Porphyrin-Based Agents and Their Applications in Cancer Imaging and Therapy

By

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A thesis submitted in conformity with the requirements for the degree of Doctorate of Philosophy

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Doctor of Philosophy

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Abstract

Porphyrs represent one of the oldest, most widely studied chemical structures, both in nature and in biomedical applications. Due to their tumor avidity and favorable photophysical properties, such as long wavelength absorption and emission, easy derivatization, high singlet oxygen quantum yield and low in vivo toxicity, porphyrins have found particular success for photodynamic therapy and fluorescence imaging of cancer. Additionally, they are excellent metal chelators, forming highly stable metallo-complexes, making porphyrins an efficient delivery vehicle for radioisotopes. Thus, there is great potential in the applications of these multi-modal porphyrin-based agents for cancer imaging and therapy. I have investigated the characteristics of various porphyrin-based probes and their potential application in different clinically relevant models. Here, I will discuss three types of porphyrin-based agents: 1) photodynamic molecular beacons (PP\textsubscript{MMPB}), 2) targeted peptide porphyrins (PPF) and 3) porphyrin-lipid nanovesicles, porphysomes. I will demonstrate that all of these porphyrin-based agents have potential clinical applications in various fields of cancer imaging and therapy. Although these three
agents differ greatly, they all aim to increase the signal-to-background ratio of tumor to healthy tissue uptake of porphyrins, thereby increasing our ability to detect tumor tissue and better preserve healthy tissue during therapy.
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Abbreviations

%ID/g – percent injected dose per gram

22RV1 – human hypoxic prostate cancer cell line

$^{64}$Cu – copper-64

$^{64}$Cu-PPF – copper-64 labeled porphyrin (Pyropheophorbide)-peptide-folate

$^{99}$Tcm – technecium-99m

$^{109}$Pd – palladium-109

$^{111}$In – indium-111

Ace-1 – metastatic canine prostate cancer cell line

AE1/AE3 – Pan Cytokeratin antibody

Bchl – Bacteriochlorophyll

BHQ3 – black hole quencher 3

DAPI – 4',6-diamidino-2-phenylindole

DLS – dynamic light scattering

DMEM – Dulbecco’s Modified Eagle’s Medium

DMSO – dimethyl sulfoxide

EDTA – ethylenediamine tetraacetic acid

FR – folate receptor

FRET – Förster resonance energy transfer

GFP – green fluorescent protein

H&E – haematoxylin and eosin

hEGFr – human epidermal growth factor receptor

HPLC-MS – high performance liquid chromatography-mass spectrometry

HT1080 – low FR-expressing human epithelial fibrosarcoma cell line

i.v. – intravenous
i.p. – intraperitoneal

IVD – intervertebral disc

KB – FR-positive human epidermal cancer cell line

LED – Laser emitting diodes

Luc – luciferase

MMP – matrix metalloproteinases

MT-1 – human metastatic breast cancer cell line

MTT – 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide

NIR – near-infrared

PC3 – human prostate cancer cell line

PDI – polydispersity index

PDT – photodynamic therapy

PET – positron emission tomography

PPF – porphyrin (Pyropheophorbide)-peptide-folate probe

PPF488 – Fluorescein-peptide-folate probe

PPF750 – porphyrin (Bacteriochlorophyll)-peptide-folate probe

PP\textsubscript{MMP}B – photodynamic molecular beacon activated by matrix metalloproteinases

PP\textsubscript{scrambled}B – non-activatable (scrambled peptide linker sequence) photodynamic molecular beacon

PS – photosensitizer

PSA – prostate specific antigen

Pyro – Pyropheophorbide

RPMI media – Roswell Park Memorial Institute media

ROI – region of interest

SC – spinal cord

SOC – serous ovarian cancer
$t_{1/2}$ – half-life

TRAP – tart rate resistant acid phosphatase

VB – vertebral body
Chapter 1
Porphyrrins for Cancer Imaging and Therapy

1.1. Porphyrrins

Porphyrrins represent one of the oldest, most widely studied chemical structures, both in nature and in biomedical applications\(^1\). The origin of the word porphyrin dates back to ancient Greece and even earlier as Phoenicians used this word to describe the colour purple\(^2\). Porphyrrins actually represent many colours found in life and are a family of tetrapyrrrole rings essential for the biological activity of all living organisms\(^3\)\(^-\)\(^5\). Various classes of porphyrin exist, which are responsible for controlling many processes involved in sustaining life. For example, porphyrrins are a major constituent of hemoglobin and play a key role in the cytochrome chain, both of which are involved in aerobic metabolism\(^2\)\(^,\)\(^3\)\(^,\)\(^6\). Errors in porphyrin metabolism can lead to severe disorders in hemoglobin or a family of genetically inherited diseases associated with enzyme deficiencies known as porphyrias\(^2\)\(^,\)\(^6\). What makes porphyrins unique is that they contain a highly conjugated, heterocyclic macrocycle, a porphin structure – four pyrrole subunits linked together by four methine bridges, which may also contain central metallic atoms\(^2\)\(^-\)\(^5\). This porphin structure has a 22\(\pi\)-electron system which gives rise to their many photonic properties\(^3\) such as fluorescence or photodynamic therapy (PDT) – a photochemical reaction that combines light with a light activatable drug, photosensitiser, producing cytotoxic effects. A molecule can be excited to the first or higher excited states by the absorption of light (Figure 1.1). Excited states \((S_1)\) can return to the ground state \((S_0)\) by radiative decay such as fluorescence (a wavelength of light equal or longer than that of the excitation light), or heat (non-radiative decay). However, because of the \(\pi\)-electron system in porphyrrins, they have longer-lived triplet states \((T_1)\). Thus, when porphyrrins are in their excited singlet state, they can also undergo intersystem crossing producing an excited triplet state. In PDT, the triplet state can react in two ways: either a Type I PDT mechanism whereby the triple state transfers an electron directly to a
substrate forming toxic radical species (substrate•), or a Type II PDT mechanism whereby the triplet state transfers its energy to the ground-state of molecular oxygen (³O₂) producing toxic excited singlet oxygen (¹O₂) which oxidizes various substrates resulting in cell death³⁵,⁷.

The first produced porphyrin was hematoporphyrin; it was discovered by Scherer in 1841 while studying the nature of blood⁸. The fluorescence properties of hematoporphyrin were described years later in 1871⁹ followed by reports revealing the PDT capabilities in 1911¹⁰. In 1913, Meyer-Betz demonstrated the photosensitization of hematoporphyrin in humans by injecting himself with 200mg of the porphyrin¹¹. The discovery of this dual functionality of fluorescence and photosensitization marked porphyrins as the first reported intrinsically multimodal molecules.

Although porphyrins were identified in the mid-nineteenth century, it was not until the early twentieth century that their medicinal potential was realized¹². In 1924, the first observation of porphyrin’s affinity for tumor tissue was reported¹³. Following this
finding, many efforts have been made in exploiting the tissue selective localization of porphyrins combined with their fluorescent and phototoxic effects in the development of modern photodetection and PDT. There are currently 9 porphyrin-based compounds that are clinically approved for a number of clinical indications in the detection and treatment of numerous diseases (Table 1.1).

<table>
<thead>
<tr>
<th>Chemical Name</th>
<th>Trade name</th>
<th>Clinical Application</th>
<th>Approval (Country &amp; year)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hematoporphyrin derivative (HpD), porfimer sodium</td>
<td>Photofrin</td>
<td>Esophageal, lung, superficial bladder, gastric, cervical and endobroncial cancers</td>
<td>Over 120 countries since 1993</td>
</tr>
<tr>
<td>Benzoporphyrin-derivative monoacid ring A (BPD-MA), verteporfin</td>
<td>Visudyne</td>
<td>Age-related macular degeneration</td>
<td>Over 70 countries since 2001</td>
</tr>
<tr>
<td>Meta-tetra hydroxyphenyl chlorin (m-THPC), tempoporfin</td>
<td>Foscan</td>
<td>Head and neck cancer</td>
<td>European Union (EU), Norway and Iceland since 2001</td>
</tr>
<tr>
<td>5-Aminolevulinic acid (ALA)</td>
<td>Levulan</td>
<td>Actinic keratoses</td>
<td>USA since 1999</td>
</tr>
<tr>
<td>Methyl aminolevulinate (MAL)</td>
<td>Metric</td>
<td>Actinic keratosis and basal cell carcinoma</td>
<td>EU and Australia since 2001</td>
</tr>
<tr>
<td>Hexyl aminolevulinate (HAL)</td>
<td>Hexvix</td>
<td>Fluorescence diagnosis of bladder cancer</td>
<td>Sweden since 2004 and EU since 2005</td>
</tr>
<tr>
<td>Mono-L-aspartyl chlorine 6, talaporfin sodium, laserphyrin</td>
<td>NPe6</td>
<td>Early lung cancer</td>
<td>Japan since 2004</td>
</tr>
<tr>
<td>Aluminium phthalocyanine disulphonate (AlPcS2a)</td>
<td>Photosense</td>
<td>Head and neck cancer</td>
<td>Russia since 2001</td>
</tr>
<tr>
<td>Tin ethyl etiopurpurin (SnET2)</td>
<td>Purlytin</td>
<td>Age-related macular degeneration</td>
<td>FDA approved fast track status since 1998</td>
</tr>
</tbody>
</table>

Table 1.1: Clinically approved porphyrin-based drugs\(^3,5,7,12,14\).

Due to their favorable photophysical properties, such as long wavelength absorption and emission, easy derivatization, high singlet oxygen quantum yield and low in vivo toxicity, porphyrins have found particular success for cancer imaging and therapy\(^4,15-20\). The role of porphyrins in fluorescence, PDT and other applications beyond photonics and strategies for improving upon their application in oncology will be discussed in the following sections.
1.2. Imaging with Porphyrins

The fluorescent properties of porphyrins were realized in the late 19th century\textsuperscript{9}. The selective appearance of porphyrin fluorescence in tumors was first observed in 1924\textsuperscript{13}. Following this tumor-localizing discovery, the use of porphyrin fluorescence as a means to identify cancerous tissue was evaluated for most of the past century in preclinical and clinical studies. In addition to the preferential accumulation of porphyrins in malignant tissue, porphyrins are ideal fluorophores as they absorb light, generally, within the “optical window” of living tissue, between 600nm (above the absorption of heme) and 1200nm (below the absorption of water). The advantage of using porphyrins is that they are excited within this “optical window”, at near infrared (NIR) wavelengths (650-900nm) of light, which penetrates deeper into tissues than visible light. NIR wavelengths of light can penetrate several centimeters since NIR light entering tissue is not absorbed as highly by dominant chromophores, such as hemoglobin, melanin and water\textsuperscript{7,14,21-23}. Furthermore, NIR light reduces tissue autofluorescence, (the intrinsic fluorescence of tissues due to endogenous fluorophores such as flavins, collagen and elastin) which optimizes the signal to background ratio\textsuperscript{21-23}. On average, porphyrins accumulate in tumor tissue 2-3 fold higher than in normal surrounding tissue\textsuperscript{5}; however, some preclinical indications have demonstrated much higher uptake (~7 fold increase) in malignant tumors (e.g. brain tumors)\textsuperscript{24,25}.

The first attempt in the utilization of porphyrin fluorescence in humans was for the detection of head and neck malignancies\textsuperscript{26,27}. These studies demonstrated that tumor fluorescence increased in proportion to porphyrin dose, supporting the potential of porphyrin-based photodiagnosis. With these promising results, the phenomenon of porphyrin fluorescence was further evaluated to assess its potential application in guiding surgery. Using patients undergoing surgical excision of a variety of benign and malignant lesions, selective porphyrin fluorescence was observed in a patient with cervical carcinoma\textsuperscript{28}. However, tumor fluorescence was only observed after using significantly high doses, 1000mg, of an intravenous infusion of hematoporphyrin\textsuperscript{27,28}. This significant dose, which was necessary to achieve consistent uptake in tumors, proved to be a major drawback, resulting in unacceptable photosensitivity in patients\textsuperscript{3,12}. Promisingly, even at
these high doses, porphyrins remained relatively nontoxic if unexposed to light\textsuperscript{27,28}. Regardless, these studies jump-started subsequent work in attempting to exploit the fluorescence of porphyrins to aid in tumor diagnosis by better visualizing and delineating neoplastic tissue.

The use of porphyrin-based compounds in photodetection was solidified when hexylaminolevulinate, Hexvix, was clinically approved for fluorescence diagnosis of bladder cancer\textsuperscript{29,30}. The approval of porphyrins as a diagnostic tool for the detection of other indications is to be expected in the future. Porphyrin-based photodiagnosis has been evaluated in patients undergoing bronchoscopy or esophagoscopy for suspected malignant disease\textsuperscript{31-34}, detection of early cancers and premalignant changes in the cervix\textsuperscript{35,36}, adenocarcinomas, squamous carcinoma and sarcomas\textsuperscript{37}, head and neck tumors\textsuperscript{38}, the detection of early-stage lung cancer\textsuperscript{39-42} and in guiding surgical resection of glioblastomas\textsuperscript{43}. It was further recognized that porphyrin fluorescence of resected specimens positively correlated to the presence of tumor on histological examination, demonstrating its potential use to microscopically identify diseased tissue in real-time\textsuperscript{44}. However, continued work in this field found that precancerous and cancerous lesions were extremely difficult to distinguish from normal tissues, partly because of the presence of inflammation and/or autofluorescence\textsuperscript{12}. Although the use of porphyrins in photodetection demonstrates promise and has been evaluated in numerous clinical applications, the clinical impact of porphyrins as a diagnostic tool for the detection of cancers still requires further development of these molecules to ensure reliable identification of disease.

1.3. Porphyrin-based PDT

The therapeutic effects of light have been known for well over three thousand years. The ancient Egyptians, Indians and Chinese used light to treat diseases, including psoriasis, rickets, vitiligo and skin cancer\textsuperscript{2-5}. It was not until the beginning of the twentieth century that the therapeutic potential of porphyrins was recognized, whereby the manipulation of light in combination with porphyrins resulted in photoreactions in biological systems. The first report of PDT was in 1903, when topical eosin and visible light were used to
treat a skin tumor\textsuperscript{3}. PDT involves two individually non-toxic components that are combined to induce cytotoxic effects. Once a photosensitizer (PS), a light activated molecule, is combined with the administration of light of a specific wavelength that activates the PS, reactive oxygen species are produced. These reactions occur in the immediate locale of the light-absorbing PS\textsuperscript{2-5}. The potential of porphyrins as powerful PSs was recognized in 1912 and this class of molecules has since been extensively studied\textsuperscript{4,11,14}. However, although the medicinal use of PDT was postulated early on, it was not until several decades later that the combination of tumor-localizing and phototoxic properties of porphyrins were exploited to produce an effective treatment for cancer\textsuperscript{45}. In 1975, Dougherty et al. reported the first PDT induced complete tumor cure following administration of porphyrins and red light in the treatment of animal tumors\textsuperscript{46}. A year later, the first human study of the effects of porphyrin PDT was evaluated in patients with bladder cancer during cystoscopy. As expected, porphyrin fluorescence occurred and was selective for malignant and premalignant lesions\textsuperscript{47}. In this study, only a single patient, who had failed to respond to resection, radiotherapy and chemotherapy, was treated with PDT. Promisingly, tumor necrosis was observed in the treated area, while no effects were seen in unexposed areas\textsuperscript{47}. Shortly following this, another clinical study evaluating PDT as a treatment regime for patients with skin cancer was reported. Of the 113 skin lesions treated, 98 lesions showed complete regression, 13 demonstrated partial response and only 2 were resistant to porphyrin PDT\textsuperscript{48}. However, several side effects were observed following treatment, including sunburns, erythema, edema and skin necrosis\textsuperscript{48}. Due to the superficial location and ease of light application in skin tumors, many more studies followed demonstrating the effectiveness of porphyrin PDT in this clinical indication\textsuperscript{49-51}.

The parallel advance of light source technologies, starting in the 1970s, also had a large impact on the application of PDT. The light source and aimed light delivery are of the utmost importance. There are currently 3 main classes of clinical PDT light sources: filtered lamps, light emitting diodes (LED) and lasers\textsuperscript{7}. Filtered lamps have the advantage of being spectrally filtered to match any PS. However, the broad emission spectra have a few drawbacks, including thermal effects, difficulty in determining light dosimetry and are limited to surface treatments\textsuperscript{7,14}. Filtered lamps are the most frequently applied light
sources for PDT in dermatological indications. LEDs have also emerged as a viable technology in PDT, albeit for easily accessible tissue surfaces. LEDs have the advantage of low cost and ease of creating LED arrays into different irradiation geometries. The main limitation with LEDs is their relatively low electrical-to-light conversion efficiency resulting in heat generation. Lasers produce high intensity, coherent, monochromatic light with high efficiency where this light can be focused through optical fibers. This allows for endoscopic and interstitial light delivery directly to the target where their monochromaticity allows maximum efficiency of photoactivation. The main limitation of diode lasers is that they only emit at a single wavelength and thus, separate devices are required for PS with different excitation wavelengths. A variety of lasers have been used for PDT, including argon-dye, potassium-titanium-phosphate dye, metal vapour, copper, gold and most recently diode lasers. With the advancements in both light source and fiber optic technology, it was quickly realized that PDT could be used for endoscopic and bronchoscopic indications. It demonstrated dramatic improvement in symptoms and pulmonary function in patients with inoperable obstructing lung tumors as well as early stage lung cancer not suitable for resection. Further studies demonstrated successful treatment and retreatment of patients with bladder tumors. These positive results were mirrored in the treatment of esophageal and early gastric cancer, recurrent gynecological tumors, intra-ocular lesions, brain tumors, head and neck lesions and rectal cancer. Today, PDT has been approved in the clinic for some cancer indications, precancerous lesions as well as age-related macular degeneration. PDT was first approved in Canada in 1993, using Photofrin for the prophylactic treatment of bladder cancer.

In these early preclinical and clinical studies of PDT in oncology, the PSs used were mostly porphyrin-related compounds. Currently, all clinically approved PS, with the exception of methylene blue, are based on or related to porphyrins. Numerous porphyrin-based PS have been in development ever since the therapeutic potential of hematoporphyrin was recognized. The need to purify hematoporphyrin resulted in the development of hematoporphyrin derivative, Photofrin – the first clinically approved PS. Photofrin was superior to hematoporphyrin but still suffered from several drawbacks, including skin photosensitivity that lasts for several weeks, dark toxicity, long drug-light
interval and its heterogeneous mixture of compounds. Table 1.2 summarizes the disadvantages of several porphyrin-based PSs that have emerged since.\textsuperscript{3-5,70}

<table>
<thead>
<tr>
<th>Photosensitizer</th>
<th>Chemical Name</th>
<th>Absorption (nm), Drug-light interval</th>
<th>Advantages</th>
<th>Disadvantages</th>
<th>Clinically approved</th>
</tr>
</thead>
<tbody>
<tr>
<td>Photofrin</td>
<td>Hematoporphyrin derivative</td>
<td>630 24-48h</td>
<td></td>
<td>Heterogeneous mixture, erythema, long photosensitivity (several weeks), mild constipation, long drug-light interval</td>
<td>Yes</td>
</tr>
<tr>
<td>Foscan</td>
<td>Meta-tetrahydroxyphenylchlorin</td>
<td>652 96h</td>
<td>High singlet oxygen yield, chemically pure</td>
<td>Swelling, bleeding, ulceration, scarring, long photosensitivity (several weeks), long drug-light interval, hydrophobic</td>
<td>Yes</td>
</tr>
<tr>
<td>Lutrin</td>
<td>Motexafin lutetium</td>
<td>732 3h</td>
<td>Long wavelength resulting in deeper tissue penetration, soluble, chemically pure</td>
<td>Photosensitivity, severe pain during PDT treatment, multistep synthesis for formulation</td>
<td>No</td>
</tr>
<tr>
<td>Levulan</td>
<td>5-Aminolevulinic acid</td>
<td>635 4-6h</td>
<td>Chemically pure, systemic and topical administration, soluble</td>
<td>Stinging, burning, itching and pain during PDT treatment, erythema, limited depth penetration due to shorter wavelength</td>
<td>Yes</td>
</tr>
<tr>
<td>Metvix</td>
<td>Methyl aminolevulinate</td>
<td>635 3h</td>
<td>Chemically pure, systemic and topical administration, soluble</td>
<td>Burning sensation and some pain during PDT treatment, redness, scabbing, limited depth penetration due to shorter wavelength</td>
<td>Yes</td>
</tr>
<tr>
<td>NPe6</td>
<td>Mono-L-aspartyl chlorine 6</td>
<td>664 2-4h</td>
<td>Chemically pure, rapid clearance</td>
<td>Questionable tumor localizing ability</td>
<td>Yes</td>
</tr>
<tr>
<td>Purlytin</td>
<td>Tin ethyl etiopurpurin</td>
<td>664 24h</td>
<td>Chemically pure</td>
<td>Photosensitivity (~2 weeks), insoluble, dark toxicity</td>
<td>FDA approved fast track status</td>
</tr>
<tr>
<td>TOOKAD</td>
<td>Palladium bacterio-pherophorbide</td>
<td>763 15min</td>
<td>Short half-life, long wavelength resulting in deeper tissue penetration, high singlet oxygen yield, soluble, chemically pure, rapid clearance</td>
<td>No</td>
<td></td>
</tr>
</tbody>
</table>

Table 1.2. Characteristics of selected porphyrin-based photosensitizers compared to Photofrin.\textsuperscript{3-5}

Clearly, these new porphyrin-based PSs have improved upon some of the limitations of Photofrin but they still suffer from several drawbacks. Although these PSs preferentially accumulate in neoplastic tissue compared to the surrounding normal tissue, it is only between 2 – 3 fold higher, mirroring the same difficulty found in photodetection: the
difficulty in distinguishing between precancerous and cancerous lesions from normal tissue, which results in non-target, healthy tissue effects. In addition, the mechanisms driving the selective accumulation of porphyrins in tumors are complex and still not fully understood. Dolmans et al. postulate that porphyrin accumulation in tumors is due to the “high vascular permeability of the agents, as well as their affinity for proliferating endothelium and the lack of lymphatic drainage in tumors”\(^4\). Currently, Photofrin is still viewed as the gold standard in PDT. It should be noted that TOOKAD has shown superior photochemical and pharmacological characteristics to Photofrin and the various other PSs developed, representing a promising PS in PDT-based treatment of cancer. The development of nonporphyrin-based PSs has lagged considerably behind, due to the limited potency and various side effects (e.g. pain during PDT treatment, significant dark toxicity, poor tumor localization, metabolic inactivation and rapid excretion) associated with these nonporphyrin-based PS\(^3\). Consequently, future work on the development and improvement of PSs, should focus on increasing the therapeutic efficacy and selectivity for malignant tissue, while minimizing side effects such as the use of targeting strategies (e.g. antibodies or nanoparticles to enhance selective tissue localization\(^3\-5,12\)).

