin with mild acid, specifically 1-2% trifluoroacetic acid. This provides two benefits: (a) all of the imaging and PS dyes we tested are stable in this medium, allowing synthesis by our modular method, and (b) after finding side-chain protecting groups for lysine, tyrosine and glutamic acid that are labile to mild acid the final cleavage step has been shown to be exceptionally clean with little or no bi-products that are customarily seen in strong acid.

The eleven-step SPPS synthesis including the starting peptide, shown in Figure 9 has been optimized and scaled up.

**Figure 9:** Solid phase synthesis (SPPS) of BrCa targeting peptide 18-4 on polymeric Sieber resin.\(^{43}\)
The first target for binding studies by CFM, was the peptide 18-4 with the Cy5.5-3S dye attached to the lysine in the 3-position as shown below. This was prepared as shown in Figure 10 below. While other students including Xinyu Xu, Matt Law, and Basant Kaur had worked out the synthesis of the peptide on solid phase resin, the cleavage step yielded a mixture of products.\textsuperscript{43-45}

Therefore, it was necessary to first optimize cleavage of peptide from the solid phase synthesis resin. After this was optimized, the second step was to attach the Cy5.5-3S dye to the lysine side chain as shown in Figure 10 below. After attachment of the Cy5.5-3S, the final step was to remove the Fmoc protecting group, followed by purification of the final TMIA.

![Synthesis of a single-modal TMIA containing Cy5.5-3S on the 3\textsuperscript{rd} lysine residue of 18-4.](image)

**Figure 10:** Synthesis of a single-modal TMIA containing Cy5.5-3S on the 3\textsuperscript{rd} lysine residue of 18-4.

The proposed synthesis design of the MTPS probe, shown in Figure 11, will bring in the PS dye HPPH in an analogous manner to the synthesis shown in Figure 10. In this case a PDT module (in black) is constructed then coupled to peptide 18-4 following the SPPS. It is important to note that we addressed water solubility concerns, and have shown that the peptide imparts solubility at the 50-100 micromolar (µM) level for biological testing.
Figure 1: Synthesis and structure of proposed chlorin conjugate, an MTPS for PDT of BrCa for irradiation at 660 nm.

A major advantage of the modular approach is that it can also be applied to the synthesis of dual-modal probes. Two modules, one for OMI and one for PDT can thus be coupled sequentially in the same SPPS synthesis to provide the dual TMIA-MTPS probe in one process as shown in Figure 12.

Figure 12. Dual modal MTPS-TMIA with Cy5.5-3S at the third lysine residue and HPPH on an added 11th lysine residue of the 18-4 peptide.
3. Results and Discussion

3.1 Synthesis of Photosensitizers

This project first focused on the synthesis of a molecularly targeted photosensitizer (MTPS). The PS dye class we first chose was the chlorin core structure based on chlorin e₆ trimethyl ester shown in Figure 13.

![Chlorin e₆ trimethyl ester](image)

**Figure 13.** Chlorin e₆ trimethyl ester, the chlorin chosen as our starting material for chlorin-based photosensitizer synthesis.

The first process of the synthesis, as described in the literature, and shown in Figure 14, involved the addition of potassium tert-butoxide (1M in tert-butyl alcohol) to chlorin e₆ trimethyl ester with 2,4,6-collidine used as a basic pyridine-like solvent.³² The reaction was allowed to stir at room temperature under argon for 20 minutes before being quenched with glacial acetic acid and then vacuum distilled. Fresh collidine was added back and then the reaction was heated to reflux (about 190 °C) under argon for 2 hours.
Based on the literature, this step was expected to cause the chlorin to undergo a Dieckmann condensation as well as a subsequent thermal decarboxylation and then saponification of the methyl ester of the pheophorbide system to result in the intermediate pyropheophorbide-a.

While we observed the Dieckmann and the subsequent decarboxylation product, we were unable to replicate the saponification product as described by Pallenburg et al.\textsuperscript{32} Thus, our results provided only the ester, methyl pyropheophorbide-a (1) as shown in Figure 15.

Figure 14. The two-pot synthesis of HPPH as described in previous literature.

Figure 15. The structure of the methyl pyropheophorbide-a (1), the product obtained from the Dieckmann condensation followed by a thermal decarboxylation of chlorin \textit{e}_6 trimethyl ester.
It is important to note that the literature procedure reported for the Dieckmann condensation followed by decarboxylation and saponification did not provide the acid product as reported. In optimizing the first step, we opted to use potassium acetate, instead of the potassium tert-butoxide followed by acetic acid, and heated this reaction to reflux with a condenser to an increased time of overnight for 2 nights (approximately 42 hours) to observe greater product formation of methyl pyropheophorbide-a (1). This process proved to be extremely repeatable and yielded significant product with minimal side products and residual starting material even when increased to scales of 200 mg and 250 mg.

![Chemical structure of methyl pyropheophorbide-a (1)](image)

**Figure 16.** The optimized synthesis of methyl pyropheophorbide-a (1) from chlorin e6 trimethyl ester.

The optimization of this conversion could prove very valuable if a future student in our group were able to refine the saponification step. As we were unable to replicate the saponification occurring during the reflux process, we transitioned to hydrolyze the methyl ester of methyl pyropheophorbide-a (1) in an independent process.
Based upon the conditions we observed in the literature for this process as well as similar processes for related compounds, we attempted various basic routes of hydrolysis.\textsuperscript{32,46,47} We utilized LiOH and KOH as bases along with combinations of tetrahydrofuran (THF), methanol, and water as solvents to induce hydrolysis. All of these hydrolysis efforts were unable to create a sufficient yield that could be isolated.