### 1.4. Metalloporphyrins

Following the preferential affinity porphyrins demonstrated for malignant tissue, it was quickly hypothesized that the application of porphyrins may be extended to nuclear medicine as they may be good delivery vehicles for radioisotopes. This was based on the idea that metals chelate stably and easily with porphyrins with minimal loss \textit{in vivo}, where the porphyrins themselves act as the radioisotope chelator\(^6,71-73\). The early studies of radiolabeled metalloporphyrins demonstrated that porphyrins were among the earliest chelating agents to undergo nuclear medicine evaluation\(^6\). The interest in the differential localization of metalloporphyrins in cancerous tissue for delivery of radioactive metals was realized as early as 1948\(^26\). Copper-64 (\(^{64}\)Cu) was one of the metal radioisotopes initially evaluated due to its well established stable chelation of Cu\(^{2+}\) to porphyrins. The potential use of \(^{64}\)Cu-porphyrin for the detection of cancer in patients was recognized well over half a century ago\(^74\). The radioactive \(^{64}\)Cu-porphyrin is extremely stable to demetallation and was first used \textit{in vivo} in 1951 to develop coincident scintillation
counters, permitting more accurate localization of radioisotopes\textsuperscript{75}. Despite the recognition of $^{64}$Cu as a suitable radionuclide (t\textsubscript{1/2} = 12.7 h, $\beta^-$: 17.4\%, E\textsubscript{\beta-max} = 656 keV; $\beta^-$: 39\%, E\textsubscript{\beta-max} = 573 keV) in positron emitting tomography (PET) imaging\textsuperscript{76-78}, there have been few efforts and even less promising results to date for using $^{64}$Cu-labeled porphyrins as PET probes\textsuperscript{79-81}. In large part this is due to their poor tumor targeting, as well as the technical limitations of earlier PET scanners. The field went into hiatus until the 1980s when Wilson \textit{et al.} attempted to detect brain tumors using $^{64}$Cu-porphyrin with the emerging technology of PET. This effort failed to generate wide interest, due to the limited tumor localization of the particular $^{64}$Cu-porphyrin and the poor spatial resolution (8 mm) of PET scanners at that time\textsuperscript{79,80,82}. Over the past several decades, the stable chelation of different radioisotopes, such as palladium-109 ($^{109}$Pd), indium-111 ($^{111}$In) and technecium-99m ($^{99m}$Tcm), to a variety of porphyrins and their abilities to delineate tumors was evaluated\textsuperscript{83-93}. Some of these complexes demonstrated better tumor-to-tissue ratios in select tissues. However, none of the radiolabeled porphyrins demonstrated true tumor selectivity. There have been a few attempts at improving the uptake of radiolabeled metalloporphyrins by conjugating targeting moieties such as antibodies\textsuperscript{94-96}. Unfortunately, targeted or non-targeted complexes demonstrated high uptake in organs such as the liver, kidneys and spleen, limiting their usefulness for imaging in the abdomen and casting doubt on their potential applications in humans. In spite of this, selective metalloporphyrin accumulation was observed in the lymph nodes and inflammation sites, which has become an active area of research\textsuperscript{97-102}.

Even with the large variety of suitable radioactive metals and numerous possible porphyrins, there is no general strategy for the development of radiolabeling the tetrapyrrrole ring\textsuperscript{73}. Although the field of metalloporphyrins as radiovehicles was not as fruitful as anticipated, it was observed that porphyrins and metalloporphyrins share the tissue distribution properties of the chelating moiety; the insertion of a metal ion into the tetrapyrrrole ring does not affect the \textit{in vivo} characteristics of the porphyrin itself\textsuperscript{26,74,75,79,80,103,104}. Porphyrin’s intrinsic ability to chelate metals will be re-evaluated, particularly copper-64 ($^{64}$Cu), and their potential as contrast agents for positron emission tomography in the later chapters of this thesis.
1.5 Porphyrin-based probes

1.5.1. Pyropheophorbide and Bacteriochlorophyll

In this thesis, two porphyrins, specifically Pyropheophorbide-α (Pyro) and Bacteriochlorophyll-α (Bchl) are evaluated throughout this work to improve upon the tumor selectivity of these multifunctional molecules (Figure 1.2).

![Pyropheophorbide-α and Bacteriochlorophyll-α](image)

Figure 1.2. General chemical structure of Pyropheophorbide-α (Pyro) and Bacteriochlorophyll-α (Bchl).

Pyro is a prototype of the chlorin class of natural products and is derived from *Spirulina* algae\(^{105}\). Pyro is attractive as it can be easily obtained in multigram quantity\(^{105}\). It is a potent PS with 660-740 nm excitation and emission profile, demonstrating potential for optical imaging. Much work has been done in studying the photophysical and biological properties of Pyro. It was demonstrated that variation of substituents in the parent Pyro molecule makes a remarkable difference in biological activity\(^{105}\). Bchl is one of the most widely distributed bacteriochlorin pigments and is extracted from the bacteria, *Rhodobacter Sphaeroids*\(^{70,106}\). It is a near-infrared PS, with 750-850 nm excitation and emission profile, making it highly attractive for optical imaging\(^{17,18,107,108}\). Despite its favourable characteristics, early Bchl studies demonstrated limited application in PDT and optical imaging due to its unstable nature. Many efforts have been made to address this instability including modification of the isocyclic ring\(^{70,109}\), serine stabilization\(^{110}\),
fluorinated stabilization\textsuperscript{111}, inserting palladium to form a more stable complex\textsuperscript{112,113} and incorporation into nanoparticles\textsuperscript{106}. Even with the numerous derivatives and analogs of porphyrins that have been synthesized and investigated, little success has occurred in improving these molecules’ tumor selectivity and specificity. Here, three different strategies will be presented – 1) photodynamic molecular beacons, 2) peptide-targeted porphyrins, and 3) porphyrin-based nanoparticles – that can increase the signal-to-background ratio of tumor to healthy tissue uptake of porphyrins, thereby increasing our ability to delineate tumor tissue and better preserve healthy tissue during therapy.

1.5.2. Photodynamic Molecular Beacons

Photodynamic molecular beacons are an expansion of classic peptide-based molecular beacons. Molecular beacons offer control of fluorescence emission in response to specific cancer targets and are useful tools for \textit{in vivo} cancer imaging. With our increasing knowledge about the human genome in health and disease, peptide-based “smart” probes are continually developed for \textit{in vivo} optical imaging of specific molecular targets, biological pathways and cancer progression and therapeutics. In the 1940’s, Theodor Förster published a series of papers that contributed to the formulation of Förster resonance energy transfer, FRET, theory\textsuperscript{114,115}. FRET describes the process where a chromophore (donor) in its excited state non-radiatively transfers its energy to another chromophore (acceptor) in the ground state through long range dipole-dipole interaction\textsuperscript{115}. This results in quenching of the fluorescence of the donor and/or appearance of the characteristic fluorescence of the acceptor. The efficiency of FRET depends on the overlap between the emission spectrum of the donor and absorption spectrum of the acceptor\textsuperscript{116,117}. By combining the mechanisms of protease-targeting and FRET-based activation, probes with an extremely high level of target specificity, namely peptide-based molecular beacons, were created. Classic protease molecular beacons (MBs) consisted of two chromophores attached to the same molecule by an enzymatic substrate (Figure 1.3)\textsuperscript{116}. An intramolecular interaction occurred wherein the excited chromophore (the donor) transferred its excitation energy to another chromophore (the acceptor), resulting in quenching of the fluorescence of the donor, and appearance of the acceptor characteristic fluorescence. Enzymatic cleavage of these compounds resulted in
the separation of the donor and acceptor moieties by diffusion, decreasing the yield of energy transfer, enhancing the fluorescence of the donor (and reduction of the fluorescence of the acceptor). MBs use FRET to deactivate/quench fluorophores. Therefore, the selection of an efficient donor/acceptor pair is critical for obtaining a large change in fluorescence signal for each activated beacon\textsuperscript{116,117}. These first classical protease probes were primarily used in kinetic assays studying enzymatic cleavage characteristics\textsuperscript{116}. Proteolytic activity can be continuously monitored, using molecular beacons, by the increase in fluorescence intensity over time. Beacons are attractive, as their activation is confined to tissues overexpressing the protease target, whereas they remain inactive in non-expressing tissues. Other than the specificity of MBs, another advantage of protease activation is the high signal amplification, as a small amount of enzyme can continually (catalytically) cleave, resulting in the activation of a large number of beacons. Protease-activated probes create a reduced background as a result of the quenching of the inactive form. Protease specificity is achieved by virtue of different peptide substrates and the MB template can be applied to various enzymes\textsuperscript{18,118}.

Figure 1.3. Schematic diagram of the classic activatable peptide-based molecular beacon. When the beacon is intact (A), minimal fluorescence is detectable until the beacon comes in contact with a target protease (scissors), which cleaves the peptide-linker, activating the beacon (B) resulting in a distinct fluorescence emission.
Not until 1999, were MBs applied *in vivo* and the true potential of MBs realized\textsuperscript{119}. Not only can MBs image specific tumor-associated protease activity in tumors, they can be used to assess protease inhibitor therapy at a molecular level, used as an image guidance tool distinguishing tumor from healthy tissue, and to elucidate the functional contribution of specific proteases involved in tumorigenesis, metastatic spread and angiogenesis\textsuperscript{118-120}. MB imaging has also been aided by the use of NIR fluorophores and the concurrent development of *in vivo* NIR imaging systems\textsuperscript{22,118}. Protease-activatable probes have been generating a great amount of promise for cancer imaging. They offer control of fluorescence emission in response to specific cancer targets and are useful tools for *in vivo* imaging. Classically, molecular beacons consisted of a NIR fluorescent emitter, e.g. Cy 5.5, and a NIR fluorescent quencher, e.g. NIRQ820\textsuperscript{121,122}. However, by exploiting the intrinsic multimodal NIR fluorescent and PDT properties of porphyrins, we can introduce an additional therapeutic mechanism to molecular beacons and create photodynamic molecular beacons\textsuperscript{123}. Photodynamic molecular beacons still maintain the fluorescent imaging capabilities of classic protease activatable probes but, because of the additional therapeutic mechanism, PDT, provided from using a porphyrin as the donor, the potential applications of these beacons increase. These beacons comprise a porphyrin and a quencher moiety, linked, in the present case, by an enzyme-cleavable peptide. They remain ‘optically silent’, i.e. photodynamically and optically inactive, until transformed into an activated state through cleavage of the linker, upon which both the PDT activity and fluorescence are restored\textsuperscript{123}. This is illustrated in Figure 1.4.
Figure 1.5. Schematic diagram of photodynamic molecular beacons. In the intact state (A), photodynamic molecular beacons are both photodynamically and optically silent. Once in the presence of a target enzyme, the peptide-linker is cleaved, activating the photodynamic molecular beacon (B) restoring both the photodynamic and fluorescent activity.

Not only do photodynamic molecular beacons provide an additional mechanism of selectivity in PDT over and above the targeting afforded by conventional PS and specific light delivery, but the combination of photonic imaging and therapeutic properties that are restored upon beacon activation provide real-time image-guided therapy. Chapter 2 will evaluate the advantages and pitfalls of photodynamic molecular beacons and their promising application in the treatment of breast cancer vertebral metastases.

1.5.3. Targeted Porphyrin Probes

Recent efforts to develop tumor-specific imaging and therapeutic agents have focused on the use of targeting ligands that deliver attached agents to receptors that are over-expressed on cancer cells. The high selectivity and affinity of receptor ligands enable the use of low (as low as nanomolar) doses of compounds, resulting in high signal-to-background ratios. Furthermore, receptor targeted contrast agents allow for the
characterization of biological changes on a molecular level. Initially, antibodies were used as targeting molecules. However, the immunogenicity and long plasma half-life of these molecules were detrimental\textsuperscript{124}. Consequently, the uses of small molecules, such as folic acid to target the folate receptor, instead of antibodies or antibody fragments, were employed to try and eliminate these shortcomings. Small molecules are non-immunogenic and combine high affinity and selectivity for receptors with more desirable pharmacokinetic characteristics. As previously mentioned, to date, conventional porphyrin-based PSs display only a slight preference for malignant cells leading to significant skin photosensitivity and high uptake by healthy tissues. The use of conjugating these small molecule targeting moieties to PS has been explored in order to improve preferential tumor uptake, resulting in improved efficiency\textsuperscript{125}.

The success of small molecule targeting strategies has depended on both the specificity of the targeting ligand for malignant cells and the capacity of the tumor-specific receptor to bind sufficient quantities of targeted agents to achieve contrast. The folate receptor (FR) has emerged as a viable target for both imaging and therapy, as a variety of human cancers have demonstrated FR overexpression, including cancers of the breast, ovaries, endometrium, lungs, kidneys, colon and brain, while healthy tissues demonstrate limited FR expression\textsuperscript{126-129}. In addition, folic acid and folate-linked drugs demonstrate a high affinity (Kd \textasciitilde100pM) for FRs\textsuperscript{129}. This overexpression of FR on cancer tissues can be exploited to target folate-linked imaging and therapeutic agents specifically to FR-expressing tumors with high affinity, thereby avoiding uptake by most healthy tissues.

We have previously developed a FR-targeted optical imaging and PDT agent, porphyrin-GDEVDGSGK-folate (PPF, Figure 1.5)\textsuperscript{130}. PPF is composed of three modules: 1) a near-infrared fluorescent porphyrin, 2) folate for targeted delivery of porphyrin to FR-expressing cancer cells, and 3) a short peptide as a linker.
Figure 1.5. Three components of PPF: Multifunctional porphyrin, a peptide linker and a targeting moiety (e.g. Folate). PPF is multi-modal: Near-infrared tunable fluorescence imaging, PDT and PET imaging. Reprinted (adapted) by permission from Theranostics (Liu et al. Theranostics. 2011;1:354-62.), copyright (2011).

We have demonstrated, both in vitro and in vivo, that the use of three functional modules significantly improved tumor uptake efficiency, pharmacokinetics and biodistribution of the porphyrin moiety itself. An attractive feature of PPF is the ability to optically tune its fluorescence by simply conjugating different porphyrins to the probe. We will demonstrate in chapter 3 that it is feasible to target Bchl to the folate receptor (PPF750), using a strategy that also overcomes its stability issues. We will also discuss extending the already multifunctional capabilities of PPF, fluorescence imaging and PDT, beyond photonics by incorporating $^{64}$Cu into the porphyrin moiety of PPF ($^{64}$Cu-PPF). This will effectively switch PPF from a targeted fluorescent/PDT agent into a PET probe for cancer imaging. Porphyrins have several ideal characteristics as $^{64}$Cu chelators: their aforementioned stable $^{64}$Cu-chelating ability, the clinically-validated minimal toxicity of $^{64}$Cu-porphyrin, the compatible half-lives of $^{64}$Cu and the pharmacokinetics of porphyrin, and the critical fact that $^{64}$Cu-chelation does not alter the biodistribution of the host porphyrin. There are several advantages to having multimodal applications.
in a single probe, as it ensures the same pharmacokinetics and co-localization of the probe for all applications. This also avoids placing additional stress on the body’s blood clearance mechanisms, a problem often caused by the administration of multiple doses of agents\textsuperscript{133}. Thus, the use of a single agent eliminates variability in the tumor uptake specificity, pharmacokinetics and pharmacodynamics. With the complementary nature of PET and optical imaging, a multimodal PET/fluorescent probe is an attractive combination. PET provides noninvasive whole-body imaging with deep tissue penetration and quantitative probe distribution\textsuperscript{134} not achievable by fluorescence. In contrast, fluorescent imaging has the ability for real-time, high resolution imaging\textsuperscript{108}. Chapter 3 presents how these multimodal features of porphyrins (optical tunability, \textsuperscript{64}Cu-chelation and PDT), coupled with the promising tumor targeting ability of PPF and the high resolution and deep tissue imaging capability of modern PET technology\textsuperscript{134-136}, will enable \textsuperscript{64}Cu-PPF to 1) become a novel cancer-targeted PET and fluorescent imaging probe, 2) facilitate the development of PSs via quantitative biodistribution and pharmacokinetics assessment, 3) serve as a novel means to monitor porphyrin tumor uptake in patients receiving PDT by: pre-treatment PET scanning, administering a cocktail of labeled and unlabelled porphyrin and/or during treatment using fluorescence image-guidance and, most importantly 4) open the door to transform a variety of porphyrin-based PSs into PET probes\textsuperscript{123,137}.

1.5.4. Porphyrin-based Nanoparticles

The field of nanomedicine has been gaining momentum over the past decade. Organic nanoparticles have favourable characteristics for medical applications, such as long circulation times, high payload delivery, multimodal functionalization potential and tunable size\textsuperscript{138-141}. Since porphyrins are a unique platform for the development of multifunctional imaging agents, there has been much effort in incorporating porphyrins into nanostructures. Not only would porphyrin-based nanoparticles achieve high payload, targeted delivery of a large number of porphyrin molecules, they would also introduce optical properties to organic nanoparticles. However, the lack of stability, solubility and/or biological utility have limited the field\textsuperscript{142}. Our lab has recently developed porphysomes (Fig. 1.6): all-organic, non-toxic, biodegradable nanovesicles formed from
aqueous self-assembly of porphyrin-lipid conjugates into liposome-like bilayers\textsuperscript{143}. Porphysomes possess all the functionality of liposomes, such as drug delivery capabilities and an easily functionalized surface, while exhibiting strong photonic properties due to the high-density packing of tens of thousands of porphyrin moieties in the lipid bilayer\textsuperscript{143}. This has two consequences: firstly, the optical extinction of the porphysomes is extremely high (\(~ 10^9 \text{cm}^{-2} \text{M}^{-1} \text{ at visible/near-infrared wavelengths}) and secondly, they show structure-dependent optical quenching. Hence, the intact porphysomes convert absorbed light energy into heat with high efficiency, giving photothermal and photoacoustic properties rivaling those of gold nanoparticles\textsuperscript{143}. Uptake into cells then causes dissociation of the nanovesicles, yielding unquenched porphyrins, thereby enabling fluorescence imaging or treatment with photodynamic therapy\textsuperscript{143}.

![Figure 1.6](image)

**Figure 1.6.** Porphysomes are optically active nanovesicles formed from porphyrin bilayers. A) Schematic representation of porphyrin-lipid porphysome where the phospholipid head group (red) and porphyrin (blue) are highlighted in the subunit and assembled nanostructure. B) Electron micrographs of negatively stained porphysomes\textsuperscript{143}. Reprinted (adapted) by permission from Macmillan Publishers Ltd: Nature Materials (Lovell et al. *Nature Materials*. 2011;10:324-32), copyright (2011).

In chapter 4, the potential to expand the multifunctional properties of porphysomes beyond photonics by exploiting the stable $^{64}\text{Cu}$ chelation of porphyrins within the nanoparticle structure will be discussed. Not only are PET imaging capabilities introduced into the nanovesicle, but $^{64}\text{Cu}$-porphysomes potentially address one of the current challenges in the translation of nanomedicine from bench top to beside. One of the major challenges, identified by the Food and Drug Administration and the Alliance for NanoHealth, is our current difficulty in determining and imaging the distribution of
nanoparticles in vivo: “One of the top priorities is the determination of the distribution of nanoparticulate carriers in the body following systemic administration through any route”\textsuperscript{144}. Currently, the only technique providing whole-body quantitative information is radiolabeling, for which there are a number of approaches presently used for nanoparticles, as illustrated in Figure 1.7: adding an exogenous chelator to attach the radionuclide to the nanoparticle surface, entrapping the radionuclide in an enclosed compartment, or manufacturing nanoparticles from pre-radiolabeled building blocks.

![Figure 1.7](http://onlinelibrary.wiley.com/doi/10.1002/anie.201206939/abstract)

Each method suffers from some combination of *in vivo* instability, low specific activity (activity per unit mass), or restrictive radiolabeling procedures with low radiochemical yields, long and complicated procedures, and narrow ranges of often-low concentrations\(^{133,144,146-160}\). Further, having to manufacture nanoparticles from pre-labeled building blocks on-site adds a significant burden to the end user, while the *in vivo* instability of exogenous chelators and entrapped radionuclides leads to concerns that the label is not faithful to the nanostructure or alters it such that the *in vivo* behavior of the radiolabeled nanoparticle differs from the same parent nanoparticles without the radiolabel\(^{144}\). Here, we introduce a novel, exogenous-chelator free nanoplatform with intrinsic radiotracking, in which the sole modification is the inclusion of a radionuclide directly into the building blocks of pre-formed nanoparticles. This strategy takes advantage of the unique properties of porphysomes and extends the capability of porphysomes by exploiting the aforementioned intrinsic ability of porphyrins to form stable, high affinity complexes with \(^{64}\)Cu. We directly label the building blocks of pre-formed porphysomes with \(^{64}\)Cu without altering their behavior *in vivo* yielding photonic nanoparticles with intrinsic and highly stable radiotracking. By including the radionuclide directly into the building blocks, the nanoparticles can be faithfully tracked *in vivo* while ensuring that the pharmacokinetics and biodistribution are not affected\(^{79,80}\). By virtue of being composed of a single, biodegradable building block, \(^{64}\)Cu-porphysomes achieve a high level of multifunctionality while being free of the complexity and toxicity plaguing other multifunctional nanoparticles with promising applications in oncology; all of this will be discussed in chapter 4.

In the following chapters, the three types of porphyrin-based agents introduced in section 1.5 and their potential application in cancer imaging and therapy will be discussed in detail; chapter 2 will focus on photodynamic molecular beacons, chapter 3 will discuss targeted porphyrin probes and chapter 4 will concentrate on porphyrin based nanoparticles. The final conclusion chapter will delve into the future outlook and limitations of these porphyrin-based strategies.
Chapter 2
Photodynamic Molecular Beacons

2.1 Introduction

2.1.1. Acknowledgements

In large part, chapter 2 is a reformatted version of the manuscript entitled “Imaging of specific activation of photodynamic molecular beacons in breast cancer vertebral metastases” published in Bioconjugate Chemistry\textsuperscript{161}. Under the supervision of Drs. Brian C. Wilson and Gang Zheng, my contributions to this work included designing and carrying out the experiments, analyzing and interpreting the data and writing the manuscript text. I would like to thank Drs. Juan Chen and Pui-Chi Lo for instructing and assisting with compound synthesis and purification, and in vitro studies, Dr. Margarete K. Akens for teaching and assisting with the animal model and in vitro experiments, Dr. Lisa Wise-Milestone for assistance with in vivo experiments and to John Duhig, Laura Burgess and Joseph Gold for their assistance with the animals during in vivo experiments. In this chapter, I will discuss my work in evaluating the application of photodynamic molecular beacons as a potential treatment option for breast cancer vertebral metastases.

2.1.2. Breast Cancer Vertebral Metastases

Breast cancer is the second leading cause of cancer-related death in women. The metastatic spread of tumor cells is the most devastating attribute of cancer and is often the cause of mortality. Due to the unique microenvironment of the bone, it is the most common site of distant metastases from cancers of the breast, lung, kidney, thyroid and prostate\textsuperscript{162}. The bone provides a fertile environment for the growth of cancer and promotes the aggressive behavior of tumor cells, since the mineralized bone matrix houses many growth factors, bone-destroying osteoclasts and bone-forming osteoblasts\textsuperscript{162}. Approximately 85\% of breast cancer patients with metastatic disease will develop bone metastases, in which the vertebral column is the most common site for
metastatic formation\textsuperscript{163,164}. Matrix metalloproteinases (MMPs) are a family of structurally-related, zinc-dependent endopeptidases implicated in the invasion and metastasis of cancer through the degradation of the basement membrane and collagen-rich extracellular matrix\textsuperscript{165-168}. Furthermore, MMPs are exquisite regulators of the tumor microenvironment by virtue of their ability to process many biological modulators, such as cytokines and growth factors within the bone matrix\textsuperscript{165-167}.

Breast cancer spinal metastases are predominantly osteolytic (bone destructive), which is associated with an increase in osteoclast activity\textsuperscript{162}. Metastasis disrupts the dynamic balance between bone resorption by osteoclasts and bone formation by osteoblasts, inducing a vicious cycle whereby tumor cells reprogram osteoclasts, leading to osteolysis and promotion of tumor growth\textsuperscript{162,163}. MMPs play a vital role in the signaling cascades involved in metastatic growth, and their upregulation leads to the processing of many growth factors, including TGF-\(\beta\),\textsuperscript{169} IGFs,\textsuperscript{170} E-cadherin\textsuperscript{171} and RANKL\textsuperscript{172} that are all essential for metastatic tumor progression within the vertebral body\textsuperscript{168,173,174}. MMPs themselves also directly destroy healthy tissue, particularly in the bone, since the matrix is composed primarily of mineralized fibrillar type I collagen\textsuperscript{168}, a main substrate of many MMPs. The upregulation of MMPs is thus critically involved in the destruction of the delicate balance between bone formation and degradation, reducing bone integrity (Figure 2.1)\textsuperscript{162,163}. Hence, MMPs not only aid the spread of tumor cells to distant sites, but are also involved in the local dissolution of the vertebral body and promote tumor progression within the vertebrae.
Figure 2.1. MMP involvement in the tumor-bone vicious cycle. MMPs directly promote tumor progression but also indirectly promote tumor progression by activating osteoclasts and suppressing osteoblasts\textsuperscript{161}. Reprinted (adapted) with permission from (Liu et al. *Bioconjug Chem.* 2011;22:1021-30.). Copyright (2011) American Chemical Society.

The resulting osteolysis causes bony pain, vertebral pathological fractures, progressive deformity, hypercalcemia and spinal instability\textsuperscript{162,163}. Patients with spinal metastases have a high risk of spinal cord compression, resulting in motor dysfunction, neurological compromise and an overall poor prognosis\textsuperscript{162,163}. The associated 5-year survival rate in patients with spinal metastases is below 20\% compared to a 5-year survival rate over 85\% in patients with early stage breast cancer\textsuperscript{175}. Regardless of the symptoms, all patients suffering from spinal metastases experience a substantial decrease in their quality of life. Surgery and radiation therapy are the main treatment options for these patients. Surgical treatments, however, carry a high risk of morbidity due to the proximity of the spinal cord (50\% increase in neurological decompistion after surgery), while radiation therapy is limited to a level below the optimal therapeutic dose because of the low tolerance of the spinal cord (a dose of 10Gy delivered to less than 10\% of the spinal cord has been suggested to be safely tolerated)\textsuperscript{176}. Clinical studies of radiotherapy have reported recurrence of symptoms, and pain relief is not experienced until at least 3 months after treatment\textsuperscript{177}. Lastly, although pain relief and, in part, tumor regression are addressed by radiation treatment, spinal instability is not. Therein lies the need for improved therapies
that specifically target metastatic tumor cells while preserving the spinal cord to address pain relief, tumor regression and mechanical instability of the spine.

2.1.3. PDT and Vertebral Metastases

PDT has been proposed as a promising therapeutic strategy for patients suffering from spinal metastases. It is an approved cancer treatment modality with several potential advantages over current cancer treatments due to its minimally-invasive nature, selectivity, ability to treat patients with repeated doses without initiating resistance or exceeding total-dose limitations, fast healing that results in little or no scarring, the ability to administer in an outpatient setting, minimal associated side effects and lack of contraindication with other modalities\textsuperscript{7,16,178}. Wilson \textit{et al.} have previously proposed and reported several preclinical studies on the concept of using PDT for destroying spinal metastases, particularly to debulk lesions as an adjuvant to vertebroplasty or kyphoplasty in order to mechanically stabilize weak or fractured vertebrae\textsuperscript{179-184}. These surgical procedures involve, respectively, injection of a plastic compound or placement of an inflatable balloon into the vertebral body, which is often limited by the space-occupying tumor mass. We have shown that, not only can PDT (using the clinical photosensitizer benzoporphyrin derivative A, Visudyne\textsuperscript{®} (QLT Inc, Vancouver, BC, Canada)) ablate spinal metastatic tumors\textsuperscript{179}, but unexpectedly it also enhances vertebral mechanical stability\textsuperscript{183,184}. This has led to a Phase I clinical trial with Visudyne, in which the objective is to debulk the intravertebral space-occupying tumor mass that often impedes these surgical approaches. However, current photosensitizers are limited by their non-specific accumulation in normal tissues, e.g. Visudyne has non-specific uptake in the spinal cord, limiting the therapeutic window which, in turn, reduces the aggressiveness of treatment in order to stay well within safe dose limits\textsuperscript{179,180}. Clearly, preservation of spinal cord structure and function is critical in the management of vertebral metastases.

2.1.4. Photodynamic Molecular Beacons and Vertebral Metastases

The overexpression of MMPs in vertebral metastases and the current clinical need for improved palliative therapies, led to the concept of photodynamic molecular beacons to
provide an additional mechanism of therapeutic selectivity aiding the management of breast cancer patients suffering from vertebral metastases. By utilizing a peptide sequence selectively cleaved by MMPs, we may improve the selectivity of this therapeutic approach and increase the therapeutic window while better preserving healthy normal tissue. Here, the potential of photodynamic molecular beacons is evaluated as an image-guided therapeutic approach for treating vertebral metastases. The photodynamic molecular beacon used comprises a fluorescent PS, Pyropheophorbide-α (Pyro) and a quencher moiety, black hole quencher 3 (BHQ3), linked by a MMP-cleavable peptide, GPLGLARK (PP\textsubscript{MMP}B). PP\textsubscript{MMP}B potentially addresses the limitation observed in PDT treatment of vertebral metastases using Visudyne: since PP\textsubscript{MMP}Bs are only activated in the presence of these secreted tumor-specific proteases, normal tissues, including the spinal cord, should remain relatively protected. The potential for improved selectivity of PP\textsubscript{MMP}Bs is illustrated in Figure 2.2.

Figure 2.2. Schematic of photodynamic molecular beacon (PP\textsubscript{MMP}B) activation in tumor involved vertebrae. The beacon accumulates in tissue but remains photodynamically and optically silent (A) until cleaved by MMPs at sites of metastases (B), which restores both its fluorescence for imaging and generation of singlet oxygen for treatment (C)\textsuperscript{161}. Reprinted (adapted) with permission from (Liu et al. Bioconjug Chem. 2011;22:1021-30.). Copyright (2011) American Chemical Society.
The metastases-specific activation of PP\textsubscript{MMP}B with its ability to remain inactive in critical normal tissues (i.e. spinal cord), combined with the potent PDT capability of Pyro, makes PP\textsubscript{MMP}B highly attractive by providing an additional mechanism of selectivity in PDT \cite{123,161,185}. One concern is that uptake of activated PP\textsubscript{MMP}Bs by the spinal cord may occur if activated beacons are allowed sufficient time to diffuse from the tumor to normal tissues. Here, as a first step in implementing PP\textsubscript{MMP} Bs as a therapeutic strategy for the management of vertebral metastases, the activation of PP\textsubscript{MMP}B over time is evaluated and the metastasis-selective mechanism of PP\textsubscript{MMP}Bs is validated, demonstrating the ability of PP\textsubscript{MMP}Bs to target specifically and subsequently destroy breast cancer vertebral metastases, thereby differentiating tumor and healthy tissue and enlarging the therapeutic window for PDT.

2.2. Materials and Methods:

\textit{Study Design}: PP\textsubscript{MMP}B activation was validated \textit{in vitro} using the human MT-1 metastatic breast cancer cell line. Various breast cancer tissue and cell lines have been shown to overexpress MMPs \cite{168,186,187}. Evidence of MMP expression in MT-1, a metastatic human breast cancer cell line is demonstrated in Figure 2.3.

![Figure 2.3](image)

\textit{MT-1}

MMp7

MMp10

MMp12

B2M

Figure 2.3. Preliminary reverse transcription polymerase chain reaction (RT-PCR) analysis of MMP gene expression in MT-1 cells. Beta-2-microglobulin (B2M) was used as a control. MMP primer sequences and RT-PCR protocol were previously described by Giambenardi \textit{et al.} \cite{187}. Reprinted (adapted) with permission from (Liu et al. \textit{Bioconjug Chem}. 2011;22:1021-30.). Copyright (2011) American Chemical Society.

Preliminary \textit{in vivo} activation was demonstrated in MT-1 xenografts grown subcutaneously on the flank of athymic mice. A scrambled beacon, PP\textsubscript{scrambledB}, in which
the peptide linker sequence is not cleavable by MMPs[20], was used to validate the specific activation of $\text{PP}_{\text{MMP B}}$ by the MT-1 cells. As a relevant preclinical model of vertebral metastases, MT-1 cells were injected intra-cardially to establish metastases in athymic rats. The activation of $\text{PP}_{\text{MMP B}}$ within the spinal column was assessed at each of 2, 4, 12 and 24h after intravenous injection of $\text{PP}_{\text{MMP B}}$. $\text{PP}_{\text{MMP B}}$ activation was further compared in tumor-bearing versus healthy animals, at each of 2 and 4h post injection. The fixed dose of $\text{PP}_{\text{MMP B}}$ was 3mg/kg bodyweight, equivalent to that using Visudyne in previous studies with this model $^{179}$.

$\text{PP}_{\text{MMP B}}$ Synthesis: The $\text{PP}_{\text{MMP B}}$ consists of the photosensitizer Pyropheophorbide-α (PS) and black hole quencher 3 (Q), linked by the amino acid sequence GPLGLARK, which is an MMP-cleavable peptide: italics indicate the cleavage site. It was synthesized as described previously $^{123}$. $\text{PP}_{\text{scrambled B}}$ comprises the same PS and Q but with a linker sequence, tsgpnqeek, composed of d-amino acids, which is not cleaved by MMPs $^{185}$.

Cell Line: MT-1 cells, a human metastatic breast cancer cell line, were kindly provided by Dr. O. Engebraaten, Norwegian Radium Hospital, Norway $^{188,189}$. Cells were grown and maintained in Roswell Park Memorial Institute (RPMI) 1640 media supplemented with 10% fetal bovine serum at 37°C in an atmosphere of 5% CO$_2$ in a humidified incubator. The MT-1 cells were stably infected with a double-fusion plasmid (luciferase, Luc, and green fluorescent protein, GFP) kindly provided by Dr. Joseph Wu (Stanford University) and a lentivirus kindly provided by Dr Ren-Ke Li, (University of Toronto). The expression of GFP and luciferase provides a means to identify the cells and assess their viability, respectively $^{179,180,183,184,190,191}$.

In vitro Studies: In vitro activation of $\text{PP}_{\text{MMP B}}$ was evaluated by both confocal microscopy and flow cytometry. $\text{PP}_{\text{MMP B}}$ (2nmol) or $\text{PP}_{\text{scrambled B}}$ (2nmol) was first dissolved in 2% dimethyl sulfoxide, DMSO (Sigma Aldrich) and 0.025% Cremophore® $^{123}$. The solutions were then diluted with culture medium to a final concentration of 10µM (molecular weight of the $\text{PP}_{\text{MMP B}}$ is 1856 g/mol). 2×10$^4$ cells in 0.4mL of culture medium per well were seeded in Nunc Laboratory-TekII-CC2 8-well chamber slides and incubated for 2 days at 37°C under 5% CO$_2$ in a humidified incubator
to grow to 80% confluency. PP$_{\text{MMP}}$B activation after 6h incubation of MT-1 cells was assessed using a laser scanning confocal microscope (Olympus FluoView 1000: 633nm excitation, > 650nm detection). To quantify PP$_{\text{MMP}}$B fluorescence activation and uptake in MT-1 cells, 2\times10^5 cells in 2mL of medium per well were inoculated in 6-well plates and incubated for 2 days at 37°C under 5% CO$_2$ in a humidified incubator. The cells were then incubated with 1mL of PP$_{\text{MMP}}$B or PP$_{\text{scrambled}}$B solution (10µM) for 1 and 6h respectively (similar to previous studies$^{123}$), at 37°C under 5% CO$_2$. The beacon solution was then removed after the above incubation times and assessed by HPLC-MS, high performance liquid chromatography-mass spectrometry, to confirm the beacon cleavage. The cells were trypsinized from the well plates and transferred to a 15mL centrifuge tube containing 2mL PBS. The cells were centrifuged at 1000 rpm for 6 min and the PBS was removed. This rinsing procedure was repeated 3 times. The cells were fixed by a 2% paraformaldehyde solution for 10min, after which they were centrifuged and rinsed twice with PBS. The fluorescence intensities of the cells were measured by flow cytometry (CytomicsFC 500, Beckman Coulter, CA, USA: 633nm excitation, 660-690nm detection). The maximum cell count was approximately 10,000 in each sample.

In vivo Xenograft Model: All animal studies were carried out under institutional approval (University Health Network, Toronto, Canada). Adult athymic female nude mice (Hsd:Athymic Nude-Foxn1$^{\text{nu}}$; Harlan, Indianapolis, IN, USA) were inoculated subcutaneously with 1x10$^6$ MT-1 cells in 200µL of media in both the left and right flanks for intratumoral injection studies of PP$_{\text{MMP}}$B. To evaluate the activation of PP$_{\text{MMP}}$B using a systemic injection, mice were inoculated only on the right flank. Animals were maintained in pathogen-free conditions in autoclaved microisolator cages. After 2 weeks, by which time the tumors were 5-10mm in diameter, the mice were fed a low-fluorescence diet (Harlan Tekland) for 4 days to reduce the autofluorescence background. A 2mg/kg dose of PP$_{\text{MMP}}$B or PP$_{\text{scrambled}}$B (corresponding to the dose used in previous studies$^{123}$) was formulated in 30µL of aqueous solution with 2% DMSO and 0.5% Tween-80$^{123}$. Under general anesthesia (isofluorane in oxygen), a 27G needle was used to inject this in multiple locations in the tumor to improve the distribution. A 2mg/kg intravenous injection (tail vein) of PP$_{\text{MMP}}$B was formulated in 150uL of aqueous solution
with 2% DMSO and 0.5% Tween-80. Whole-body \textit{in vivo} fluorescence imaging was performed before and at multiple time points after injection (Maestro\textsuperscript{TM}, CRi: 650nm excitation, ≥ 700nm detection, autoexposure integration time).

\textit{Ex vivo Xenograft Studies:} MT-1 tumors harvested from mice 24h following intratumor injections of beacon were snap-frozen in liquid nitrogen and stored at -70 °C. Frozen sections (10µm) were cut on a cryostat. After 5min at room temperature the slides were immersed in PBS for another 5min, then dried, and 4µL of mounting solution with DAPI, 4',6-diamidino-2-phenylindole (Vector laboratories. Inc.), was added as a nuclear stain. The sections were covered by a cover slip and imaged by confocal microscopy, similar to the \textit{in vitro} studies above.

\textit{In vivo Vertebral Metastases Model:} Female 5–6 week old athymic rats (nu/nu; Harlan Sprague Dawley, Indianapolis, IN, USA) were used as a model in which to mimic vertebral metastatic spread. The activation of PP\textsubscript{MMPB} was compared in the spinal column in tumor-bearing and healthy animals. Under general inhalation anesthesia (isofluorane in oxygen), MT-1 cells were injected intra-cardially at a concentration of 2 x 10\textsuperscript{6} in 0.2ml of RPMI 1640 media. Fourteen days later \textit{in vivo} bioluminescence imaging was performed as follows to confirm the establishment of metastases. Luciferin (Xenogen Corp., MA, USA) was dissolved in 0.9% NaCl at a concentration of 30mg/ml and a single dose of 60mg/kg was injected intraperitoneally into anaesthetized animals. Bioluminescent images were taken 15 min later, where the animals were placed ventrally in a whole-body bioluminescence imager (IVIS Spectrum: Caliper Life Sciences, CA, USA), integrating the counts over 30sec (Fig. 2.4A). Following this, 3mg/kg of PP\textsubscript{MMPB} (corresponding to equivalent Visudyne\textsuperscript{®} dose used in previous PDT studies\textsuperscript{179,183,184}) in 2.5% DMSO and 0.5% Tween80 was injected through the tail vein and, at each time point above, cohorts were euthanized by pentobarbital overdose (Euthanyl \textsuperscript{®}, Bimeda-MTC Animal Health Inc., Cambridge, ON, Canada).

\textit{Ex vivo Vertebral Metastases Studies:} At the time of necroscopy in these animals, the T13- L4 vertebrae were harvested intact, transferred to PBS and immediately cut in the sagittal plane using a low-speed precision saw (IsoMet, Buehler, Illinois). PP\textsubscript{MMPB}
activation by MT-1 vertebral tumors was assessed by confocal microscopy of the cut surface (633nm excitation, > 650nm detection) at 10X magnification. These images were overlaid by corresponding GFP images from the MT-1 cells (488nm excitation, >520nm detection), in order to evaluate the specific activation of PP_{MMP}B \textit{in vivo} by MT-1 metastases. Using commercial software (Olympus FluoView\textsuperscript{TM} FV1000), the degree of co-localization of PP_{MMP}B and MT-1 metastatic cells within the vertebral column was assessed by calculating the Pearson coefficient between the two fluorophores (activated PP_{MMP}B and GFP), with -1 representing no overlap and +1 representing 100% concordance of localization. PP_{MMP}B activation was quantified in these samples using the Maestro imaging system (650nm excitation, ≥ 700nm detection, autoexposure integration time), normalizing the total fluorescence signal within a defined region of interest (ROI) by the exposure time and the ROI area. The values used were based on the mid-sagittal images of the fluorescence within the vertebral body and the spinal cord, and the difference between these was compared using a Student t-test. To confirm metastatic formation within the vertebral column, the spines were then fixed in 10% buffered formalin, decalcified using ethylenediamine tetraacetic acid (EDTA, Sigma Aldrich, St. Louis, MO) for 2 weeks, sectioned (10\textmu m) and stained either with H&E or immunohistochemically using a mouse-anti-human epidermal growth factor receptor (hEGFr) antibody (Zymed\textregistered, Laboratories Inc., San Francisco, CA, USA). This antibody does not cross react with rat tissues, enabling specific visualization of the metastatic human breast cancer cells within the rat vertebral bone and bone marrow compartments (Fig. 2.4B).
Figure 2.4. In vivo breast cancer vertebral metastases model. (A) Representative bioluminescent image of a MT-1 bearing rat presenting with spinal metastases. (B) Example of hEGFr immunohistology sections of vertebral column where hEGFr stains for viable tumor (T - tumor, SC - spinal cord, IVD - intervertebral disc, L - lumbar, Th - thoracic)\(^{161}\). Reprinted (adapted) with permission from (Liu et al. Bioconjug Chem. 2011;22:1021-30.). Copyright (2011) American Chemical Society.