Taking a step back and reviewing the literature, we found that the desired hydrolyzed product pyropheophorbide-\(\alpha\), CAS No.: 24533-72-0 shown in Figure 17, became available commercially from BOC Sciences. As our focus was on making a molecularly targeted photosensitizer (MTPS) and not the photosensitizer synthesis process itself, we decided to move forward using this new commercial product for the photosensitizer synthesis.

![Figure 17. Pyropheophorbide-\(\alpha\), CAS No.: 24533-72-0, the pheophorbide with an acid functionality that was commercially obtained to continue photosensitizer synthesis.](image)

The next process in the synthesis of HPPH and related analogs is the addition of HBr across the peripheral double bond followed by the substitution of Br by a hexyloxy group. The addition of HBr is done by treating the pyropheophorbide-\(\alpha\) with 30\% HBr in acetic acid at room
temperature for 2 hours before removing the solvent by vacuum. The resulting solid is then
redissolved in dichloromethane (DCM) with potassium carbonate to make the reaction mixture
basic and hexanol is then added and allowed to stir for 45 minutes under argon atmosphere. The
structure of the hydrobromination product is as shown in Figure 18.

![Figure 18. The hydrobromination product resulting from the addition of HBr across the peripheral double bond of
pyropheophorbide-a.]

Attention had to be given to the solvents used in both the gradient and the sample
preparation for the analysis as polar solvents could undergo a substitution reaction. This was
fortuitously observed with methanol where the methoxy group readily replaced the leaving group
of the bromine. Thus, the methyl ether analog shown in Figure 19a was observed upon treatment
of a small sample with methanol, and we expected this to show much promise in the addition of
the hexyloxy with hexanol to create HPPH.

However, the removal of the HBr and acetic acid proved troublesome when done on the
whole reaction. Although a very small amount of HPPH (Figure 19b) was prepared, we learned
that this class of compounds was water insoluble and that for purposes of an injectable MTPS that had the hexyloxy group on the side chain would not be advisable.

Figure 19. The methoxy substitution (a, left) and the hexyloxy substitution (b, right) of the hydrobromination product facilitated with methanol and hexanol respectively.

For these reasons, rather than focus on this reaction, we turned to the synthesis of a hydrogenated analog as our main focus, and this reaction was handed off to a new graduate student in the lab to allow my work to focus on advancing to the attachment of porphyrins to the peptide.

In order to move forward in the development towards a porphyrinoid photosensitizer without unwanted side reactions, it was decided to reduce the peripheral double bond of pyropheophorbide-a through a hydrogenation reaction. The hydrogenation was facilitated by stirring the pyropheophorbide-a in acetone with 20% Pd on carbon black under a hydrogen atmosphere with a hydrogen balloon as shown in Figure 20.
Figure 20. The reaction scheme for the hydrogenation of pyropheophorbide-a.

This hydrogenation resulted in a near perfect conversion to the reduced form shown below in Figure 21 with no detectable byproducts. Following the naming conventions of a similar reduction of methyl pyropheophorbide-a that has been performed by Smith et al, this compound has been referred to as meso pyropheophorbide-a (mPPa, 2).48

Figure 21. The structure of meso pyropheophorbide-a (2), resulting from the hydrogenation of pyropheophorbide-a.
Given the success in producing an HPPH-like chlorin that contained the requisite acid for conjugation, and a reduced, non-reactive double bond could now focus our efforts on conjugation to the targeting peptide. Apart from the chromatography and mass spectroscopy results reported in the experimental section for (2), the compound gave the absorption results shown in Figure 22 which are important for the optical properties useful for being a photosensitizer dye for photodynamic therapy.

Figure 22. The absorption spectra for mPPa (2), with maximums at 407 nm and 660 nm

In discussion with our collaborators, the fluorescence properties of chlorin dyes, when irradiated at 400 nM were contemplated for use in confocal fluorescence microscopy. While the extinction coefficient is low, and the reported quantum yield is also low, a single modal compound containing this dye might suffice as an imaging agent along with also being a photosensitizer dye for PDT.49
It was surprising, but consistent with literature results that irradiation at 418 nM as shown in Figure 23 yielded an emission band at 661 nm thereby exhibiting a Stoke’s shift of 243 nM which is very large. This may lead to a further query that the emission at 661 nm being close to the Q band of absorption at 660 nm could “self-excite” via irradiation at 418 nm.

**Figure 23.** The fluorescence spectra for mPPa (2), with a maximum emission at 661 nm.

### 3.2 Preparation of BrCa Targeting Peptide 18-4

The BrCa targeting peptide, 18-4, was synthesized by solid phase peptide synthesis (SPPS) previously developed by Xinyu Xu and further modified by Matt Law and Basant Kaur as a scaffold for TMIAs.\(^{43-45}\) When fully synthesized, the targeting peptide is still tethered to the resin used in the SPPS and is fully protected on the necessary side groups to prevent side reactions during SPPS.
To use the peptide in further solution phase syntheses, the peptide must be cleaved from the resin and this step has been far from optimal in our lab. In this step, the protecting groups that are prone to acid treatment are also cleaved (deprotected) from the peptide itself. While these groups are base stable through the process of SPPS, they are cleavable in mild acid. Thus, both the cleavage from the resin and the removal of side protecting groups are accomplished by treating the 18-4 peptide TFA in DCM. Moreover, by using the Sieber Resin, a milder procedure using a low percentage of TFA in DCM (1-2%) is carried out.