**PP\(_{\text{MMPB}}\)-PDT Treatment Studies:** The PP\(_{\text{MMPB}}\)-PDT efficacy was determined using the in vivo vertebral metastases model comparing PP\(_{\text{MMPB}}\)-PDT treatment group to control groups that included light-only and PP\(_{\text{MMPB}}\)-only animals. During the bioluminescent imaging to detect metastatic formation at 14 days post MT-1 cell intracardial injection, the area along the spine with the highest bioluminescence signal was marked on the skin, for which the brindled skin aided in orientation. These images also served as the prescan prior to treatment. PDT treatment was administered as follows: under general anesthesia, PP\(_{\text{MMPB}}\) was injected intravenously and the rat was placed in a lateral position on a warming blanket. Under X-ray fluoroscopic guidance, an 18g needle was placed adjacent to the marked vertebra with the highest bioluminescence signal (Fig. 2.5). A 400\(\mu\)m optical flat-cut fiber, coupled to a 300mW 670nm diode laser was placed into the lumen of the needle.
Figure 2.5. PDT treatment setup in vertebral metastases model. PDT was performed using a 670nm CW laser whereby the fiber was placed adjacent to the targeted vertebrae, photograph (right), guided by a fluoroscan image (left).

The light treatment occurred 2h after the end of the beacon injection. This drug-light interval was selected from the imaging validation studies. Following treatment, the fiber and needle were removed and the rats were monitored for neurological signs, including paresis (i.e. lameness) or paralysis using an established locomotor rating scale of neurologic injury (Basso-Beattie-Bresnahan). Bioluminescent imaging was repeated 48h after PPMPB-PDT treatment immediately prior to euthanasia, and the ratio of signal intensity (photons*s⁻¹*cm⁻²) from the pre-treatment and 48h post-treatment measurements over the treated locations were quantified. The data was analyzed by GraphPad Prism (GraphPad Prism for Windows, La Jolla, CA, USA). Descriptive analysis was first carried out for all treatment groups. Kolmogorov-Smirnov normality test was performed and Kruskal-Wallis tests were employed to determine whether statistically significant treatment effects were produced. The level of significance was set at 5% (p<0.05). Dunn’s Multiple Comparison test was used for pair wise comparison. Animals were euthanized using a pentobarbital overdose (Euthanyl ®, Bimeda-MTC Animal Health Inc., Cambridge, ON, Canada) or earlier if they showed signs of significant neurological deficit. At the time of necroscopy, five vertebrae centered over the PDT target were harvested intact, fixed in 10% buffered formalin, decalcified and stained with haematoxylin and eosin (H&E) or by immunohistochemistry using a mouse-anti-human epidermal growth factor receptor (hEGFr) or tartrate-resistant acid phosphatase (TRAP). The routine pathologic examination of the H&E-stained slides
included the appearance of the tumor cells and the presence of polymorphonuclear cells, apoptotic and necrotic tumor cells, as well as hemorrhage and signs of vascular changes in the vertebrae. Additionally, the spinal cord was examined for histological signs of pathological changes due to PDT treatment.

*Intramuscular VX-2 tumor rabbit model:* The activation kinetics of PP$_{\text{MMP}}$B by VX-2 tumors was first evaluated using tumors grown in the muscle. NZW rabbits weighing 3-4kg were anesthetized by an intramuscular injection of ketamine (20mg/kg) and xylazine (2mg/kg) using a 25g needle. A VX-2 tumor cell suspension of approximately 1x10$^6$ cells, was then injected into the quadriceps muscle using a 25g needle. Two weeks after injection, the tumor size reached approximately 2cm x 1cm. Under general inhalation anesthesia of 1-3% isoflurane in oxygen (2L/min), the area around the tumor was surgically prepared. A background fluorescence image was first taken using a confocal laser microendoscope (Leica FCM1000, Concord ON). An 18G needle was placed into the centre of the tumor and the 650µm in diameter flat tip confocal probe was inserted into the needle until in contact with the tumor. Using the same insertion site, an intratumor injection of 100nmol PP$_{\text{MMP}}$B (10µL DMSO, 2.5µL Tween80 in 300µL of PBS) was administered. Immediately after injection, the confocal fiber was re-inserted into the tumor and PP$_{\text{MMP}}$B activation was assessed based on the increase in the fluorescence signal over time. The animals were then euthanized by an overdose of pentobarbital (Euthanyl 120mg/kg) and the VX-2 tumor and some healthy muscle were excised and fluorescently imaged (Maestro, 650nm excitation, $\geq$ 700nm detection, autoexposure integration time) to assess PP$_{\text{MMP}}$B activation. Both the VX-2 tumor and muscle tissue samples were then snap-frozen in liquid nitrogen and stored at -70°C.

*VX-2 Femur Tumor Rabbit Model:* Using a VX-2 tumor grown within the femur, the efficacy of PP$_{\text{MMP}}$B-PDT treatment following a local injection of PP$_{\text{MMP}}$B and local light delivery was also assessed. NZW rabbits weighing 3-4kg were anaesthetized by intramuscular injection of ketamine (50mg/kg) and xylazine (5mg/kg). The rabbits were then intubated and anesthesia was maintained using 1-3% isoflurane in oxygen (2L/min). The skin around the knee joint was surgically prepared and a small incision (3cm) was made lateral of the patellar ligament. After opening the joint, the patella was dislocated
medically to gain access to the distal femur. A small hole (1.2mm diameter) was drilled between the femoral condyles, and the VX-2 cells (4x10^6 cells in 300µL) were injected using a 20G plastic catheter. The hole was closed with bone wax, the patella repositioned and the incision closed in a serial fashion using absorbable sutures. The rabbit received analgesics (Metacam – 0.2mg/kg or Temgesic – 0.1mg/kg) directly after surgery for 2 days and thereafter as required by intramuscular injection. After 14 days, the tumor reached a size of approximately 1cm x 2cm in the distal end of the femur. Tumor development was confirmed by MRI imaging (7T) under general anesthesia (Fig. 2.6A). Thereafter, for the PDT treatment, access to the femur was prepared as for the cell injection, using the same incision. PP_{MMP}B-PDT treatment was administered as follows: a background fluorescence image was first taken with the confocal fiber system. Then 100nmol of PP_{MMP}B (10μL DMSO, 2.5μL Tween80 in 300μL of PBS) was administered locally through the drill hole. The confocal fiber was then re-inserted into the drill hole to validate PP_{MMP}B activation based on fluorescence. Once fluorescence was detected, a sterile flexi needle was inserted through the drill hole into the tumor under fluoroscopic control as a sheath for a 1cm long diffusing fiber (0.8 cm diameter) coupled to a 300mW 670nm diode laser (Fig. 2.6B). A 112.5J/cm PDT light dose was administered. After treatment, the incision was closed and the rabbit received analgesics (Metacam – 0.2mg/kg or Temgesic - 0.1 mg/kg). One day after treatment, the animals were euthanized with an overdose of pentobarbital (Euthanyl 120mg/kg) under anesthesia. The femur was harvested intact, fixed in 10% buffered formalin, decalcified and stained with H&E and pan cytokeratin, cytokeratin AE1/AE3, antibody for immunohistological examination to assess the extent and effect of the treatment to the tumor and surrounding tissue.
2.3. Results

2.3.1. In vitro validation of $PP_{MMPB}$ specific activation

The specific activation of $PP_{MMPB}$ by MT-1 cells was evaluated using confocal imaging, flow cytometry and HPLC-MS analysis. By imaging the fluorescence release resulting from beacon activation, the ability of MT-1 cells to specifically activate $PP_{MMPB}$ was compared with that of the scrambled beacon ($PP_{scrambledB}$), in which the linker is not MMP cleavable, as illustrated by confocal fluorescence microscopy: Figure 2.7.

Figure 2.7. In vitro confocal microscopy of $PP_{MMPB}$ activation in MT-1 cells. In vitro fluorescence and corresponding brightfield images of MT-1 cells after 6h incubation with A) 10uM $PP_{MMPB}$ and B) 10uM $PP_{scrambledB}$ (n=3 experimental replicates)\textsuperscript{161}. Fluorescence is only detected when MT-1 cells are incubated with $PP_{MMPB}$. Reprinted (adapted) with permission from (Liu et al. Bioconjug Chem. 2011;22:1021-30.). Copyright (2011) American Chemical Society.
HPLC-MS of the MT-1 culture media and cell lysate following PP_{MMPB} incubation shows 2 peaks corresponding to the expected MMP cleaved fragments of PP_{MMPB}, Pyro-GPLG (PS-fragment) and LARK-BHQ3 (Q fragment) (Fig. 2.8). This further demonstrates the specific activation of PP_{MMPB} by MT-1 cells.

Figure 2.8. High performance liquid chromatography-mass spectrometry (HPLC-MS) analysis of PP_{MMPB} cleavage in A) cell media and B) cell lysate evaluated at 665nm (n=3 experimental replicates). In both MT-1 cell media and cell lysate, intact PP_{MMPB}, and the two expected PP_{MMPB} fragments (PS and Q fragment) due to specific PP_{MMPB} activation, are detected. Reprinted (adapted) with permission from (Liu et al. Bioconjug Chem. 2011;22:1021-30.). Copyright (2011) American Chemical Society.

Examples of flow cytometry quantifying the fluorescence intensity of PP_{MMPB} and PP_{scrambledB} in cells incubated for different times are shown in Figure 2.9A. At all time points the fluorescence intensity was 5- to 10-fold higher in cells incubated with PP_{MMPB} than with the scrambled beacon (p< 0.001).
These data demonstrate that specific activation (enzymatic cleavage resulting in unquenching of the PS fluorescence) of PP_{MMPB} is mediated by MT-1, metastatic human breast cancer cells, that express MMPs.

### 2.3.2. In vivo validation of PP_{MMPB} specific activation

The activation of PP_{MMPB} in vivo was first examined in subcutaneous MT-1 xenografts, following intravenous administration of 2mg/kg PP_{MMPB}, monitoring fluorescence release from PP_{MMPB} activation. A strong fluorescence signal localized within the MT-1 tumor (Figure 2.10A), demonstrating in vivo PP_{MMPB} activation. To further validate the in vivo MMP specificity, mice bearing MT-1 tumors on both flanks were injected intratumorally with 2mg/kg of either PP_{MMPB} (left) or PP_{scrambledB} (right) and followed over time (n=5 animals). Initially, little fluorescence was observed in either tumor. However, at 6 h a 9-fold increase in tumor fluorescence was observed with PP_{MMPB} (3.5
± 0.02 average fluorescent counts/(sec*area)) compared with $\text{PP}_{\text{scrambled}}$B (0.4 ± 0.09 average fluorescent counts/(sec*area)), the latter showing minimal change in fluorescence (Figure 2.10B). Even after 24h, confocal images of frozen sections of harvested tumors confirmed that fluorescence was only detectable in tumors injected with $\text{PP}_{\text{MMP}}$B (Figure 2.10C and D).

Figure 2.10. *In vivo* fluorescence images in the MT-1 subcutaneous xenograft tumor model. Representative fluorescent images post (A) intravenous injection of 2 mg/kg $\text{PP}_{\text{MMP}}$B (i) before and (ii) 17 h after, and post (B) intratumor injection of 2mg/kg $\text{PP}_{\text{MMP}}$B (left) or $\text{PP}_{\text{scrambled}}$B (right) at (i) 10 min, (ii) 6h and (iii) 24h post injection. C) Fluorescence micrographs and corresponding differential contrast images of $\text{PP}_{\text{MMP}}$B in MT-1 frozen tissue sections, and D) corresponding $\text{PP}_{\text{scrambled}}$B images (n=5) 24h post injection (Green – activated $\text{PP}_{\text{MMP}}$B fluorescence, DAPI – blue nucleus stain)\textsuperscript{161}. Reprinted (adapted) with permission from (Liu et al. *Bioconjug Chem*. 2011;22:1021-30.). Copyright (2011) American Chemical Society.
2.3.3. In vivo Validation of \( PP_{MMPB} \) Specific Activation by MT-1

Vertebral Metastases

Although these results are promising, evaluation of \( PP_{MMPB} \) specific activation in vertebral metastases is necessary to validate the PDT beacon concept for this particular clinical application. Following intracardiac injection of MT-1 cells, metastatic spread to the spine was seen in all animals using bioluminescent imaging. \textit{Ex vivo} fluorescence imaging of the sagitally-cut spine over 24h evaluated \( PP_{MMPB} \) activation in vertebral metastases. As shown in Figure 2.11, the highest fluorescence signal was between 2 and 4h and then significantly decreased (p<0.05) at 12 and 24h. Importantly, no fluorescence signal from activated beacons was detected above background within the spinal cord at any time point (Fig. 2.11B and C). Weak fluorescence was observed within the intervertebral discs (Fig. 2.11A). The strongest fluorescence was within the vertebral body, where metastases most commonly form. At all imaging time points, the fluorescence intensity was 6 to 9-fold higher in the vertebral body versus the spinal cord (p<0.05) (Fig. 2.11B), demonstrating that \( PP_{MMPB} \) activation is substantively confined to sites where vertebral metastases develop, with rapid onset of beacon activation after administration. Thus, either the beacon is not significantly taken up in or activated by the spinal cord or, at least over the time period of observation, there is no significant diffusion of cleaved \( PP_{MMPB} \) activated by tumors (local or distant) into the spinal cord\textsuperscript{161}. 
Figure 2.11. Time-dependent activation of PP\textsubscript{MMP}B by vertebral metastases \textit{ex vivo}. (A) Representative composite fluorescence in \textit{ex vivo} tissue at 2, 4, 12, 24h post 3mg/kg PP\textsubscript{MMP}B i.v. injection (SC – spinal cord, VB – vertebral body, IVD – intervertebral disc). (B) Average fluorescent signal comparing the vertebrae and spinal cord. There is a statistically significant difference in the PP\textsubscript{MMP}B activation in the vertebrae vs. spinal cord (p<0.05) at all time points. Statistically significant difference, * (p<0.05), in PP\textsubscript{MMP}B activation in the vertebrae at 2h compared to 12h and 24h (n = 4 for 2 and 4 h time points, n = 2 for 12 and 24 h). (C) Autofluorescence within the spinal cord in tumor bearing versus healthy animals (note: healthy animals were not evaluated at 12h and 24h)\textsuperscript{16}. Reprinted (adapted) with permission from (Liu et al. \textit{Bioconjug Chem.} 2011;22:1021-30.). Copyright (2011) American Chemical Society.
To ensure that PP$_{MMP}B$ activation was a result of the vertebral metastases expressing MMPs and not due to normal bone tissues, spines from tumor-bearing and healthy animals were imaged \textit{ex vivo} at 2 and 4h after i.v. administration of PP$_{MMP}B$. No fluorescence was observed in the spinal cord in either case. Under confocal microscopy, in which the unquenched beacon fluorescence was overlaid with the GFP signal of the cells, sites with positive GFP showed detectable activation (Figs. 2.12A and B), whereas no activation was detected in the healthy animals (Figs. 2.12C and D). Furthermore, no PP$_{MMP}B$ activation was seen within the spinal cord. The Pearson coefficients for the degree of overlap between PP$_{MMP}B$ activation and MT-1 vertebral tumors (Figs. 2.12A, B, E and F) were 0.76 and 0.68 at 2 and 4h post injection, respectively. This was not applicable in the healthy animal cohort, since no signal was detected in either imaging channel (tumor or cleaved beacon).
Figure 2.12. Micrographs of vertebrae at 2h post i.v. injection of 3mg/kg $\text{PP}_{\text{MMP}}\text{B}$ in (A, B) MT-1 tumor-bearing rats, (C, D) healthy rats. (i) brightfield image, (ii) GFP image showing the MT-1 cells, (iii) activated $\text{PP}_{\text{MMP}}\text{B}$, (iv) merged images of GFP and cleaved beacon: VB – vertebral body, IVD – intervertebral disc, SC – spinal cord. (v) hEGFr and vi) H&E stained sections (T – tumor, N – normal, 5x magnification). Note in the merged images in panel iv that $\text{PP}_{\text{MMP}}\text{B}$ activation extends beyond the GFP, indicating uptake/activation by surrounding tumor stroma (white arrows). Colocalization image analysis of (E) 2h $\text{PP}_{\text{MMP}}\text{B}$ activation and (F) 4h $\text{PP}_{\text{MMP}}\text{B}$ activation in vertebral metastases bearing animals. (i) Brightfield image, (ii) GFP (MT-1 cells) colocalization signal, (iii) Activated $\text{PP}_{\text{MMP}}\text{B}$ colocalization signal, (iv) colocalization images where VB – vertebral body and SC – spinal cord$^{161}$. Reprinted (adapted) with permission from (Liu et al. *Bioconjug Chem*. 2011;22:1021-30.). Copyright (2011) American Chemical Society.
2.3.4. In vivo $\text{PP}_{\text{MMPB-PDT}}$ Efficacy

After confirming the specific activation of $\text{PP}_{\text{MMPB}}$ by vertebral metastases, the $\text{PP}_{\text{MMPB-PDT}}$ efficacy was evaluated. Only animals with confirmed vertebral metastases based upon bioluminescent imaging 14 days post MT-1 intracardiac injections were used. The efficacy of $\text{PP}_{\text{MMPB-PDT}}$ was evaluated using the following treatment parameters: a 150J light dose, a 3mg/kg $\text{PP}_{\text{MMPB}}$ concentration and an 80mW fluence rate (n=5 animals treated). The $\text{PP}_{\text{MMPB-PDT}}$ treatment group was compared to 3 control groups: MT-1 tumor growth, 150J light-only, and 3mg/kg $\text{PP}_{\text{MMPB}}$-only (n=5 animals per group). Bioluminescent imaging occurred again 48h post treatment. By comparing the bioluminescent signals pre and post $\text{PP}_{\text{MMPB-PDT}}$ treatment, the efficacy of this therapeutic approach was assessed (Fig. 2.13A). No side effects, including neurological damage or paresis, were observed in any of the animals from the control groups, which used the highest light dose and beacon concentration. The bioluminescent signals decreased in the treated area over 48h in only the $\text{PP}_{\text{MMPB-PDT}}$ animals. This small experiment suggests that $\text{PP}_{\text{MMPB-PDT}}$ demonstrates potential efficacy but statistically significant differences ($p<0.05$) were observed between the light-only group and the $\text{PP}_{\text{MMPB-PDT}}$ treatment group when the 48h bioluminescent images were normalized to the pre-treatment bioluminescent images. This ratio is representative of the metastatic growth and used to determine the efficacy of the $\text{PP}_{\text{MMPB-PDT}}$ treatments (Fig 2.13B). Furthermore, statistically significant differences (Student t-test, $p<0.05$) were observed for the $\text{PP}_{\text{MMPB-PDT}}$ treatment group when comparing treated area versus untreated area in the spine by normalizing the 48h bioluminescent images to the pre-treatment bioluminescent images (Fig 2.13C).
Figure 2.13. PP_{MMPB}-PDT treatment efficacy summary. (A) Representative bioluminescent image of MT-1 vertebral metastases pre PP_{MMPB}-PDT and 48 h post PP_{MMPB}-PDT where red circle indicates treated vertebrae and black arrows indicate untreated areas (vertebrae and femur). (B) Scatter-plot of the relative changes in bioluminescence following PDT in vertebral metastases compared to pre-treatment levels. The bars represent the mean where * denotes statistical significance compared to all light-only group where p < 0.05 using a Kruskal-Wallis test. (C) Bar graph of the average change in bioluminescence in PDT treated and untreated vertebrae where * denotes statistical significance p<0.05 (Student t-test). Data are expressed as mean values + standard error, n=4 replicates.

Within 48h post PP_{MMPB}-PDT treatment, only 1 animal demonstrated complete hind limb paralysis and was immediately euthanized. Unfortunately, because of the aggressiveness of the model, long term recovery and overall survival could not be evaluated and thus whether the paresis was caused by PP_{MMPB}-PDT induced side effects or inflammation that would eventually rectify itself cannot be confirmed. It should be noted that there was considerable variability in the bioluminescent signal intensity of the vertebrae targeted for treatment as well as variability in the rate of metastatic growth.

Histology of the spines after PP_{MMPB}-PDT treatment confirmed PDT-induced therapeutic effect, evident from the loss of hEGFr membrane staining that indicated destroyed tumor cells (Fig. 2.14). Additionally, necrotic and apoptotic cells were detected, as well as
invasion of polymorphonuclear cells. Surprisingly, TRAP staining of osteoclasts revealed that, in areas with PDT-induced tumor cell damage, osteoclasts were also destroyed. In contrast, areas with minimal PDT damage stained for numerous osteoclasts.

Figure 2.14. Histology of spines after PDT treatment confirms PDT-induced therapeutic effect evident from H&E, hEGFR immunohistological and titrate-resistant acid phosphatase (TRAP) staining of a treated vertebrae; (i) full vertebra, (ii) zoomed PDT affected area, (iii) zoomed unaffected area. Note: A - apoptotic and necrotic tumor cells, TC - intact tumor cells and → - osteoclasts.

Although the efficacy of PP\textsubscript{MMP}B-PDT in specifically ablating vertebral metastases in an MT-1 vertebral metastases model shows promise, a limitation of the small murine model is the inability to clinically mimic the PDT treatment procedure. Thus, the use of larger animals, such as rabbits, allows for the evaluation of a local PDT procedure, which better mimics the clinical treatment scenario. In addition, the local injection of beacons may be evaluated by using larger animal models, which may further increase the therapeutic window and increase the efficacy of the PP\textsubscript{MMP}B-PDT treatment as well as better preserving normal non-target tissues. First, the activation of PP\textsubscript{MMP}B was confirmed using an intramuscular VX-2 tumor in a rabbit (Fig. 2.15). Minimal background fluorescence was detected (Fig. 2.15Ai) initially. Within 10 min post PP\textsubscript{MMP}B injection, strong Pyro fluorescence, indicative of PP\textsubscript{MMP}B activation, was detected (Fig. 2.15Aii).
When the VX-2 tumor was excised, it was apparent that some PP_{MMP}B had been injected into the muscle and not only the tumor, evident from the green accumulation in figure 2.15Bii. However, based upon ex vivo Maestro imaging, PP_{MMP}B fluorescence was detected primarily within the VX-2 tumor (Fig. 2.15Bi). Histological analysis confirmed that the tissues imaged were muscle and tumor sections (Fig 2.15C).