However, these cleavage steps have been highly problematic in our hands. Though they many cleave well from the resin and yield completely deprotected peptide, save for the Fmoc group which is acid stable, it is common to see that some of the side protecting groups, such as t-butyl, will cleave from the peptide and they can add back to the backbone. This leads to well-known side-products. In addition, impurities thought to result from the polymer resin itself were observed, with some of these reacting with the peptide. This resulted in impurities, some of which are simply incomplete deprotection of some groups, while others are adducts containing unknown groups. These resulted in a much lower yield of usable peptide for further synthesis.

In past syntheses to make TMIA:s, this was partly solved by allowing the cleavage reaction to stir for an hour before being filtered, then TFA being removed by vacuum, and then fresh TFA solution being added back and continued to be stirred overnight. This was substantially worse when the entire cleavage process was run in the vessel containing the resin. Therefore, it was suspected that the resin either plays some role in or exacerbates the partial cleavage.

Given these difficulties in preparing completed 18-4 peptide, new methods were employed for the cleavage step. It was hypothesized that if the peptide is eluted as it is being cleaved from the resin it can be separated from the troublesome resin immediately on cleavage, which would minimize reaction with polymer bi-products.
We thus developed the method we termed as continuous flow cleavage. This method was used for Basant Kaur’s M3 TMIA product previously because the dye in the TMIA appeared to bind to the resin during cleavage. This was mitigated by providing a fresh and continuous flow of 1% TFA, thereby giving less of an opportunity for the dye to bind to the resin as it is being eluted and collected perpetually as it is cleaved.

In the same way, it was found that this method of continually providing a fresh flow of TFA solution was far better for cleaving the 18-4 peptide itself (even without a dye). Unlike the TMIA containing a dye, in this case there was no visual indicator for when the cleavage was complete. Peptide 18-4 by itself has no color, so it was necessary to assay numerous consecutive fractions by LC-MS to determine when the cleavage was complete.

To further optimize this step, it was determined that instead of concentrating the fractions immediately, it was necessary to allow them to sit at room temperature until the cleavage of all side protecting groups was verified by LC-MS. It was found that allowing the fractions to sit overnight was the optimal time for deprotection. Longer times than this were observed to cause further degradation.

After sitting overnight, the TFA solution was removed under vacuum. The peptide was then purified by solid phase extraction (SPE) to remove any partially protected intermediates and other impurities using 5 mM ammonium acetate (AmAc) as the aqueous mobile phase and a 5-100% acetonitrile (ACN) step gradient. The gradient was increased in 5% increments until 30% ACN and then increased in 10% increments for the remainder of the gradient. Pure fractions were identified by LC-MS, combined, and solvent removed under vacuum and then freeze dried three times. It was necessary to freeze dry multiple times to remove the AmAc as it can inactivate the N-Hydroxysuccinimide (NHS) esters used in subsequent coupling modules to the peptide.
With the peptide created by optimal cleavage conditions in hand, and a new SPE purification procedure in hand, we were ready to explore the attachment of fluorescent imaging dyes and dyes for photodynamic therapy (PDT) to peptide 18-4.

### 3.3 Synthesis of Targeted Agents

Using the purified peptide 18-4, both a MTPS using mPPa and a fluorescence TMIA using Cy5.5-3S were synthesized with the respective dyes on the d-Lysine at the third residue of the 18-4 peptide chain. The coupling reactions to make these agents were modeled after similar coupling conditions that have been used previously in our lab. Such coupling conditions were used to prepare the imaging modules as published.\(^3^9\)

These coupling conditions could then be used to conjugate the dyes to the 3 dLys position, or to the N-terminal free amine of Fmoc-D-Lys, to form “puzzle pieces” that are then added to the N-terminal of the peptide chain in the same manner as SPPS. This is done by activating the respective dye by making an NHS ester at the coupling site and then running the coupling reaction under basic conditions.

N,N-Diisopropylethylamine (DIPEA) is used as the base in these reactions and TSTU (N,N,N',N'-Tetramethyl-O-(N-succinimidyl)uronium tetrafluoroborate) was used as the N-succinimide reagent used to create the NHS ester at an acid on the respective group to be conjugated to the peptide. The NHS ester acts as an activating group for attaching these dyes to the side chain of lysine under these conditions.

Both mPPa and Cy5.5-3S were each coupled to the third dLys residue of 18-4 in this manner. The coupling of mPPa to 18-4 via the mPPa NHS ester (3) is shown below in Figure 22.
Figure 22. The NHS ester activated coupling of mPPu to 3-dLys of 18-4 targeting peptide to yield 6.

The coupling of Cy5.5-3S to 18-4 via the Cy5.5-3S NHS ester is shown below in Figure 23.
In the final step to reach the final desired MTPS and TMIA products, 6 and 8 were reacted with diethylamine (DEA) as a base for the removal of the base-labile protecting group of fluorenylmethoxycarbonyl (Fmoc) from the N-terminus of the peptide. The deprotected final MTPS (7), shown below in Figure 24a, was purified by SPE by the same gradient as is done for the 18-4 peptide. The deprotected final TMIA (9), shown below in Figure 24b, was then purified via prep-HPLC using a 10-100% ACN gradient in 0.1 M AmAc as the aqueous mobile phase. Both products are freeze-dried for removal of the AmAc buffer.