![Image](image1.png)

Figure 2.15. Validation of PP_{MMP}B activation by VX-2 tumors. (A) Confocal fiber fluorescent imaging of PP_{MMP}B activation (i) prior and (ii) 10min post local PP_{MMP}B injection. (B) Ex vivo (i) fluorescent imaging and (ii) photograph of excised tissue where PP_{MMP}B accumulation (green) is evident in the photograph (n=1). (C) Histology of (i) muscle and (ii) tumor using H&E staining.

Following the validation of PP_{MMP}B activation by VX-2 tumors, PP_{MMP}B-PDT efficacy was then evaluated using a VX-2 femur tumor model (n=3 animals). Similar to the VX-2 intramuscular tumor, activated PP_{MMP}B fluorescence was detected within 10min (Fig.
2.16A) but only in one animal. Histology of the femurs after PP_{MMPB}-PDT confirmed that only one animal developed a VX-2 tumor within the femur, the one that demonstrated activated PP_{MMPB} fluorescence. However, in this animal, PDT-induced therapeutic effect was evident from the loss of AE1/AE3 staining indicating destroyed tumor cells (Fig 2.16B and C). Additionally, necrotic and apoptotic cells were detected. No side effects were observed in any of the animals following PP_{MMPB}-PDT treatment.

Figure 2.16. PP_{MMPB}-PDT treatment in VX-2 rabbit femur model. (A) Fluorescent confocal imaging of PP_{MMPB} activation in VX-2 femur tumors (i) prior and (ii) 10 min post PP_{MMPB} injection (n=3). Histology of femurs 24h after PP_{MMPB}-PDT treatment using (B) cytokeratin AE1/AE3 and (C) H&E staining confirms PDT-induced therapeutic effect; zoomed (i) unaffected area and (ii) PDT affected area in femur. Note: A-apoptotic and necrotic tumor cells and TC – intact tumor cells. Scale bars in represent 500µm.
2.4. Discussion

These data demonstrate that specific activation (enzymatic cleavage resulting in unquenching of the photosensitizer fluorescence) of \( \text{PP}_{\text{MMP}} \)Bs is mediated by breast cancer vertebral metastases that express MMPs. Among the MMPs, in addition to MMP 7, the \( \text{PP}_{\text{MMP}} \)B peptide linker sequence, GPLGLA, can also be specifically cleaved by MMP 10 and MMP 12, although at a lower rate than MMP7 (Fig 2.17). However, this additional cleavage would not be a disadvantage in this application, since both MMP 10 and 12 also play roles in bone development and bone matrix solubilization.\(^{192-195}\) Since tumor cells have already hi-jacked the normal osteolytic cycle, MMP 10 and 12-mediated beacon activation may then aid in further disrupting the vicious tumor-bone cycle.

![HPLC analysis of \( \text{PP}_{\text{MMP}} \)B cleavage by various MMPs.](image)

Figure 2.17. HPLC analysis of \( \text{PP}_{\text{MMP}} \)B cleavage by various MMPs. The blue line represents elution peak of intact \( \text{PP}_{\text{MMP}} \)B whereas red line represents the elution peak of the cleaved beacon by different MMPs. Red boxes highlight the MMPs that cleave \( \text{PP}_{\text{MMP}} \)B. In addition to MMP7 as previously reported\(^{123}\), \( \text{PP}_{\text{MMP}} \)B is also specifically cleaved by MMP3, MMP9, MMP10 and MMP12.

Further development in the design of beacons is currently underway to increase specificity and sensitivity such as including using a different enzyme specific target\(^{185}\) or a “zipper” mechanism\(^{196}\). The “zipper” mechanism comprises an asymmetrical...
polyarginine and polyglutamate attached to each end of the peptide linker and to Pyro and BHQ3 respectively (Fig 2.18)\textsuperscript{196}. Thus, the electrostatic attraction between the polyarginine/polyglutamate forms a “zipper” structure.

![Diagram of the zipper structure](image)

Figure 2.18. The “zipper” is composed of a pair of polycation (polyarginine) and polyanion (polyglutamate) arms holding Pyro (D) and Black Hole Quencher 3 (Q) in close proximity due to electrostatic attraction. This results in silenced photoactivity independent of peptide linker variations. Upon specific enzymatic cleavage of the linker, Pyro and quencher dissociate, resulting in Pyro photoactivity and unleashing the polycation, which increases cellular uptake\textsuperscript{196}. Reprinted (adapted) with permission from (Chen, Liu et al. *Bioconjug Chem*. 2009; 20(10):1836-42.). Copyright (2011) American Chemical Society.

As shown in figure 2.18, the zipper provides several potential advantages: 1) the formation of the polycation/polyanion “zipper” through electrostatic attraction improves the silencing of the beacon by bringing Pyro and quencher into closer contact, 2) a “hairpin” conformation of the substrate sequence occurs as a result of the zipper, improving the cleavage rate of the enzyme-specific linker, 3) the polyanionic arm of the zipper prevents the probe from entering cells, by blocking the cell-penetrating function of the polycation, 4) the polycationic arm enhances cellular uptake of Pyro after linker cleavage, and 5) quenching is no longer dependent upon the natural folding of the peptide linker, since the zipper is solely responsible for the “dormant” state\textsuperscript{196}. In the presence of a target protease, the peptide linker is first specifically cleaved; the quencher-attached
polyanion then dissociates from the Pyro-attached polycation, becoming photoactive, and unleashes the polycation, which enhances the delivery of activated Pyro locally into the target cells. This ZMB concept is a generalizable approach to improve the functionality of a wide range of diagnostic/therapeutic probes through a simple switching of substrate sequences, which eliminates the beacon’s quenching dependence upon the natural conformation of the peptide linker.

The in vitro studies confirm the specific activation by the MT-1 breast cancer cells. The in vivo experiments in MT-1 xenografts demonstrate specific activation of PP<sub>MMP</sub>B by these tumors, using both systemic (i.v.) and intratumoral injection of the beacon. In the more clinicallyrelevant metastatic model, fluorescent imaging also confirmed tumor-specific activation of PP<sub>MMP</sub>B. There are, however, limitations with this model. The attenuation of the fluorescent signal by the overlying bone and soft tissue confounds quantitative in vivo imaging to determine the activation kinetics. Nevertheless, this could be determined by imaging the sectioned vertebrae ex vivo, albeit at only select time points, and showed that the beacon can reach and target the intravertebral tumor tissue. In addition, it remained inactive (uncleaved) within the spinal cord, which is the critical dose-limiting tissue. By using larger animals, rabbit VX-2 tumor models, a local injection of PP<sub>MMP</sub>B demonstrated specific and fast activation (fluorescence was detectable as early as 10 min post injection) by VX-2 bone tumors. It may be anticipated then that PP<sub>MMP</sub>Bs may serve as a vertebral metastases-specific imaging agent. Surgical debulking is currently one of the main treatment options for patients suffering from spinal metastases, but recurrence is common due to incomplete resection. The strong fluorescence signal and specificity of PP<sub>MMP</sub>B could enable intra-operative image guidance to increase the completeness of resection and to aid in the intraoperative detection of small metastatic lesions that are otherwise not visible. Thereby, the risk of recurrence would be reduced. This is also analogous to fluorescence image-guided tumor resection in other organs. Originally, protease-activatable probes were developed to differentiate tumor versus healthy tissue, better defining surgical margins. Thus, the initial in vivo and clinical application of beacons were intended for fluorescence image-guided tumor resection. We have demonstrated tumor-specific activation for both systemic and local administration in tumors. Intratumoral injection
was not technically feasible in the vertebral metastasis model due to the size limitation, but demonstrated fast (10 min post injection) activation in VX-2 femur tumors, which develop similarly to vertebral metastases. It is encouraging that activation occurs rapidly, so that the beacon could be administered shortly before or during surgery, assuming comparable activation kinetics in humans after a local injection at the tumor site. Furthermore, since Pyro is a potent PDT agent\textsuperscript{123,185} in addition to being a fluorescence marker, PDT could be used as a means to ‘clean up’ the surgical bed following resection, as has been used in gliomas with conventional photosensitzers\textsuperscript{197,206}. The high degree of quenching in the intact beacon (>99%) and the tumor specificity of activation are then distinct advantages, by markedly reducing the risk of collateral damage to normal tissues that are within range of the photoactivating light. Thus, PDT treatment of the entire surgical bed post-resection could result in the eradication of any residual tumor mass and microscopic vertebral metastatic deposits, while preserving normal tissues, specifically the spinal cord.

Based upon the confocal imaging of tumor cell GFP and beacon activation in vertebral metastases (Figs. 2.12Aiv and Biv), there is evidence of beacon uptake by tumor stroma as well as by the tumor cells themselves. This is not too surprising, since it is known that MMPs are also secreted by tumor stroma\textsuperscript{165,166}. However, this may also be a result of beacon diffusion from target site and uptake by the stroma. Regardless, in this particular clinical scenario, this off target diffusion of PP\textsubscript{MMP}B may not a disadvantage, as the goal is to disrupt the osteolytic cycle and destroy as much of the compromised tissue within the vertebral body as possible in order for vertebroplasty or kyphoplasty to be successful. Furthermore, histological evaluation of the vertebral bodies at 48h post PP\textsubscript{MMP}B-PDT treatment demonstrated that not only were tumor cells destroyed but osteoclasts as well. This multi-compartment targeting provides a further means to interrupt the signaling between the stroma and adjacent cancer cells by identifying cells hijacked by the tumor to promote osteolysis. Hence, eradication of the tumor microenvironment could result in further synergistic effects.

The metastases-specific activation of PP\textsubscript{MMP}B with its ability to remain inactive in critical normal tissues (i.e. spinal cord), combined with the potent PDT capability of Pyro in
beacons, makes $PP_{MMP}$B highly attractive by providing an additional mechanism of selectivity in PDT$^{123,185}$. $PP_{MMP}$B potentially addresses the limitation of off-target PDT effects from conventional PS and may be a key component in establishing PDT as a new, minimally-invasive, safe and effective therapy for the management of patients with spinal metastases. In our initial study, we are able to increase the therapeutic window for treating vertebral metastases. We are able to achieve significant reduction in tumor volumes using a 3mg/kg dose of $PP_{MMP}$B and a 150J light dose delivered at a power of 80mW. Here, we demonstrate that, in comparison to Visudyne®, we are able to increase the light dose used to treat the thoracic spine by 3 fold and by 2 fold when treating the lumbar spine. Although higher light doses than 150J were not used, there is no indication that higher light doses could not be achieved. A limitation of these studies is that animals only survive for a few weeks when using the intracardiac-injection metastatic model$^{179}$ due to widespread disease, so that long-term treatment responses cannot be evaluated. Due to the small size of the animals, evaluation of the PDT effect due to an intratumoral injection of $PP_{MMP}$B or specific delivery of light within the vertebrae is not possible. However, using the VX-2 femur tumor model in rabbits, we were able to demonstrate the efficacy of both the specific delivery of the PDT light dose, as well as the local injection of $PP_{MMP}$B. We have established, using two clinically relevant preclinical models, that using photodynamic molecular beacons in the PDT treatment of vertebral metastases have the potential to destroy tumors, disrupt the osteolytic cycle and better preserve critical organs with an increased therapeutic window demonstrating prospective as a feasible clinical approach in management of these patients.

2.5. Conclusion:

In summary, we have demonstrated specific activation of $PP_{MMP}$B by MMP-expressing vertebral metastases and VX-2 bone tumors in relevant preclinical models illustrating the rapid kinetics and ability to target intravertebral metastatic tumors with minimal uptake or activation in normal tissue. The efficient and specific photocytotoxicity of $PP_{MMP}$B in tumors was established, confirming their capabilities to destroy vertebral metastases within the vertebral body while preserving normal tissue (i.e. spinal cord). The specificity of $PP_{MMP}$B-PDT increases the therapeutic window of this treatment approach in
comparison to using conventional PS, such as Visudyne®. Furthermore, immunohistological analysis post PP_{MMPB}-PDT indicates that, not only are vertebral metastases destroyed, but also osteoclasts, potentially demonstrating the ability of PP_{MMPB}-PDT to disrupt the osteolytic cycle. Using a VX-2 bone tumor model, the specific activation and photocytotoxicity of PP_{MMPB} were further demonstrated using a local injection and local PDT treatment that mimics what may occur in the clinic. This is a first step in the further development of such beacons and their potential translation into useful clinical tools for a range of applications.
Chapter 3
Targeted Porphyrin Probes

3.1. Introduction

3.1.1. Acknowledgements

In this chapter, the multimodal theranostic capability of PPF, its FR targeting capability and its utility for fluorescence and PET imaging and PDT are validated. PPF is also evaluated in preclinical models of ovarian cancer demonstrating its clinical potential. Chapter 3 is a reformatted version of the manuscripts entitled “Multimodal Bacteriochlorophyll Theranostic Agent” and “Transforming a Targeted Porphyrin Theranostic Agent into a PET imaging Probe for Cancer” both published in Theranostics \( ^{103,131} \) and “Biologically-Targeted Detection of Primary and Micro-Metastatic Ovarian Cancer” currently under review to Clinical Cancer Research with Jocelyn Stewart as co-author. Under the supervision of Drs. Brian C. Wilson and Gang Zheng, my contributions to this work included designing and carrying out the experiments, analyzing and interpreting the data and writing the manuscript text. A huge thank you to Jocelyn Stewart for her collaboration in conceiving, designing, and performing the experiments evaluating PPF’s application as an imaging agent for ovarian cancer. Thank you to Dr. Benjamin G. Neel for providing critical analysis and revisions to the manuscript, Dr. Jiyun Shi for teaching and assisting with radiolabeling studies, Dr. Juan Chen for instructing and assisting with compound synthesis and purification, TD MacDonald for his assistance with radiolabeling procedures and to Laura Burgess and Joseph Gold for their assistance with the animals during \textit{in vivo} experiments.

3.1.2. Expanding PPF

We originally developed a folate receptor (FR)-targeted optical imaging and PDT agent using the porphyrin, Pyro (PPF). PPF is composed of 3 modules: 1) Pyro, 2) folate for
targeted delivery to FR-expressing cancer cells and 3) a short peptide, GDEVDSGK, linker. We demonstrated, both in vitro and in vivo, that the use of these 3 functional modules significantly improved tumor uptake, efficiency, pharmacokinetics and biodistribution of Pyro$^{130}$. In this chapter, I will explore the potential of expanding the functionality of PPF using 2 strategies. First, the optical tunability of PPF will be evaluated by demonstrating the ease in conjugating a different porphyrin, Bchl, to the construct; the conjugation of different porphyrin moieties does not influence the tumor targeting or biodistribution of PPF significantly. To the best of my knowledge, this is the first targeted multimodal Bchl probe (PPF750)$^{131}$. The multimodal capability of PPF750, its FR targeting capability and its utility for both optical and therapeutic purposes will be evaluated. Secondly, the functionality of PPF will be expanded beyond photonics by introducing PET imaging capabilities. Incorporating $^{64}$Cu into the Pyro moiety of PPF ($^{64}$Cu-PPF) will effectively switch PPF from a targeted fluorescent/PDT agent into a PET probe for cancer imaging$^{103}$. Porphyrins have several ideal characteristics as $^{64}$Cu chelators: their aforementioned stable $^{64}$Cu-chelating ability$^{75}$, the clinically-validated minimal toxicity of $^{64}$Cu-prophyrin$^{74}$, the compatible half-lives of $^{64}$Cu and the pharmacokinetics of porphyrin$^{79,80}$, and the fact that $^{64}$Cu-chelation does not alter the biodistribution of the host porphyrin$^{79,132}$. A stable and facile preparation of $^{64}$Cu-PPF with promising PET imaging capabilities will be demonstrated.

3.1.3. The Folate Receptor

The folate receptor (FR) is a membrane-bound protein linked to the cell surface via a glycoprophatidylinositol anchor and internalizes folates by receptor-mediated endocytosis$^{126-129}$. FR has high affinity for binding and transporting physiologic levels of folate into cells$^{126-129}$. FR is over expressed in a variety of epithelial cancers, particularly in ovarian, head and neck, brain, breast, colon, renal and lung carcinoma, with limited expression in normal tissues$^{126-129}$. Folate is a basic component of cell metabolism and DNA synthesis and repair$^{128}$. Since rapidly dividing cells have an increased need for folate in order to maintain DNA synthesis and replication, cell division, and growth and survival, it is not surprising that cancer cells upregulate FR expression$^{128}$. Overexpression of FR may possibly enable a growth advantage to tumors by modulating folate uptake
from serum or by generating regulatory signals. In addition to the promise of FR as an oncologic biomarker, FR are also over expressed and accessible on activated macrophages (i.e. associated with many inflammatory diseases, including rheumatoid arthritis, Crohn’s disease and atherosclerosis).

3.1.4. Ovarian Cancer

Epithelial ovarian cancer is the leading cause of morbidity/mortality from gynecologic malignancy, with the high-grade serous ovarian cancer (SOC) histotype representing the largest proportion of cases (65%). SOC frequently presents at advanced stages and has a poor overall survival (5-year survival of less than 30%), largely due to the inability to monitor the anatomical location of the ovaries, deep within the pelvis, combined with few persistent and usually subtle symptoms. Consequently, almost 90% of patients are diagnosed with an advanced stage of disease (stage III/IV), with widespread peritoneal carcinomatosis, and an associated five-year survival of less than 30%. Therefore, detecting small-volume disease, although essential, is difficult due to the propensity of ovarian cancer to disseminate in the peritoneum and its aggressive progression. Most patients respond to current therapies, including cytoreductive surgery and chemotherapy. However, the majority of patients recur and eventually die of their disease (70-90%). Prognostic factors include: age, stage, and residual tumor volume after surgery. Increased residual tumor volume after surgery increases the risk of relapse and decreases survival of SOC patients. Currently, there is no single accurate test to detect primary or recurrent SOC. Methods that enhance the detection of SOC, before, during and after surgery, might improve the prognosis of patients with this deadly disease.

3.1.5. Folate Receptor and Ovarian Cancer

Several studies have revealed that up to 90% of human ovarian tumors, particularly those of the SOC subtype, overexpress folate receptor (FR). By contrast, most normal tissues express low to negligible levels of FR, raising the possibility that reagents that target this receptor might be useful for imaging and/or drug delivery. Indeed, several studies have shown the efficacy and capability of using folate as a vehicle to shuttle
therapeutic and imaging agents directly to FR-overexpressing tumors\textsuperscript{216-223}. One of these studies demonstrated that the use of a folate-targeted imaging agent can identify patients with advanced ovarian cancer who are most likely to benefit from folate-targeted therapy\textsuperscript{223}. This suggests a personalized medicine application for this tool in that it identifies a subgroup of patients with increased likelihood of treatment response\textsuperscript{223}. Recently, findings from a phase II study in platinum-resistant ovarian cancer have shown a two-fold increase in progression-free survival when treated with pegylated liposomal doxorubicin (PLD); with strongest benefit in patients with FR-positive lesions\textsuperscript{219} showing evidence that a sub-population of FR-positive ovarian cancer patients exists and can be screened using companion FR-imaging agents. The first in-human clinical use of a folate-targeted FITC small molecule for intraoperative FR-specific fluorescence imaging has also been reported\textsuperscript{222}. Based on this evidence, FR has clinical potential as an imaging and therapeutic target for SOC. However, improvements to probe design that allow the use of multiple imaging modalities could increase the clinical applicability of FR-targeted agents.

Previously described folate receptor-targeted probes are either fluorescence- or SPECT-based\textsuperscript{218,220,222,223}. However, PET probes have multiple advantages, including: 1) shorter half-lives of most PET radioisotopes, allowing for faster clearance and higher dose administration; 2) ease in the ability to calculate attenuation corrections, which produces better resolved images; 3) increased sensitivity (by 2-3 orders of magnitude), providing greater accuracy; and 4) wider clinical acceptance\textsuperscript{224}. In contrast, PET lacks the ability to image patients in real-time with high resolution. Hence, there is increasing interest in dual-modality imaging agents to aid in pre-operative, intra-operative and post-operative treatment management. By combining the non-invasive sensitivity of radionuclide imaging with the real-time, high sensitivity and high resolution of optical imaging, pre-operative and post-operative assessment of tumor burden by PET could initially be used to map disseminated lesions, and fluorescence imaging could then aid in image-guided surgery to more precisely delineate tumor margins. PPF, a dual modality, PET and optical, targeted contrast agent possesses these capabilities for use in ovarian cancer management.
In this chapter, the multimodal imaging (fluorescence and PET) properties of PPF will be confirmed and evaluated in preclinical models of ovarian cancer. PPF is unique in that it has the ability to simultaneously address the current clinical challenges in ovarian cancer – pre- and post-operative noninvasive assessment of disease involvement that can be translated into real-time, intra-operative fluorescence guided therapy. I will demonstrate PPF’s ability to noninvasively identify FR positive SOC using PET and fluorescence imaging of diseased tissue in different primary human ovarian cancer models. This is the first report of a multimodal-imaging agent, PPF that delineates primary human SOC and micro-metastases in the peritoneum using both PET and fluorescence.

3.2. Materials and Methods

General Materials: The activating agents 1-hydroxybenzotriazole (HOBt) and (benzotriazol-1-yl)-N,N,N′,N′-tetramethyluronium hexafluorophosphate (HBTU) were received from Sigma-Aldrich and Novabiochem, and used without further purification. The Rink amide resins and all the 9H-fluoren-9-ylmethoxycarbonyl (N-R-Fmoc)-protected amino acids were purchased from Novabiochem. Pyropheophorbide-α (Pyro), Bacteriochlorophyll-a (Bchl acid) and folate succinimide (Folate-NHS) were synthesized as previously described\textsuperscript{106,130}.