Figure 23. The NHS ester activated coupling of Cy5.5-3S to 3-dLys of 18-4 targeting peptide to yield 8.
A MTPS was also synthesized using the puzzle piece method, in which the mPPa NHS ester (3) was coupled to Fmoc-dLys-OH to create the PDT module or puzzle piece. Similarly to the activation of mPPa by the NHS ester, the PDT puzzle piece was activated by making the NHS ester at the C-terminus of the lysine as shown below.

Figure 24. a) The final MTPS 7 after Fmoc deprotection. b) The final TMIA 9 after Fmoc deprotection.
In order to allow for this puzzle piece to be conjugated to the N-terminus end of the 18-4 peptide, the third dLys residue must be protected to protect conjugation at that position. Instead of adding a protecting group back to the third dLys residue after cleavage, we decided to utilize d-lysine with a protecting group that is both acid and base stable in the 18-4 synthesis and would remain after cleavage in 2% TFA.

Thus, instead of using the usual \(4\)-methyltrityl (Mtt) protected d-lysine, a \(1\)-(4,4-dimethyl-2,6-dioxacyclohexylidene)ethyl (Dde) protected d-lysine was used in the construction of a new 18-4 analog, containing Dde in the 3-position. After cleavage by continuous flow with 2% TFA on the complete decapeptide, the peptide was then Fmoc-deprotected to make the N-terminus accessible for conjugation, and then purified by SPE and then the following product 11 was isolated and its structure was verified by LC-MS.

**Figure 25.** The NHS-ester activated mPPa puzzle piece for addition to the N-terminus of 18-4.
Figure 26. The Dde protected 18-4 peptide prepared for conjugation at the N-terminus.

The activated puzzle piece can then be coupled to the peptide to produce a MTPS with the photosensitizer attached to at the added eleventh residue of d-lysine as shown below.

Figure 27. The MTPS product resulting from the coupling of the activated mPPa puzzle piece and Dde protected 18-4.

The synthesis of the MTPS was halted at 12 without deprotection of the Fmoc or the Dde protecting groups due to the small amount of yielded product. However, in the experience of Matt
Law in our lab that has worked more familiarly with Dde, its removal, which is facilitated by hydrazine, is not ideal in difficulty and byproducts.

Ultimately, it was determined that Dde is not a suitable protecting group for this purpose. The conditions for its removal caused degradation of the peptide, resulting in very low yields of deprotected product after purification. Thus, the value in testing this strategy was ultimately to spare future students who follow in similar synthesize and to steer them into a more viable strategy. The advice for future researchers is that it would be wise to either make use of a protecting group that is more easily removed, or to add a dye at that position first in the synthesis of a dual-modal molecule in future syntheses, or to simply add a blocking group such as a acetyl or phenyl amide which should have no effect on binding.

4. Conclusions

The synthetic methods developed in this research has proven to be useful and practical in the synthesis of single modal MTPS and TMIA agents and will provide methods useful to those synthesizing future dual modal MTPS-TMIA agents.

The first aim of this research, to synthesize a chlorin-type photosensitizer dye, was successfully achieved. HPPH was used as a model for the photosensitizer synthesis and though it was not isolated specifically, an equally useful and less lipophilic analog of HPPH called mPPa was successfully synthesized in a robust and reproducible manner.

Next, the cleavage and purification of the 18-4 peptide from the Sieber resin was modified and optimized through the use of continuous flow method and an SPE method to provide vastly improved yields of pure peptide without byproducts.
With the synthesis of a suitable PS dye, and the vastly improved synthesis of 18-4 peptide, the stage was set for the synthesis of two novel MTPS agents and one TMIA agent, each containing the 18-4 peptide to target breast cancer cells. Utilizing analogous techniques for solution phase coupling and deprotection, all three targeted agents were successfully synthesized and characterized by HPLC-MS.

However, when we began this work, the peptide was available in meager quantities, resulting in the need to work at small scales (10-20 mg peptide). The resulting yield lost in purification was significant on this small scale. However, it is comforting to know that through the modifications developed, the current students working on this have been able to prepare much larger quantities of 18-4 peptide.

Though the cleavage and purification were optimized for greater yield of pure peptide, it could be even further improved upon in the future. Another possible cause for loss of yield is the potential degradation of the PS dye in the presence of oxygen and white light conditions due to its photochemical properties. We speculate this based on the loss of yield during purification procedures (from 10 mg to 1 mg product after purification for example, when the HPLC-MS results indicate there should be significantly more yield).

In addition, we found that both MTPS agents we developed were too hydrophobic to effectively dissolve in water, requiring 5 % DMSO, thus likely not being ideal for use in human cells. However, they provide a foundation upon which more water-soluble MTPS agents can be synthesized and optimized for future use in the treatment of BrCa by PDT.

Furthermore, the methods developed here will be useful to combine TMIA with an MTPS in this modular synthetic approach to produce a dual modal MTPS-TMIA agent which can potentially be synthesized to both visualize and treat cancer. This is an anticipated future goal of
our lab using the 18-4 peptide, a fluorescence dye, and a chlorin-type PS to create a dual modal agent for targeted breast cancer visualization and treatment.