General HPLC Methods: Reverse-phase analytical high performance liquid chromatography (HPLC) experiments were performed on a XBridge-C18 column (2.5µm, 4.6mm \times 50mm) using a Waters 2695 controller with a 2996 photodiode array detector and a Waters ZQ mass detector. The conditions were as follows: solvent A - acetonitrile; solvent B - 0.1% trifluoroacetic acid (TFA); gradient, from 20% A + 80% B to 0% A + 100% B in 12min; flow rate, 0.8mL min\textsuperscript{-1}.

Synthesis of PPF: A peptide sequence with D amino acid backbone, Fmoc-gd(OtBu)e(OtBu)vd(OtBu)gs(tBu)gk(Mtt), was synthesized on Rink resin using a Fmoc chemistry protocol. After removing the last Fmoc group, one of the two porphyrins, either Pyropheophorbide-α (PPF) or Bacteriochlorophyll-a (PPF750) or Fluorescein (PPF488), was coupled to the N-terminal of the peptide on resin at room temperature.
([porphyrin/HOBt/HBTU/peptide 3:3:3:1]). The porphyrin-peptide-resin was then treated with a cleavage cocktail (TFA: triisopropylsilane: water = 95:2.5:2.5) for 1h at room temperature to remove the resin and cleave the protected groups. The acquired porphyrin-peptide (PP) was divided into two parts. One part was purified by HPLC and used as a folate-free control in the following studies. The other part was conjugated with folate-NHS according to the previously reported protocol\textsuperscript{130}. The acquired porphyrin-peptide-folate (PPF) was purified by HPLC. The UV-visible spectrum of PPF670 and PPF750 was measured using a Varian Cary 50 UV-visible spectrophotometer. PPF670 and PPF750 were prepared in DMSO at a concentration of 1\(\mu\)M.

**Cell Lines and Culture Conditions:** Epithelial carcinoma cells with differing FR expression, KB (FR positive, nasopharyngeal) and HT1080 (FR negative, fibrosarcoma), were grown and maintained in Minimum Essential Medium Eagle (MEM) media supplemented with 10\% fetal bovine serum at 37 °C in an atmosphere of 5%\(\text{CO}_2\) in a humidified incubator. High-grade serous ovarian cancer (SOC) tissue samples and ascites were obtained from the University Health Network Tissue Bank with patient consent and Research Ethics Board approval and were verified by a pathologist following a previously reported protocol\textsuperscript{225}. Tumors were procured within 2–4h of excision. Solid tumors were minced and digested with collagenase/hyaluronidase (Stem Cell Technologies) in Dulbecco’s Modified Eagle’s Medium (DMEM) at 37°C for 2h. Red blood cells were lysed in 0.16 M ammonium chloride, and the remaining cells were filtered through a 70\(\mu\)m mesh and counted. Ascites cells were collected by centrifugation at 300g and red blood cell lysed as above. Cells can be revived and still form tumors *in vivo* after viable freezing.

**In vitro Studies:** For in vitro PDT studies, approximately \(2 \times 10^4\) cells per well (200\(\mu\)L) were seeded in Nunc Laboratory-TekIIICC2 96-multiwell plates and incubated for 2 days at 37°C under 5%\(\text{CO}_2\). The cell medium was changed to folate-free Roswell Park Memorial Institute (RPMI) 1640 medium 24h prior to treatment. PPF750 (5\(\mu\)M) was dissolved in 2\% dimethyl sulfoxide, DMSO (Sigma Aldrich), and 0.01\% Tween-80 in 200\(\mu\)L of folate-free RPMI 1640 media and incubated with cells for 16h at 37°C under 5\% \(\text{CO}_2\). The cells were then rinsed with PBS, resuspended with 150\(\mu\)L of the MEM
medium and illuminated by light. The light source consisted of a 740nm light box consisting of 48 LED diodes (Roithner Lasertechnik, Vienna, Austria). The fluence rate was 6.3mW/cm². Cell viability was then determined by means of the colorimetric MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) assay. Briefly, after illumination, the cells were incubated at 37°C under 5% CO₂ for 24h. The medium was removed and 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (Invitrogen, Burlington, ON) solution in medium (0.5mg/mL, 150µL) was added to each well followed by incubation for 2h under the same environment. 150µL of a 1:1 ratio of DMSO to 70% isopropyl alcohol in 0.1M HCl (10% by weight, 100µL) was then added to each well. The plate was agitated on a Spectra Max Plus microplate reader (Molecular Devices Corporation) for 5sec before the absorbance at 570nm at each well was taken.

For all the in vitro SOC studies, PPF was used for fluorescence in both confocal microscopy and flow cytometry. PPF (2nmol) was first dissolved in 2% DMSO, and 0.25% Tween80. The solutions were then diluted with culture medium to a final concentration of 10µM (molecular weight of the PPF is 1800 g/mol). Confocal microscopy studies used 2×10⁴ SOC cells seeded in 0.4mL of culture medium in Nunc Laboratory-TekII-CC2 8-well chamber slides and incubated for 2 days at 37°C under 5% CO₂ in a humidified incubator to 80% confluency. PPF uptake after 3h incubation with primary human SOC cells was assessed using a laser scanning confocal microscope (Olympus FluoView 1000: 633nm excitation, > 650nm detection). For flow cytometry, primary tumor/ascites or xenograft cells were thawed and recovered in defined serum-free/folate-free media (DMEM/F12 with EGF, bFGF, B27 Supplement and antibiotics) for 1h at 37°C. 10⁶ cells were incubated with or without excess folate for 30min at 37°C. They were then incubated with 50µM PPF for an additional 30min at 37°C. Cells were washed and stained for CD45 (BD Biosciences) to stain for immune cells and propidium iodide to exclude dead cells. Data were acquired on a BD LSRII Cytometer.

In vivo model: All animal studies were carried out under institutional approval (University Health Network, Toronto, Canada). Adult athymic female nude mice, weighing 20g, were inoculated subcutaneously with 2 x 10⁶ KB or HT1080 cells in 200µL of PBS on the right or left flank under general anesthesia (isofluorane in oxygen) for the PPF750 studies. Adult athymic female nude mice, weighing 20g, were inoculated
subcutaneously with 1x10^6 of KB (FR-positive human epidermal cancer) or MT-1 (FR-positive human breast carcinoma) cells in 200μL of media in the left flank under general anesthesia with 2% isoflurane in oxygen for the ^{64}\text{Cu}-PPF studies. Animals were maintained in pathogen-free conditions in autoclaved microisolator cages. After 2 weeks, the tumors were 5-10mm in diameter. For all the SOC in vivo studies, CD45-depleted cells (10^6) in 1:1 HBSS:growt factor-reduced Matrigel (BD Biosciences) were injected into the mammary fat pad (xenograft model) or peritoneum (ascites model) of Non-Obese Diabetic/Severe Combined Immunodeficient (NOD/SCID) or NOD/SCID/Il2rγ^-^ (NSG) mice. Mice were monitored for tumors for up to 6 months post-injection or until moribund. Animals were euthanized and tumors were harvested for subsequent studies or passaging and cells were harvested as described above.

In vivo optical imaging studies: A concentration of 1.8 – 2.25mg/kg of PPF670, PPF750 or PPF488 was formulated in 150μL of aqueous solution using 5μL of DMSO and 1.5μL of Tween80. When the tumor size reached 5-10 mm in diameter, mice were intravenously injected via tail vein with PPF under general anesthesia. Whole-body in vivo fluorescent imaging was performed before and at multiple time points (For PPF750 - 10min, 3h, 5h, 24h and 48h, for SOC imaging - 30min, 2h, 6.5h and 24h) after injection (Maestro™, CRi: PPF – 661nm (641-681nm) excitation, 700nm longpass detection, PPF750 – 704nm (684-729nm) excitation, 745nm longpass detection and PPF488 – 455nm (435-480nm) excitation, 490nm long pass detection, autoexposure integration time, total fluorescence signals normalized by exposure time and ROI area (total signal/(msec*pixels)). Comparison between two different probes was made using the Student t-test with the level of significance set at p<0.05. Comparison of probe uptake over time was made using a paired Student t-test with the level of significance set at p<0.05.

Ex vivo xenograft studies: KB and HT1080 tumors were harvested from mice 48h following PPF750 or PP750 injection and imaged as described in the in vivo studies.. SOC tumors were harvested from mice 24h following PPF injections and were snap-frozen in liquid nitrogen and stored at -70°C. Frozen sections (10μm) were cut on a cryostat. After 5 min at room temperature the slides were immersed in PBS for another 5 min, then dried, and 4μL of mounting solution with DAPI, 4',6-diamidino-2-phenylindole
(Vector Laboratories, Inc.) was added as a nuclear stain. The sections were covered by a cover slip and imaged by confocal microscopy similar to the _in vitro_ studies reported above. Comparison between two different probes was made using the Student t-test with the level of significance set at _p_ < 0.05.

**In vivo PDT efficacy:** Mice bearing KB tumors were intravenously injected with 50nmol of PPF750 (n=5) under general anesthesia. PPF750 uptake was monitored by _in vivo_ fluorescence imaging, and at 3h after injection PDT treatment was given to the tumor. Using a 760nm continuous wave pigtail diode laser, tumors were treated with a single PDT light dose of 137J using surface irradiation and fluence rate of 75mW/cm^2_. The mice were then monitored daily. Drug-free control animals (n=5) was treated with the same light parameters without PPF750. The control animals were sacrificed after 7 days as the tumor size had reached the experimental endpoint.

**^{64}Cu-Radiolabeling:** In a 1.5mL Eppendorf tube, 2µL DMSO was added to dissolve 50µg (~30nmol) of PPF (Pyro-Peptide-Folate). 0.1mL of 0.1M NH_4_OAc buffer (pH = 5.5) was added and vortexed, producing a dark green solution. 0.10mL of ^{64}Cu(Acetate)\_2 solution (0.5 - 5.0mCi) was then added and the reaction mixture was heated in a water bath at 60°C for 20min. After cooling to room temperature, a sample of the resulting solution was analyzed by radio-UPLC. The radiolabeling yield was > 99.9% and the radiochemical purity of ^{64}Cu-PPF was > 98% (this depends on the purity of the starting material, PPF) and the specific activity was 2.66 × 10^6 GBq/mol.

**The radio-UPLC method:** The radio-UPLC method used the Acquity™ Waters UPLC system (Waters Corp., Milford, MA) equipped with PDA detector and Bioscan radioactive detector and Acquity BEH C18 column (2.1 × 100mm, 1.7µm; Waters). The flow rate was 0.8mL/min. The mobile phase was isocratic with 80% solvent A (0.1M triethylammonium acetate, TEAA, pH 7) and 20% solvent B (acetonitrile) at 0 min, followed by a gradient mobile phase shifting from 20% solvent B at 0 min to 100% solvent B at 12 min and back to 20% solvent B at 13-15 min.

**Purification:** Purification with a Sep-Pak C18 cartridge was done according to the following procedure: 1) Attach a syringe to the Sep-Pak C18 cartridge. 2) Flush the
column with 5mL of ethanol and flush the column with 10mL of saline to equilibrate the column. 3) Load the sample onto the column and wash the sample with 10mL of saline. 4) Elute with 400µl of 80% ethanol, collect the fractions of purified sample. 5) Dry samples using a speed-vacuum and resuspend in saline. A certain amount of radioactive is washed down in step 3 if unlabeled free $^{64}$Cu is observed in the system. With the natural dark green color of Pyro and the natural dark purple colour of Bchl, the elution of porphyrin-conjugates could be easily and directly monitored visually in step 4, and the fractions with the deepest color contain the highest concentration of labeled and unlabeled porphyrin-conjugate.

**Dose Preparation:** $^{64}$Cu-PPF was prepared and administered without any further purification. The dose was prepared by dissolving the radiotracer in saline to a concentration of 2.5 - 5.0mCi/mL for MicroPET imaging, and diluted to a concentration of 0.1 - 0.5mCi/mL. The resulting solution was filtered with a 0.20 µMillex-LG filter before being administered to the animals. Each tumor-bearing mouse was injected via the tail vein with 0.1 - 0.2mL of the filtered solution.

**Solution Stability:** For *in vitro* solution stability studies, $^{64}$Cu-PPF was prepared and used without any further purification. The $^{64}$Cu-PPF was dissolved in a saline or serum solution (10% FBS in saline) to a final concentration of 1mCi/mL and left at room temperature. Samples were analyzed by radio-UPLC at 0, 6, and 24h. The samples from the serum solution were centrifuged before UPLC injection.

**In vivo MicroPET/CT Imaging:** MicroPET imaging was performed using a Siemens Focus 220 MicroPET scanner (Siemens, Munich, Germany). The tumor-bearing mice were anesthetized with 2% isoflurane in oxygen, and injected with ~500µCi of $^{64}$Cu-PPF via the tail vein, and placed near the center of the field of view where the highest resolution (1mm) and sensitivity are obtained. A 10-min static PET image was obtained at 4h post injection and 40 min static PET images were acquired at 24h post injection. Throughout the imaging, mice were kept anesthetized and directly transferred to the GE Locus Ultra microCT scanner (GE Healthcare, Little Chalfont, UK), together with the supporting bed, without any movement. CT scanning was carried out immediately after
each PET imaging session. An inhibition study was conducted where a mouse bearing a KB xenograft was injected with 500μCi of $^{64}$Cu-PPF along with 500-fold excess free folic acid (n=1). The static PET images were then acquired with same parameters at 4 and 24h post injection.

**Biodistribution Studies:** The $^{64}$Cu-PPF radiotracer (~12.5μCi in 0.1mL saline for $^{64}$Cu-PPF studies and ~500μCi in 0.1mL saline for $^{64}$Cu-PPF SOC studies) was administered into each animal via the tail vein. Animals were euthanized by 2% isoflurane, exsanguinated, and the thoracic cavity was opened at 4 or 24h post injection. Blood samples were withdrawn from the heart through a syringe. Organs were excised, washed with saline, dried with absorbent tissue, weighed and counted on a γ-counter (Perkin-Elmer Wizard-1480). Organs of interest included the tumor, heart, spleen, lungs, liver, kidneys, adrenal, stomach, intestine, muscle, bone and brain. Organ uptake was calculated as a percentage of the injected dose per gram of tissue (%ID/g). For the inhibition study, each animal was administered with ~12.5μCi of $^{64}$Cu radiotracer, along with more than 500-fold excess folic acid, and animals were sacrificed at 4 or 24h post injection for biodistribution studies (n=1). The biodistribution data and target-to-background ratios are reported as the mean and standard deviation based on results from three animals at each time point. Comparison between two different radiotracers was made using the two-way ANOVA test (GraphPad Prim 5.0, San Diego, CA). The level of significance was set at p<0.05.

**Ascites imaging studies:** Ascites fluid was first drained from the abdominal cavity using a 27G needle. Approximately 0.5-1mL of ascites fluid was removed from each animal. A mixed solution of $^{64}$Cu-PPF and PPF was administered using an intraperitoneal injection to ascitic and healthy animals. A 10-min static PET image was obtained 1h post injection. Animals were then euthanized and *ex vivo* fluorescence imaging of the intraperitoneal cavity was performed as described in the in vivo optical imaging section. Comparison between small metastatic studding and background signal was made using the Student t-test with the level of significance set at p <0.05.
Omentum optical imaging: Omentum samples were imaged prior to incubation with 10µM of PPF at 37°C. Ex vivo fluorescence imaging was performed at multiple time points (30 min, 1, 2, 4, 5.5 and 24h) similar to the in vivo optical imaging protocol above. At 24 h, the omentum was snap-frozen in liquid nitrogen with OCT media and stored at -70°C. Frozen sections (10µm) were cut on a cryostat. Frozen tissue slices were immersed in PBS for 5 min, dried, and DAPI stained. The sections were covered by a cover slip and fluorescently imaged (Olympus Upright Tiling Microscope, BX50; excitation 410±70nm, emission 685±40nm). Adjacent sections were H&E stained to confirm the presence of peritoneal carcinomatosis.

3.3. Results:

3.3.1. Optical tunability of PPF<sup>131</sup>

The optical tunability of PPF was first evaluated to ensure that conjugation of a different porphyrin did not interfere with the FR targeting ability of PPF. This was demonstrated by designing the first prototype targeted Bchl probe based on the following principles: 1) Bchl is an effective imaging and therapeutic agent, 2) the Caspase-3 peptide sequence is a stable linker and improves the delivery efficiency of Bchl and 3) folate is an effective homing molecule targeting FR-expressing tumors. On the basis of this design, PPF750 (Bchl-GDEVDGSGK(folate)), was synthesized. The final product was purified by HPLC (Fig. 3.1A) and characterized by UV-visible spectroscopy (Fig. 3.1B) and ESI mass spectrometry (Fig. 3.1C). With the purified PPF750 product, preliminary in vitro PDT studies were first conducted to evaluate the photocytotoxicity of PPF750 in FR-expressing KB cells. KB cell viability was investigated after a 16h incubation with 5µM of PPF750 in the absence and presence of light (Fig. 3.1D). The formulation used (DMSO and Tween-80) was non-toxic to cells. Using 10 and 20J/cm<sup>2</sup> light doses and 5 and 15µM PPF750, a gradient in cell viability was seen, depending on both light dose and PPF750 concentration, as expected (Fig. 3.1D). A PPF750 concentration of 5µM with a light dose of 10J/cm<sup>2</sup> reduced cell viability to 70.8% ± 1.9%. When the light dose was increased to 20J/cm<sup>2</sup>, cell viability was reduced to less than 34.7% ± 9.3%. When the
concentration of PPF750 was increased to 15μM, cell viability was reduced to less than 30% and 5% for a 10 and 20J/cm² light doses, respectively. This study demonstrates the PDT efficacy of PPF750 in vitro.

Figure 3.1. PPF750 Characterization: (A) HPLC chromatogram at 748nm, (B) absorbance spectra, (C) PPF750 structure and molecular weight ([M]+) is 1878g/mol, half mass ([M]_2+) = 939, and (D) in vitro PDT efficacy of PPF750 in KB (FR positive) cells. Data are expressed as means ± SEM of three independent experiments, each performed in triplicate. Reprinted (adapted) by permission from Theranostics (Liu et al. Theranostics. 2011;1:354-62.), copyright (2011).
To validate the tumor specificity and FR mediated uptake of PPF750, PPF750 versus PP750 (no folate) tumor uptake was evaluated in vivo with nude mice bearing KB (high FR expression) on their right flank and HT1080 (low FR expression) on their left flank. Prior to Bchl injection, minimal autofluorescence was seen in the animals. After a 25nmol intravenous injection of PPF750 or PP750, whole body fluorescence was detectable within 10min (Fig. 3.2). Within 3h, strong fluorescence signal was detectable in KB tumors and remained at 48h, whereas only a weak fluorescent signal was detectable in HT1080 tumors (Fig. 3.2A and C). At all time points for the animals administered PPF750, the fluorescent signal in KB tumors was significantly higher (p<0.05) than the fluorescent signal in HT1080 tumors (Fig 3C). Animals injected with PP750 showed strong fluorescence signal in both KB and HT1080 tumors, regardless of their FR expression, but not until 5h (Figs 3.2B and D).

Figure 3.2. Representative fluorescence images of tumor-bearing mice with KB (high FR expressing, right flank) and HT1080 (low FR expressing, left flank) xenografts. Mice were administered 25nmol (in 200µL) (A) PPF750 and (B) PP750 and imaged (i) 10 min, (ii) 3h, (iii) 5h, (iv) 24h, (v) 48h post intravenous injection. The in vivo time dependent KB versus HT1080 tumor uptake of (C) PPF750 and (D) PP750 is quantified based on the average fluorescent signal (counts/sec*area). Data are expressed as mean values + SEM (n=5) where * indicates p<0.05. Reprinted (adapted) by permission from Theranostics (Liu et al. Theranostics. 2011;1:354-62.), copyright (2011).
After 48h, animals were euthanized and tumors were harvested. *Ex vivo* fluorescent imaging of KB and HT1080 tumors mirrored the *in vivo* imaging. Again, PPF750 preferentially accumulated in FR expressing KB tumors significantly more (p<0.05) than in HT1080 tumors, while there was no significant difference in the uptake of PP750 between KB and HT1080 xenografts (Fig. 3.3).

![Ex vivo tumor uptake](image)

**Figure 3.3.** (A) Fluorescent imaging of excised KB and HT1080 tumors from animals administered 25nmol of PPF750 or PP750 after 48h. (B) Corresponding average fluorescent signals. Data are expressed as mean values ± SEM (n=5) where * depicts statistical significance at p<0.05. Reprinted (adapted) by permission from Theranostics (Liu et al. *Theranostics*. 2011;1:354-62.), copyright (2011).

The *in vivo* photocytotoxicity of PPF750 was then evaluated in mice bearing KB tumors on their right flank. The dose of PPF750 (3.6mg/kg) was double that used for fluorescence imaging to ensure PDT efficacy, but at 3.6mg/kg is lower than the 5mg/kg photosensitizer doses of Bchl analogs (for example, TOOKAD) used in previous xenograft PDT studies. Three hours after an i.v. injection of PPF750, using a 760nm CW laser at 75mW/cm² with 1cm in diameter surface irradiation, KB tumors were treated
with a total light dose of 137J. Drug free control animals were treated with the same light conditions without the injection of PPF750. The treated tumor in animals administered PPF750 became swollen within 24h and signs of tumor necrosis were evident at 48h (Fig 3.4A). All treated tumors in the mice injected with PPF750 (n=5) reduced in size within two weeks and at 30 days after PDT, 3 mice showed complete tumor regression without signs of tumor regrowth (Fig 3.4A v and vi) and 2 animals showed slight regrowth, possibly due to inefficient light treatment as a result of the difficulty in visualizing light at 760nm to ensure complete light delivery to the tumor site. In contrast, the tumors in the drug-free control animals continued to grow after PDT and within 7 days, were oversized (1.5 x 1.5cm) and the animals were sacrificed (Fig 3.4B). These results clearly demonstrate tumor accumulation of PPF750 and its photodynamic ability to cause tumor regression. Cumulatively, these data clearly demonstrate that the conjugation of Bchl using the PPF platform targets Bchl to FR, while retaining its fluorescent and photodynamic properties.