5. Experimental Procedures

5.1 Materials and Methods

Chemicals were purchased from Sigma Aldrich, Acros Organics, Fisher Scientific, Alfa Aesar, Beantown Chemical, Macron, TCI, VWR, Creosalus, AnaSpec Inc., and were used as received unless otherwise stated. All were HPLC or American Chemical Society grade. Photosensitizer starting materials were purchased from Frontier Scientific and BOC Sciences. The HPLC-MS used was a Waters 2695 Alliance HPLC with a Waters 2998 Diode Array Detector and a Waters 3100 SQ Mass Spectrometer. The Ultra High Performance Liquid Chromatography-Mass Spectrometer (UHPLC-MS) used is a Shimadzu LCMS2020. Mass spectra from these instruments were recorded at unit resolution with positive and negative modes at 35 V cone voltages. All aqueous mobile phases for HPLC-MS and UHPLC-MS are 0.1M ammonium acetate unless otherwise noted.

Preparative HPLC (prep-HPLC) was carried out with a Waters 600E system controller, and Waters 600 multi-solvent delivery system using a 30 mL/min flow rate with 0.01 M ammonium acetate used for aqueous mobile phase. For SPE purification, a 2 and 20 g C-18 Sep-pack Varian Mega Bond Elut SPE cartridges were utilized for 18-4 as well as TMIA and MTPS purification. The aqueous mobile phase for SPE used was 5 mM ammonium acetate unless otherwise stated. The SPE cartridges were conditioned with their initial gradient concentration and gradients were performed in 5% increasing to 10% increments with 5-10 mL fractions each unless otherwise noted.
High resolution mass spectra (HRMS) were obtained on a Waters Synapt G2Si (School of Chemical Sciences, University of Illinois at Urbana-Champaign) using the following parameters: Flow injection at flow rate of 0.1 ml/min, H2O/ACN/0.1% Formic Acid, positive and negative mode ESI, Cone voltage = 25 V, capillary voltage = 3.0, ion source temperature = 100°C, desolvation temperature = 180°C, nebulizing gas (N2) flow = 200 L/h, cone gas (N2) flow = 5L/h.

5.2 Experimental Procedures

**Methyl Pyropheophorbide-a (1).** Chlorin e6 Trimethyl Ester (250 mg, 0.401 mmol) was dissolved in 2,4,6-collidine (25 mL) and the potassium acetate (197 mg, 2.01 mmol) was added to the flask. The reaction was run under argon and heated at ~190°C with a condenser and allowed overnight for 2 nights (~42 hrs). The reaction was distilled to dryness and then redissolved in dichloromethane to be purified via flash chromatography. The mobile phase system used was dichloromethane and with a 0-6% ethyl acetate gradient. Like dark fractions were combined and solvent removed by rotovap. The resulting product was dried under vacuum to yield a dark near-black solid and was assayed by LC-MS with 0.5 mL of methanol. Yield: 152.5 mg, 71%. UHPLC-MS (LR, ESI) = Calcd. for C34H36N4O3: m/z, found: # [M+H]+_LC-MS (HR, ESI) = Calcd. for C34H36N4O3: 549.28668 m/z, found: 549.2866 [M+H]+ (0.146 ppm).

**Meso Pyropheophorbide-a (mPPa, 2).** Pyropheophorbide-a (49.7 mg, 93.52 µmol) was dissolved in acetone (10 mL) and 20% Pd on carbon black (5.4 mg) was added. The mixture was bubbled with argon and then put under pressure with H2. The reaction was stirred for 1 hour at room temperature. The product was filtered and then rotovapped to produce a black solid with a
bluish sheen. Yield: 42.7 mg, 85%. LC-MS (LR, ESI) = Calcd. For C_{34}H_{38}N_{4}O_{3} MW 536.28; found: 537.40 [M+H]^+, 535.30 [M-H]^−.

**mPPa-NHS Ester (3).** Using oven dried glassware and syringes, mPPa (2) (28 mg, 52.17 µmol) was dissolved in 10 mL of DCM in a round bottom flask under argon atmosphere. TSTU (31.47 mg, 104.3 µmol) was then added to the reaction vessel, followed by DIPEA (136 µL, 782.6 µmol). The reaction was stirred at room temperature under argon for 15 minutes and was monitored by LC-MS. The final product was precipitated in a 50:50 mix of hexanes:ether, the decant was poured off and the resulting solid was dried under argon. Yield: 22 mg, 66%. LC-MS (LR, ESI) = Calcd. for C_{37}H_{39}N_{5}O_{5}: 634.30 m/z, found: 634.47 [M+H]^+. LC-MS (HR, ESI) = Calcd. for C_{37}H_{39}N_{5}O_{5}: 634.30306 m/z, found: 634.3027 [M+H]^+ (0.568 ppm).

**Fmoc-dLys(mPPa)-OH (4).** Fmoc-dLys(H)-OH (11.03 mg, 27.9 µmol) was dissolved in dry DMF in a dry flask. DIPEA (32 µL, 186 µL) was added, followed by addition of mPPa-NHS ester (3) (11.80 mg, 18.6 µmol). The reaction was stirred at room temperature for approximately 30 minutes and was monitored via LCMS by diluting a few drops in ACN. When the reaction was completed the reaction mixture was precipitated with 50:50 hexanes:ether, decant was poured off, and the dark solid was dried under argon. Yield: 16.5 mg, 69%. LC-MS (LR, ESI) = Calcd. for C_{54}H_{58}N_{6}O_{6} MW 886.44; found: 887.38 [M+H]^+, 885.42 [M-H]^−. LC-MS (HR, ESI) = Calcd. for C_{54}H_{58}N_{6}O_{6}: 887.44972 m/z, found: 887.4493 [M+H]^+ (0.473 ppm).

**Fmoc-dLys(mPPa)-NHS Ester (5).** Using oven dried glassware and syringes, Fmoc-dLys(mPPa)-OH (4) (11.40 mg, 12.9 µmol) was dissolved in 10 mL of DMF in a round bottom
flask under argon atmosphere. TSTU (7.75 mg, 25.7 µmol) was then added to the reaction vessel, followed by DIPEA (33.5 µL, 193 µmol). The reaction was stirred at room temperature under argon for 45 minutes to an hour, and was monitored by LC-MS. The final product was precipitated in 50:50 hexanes:ether, decant poured off, and left to dry under argon. Yield: 10.6 mg, 84%. LC-MS (LR, ESI) = Calcd. for C_{58}H_{61}N_{7}O_{8}: 984.45 m/z, found: 984.14 [M+H]^+.

**Fmoc-Trp-dNle-Glu-Ala-Ala-Tyr-Gln-dLys(mPPa)-Phe-Leu-NH$_2$ (6).** Fmoc-Trp-dNle-Glu-Ala-Ala-Tyr-Gln-dLys-Phe-Leu-NH$_2$ (8.06 mg, 5.4 µmol), was dissolved in dry DMF in a dry flask. DIPEA (15.5 µL, 90 µmol) was added, followed by addition of mPPa-NHS ester (3) (3.80 mg, 6.00 µmol). The reaction was monitored via HPLC. Solvent was removed under vacuum and the resulting solid was purified by preparative-HPLC using a 70 – 100% ACN gradient in 0.01 M ammonium acetate. Yield: 2.3 mg, 19%. LC-MS (LR, ESI) = Calcd. for C_{111}H_{134}N_{18}O_{18} MW 2007.01; found: 2006.45 [M-H]^-, 2008.22 [M+H]^+, 1004.38 [M+2H]^+/2.

**H-Trp-dNle-Glu-Ala-Ala-Tyr-Gln-dLys(mPPa)-Phe-Leu-NH$_2$ (7).** Fmoc-Trp-dNle-Glu-Ala-Ala-Tyr-Gln-dLys(mPPa)-Phe-Leu-NH$_2$ (6) (3.40 mg, 1.69 µmol), was dissolved in DMF, DEA (2.6 µL, 25.4 µmol) was added, and left to spin for 2 hours. The reaction was monitored via LCMS by diluting a few drops in ACN. When the reaction was completed, the solvent was removed under vacuum. The resulting solid was then purified by SPE. Yield: 0.5 mg (yield loss due to analysis procedures and possible degradation on the SPE column). LC-MS (LR, ESI) = Calcd. for C_{96}H_{124}N_{18}O_{16} MW 1784.94; found: 1783.57 [M-H]^-, 891.29 [M-2H]^-/2, 1785.45 [M+H]^+, 893.24 [M+2H]^+/2.
**Fmoc-Trp-dNle-Glu-Ala-Ala-Tyr-Gln-dLys(Cy5.5-3S)-Phe-Leu-NH$_2$ (8).** Fmoc-Trp-dNle-Glu-Ala-Ala-Tyr-Gln-dLys-Phe-Leu-NH$_2$ (2.87 mg, 1.9 µmol) and Cy5.5(3S)-NHS ester (1.85 mg, 1.92 µmol) were dissolved separately in 1 mL dry DMF. DIPEA (5 µL, 28.8 µmol) was added to the Fmoc-18-4, followed by addition of the dissolved Cy5.5(3S)-NHS ester. The reaction was monitored via LCMS. Yield: 3.4 mg, 76%. LC-MS (LR, ESI) = Calcd. for C$_{121}$H$_{146}$N$_{16}$O$_{26}$S$_{3}$ MW 2334.98; found: 1166.52 [M-2H]/2, 777.39 [M-3H]/3, 1168.53 [M+2H]$^+$/2.

**H-Trp-dNle-Glu-Ala-Ala-Tyr-Gln-dLys(Cy5.5-3S)-Phe-Leu-NH$_2$ (9).** DEA (1.5 µL, 14.6 µmol). was added to the reaction mixture of 8 (3.4 mg, 1.46 µmol) and left to spin for an hour. The reaction was monitored via LCMS by diluting a few drops in ACN. When the reaction was completed the reaction mixture was precipitated with 50:50 hexanes:ether, decant was poured off, and the solid was dried under argon. The product was purified by preparative HPLC loading the sample in 50:50 ACN : water and using a 10-100% ACN in 0.01 M AmAc gradient. Pure fractions were identified by LCMS without dilution, concentrated by rotovap, and then freeze dried. Yield: 1.0 mg (yield loss due to analysis procedures and possible degradation on the SPE column). LC-MS (LR, ESI) = Calcd. for C$_{106}$H$_{136}$N$_{16}$O$_{24}$S$_{3}$ MW 2112.91; found: 1055.15 [M-2H]$^-$/2, 703.26 [M-3H]$^-$/3, 1057.26 [M+2H]$^+$/2. LC-MS (HR, ESI) = Calcd. for C$_{106}$H$_{136}$N$_{16}$O$_{24}$S$_{3}$: 2113.91549 m/z, found: 1057.459 [M+2H]$^+$/2 (2.57 ppm).
**Fmoc-Trp-dNle-Glu-Ala-Ala-Tyr-Gln-dLys(Dde)-Phe-Leu-NH$_2$ (10).** Protected Dde 18-4 (300.6 mg resin weight) was cleaved from the resin using a continuous flow method with 2% TFA and collected into scintillation vials and allowed to sit overnight at room temperature. A small sample of each vial was collected and the solvent was removed by rotovap, dissolved in 0.5 mL ACN, and assayed by LCMS. The pure fractions were combined and solvent was removed by rotovap. The resulting solid was purified by SPE using a 5%-100% ACN step-wise gradient with 5 mM AmAc as the aqueous mobile phase. Yield: 12 mg. LC-MS (LR, ESI) = Calcd. for C$_{80}$H$_{112}$N$_{14}$O$_{18}$: 1653.84 m/z; found: 1654.23 [M+H]$^+$, 827.59 [M+2H]$^+$.  