Figure 3.4. PDT efficacy in KB tumor-bearing mice (high FR expressing, right flank) 3h after an i.v. injection of (A) 50nmol PPF750 or (B) PBS (control) using a 137 J PDT light dose at (i) pre PDT, (ii) 24h post PDT, (iii) 2 days post PDT, (iv) 7 days post PDT, (v) 24 days post PDT, (vi) 32 days post PDT (n=5). Reprinted (adapted) by permission from Theranostics (Liu et al. Theranostics. 2011;1:354-62.), copyright (2011).
3.3.2. Transforming PPF into a PET Imaging Agent\textsuperscript{103}

The ability to transform PPF into a PET imaging agent by chelation of \(^{64}\text{Cu}\) was evaluated. An important condition for receptor-targeted delivery of radiolabeled agents is the development of labeling chemistry that allows for stable and facile preparation of radiolabeled biologically-active molecules. The half-life of \(^{64}\text{Cu}\) (\(t_{1/2} = 12.7\text{h}\)) provides adequate time for radiolabeling chemistry and imaging over 24-48h to accommodate PPF accumulation at targeted sites\textsuperscript{130}. The PKM linker was previously shown to enhance the water solubility of PPF (Figure 3.5), thereby improving its cancer-specificity, since water-soluble porphyrins have higher affinity for tumor tissues\textsuperscript{130,132}. We used Pyro as the porphyrin moiety in PPF in our first \(^{64}\text{Cu}\)-labeling studies.

Figure 3.5. The structure design of the PPF (Porphyrin-PKM Linker-Folate). Here the PKM linker (pharmacokinetics modifying linker) is the peptide sequence, GDEVGDGSGK\textsuperscript{103}. Reprinted (adapted) by permission from Theranostics (Liu et al. Theranostics. 2011;1:363-70.), copyright (2011).

PPF was easily dissolved in an aqueous solution with a small amount of DMSO (\(\leq 1\%)\) and was radiolabeled efficiently in 0.1M ammonium acetate buffer at 60°C for 10-20 min (Figure 3.6A). The success of the \(^{64}\text{Cu}\) labeling was determined by radio-UPLC (Ultra Performance Liquid Chromatography). By simultaneous multichannel monitoring of the Pyro-specific UV absorbance at 410nm and a radioactive signal, we were able to monitor the incorporation of \(^{64}\text{Cu}\) into PPF. As expected, no free \(^{64}\text{Cu}\) was detected during the radiolabeling procedure (Figure 3.6B). The radiolabeling yield was >99.9%, the
radiochemical purity was >98%, and the specific activity was $2.66 \times 10^6$ GBq/mol. Milder temperatures (room temperature and 37°C) were also evaluated but resulted in a lower radiolabeling efficiency (50-80%), even after 20-30 min incubation. A C_{18}-cartridge purification removed all free $^{64}$Cu and PPF was efficiently radiolabeled with $^{64}$Cu, successfully transforming the optical theranostic into a nuclear medicine tracer.

Figure 3.6. The radiolabeling procedure, quality control and stability of $^{64}$Cu-PPF. (a) Scheme for $^{64}$Cu-radiolabeling of Pyro-Conjugates, (b) quality control of $^{64}$Cu-labeled PPF by monitored by absorption and radioactivity during UPLC, and (c) in solution stability of $^{64}$Cu-PPF in saline or serum (10% FBS) solution based upon radiochemical purity, RCP (n = 3 experimental replicates)\textsuperscript{103}. Reprinted (adapted) by permission from Theranostics (Liu et al. Theranostics. 2011;1:363-70.), copyright (2011).

We next evaluated the potential of $^{64}$Cu-PPF for PET imaging of FR expression in tumors \textit{in vivo}. The FR-positive KB xenograft mouse model previously used for PPF-based optical imaging and PDT was used\textsuperscript{130}. Cu toxicity is not a concern as the dose used for PET studies (500µCi, ~18MBq) is far lower than those used for radiotherapy studies in
which a 10mCi (~370MBq) of $^{64}$Cu has been administered in murine models with no overt toxicity reported$^{229-231}$. The favorable tumor-to-background ratio of $^{64}$Cu-PPF is evident in Figure 3.7A. $^{64}$Cu-PPF easily delineates the tumor from all other tissues by PET. Inhibition imaging studies were conducted by co-injection of the radiotracer with 500-fold excess folic acid (n=1 animal). Figure 3.7B demonstrates that the uptake of $^{64}$Cu-PPF at the tumor site was significantly blocked by excess folic acid, indicating that $^{64}$Cu-PPF targeting is FR-mediated. Biodistribution studies of $^{64}$Cu-PPF in the KB xenograft models at 4 and 24h post-injection were also performed. The highest uptake was in the kidneys, in agreement with the PET images. Tumor uptake was 3.02 ± 0.55 % injected dose (ID)/g at 4h and 1.64 ± 0.33 %ID/g at 24h post injection (Figure 3.7C). The tumor-to-muscle ratio of $^{64}$Cu-PPF was 3.47 ± 0.47 at 4h and 8.88 ± 3.60 at 24h post injection (Figure 3.7D), demonstrating the faster clearance of $^{64}$Cu-PPF in non-target tissues while being retained within the tumor. Previous studies evaluating the biodistribution of $^{64}$Cu-labelled hematoporphyrin derivatives demonstrated no higher than a 1.07 ± 0.25 %ID/g$^{80}$. Inhibition studies further demonstrated the FR-specific uptake (Figure 3.7F). Additional experiments with another FR-positive tumor, MT-1, confirmed these findings (Figure 3.7E).
Figure 3.7. MicroPET/CT imaging and biodistribution. Representative MicroPET/CT images (coronal images (top) and single transverse slices passing through the tumors (bottom)) of KB tumor-bearing mice (n = 3) at 4, 24 h after intravenous injection of (A) $^{64}$Cu-PPF and (B) with pre-injection (0.5 h earlier) of 500-fold excess folic acid for blockade (n = 1). (C) Tissue uptake of $^{64}$Cu-PPF in selected organs at 4 h (red bars) and 24 h (blue bars) after intravenous injection. (D) Ratios of tumor-to-selected organs in mice administered with $^{64}$Cu-PPF at 4 h (red bars) and 24 h (blue bars) post injection. Data are presented as means ± SD (n = 3). (E) Representative MicroPET/CT image of MT-1 tumor bearing mice (n = 2) at 4, 24 h after intravenous injection of $^{64}$Cu-PPF. (F) Tissue uptake of $^{64}$Cu-PPF in selected organs at 24 h after intravenous injection. Data are presented as mean ± SD (n = 2). Reprinted (adapted) by permission from Theranostics (Liu et al. Theranostics. 2011;1:363-70.), copyright (2011).
The feasibility of radiolabeling PPF750 with $^{64}$Cu was evaluated (Figure 3.8). Although we were able to radiolabel PPF750 with $^{64}$Cu following the $^{64}$Cu-Pyro labeling procedures$^{103}$, within 24h some demetallation was observed, indicating an unstable metalloporphyrin complex. Further studies evaluating different radio-chemistry methods for a more stable chelation of $^{64}$Cu to Bchl are necessary.

We also preliminarily explored the potential of $^{64}$Cu-PPF750 to accumulate and image FR positive tumors (Figure 3.9). Mice (25-30g) received a 500µCi (~18MBq) intravenous injection of $^{64}$Cu-PPF750. MicroPET imaging and MicroCT scans were performed at 4 and 24h post-injection. Biodistribution studies of $^{64}$Cu-PPF750 in the KB xenograft models at 4 and 24h post-injection were also performed. The highest uptake was in the kidneys, corresponding to the PET imaging results. Tumor uptake was 2.92 % injected dose (ID)/g at 4h and 1.07 %ID/g at 24h post injection (Fig. 3.9C). The tumor-to-muscle ratio of $^{64}$Cu-PPF750 was 7.68 at 4h and 6.28 at 24h post injection (Fig. 3.9D).
Figure 3.9. PET imaging and biodistribution studies of $^{64}$Cu-PPF750 in mice bearing KB xenografts. Representative PET images showing a single (i) coronal, (ii) sagittal and (iii) axial slice at (A) 4 and (B) 24h after 500µCi i.v. injection of $^{64}$Cu-PPF750. Biodistribution of $^{64}$Cu-PPF750 at 4 and 24h post injection; (C) tissue uptake (% ID/g) and (D) tumor-tissue ratio. Reprinted (adapted) by permission from Theranostics (Liu et al. Theranostics. 2011;1:354-62.), copyright (2011).

3.3.3. Application of PPF in Ovarian Cancer Imaging

Using immunohistochemical staining of a panel of early passage, primary human SOC xenografts revealed that most express FR (Figure 3.10A), although the staining intensity and percentage of positive cells showed some variability. We used confocal imaging and flow cytometry to evaluate the uptake of PPF (50 µM) by primary SOC and xenograft cells ex vivo. In both ascites (n=7) and xenograft (n=3) samples, the fluorescence intensity was 5- to 25-fold higher in cells incubated with PPF, compared with DMSO-treated control cells (Figure 3.10B), or cells incubated with PPF in the presence of excess folic acid (n=3, Figure 3.10E).
Figure 3.10. FR expression and function in primary ovarian cancer. (A) Immunohistochemical staining of FR expression in human tumor samples with corresponding sample IDs below. (B) In vitro PPF uptake by primary SOC cells, as assessed by flow cytometry. Data represent fold-change in mean fluorescence intensity after 1h incubation with PPF. (C and D) Representative confocal image of primary SOC cells after 3h incubation of 10µM PPF and control; (i) PPF fluorescence and (ii) bright field image (n=3 experimental replicates). Folate competitively inhibits PPF binding. (E) In vitro PPF uptake by primary SOC cells, detected by flow cytometry. Data indicate fold-change in mean fluorescence intensity after 1hr incubation of primary human SOC with PPF or PPF plus folate as a competitor. (F) Incubation of primary SOC or xenograft cells with PPF does not affect the viability (propidium iodide staining), as assessed by flow cytometry. Relative viability (Treatment/Control) is shown.\(^{232}\)
Cell viability was unaffected by PPF exposure (Figure 3.10F). Likewise, cells incubated with PPF showed detectable intracellular fluorescence (Figure 3.10C and D) compared with control cells (without PPF). Taken together, these observations confirm that primary human SOC cells take up PPF.

Early passage xenografts derived from primary SOC recapitulate the inter- and intra-patient heterogeneity observed in SOC\textsuperscript{233}, unlike cell line-derived xenografts\textsuperscript{234}. Therefore, we used our established primary mammary fat pad xenograft assay as a pre-clinical model to test the \textit{in vivo} efficacy of PPF in multiple patient-derived samples (n=6). The uptake of PPF (2.25mg/kg) \textit{in vivo} was examined using primary human ovarian xenografts in mice following intravenous administration and monitored by whole-body fluorescence imaging. A strong fluorescence signal localized within the tumor at 24h post injection (Figure 3.11A) and confirmed by confocal microscopy of frozen sections (Figure 3.11D). PPF is easily optically tunable by conjugating different fluorophores: Fluorescein isothiocyanate, FITC (PPF488) versus Pyro (PPF) versus Bchl (PPF740) creating probes in the green, red and near infrared range, respectively. The uptake of each of these probes following intravenous administration was determined (Fig. 3.11B and C). As expected, with increasing excitation wavelength, the tumor-to-background ratio increased, likely due to decreased auto-fluorescence and increased penetration depths of longer wavelengths of light (Fig. 3.11E). PPF488 demonstrated only a 1.5 fold increase in tumor fluorescence over background. However, using fluorophores in the red (PPF) and near-infrared (PPF740) region demonstrated a tumor-to-background ratio of 7.94 ± 3.94 and 11.21 ± 2.62 fold, respectively, which was significantly (p<0.05) higher than PPF488.
Figure 3.11. *In vivo* fluorescence of PPF in primary ovarian xenografts in the mammary fat pad. (A) Spectral reflectance fluorescence images (i) before, (ii) 15min, (iii) 2h, (iv) 6.5h and (v) 24h post 2.25mg/kg intravenous PPF injection. Spectral reflectance fluorescence images of (B) PPF740 or (C) PPF480 (i) before and (ii) 24h post 2.25mg/kg intravenous injection. Red circle indicates location of tumors. (D) Microscopic confocal images showing the localization of PPF in 10μm primary ovarian tumor frozen sections 24h post injection of (i) PPF fluorescence images (Green) overlaid with DAPI (blue) and (ii) differential interference contrast images. (E) Average tumor-to-background fluorescent fold increase comparing PPF488, PPF and PPF740 at 24h post injection. Data are expressed as mean values ± SEM (n=4) where * depicts statistical significance with a p <0.05.

As PET imaging has broader use in the clinical setting and provides more accurate and quantitative measurements of a probe’s behavior *in vivo*, we evaluated $^{64}$Cu-PPF microPET/CT imaging of primary human SOC xenografts accordingly at 4 and 24 hours post-injection (Fig. 3.12A and B). $^{64}$Cu-PPF easily delineates the tumor from all other tissues by PET imaging at 24h post-injection and the favorable tumor-to-background
ratio of $^{64}\text{Cu}$-PPF is evident in figure 3.12B in this mammary fat pad xenograft model. Biodistribution studies of $^{64}\text{Cu}$-PPF in primary human ovarian xenograft models at 4h and 24 h post injection were subsequently performed. The highest uptake was observed in the kidneys and liver, consistent with the PET imaging results (Figure 3.12C). Tumor uptake was $2.01 \pm 0.03 \%$ injected dose (ID)/g at 4 hours, and $2.06 \pm 0.31 \%$ ID/g at 24 hours, post-injection (Figure 3.12C). Although the tumor uptake of PPF was not as high as observed for previous radionuclide imaging agents$^{216,217}$, it should be noted that the animals were not given a folate-free diet prior to imaging and thus, these low uptake values may be a result of competition with endogenous folate ligands. However, the tumor-to-muscle ratio of $^{64}\text{Cu}$-PPF was $8.91 \pm 0.91$ at 24 hours, post-injection (Figure 3.12D), demonstrating the fast clearance of $^{64}\text{Cu}$-PPF in non-target tissues while $^{64}\text{Cu}$-PPF is retained within the tumor. The tumor-to-tissue ratio of $^{64}\text{Cu}$-PPF as high as 30-fold respectively, depending on the tissue being compared. The tumor-to-tissue ratio all increased at 24h compared to that at 4h demonstrating the fast clearance of PPF while being retained at the tumor site. Importantly, the tumor-to-muscle ratio of $^{64}\text{Cu}$-PPF at 24h closely matched that found for PPF fluorescence. These results demonstrate both the fluorescence and PET imaging capabilities of PPF to delineate primary human ovarian tumors.
Figure 3.12. Representative microPET/CT image of $^{64}$Cu-PPF at (A) 4h and (B) 24h post intravenous injection of i) axial, ii) coronal and iii) sagittal slices. White arrows indicate tumors. (C) Corresponding $^{64}$Cu-PPF biodistribution data at 4h (n=3) and 24h (n=6), reported as percent injected dose per gram of tissue (%ID/g). (D) Tumor-to-tissue ratio of injected dose shows an 8.91 ± 0.91-fold increase in tumor to muscle ratio uptake at 24h post $^{64}$Cu-PPF administration. Data are expressed as mean values ± standard deviation.

Although mammary fat pad xenografts recapitulated the heterogeneity of SOC, they do not model other disease manifestations, such as peritoneal studding and ascites generation. Therefore, we also generated intraperitoneal xenografts from primary SOC, and tested the ability of PPF to accumulate in small metastases. In order to decrease the time between drug administration and imaging, increase the drug concentration at target
sites, and decrease off-target accumulation, we administered PPF by a single intraperitoneal (i.p.) injection. This changed both the PET and fluorescent imaging time point to 1h post i.p. injection. A mixture of $^{64}$Cu-PPF (500µCi) and PPF (2.25mg/kg) was injected and 1h later, animals were PET/CT imaged. In figure 3.13A, a strong PET signal is evident in the abdominal cavity of only animals presenting with ascites while healthy control mice showed no evident uptake of $^{64}$Cu-PPF. Likewise, we could detect small metastatic studding (<1mm in size) on the peritoneal wall of animals with ascites by in situ fluorescent imaging after exposing the peritoneal cavity, while healthy mice demonstrated minimal fluorescence uptake (Fig 3.13B and C). Fluorescence uptake into metastases was 3.5-fold higher than into adjacent normal tissue (p<0.001, n=5; Fig. 3.13D). Fluorescence microscopy of frozen peritoneal slices also revealed the selectivity of PPF for malignant cells (Fig. 3.13E). Histological analysis of serial H&E stained sections confirmed the presence of small, fluorescent nests of malignant cells (Fig. 3.13F). These results demonstrate the ability of PPF to identify animal with peritoneal spread of SOC by PET and fluorescence imaging and reveal an excellent correlation between the non-invasive detection of peritoneal deposits by the two imaging modalities.
Figure 3.13. Tumor-specific uptake of PPF, measured by PET and fluorescent imaging in mouse ascites model. (A) Representative 3D PET/CT image of animals 1 hr post-intraperitoneal injection of $^{64}$CuPPF (500µCi) and PPF (2.25mg/kg), followed by (B) in situ composite fluorescent imaging of peritoneal cavity in: (i) ascites-bearing and (ii) control mice. (C) Ex vivo fluorescent imaging of peritoneal tissue in (i) ascites-bearing and (ii) control mice. White arrows depict small metastatic deposits in the peritoneum. (D) Average fluorescent signal of uptake of PPF versus background in micro-metastases. There is an approximately 3.5-fold increase in tumor to background ratio of PPF. Data are expressed as mean values ± standard deviation (n=5); * p <0.001. (E) Histologic confirmation of PPF uptake and selectivity in micro-metastases. Frozen peritoneal slices (10µm) were stained with DAPI (blue). Representative PPF fluorescence (red) images were compared to sequential (F) histology slices (H&E), confirming that fluorescent studding represents tumor foci. Full tissue slice is shown in inset; scale bars in (E) and (F) represents 500µm.

Finally, we evaluated the potential of PPF to be taken up by tumor deposits in the peritoneum ex vivo. Primary omentum from SOC patients (including tumor and adjacent normal tissue/stroma) was incubated in PPF (10µM). After 30 minutes, fluorescence was clearly detectable in the omentum (Fig. 3.14A and B). Fluorescence microscopy of frozen omental slices again demonstrated selective uptake of PPF into cancer cells (Fig. 3.14C),
an assessment confirmed by histological analysis (Fig. 3.14D). These data suggest that PPF also could be used intra-operatively to identify residual tumor during surgical debulking procedures using fluorescence imaging.

Figure 3.14. Uptake of PPF by primary human omentum from high-grade serous ovarian carcinoma patient. Imaging of human omentum by (A) fluorescence and (B) white light (i) before and (ii) 30 min after topical incubation with PPF (50µM). Corresponding representative (C) fluorescence (red) images were compared to sequential (D) H&E-stained slices of (i) magnified and (ii) full tissue slice, confirming microscopically the uptake and selectivity of PPF for cancerous cells. Frozen 10µm slices were DAPI-stained (blue). (Pyro excitation 410±70 nm, detection 685±40 nm)232.
**3.4. Discussion:**

These data demonstrate successful synthesis and characterization of an optically tunable targeted multimodality porphyrin probe (PPF) with efficient PET and optical imaging properties and effective PDT capabilities. *In vivo* optical imaging studies confirmed that the conjugation of Bchl using the PPF platform targets Bchl to FR-expressing tumors, while retaining its fluorescent and photodynamic properties. It is evident that the conjugation of folate-caspase 3 peptide to Bchl stabilizes this porphyrin, changing Bchl’s properties and tumor retention characteristics. Folate enhances the potential of Bchl’s own non-specific affinity to cancer cells; without folate, PP750 still demonstrated tumor accumulation, however the conjugation of folate significantly enhanced FR-expressing tumor uptake of Bchl by almost 2 fold over tumors with low FR expression. *In vitro* PDT studies demonstrated PPF750’s photocytotoxicity, which was mirrored *in vivo*, whereby complete tumor regression was achieved. Although a 16h drug-light interval was used *in vitro*, a 3h drug-light interval was used *in vivo*, as the optical imaging studies demonstrated that the highest PPF750 uptake occurred at 3h. This is not surprising, as probe uptake demonstrated *in vitro* rarely translates to similar uptake characteristics *in vivo*.