**H-Trp-dNle-Glu-Ala-Ala-Tyr-Gln-dLys(Dde)-Phe-Leu-NH$_2$ (11).** An Fmoc-deprotection was done by taking 10 (12.00 mg, 6.05 µmol) and treating it with DEA (6.25 µL, 60.5 µmol) in DMF. This reaction was spun for 4 hours under argon and monitored by LCMS. The DMF was then removed under vacuum. LC-MS (LR, ESI) = Calcd. for C$_{73}$H$_{102}$N$_{14}$O$_{16}$: 1431.76 m/z; found: 1431.08 [M+H]$^+$, 716.38 [M+2H]$^+$. LC-MS (HR, ESI) = Calcd. for C$_{73}$H$_{102}$N$_{14}$O$_{16}$: 1429.75188 m/z; found: 1429.7489 [M-H]$^-$ (2.08 ppm), 714.3701 [M-2H]$^-$/2 (2.62 ppm).  

**Fmoc-dLys(mPPa)-Trp-dNle-Glu-Ala-Ala-Tyr-Gln-dLys(Dde)-Phe-Leu-NH$_2$ (12).** Fmoc-dLys(mPPa)-NHS Ester 5 (8.42 mg, 8.56 µmol) was dissolved into enough DMF to completely dissolve with DIPEA (11.9 µL, 68.5 µmol) was added to Trp-dNle-Glu-Ala-Ala-Tyr-Gln-dLys(Dde)-Phe-Leu-NH$_2$ 11 (9.8 mg, 6.85 µmol). The reaction was allowed to stir 5 hours under argon, adding low heat ~40°C after an hour and a half. The reaction was monitored by taking a few drops of reaction mixture into about 0.25 mL of ACN in a space saver vial and
assaying via LCMS. The solvent was removed under vacuum and the resulting dark solid was purified by preparative HPLC loading the sample in 50:50 ACN:water and using a 60-100% ACN in 0.01 AmAc gradient. Pure fractions were identified by LCMS without dilution, concentrated by rotovap, and then freeze dried. Yield: 0.2 mg (yield loss due to analysis procedures and possible degradation on the SPE column). LC-MS (LR, ESI) = Calcd. for C_{127}H_{158}N_{20}O_{21}: 2300.19 m/z; found: 1150.62 [M+2H]^+ /2.

References


(15) Nava, H. R.; Allamaneni, S. S.; Dougherty, T. J.; Cooper, M. T.; Tan, W.; Wilding, G.; Henderson, B. W. Photodynamic Therapy (PDT) Using HPPH for the Treatment of Precancerous


(27) FDA Photofrin Injection Label.


Appendix I. UHPLC-MS, HPLC-MS, and HRMS Results

Compound 1: Methyl Pyropheophorbide-a

Single Wavelength Chromatogram of 665 nm for 1, main peak at 2.47 min

UV-Vis Diode array of 1 spectrum at 2.47 min with characteristic peaks at 400 and 665 nm
Extracted (Single) Ion Chromatogram (XIC) at 549.00 amu

Positive Ion Mass Spectra of 1 at 2.67 min, product 549 m/z [M+H]+
HRMS Positive Ion Mass spectra of 1, product 549.2866 m/z [M+H]^+

**Compound 2: Meso Pyropheophorbide-a (mPPa)**

UV-Vis Absorption Spectra for 2 with a Soret band at 407 nm and max Q band at 660 nm.
Fluorescence Spectra for 2, with an excitation of 418 nm and a peak emission at 661 nm

Total Diode Array Chromatogram of 2
Single Wavelength Chromatogram of 404 nm for 2, main peak at 3.00 min

UV-Vis Diode array of 2 spectrum at 3.35 min with characteristic peaks at 405 and 660 nm
Positive Ion Mass spectrum of 2 at 2.947 min, product 537.40 m/z [M+H]^+ with metastable peaks (1073.57, 1609.80 m/z)

Negative Ion Mass spectrum of 2 at 2.895 min, product 535.30 m/z [M-H]^− with metastable peaks (1071.51, 1608.94 m/z)
**Compound 3: mPPa-NHS Ester**

Total Diode Array Chromatogram of 3

Single Wavelength Chromatogram at 404 nm for 3, main peak at 6.48 min
UV-Vis Diode array of 3 spectrum at 6.483 min with characteristic peaks at 404 and 650 nm

Positive Ion Mass spectrum of 3 at 6.477 min, product 634.47 m/z [M+H]^+

**Compound 4: Fmoc-dLys(mPPa)-OH**

Total Diode Array Chromatogram for 4
Single Wavelength Chromatogram at 404 nm for 4, main peak at 3.19 min

UV-Vis Diode array of 4 spectrum at 3.308 min with characteristic peaks at 405 and 651 nm

Positive Ion Mass spectra of 4 at 3.153 min, product 887.38 m/z [M+H]^+
Negative Ion Mass spectra of 4 at 3.136 min, product 885.42 m/z [M-H]-

HRMS Positive Ion Mass spectra of 4, product 887.4493 m/z [M+H]+
Compound 5: Fmoc-dLys(mPPa)-NHS Ester

Total Diode Array Chromatogram of 5

Single Wavelength Chromatogram at 403 nm for 5, main peak at 5.05 min
UV-Vis Diode array of 5 spectrum at 4.975 min with characteristic peaks at 402 and 660 nm

Positive Ion Mass spectra of 5 at 4.986 min, product 984.14 m/z [M+H]^+

**Compound 6: Fmoc-Trp-dNle-Glu-Ala-Ala-Tyr-Gln-dLys(mPPa)-Phe-Leu-NH₂**

Total Diode Array Chromatogram of 6
Single Wavelength Chromatogram at 406 nm for 6, main peak at 3.63 min

UV-Vis Diode array of 6 spectrum at 3.592 min with characteristic peaks at 407 and 650 nm for mPPa and at 265 nm for Fmoc protecting group
Positive Ion Mass spectra of 6 at 3.564 min; product 2008.22 m/z [M+H]+,

1004.38 m/z [M+2H]+/2

Negative Ion Mass spectra of 6 at 3.684 min, product 2006.45 m/z [M-H]-

**Compound 7: Trp-dNle-Glu-Ala-Ala-Tyr-Gln-dLys(mPPa)-Phe-Leu-NH2**

Total Diode Array Chromatogram of 7
Single Wavelength Chromatogram at 406 nm for 7, main peak at 4.13 min

UV-Vis Diode array of 7 spectrum at 4.125 min with characteristic peaks at 406 and 650 nm for mPPa
Positive Ion Mass spectra of \(7\) at 4.077 min; product 1785.45 m/z \([\text{M+H}]^+\),
893.24 m/z \([\text{M+2H}]^+/2\)

Negative Ion Mass spectra of \(7\) at 4.060 min; product 1783.57 m/z \([\text{M+H}]^-\),
891.29 m/z \([\text{M-2H}]^-/2\)
Compound 8: Fmoc-Trp-dNle-Glu-Ala-Ala-Tyr-Gln-dLys(Cy5.5-3S)-Phe-Leu-NH$_2$

Total Diode Array Chromatogram of 8

Single Wavelength Chromatogram at 682 nm for 8, main peak at 4.46 min
UV-Vis Diode array of 8 spectrum at 4.375 min with characteristic peak at 682 nm of Cy5.5-3S

Positive Ion Mass spectra of 8 at 4.403 min, product 1168.53 m/z [M+2H]⁺/2

Negative Ion Mass spectra of 8 at 4.386 min; product 1166.52 m/z [M-2H]⁻/2, 777.39 m/z [M-3H]⁻/3
Compound 9: Trp-dNle-Glu-Ala-Ala-Tyr-Gln-dLys(Cy5.5-3S)-Phe-Leu-NH₂

Total Diode Array Chromatogram of 9

Single Wavelength Chromatogram at 682 nm for 9, main peak at 4.03 min
UV-Vis Diode array of 9 spectrum at 3.942 min with characteristic peak at 682 nm of Cy5.5-3S

Positive Ion Mass spectra of 9 at 3.958 min, product 1057.26 m/z [M+2H]^+ / 2

Negative Ion Mass spectra of 9 at 3.975 min; product 1055 m/z [M-2H]^-/2,
703.26 m/z [M-3H]^-/3
HRMS Positive Ion Mass spectra of 9, product 1057.9613 m/z [M+2H]+/2

**Compound 10: Fmoc-Trp-dNle-Glu-Ala-Ala-Tyr-Gln-dLys(Dde)-Phe-Leu-NH₂**

Total Diode Array Chromatogram for 10
Single Wavelength Chromatogram at 259 nm for **10**, main peak at 1.02 min

UV-Vis Diode array of **10** spectrum at 1.042 min with characteristic peak at 260 nm of Fmoc

Positive Ion Mass spectra of **10** at 1.164 min; product 1654.23 m/z [M+H]⁺,

\[827.59 \text{ [M+2H]}^+ / 2\]
Compound 11: Trp-dNle-Glu-Ala-Ala-Tyr-Gln-dLys(Dde)-Phe-Leu-NH$_2$

Total Diode Array Chromatogram of 11

Single Wavelength Chromatogram at 289 nm for 11, main peak at 1.09 min
UV-Vis Diode array of 11 spectrum at 1.108 min with characteristic peak at 289 nm of Trp

Positive Ion Mass spectra of 11 at 0.993 min; product 1431.08 m/z [M+H]^+, 716.25 m/z [M+2H]^+ / 2
HRMS Negative Ion Mass spectra of 11; product 1429.7489 m/z [M-H]−,

714.3701 m/z [M-2H]/2

Compound 12: Fmoc-dLys(mPPa)-Trp-dNle-Glu-Ala-Ala-Tyr-Gln-dLys(Dde)-Phe-Leu-NH₂
Single Wavelength Chromatogram at 405 nm for 12, main peak at 5.52 min

UV-Vis Diode array of 12 spectrum at 5.408 min with characteristic peaks at 407 and 651 nm of mPPa and characteristic peak of 261 nm of Fmoc protecting group

Positive Ion Mass spectra of 12 at 5.397 min, product 1150.62 m/z [M+2H]^+ / 2