Fluorescence imaging is proving to be a powerful tool for cancer diagnostics and treatment. It has received much attention due to its ease of implementation, operational simplicity, low cost and recent development of novel fluorescent probes\(^{16,108,133}\). The tumor targeting ability of PPF750 and its favourable excitation and emission spectra \((\lambda_{\text{excitation}} = 748\text{nm}, \lambda_{\text{emission}} = 766\text{nm})\) make it an attractive optical agent, providing efficient tissue penetration of photons and minimal background autofluorescence. The optical imaging properties of PPF750 with its tumor selectivity may enable its use as an effective image guidance tool during surgical resection and aid in the intraoperative detection of small metastatic lesions that are otherwise not visible. PPF750 could also be used as means to ‘clean up’ the surgical bed following resection. PDT treatment of the entire surgical bed post-resection could result in the eradication of any residual tumor mass and microscopic metastatic deposits, while preserving normal tissues.
Recently, interest has risen in using positron emission tomography (PET) for disease detection and treatment planning. PET is attractive as it provides quantitative drug biodistribution, and effective treatment planning, and provides non-invasive deep tissue images, key characteristics not achievable by optical imaging and/or PDT\textsuperscript{134}. Similar to optical probes, targeted radiotracer development is an important key to increasing tumor specificity and sensitivity\textsuperscript{134,235}. Thus, attempts have been made to exploit the tumor avidity of porphyrins by the incorporation of radioisotopes for targeting PET probes\textsuperscript{236,237}. Through the present study, we hope to revitalize this field of radio-metalloporphyrins, based on the demonstration of $^{64}$Cu-PPF as a targeted PET imaging probe for FR-positive tumors. We have shown the ease and efficient radiolabeling of PPF with $^{64}$Cu, while retaining its favorable biodistribution, pharmacokinetics and selective tumor uptake, characteristics that were first demonstrated optically. Clearly, the 12.7h half-life of $^{64}$Cu is compatible with the pharmacokinetics of PPF, providing adequate time for both the radiolabeling chemistry and accumulation of $^{64}$Cu-PPF at tumor sites. Here, we report the first stable chelation of $^{64}$Cu with a chlorophyll moiety, Pyro. Not only is $^{64}$Cu-PPF a promising diagnostic tool for FR-positive tumors, the use of $^{64}$Cu-PPF may be employed for prediction and quantitative measurements of photosensitizer accumulation in tumors to aid in treatment planning and monitoring of PDT treatments. Radiolabeling PPF also provides a more accurate and quantitative measurement of the probe’s \textit{in vivo} biodistribution, overcoming the difficulty of absolute quantification of fluorescence \textit{in vivo}. The ability of Bchl as a radioisotope delivery vehicle was also evaluated. As demonstrated by TOOKAD, Bchl itself may be an excellent metal chelator, forming a highly stable metallo-complex, making it an efficient delivery vehicle of radioisotopes\textsuperscript{72,74,238}. Previous studies have demonstrated that the stability of Bchl is dependent on its central metal, where the Cu-Bchl metal complex is the most stable\textsuperscript{112}. Thus, similar to how palladium stabilizes Bchl (TOOKAD), the insertion of $^{64}$Cu into Bchl’s porphyrin ring may further stabilize Bchl making $^{64}$Cu-PPF750 not only an effective, but highly stable, PET imaging probe. Unfortunately, although we were able to radiolabel PPF750 with $^{64}$Cu following the $^{64}$Cu-PPF labeling procedures\textsuperscript{103}, within 24h, some demetallation was observed, indicating an unstable metalloporphyrin complex. This may be a result of the non-planar conformation of the Bchl macrocycle. Further studies
evaluating different radiochemistry methods for a more stable chelation of radioactive metal ions to Bchl are necessary. With the multifunctional properties of porphyrins and the efficient and stable incorporation of $^{64}$Cu, into Pyro, this approach of first developing a porphyrin-based optical theranostic probe with excellent *in vivo* tumor targeting characteristics and then switching it to a targeted nuclear imaging probe through chelation of a radioisotope might be translated to any targeted porphyrin-based agent. The appeal of introducing PET properties into PPF addresses many limitations currently faced by optical imaging, including unlimited deep tissue penetration, quantitative biodistribution and an efficient non-invasive means for treatment planning. The use of a single agent avoids any variability in the tumor uptake specificity, pharmacokinetics and pharmacodynamics of PPF. Small molecule multimodal probes, such as PPF, are attractive because they are typically cleared by the renal system, avoiding any toxicity associated with long-term liver retention, and have a fast clearance time and rapid diffusion through tissue$^{4,7,17,18,108,130,133}$. Sensitivities for radiotracers and optical imaging probes are within the same range$^{133}$ and, therefore, are an attractive combination. The multimodal nature of PPF provides complimentary information with regards to tumor delineation that should be useful in treatment planning and image guidance as well as drug accumulation, pharmacokinetics and pharmacodynamics. It is particularly useful in determining the ideal drug-light interval in PDT treatments. This efficient PDT capability of PPF, combined with its optical and PET imaging properties, makes PPF a powerful imaging and therapeutic tool that may boost the clinical benefits of hybrid technologies. The complimentary nature of PET and optical imaging may provide a means to translate PET treatment planning to the surgical table using fluorescence image guidance in a single probe.

To the best of our knowledge, this is the first report of a targeted, multimodal (PET/optical) probe useful for imaging ovarian cancer. We confirm the high specificity of PPF in primary human models of SOC (cell suspensions and xenografts; Fig.3.11). We demonstrate the ability of systemically or intraperitoneally injected PPF to clearly delineate FR-positive, primary human SOC xenografts in the superficial mammary fat pad (Fig. 3.12), as well as bulk tumor and micro-metastatic studding in the peritoneum, both by PET and fluorescence imaging (Fig. 3.13). In addition, we validate the *ex vivo*
uptake of PPF by metastatic deposits in primary human omentum (Fig. 3.14), similar to a previous report showing the utility of a folate-targeted fluorescent probe \(^{222}\). PPF thus has the capacity to act as a “one size fits all” probe for detecting and monitoring SOC.

Primary human SOC cells and \textit{in vivo} models were used for all of our studies because they retain the heterogeneity and phenotype that is displayed by patients\(^{233}\). Many studies of ovarian carcinogenesis, drug response and imaging efficacy have used immortalized cell lines that have been shown to poorly recapitulate the disease\(^{234}\). By using tumor cells derived from primary patient samples to evaluate the sensitivity of our multimodal PPF probe, we expect our results to better predict response in, and applicability to, SOC patients.

The value of PET\(^{239-241}\) and optical imaging\(^{242,243}\) have been evaluated in ovarian cancer, although separately. Nevertheless, translation of such probes to the clinic requires the development of improved contrast agents to increase tumor sensitivity and specificity. Because FR is over-expressed in SOC, FR-targeted imaging and therapeutic agents have shown promise in clinical trials\(^{130,218,220,222,223}\) cementing FR as a viable molecular target in this disease. Two studies independently demonstrated the utility of FR-targeted probes, either SPECT or fluorescence, for identifying FR-positive ovarian cancer\(^{222,223}\). These promising studies showed the clinical potential of FR-targeted imaging agents in SOC management; however, they highlighted a niche for a multi-modal agent. We have developed such an agent, PPF, whose appeal and novelty lie in its: 1) complementary imaging capabilities: specifically, the non-invasive, deep tissue penetration and quantitative nature of PET, combined with high-resolution, real-time fluorescence, ideal for surgical guidance; 2) targeted uptake, increasing the signal-to-noise ratio, and retention in tumors; and 3) applicability as a single agent, reducing concerns about variability in tumor uptake specificity, pharmacokinetics and pharmacodynamics.

PPF could aid the current clinical treatment strategy for ovarian cancer patients in several ways. As a PET agent, it could be used for staging pre- or post-operatively, allowing high-resolution evaluation of the extent of disease before and after treatment. Concomitantly, the fluorescence imaging properties of PPF could aid in image-guided
surgery to precisely delineate tumor margins and/or residual disease. Optimal debulking (<10mm residual tumor) results in a significantly improved outcome for SOC patients\textsuperscript{244}, and patients with no detectable tumor at the time of resection demonstrate even greater survival\textsuperscript{245}. This demonstrated ability to identify metastatic deposits smaller than 1mm could facilitate more complete debulking than is possible currently. PPF also has potential as a disease-monitoring and recurrence-detection tool. Currently, CA125 allows detection of relapse approximately three months sooner than CT or MRI modalities\textsuperscript{246}, and combined with PET/CT, further expedites recurrence diagnosis\textsuperscript{247}. At present, patients treated immediately upon biochemical relapse show no significant improvement in survival over those detected only when bulk disease recurs \textsuperscript{246}. However, the current lack of benefit of detecting recurrence earlier most likely reflects the paucity of effective treatment options for relapsed disease. The future advent of new, targeted therapeutics and/or immunotherapies could make the diagnosis of smaller tumor bulk, not only important, but also essential. In turn, more sensitive imaging methods to improve treatment planning, response assessment and residual and/or recurrent disease detection will be needed. Moreover, 10-20\% of SOC patients do not produce CA125, and at present, can only be monitored by radiologic methods \textsuperscript{247,248}. Unfortunately, the sensitivity of CT and MRI is less than 50\% for lesions less than 1 cm in size resulting in the detection of disease at larger volumes\textsuperscript{239}. These patients also would benefit from more sensitive detection methods, including targeted PET and optical agents such as PPF.

Finally, it should be noted that Pyro and Bchl are potent photodynamic agents\textsuperscript{130,131}. Intraperitoneal PDT was evaluated in Phase II clinical trials, but did not demonstrate significant complete responses or long-term tumor control, with the ineffectiveness attributed to lack of tumor specificity in photosensitizer (Photofrin) uptake\textsuperscript{249}. The high degree of tumor specificity of PPF might circumvent this limitation, by markedly reducing the risk of collateral damage to normal tissues within the peritoneum exposed to the photo-activating light. Thus, in addition to its utility as an imaging agent, PPF might aid in the eradication of residual intraperitoneal tumor and microscopic metastatic deposits by applying a tumor-targeted PDT treatment to the entire surgical bed post-resection.
3.5. Conclusion:

Here, we report the successful synthesis and characterization of a novel multimodal FR-targeted imaging and PDT agent. All results demonstrate the FR specificity of PPF, as it only accumulates in FR-expressing tumors in vivo and serves as an efficient fluorescent and PET imaging probe for the detection of FR-positive tumors. Furthermore, PPF750 caused complete tumor regression in vivo after PDT treatment. Therefore, PPF is not only a multimodal imaging probe for FR-expressing tumors but also a promising therapeutic agent for FR-positive cancers. Finally, we report, to the best of our knowledge, the first application of a multimodal, PET and optical, folate-targeted imaging agent, PPF, for SOC imaging. We demonstrate the PET and fluorescent imaging capabilities of PPF to specifically delineate primary human SOC using both systemic and intraperitoneal administration routes. PPF offers the unique opportunity to noninvasively preoperatively identify patients with FR-expressing tumors and the extent of tumor involvement by PET followed by intra-operative fluorescence-guided surgery to localize and remove/treat small metastatic tumors. Finally, this probe will be useful for monitoring treatment response and the onset of recurrence by PET imaging. Taken together, our results demonstrate that PPF is an “all-in-one” imaging agent that could substantially improve the prognosis of patients with SOC by allowing pre-, post- and intra-operative tumor monitoring, detection and possibly also treatment throughout all stages of therapy and tumor progression.
Chapter 4
Porphyrin-based Nanoparticles

4.1. Introductions

4.1.1. Acknowledgements

Chapter 4 is a reformatted version of the manuscripts entitled “All-Organic Nanoparticles with Intrinsic Radiotracking” in Angewandte Chemie and “All-Organic Intrinsically Multimodal Nanoparticles For Prostate Cancer Imaging” currently under review in PNAS with T.D. MacDonald as co-author on both papers. Under the supervision of Drs. Brian C. Wilson and Gang Zheng, my contributions to this work included designing and carrying out the experiments, analyzing and interpreting the data and writing the manuscript text. A huge thanks you to T.D. MacDonald for his collaboration in conceiving, designing, and performing the experiments. Thanks to Cheng Jin for her assistance with producing the PC3 orthotopic model, Dr. Robert Bristow and Carla Coakley for providing the 22RV1 orthotopic model, Drs. Ming Tsao and Margarete Akens for assistance with histopathology analysis, Dr. Thomas Rosol for kindly providing the Ace-1 YFP-Luc cells, Dr. Warren Foltz for assistance with MR imaging, Tab Siddiqi and Dr. David Green for assistance with the radioUPLC, Drs. Helen Lee, Jinzi Zhang, Sandy Peng and Wing-Ki Liu for assistance with the pharmacokinetics study, and Dr. David Jaffray and the staff of the STTARR facility for assisting with obtaining $^{64}$Cu.

4.1.2. Prostate Cancer Imaging

There is a consensus among the medical community that the perfunctory use of radical therapies is leading to the widespread overtreatment of biologically-indolent prostate cancers$^{250-253}$. Alternative, non-radical, treatment strategies include active surveillance of indolent cancers, and intraprostatic focal therapies (rather than treating the entire gland to
preclude or minimize side effects of treatment)\textsuperscript{251,254-257}. At the other end of the spectrum, for advanced stage disease, there is a great need to triage patients with occult micro-metastatic disease to better systemic therapies to cure patients that have minimal systemic burden to decrease prostate cancer-related deaths in men\textsuperscript{258}. Imaging plays a critical role in all of these strategies. Therefore, improvements in prostate cancer imaging must include the ability to delineate small areas of multi-focal disease and/or micro-metastases in an accurate and precise manner. Anatomical imaging modalities such as MRI, ultrasound, and CT are the most common current imaging methods used to assess prostate cancer. Unfortunately, all three have unsatisfactorily low accuracies in detecting clinically significant disease, as cancerous tissue is often indistinguishable from healthy tissues\textsuperscript{250,259-265}. Therefore, to personalize medicine, clinicians have three critical needs for prostate cancer imaging: 1) accurate intraprostatic imaging at early stages; 2) monitoring treatment response and imaging early recurrence; and 3), imaging of prostate cancer bone micrometastases. An imaging modality that could accurately describe the disease in these three states would give clinicians the information necessary to properly classify disease extent and prognosis and plan treatment based on initial and intra-treatment response. Notable among the emerging imaging modalities are functional imaging techniques such as fluorescence imaging and positron emission tomography (PET). Optical imaging has received much attention due to its ease of implementation, operational simplicity, low cost, and ability to provide real-time information about surgical margins, thereby, extending the surgeon’s vision ensuring complete surgical resection of tumors\textsuperscript{108}. PET provides quantitative drug biodistribution, effective treatment planning and monitoring and non-invasive deep tissue images, key characteristics not achievable by fluorescence imaging\textsuperscript{134}. With the emergence of multimodal imaging strategies and development of unique contrast agents, the complementary ability of different imaging systems, such as fluorescence and PET/CT, could enable high-resolution and sensitivity in patient assessment.

4.1.3. Porphysomes

Porphyrins are a unique platform for the development of multifunctional imaging agents. Found in nature, porphyrins are exceptionally strong metal ion chelators with inherent
photonic properties\textsuperscript{266}. By chelating a positron emitting metal ion such as copper-64 ($^{64}\text{Cu}$) with a porphyrin, one can create a highly stable radiotracer\textsuperscript{74,75,79,80,267}. Alas, the paramagnetic nature of copper 2+ ions quenches the porphyrins natural fluorescence, so that multiple porphyrins are necessary to create a multifunctional probe. An elegant solution lies in the use of the porphyrin-based nanoparticles, porphysomes\textsuperscript{143}. We recently demonstrated that $^{64}\text{Cu}$ can be directly incorporated into a small fraction of the porphyrin molecules in a porphysome to create a single, simple all-organic nanostructure that is both PET and fluorescently active (Fig. 4.1)\textsuperscript{145}. $^{64}\text{Cu}$-porphysomes, which are self assembled from a single porphyrin-lipid building block, stand apart from other organic nanoparticles, as they can be radiolabeled directly without the need for exogenous chelators or other modifications. By including the radionuclide directly into the building blocks, the nanoparticles can be faithfully tracked \textit{in vivo}, while ensuring that the pharmacokinetics and biodistribution are not affected\textsuperscript{79,80}.

Figure 4.1. Schematic diagram of multimodal properties of $^{64}\text{Cu}$-porphysomes as a result of directly radiolabeling a fraction of the porphyrin-lipid bilayer of preformed photonic porphysomes\textsuperscript{268}. 
By virtue of being composed of a single, biodegradable building block\textsuperscript{269}, \textsuperscript{64}Cu-porphysomes achieve a high level of multifunctionality, while being free of the complexity and toxicity plaguing other multifunctional nanoparticles (i.e. complex multicomponent liposomes, toxic or poorly cleared inorganic nanoparticles, etc.\textsuperscript{133,270-273}). These intrinsically multimodal \textsuperscript{64}Cu-porphysomes are well suited for prostate cancer imaging given non-indolent, malignant prostate tissue is twice as vascularized as healthy prostate\textsuperscript{274}. Thus, we expect increased \textsuperscript{64}Cu-porphysome uptake and retention in non-indolent prostate tumors owing to nanoparticles ability to extravasate through malignant vasculature\textsuperscript{275}. Herein, we demonstrate the clinical potential of our multimodal PET/CT and optical \textsuperscript{64}Cu-porphysomes to address the current clinical needs in prostate cancer imaging. \textsuperscript{64}Cu-porphysomes can clearly delineate localized prostate tumors with low non-specific accumulation in normal tissues and high-tumor uptake, accurately image prostate cancer bone metastases with potential for treatment planning and monitoring treatment response and recurrence.

### 4.2. Materials and Methods

**Formation of porphysomes:** Porphysomes were synthesized using a previously reported protocol\textsuperscript{143} and comprise 65 mol\% Pyropheophorbide-\(\alpha\)-lipid, 30mol\% cholesterol oleate, 5 mol\% DSPE-PEG\textsubscript{2000}. The nanoparticle size was determined by dynamic light scattering (DLS, Malvern Instruments, Malvern, UK) and the concentration by UV/Vis spectrophotometry (Varian Inc., Palo Alto, CA). The size (z-average) was between 120-130nm with a polydispersity index (PDI) of <0.2.

**Radiolabeling Procedure:** \textsuperscript{64}CuCl\textsubscript{2} was obtained from Université de Sherbrooke, Sherbrooke, QC. \textsuperscript{64}Cu(OAc)\textsubscript{2} was obtained from Washington University, St. Louis, MO. Porphysomes in PBS were diluted 1:1 with 0.1M NH\textsubscript{4}OAC (pH 5.5), before a small volume of aqueous \textsuperscript{64}CuX\textsubscript{2} (X = OAc, Cl) solution was added and the solution was incubated at 60°C for 30 min. Radiochemical purity and yield were assessed on a radio-UPLC (Waters, Milford, MA) equipped with a UV/Vis module (monitoring 254nm and 410nm), eSatin radiation detector, and ELSD module using a size exclusion column (pore size 100nm, mobile phase H\textsubscript{2}O). \textsuperscript{64}Cu-porphysomes elute from this column early, while
any unchelated $^{64}$CuX₂ elutes later with the buffer salts, which are easily identified by their response in the ELSD channel. The labeling solution was then diluted with PBS to the desired concentration for injection.

Animal studies: All animal studies were carried out under institutional approval (University Health Network, Toronto, Canada, AUP 2164.5 and 2273.3).

Orthotopic prostate tumor model: Adult male athymic nude mice, weighing ~20-25g, were placed under general anesthesia with 2% isoflurane in oxygen. A small incision was made in the lower abdomen into the peritoneum, where the bladder, seminal vesicles and prostate were partially removed from the abdominal cavity to expose the dorsal prostate lobe. 1.5 x10⁴ prostate tumor cells (PC3-luc+ (ATCC, Manassas, VA) or 22RV1 (from Dr. Yoni Pinthus and Dr. Robert Bristow) or PBS) in 10μL of cell media were injected in the dorsal prostate lobe using a 30 gauge needle. The organs were then returned into the body cavity, and both the muscle wall and the skin were closed using a running 5-0 silk suture. Following this surgery, animals were given 0.05 mg/kg buprenorphine and saline solution (0.5mL) subcutaneously. All surgical procedures were carried out under aseptic and pathogen-free conditions in a biosafety hood. Orthotopic prostate tumor development was monitored by MRI imaging (Biospec 70/30 USR, Bruker, Billerica, MA) 14 days post inoculation. PC3 tumor development was also monitored by bioluminescent imaging (Xenogen, Caliper Life Sciences, Hopkinton, MA).

Metastatic prostate cancer model: Ace-1-YFP-Luc cells were kindly provided by Dr. Thomas Rosol (Department of Veterinary Biosciences, College of Veterinary Medicine, The Ohio State University, Columbus, OH 43210). Following the methodology reported by Leroy et al.²⁷⁶ 1x10⁵ Ace-1-YFP-Luc cells were suspended in 100μL of PBS and injected into the left ventricle of adult male athymic nude mice using a 27 gauge needle. Following the intracardiac injection, saline solution (0.3mL) and 0.05mg/kg buprenorphine were administered subcutaneously and the analgesic was repeated 24h later. 14 days post injection, metastatic formation was monitored by bioluminescent imaging (Xenogen) to assess the development of Ace-1 metastases as well as the location of metastatic formation. All tumor induction procedures were carried out under general
anesthesia, where animals were induced using 4% isoflurane in oxygen (2L/min) and maintained with 2% isoflurane in oxygen.

**PET/CT scanning:** PET imaging was carried out on a MicroPET Focus 220 (Siemens, Munich, Germany). CT imaging was conducted on a Locus Ultra microCT (GE Healthcare, Little Chalfont, UK). After intravenous (tail vein) injection of $^{64}$Cu-porphysome solution (100-150uL, 16-23MBq, 150-200nmol Pyro-lipid), mice were imaged under anesthesia (2% isoflurane in oxygen at 2L/min) at 4h or 24h post injection. PET images were captured over 10min at 4h and 40min at 24h, followed immediately by CT imaging.

**Fluorescence imaging of orthotopic prostate tumor model:** Immediately after PET/CT imaging at 24h post $^{64}$Cu-porphysome injection, animals were euthanized and ex vivo fluorescent imaging was performed (Maestro™, Caliper Life Sciences, Hopkinton, MA; CRI: 680nm excitation, ≥700nm longpass detection, autoexposure integration time). Monochrome and fluorescent images were taken sequentially without moving the animal. Region of interest (ROI) were first drawn on the monochrome image, where structures including seminal vesicles, testes and orthotopic prostate tumor are easily distinguishable (Fig. 4.2). These ROIs were then copied and pasted onto the fluorescent composite images to evaluate the fluorescence from $^{64}$Cu-porphysome accumulation in selected tissues. Comparison between the surgery control group and orthotopic prostate tumor animals were calculated based on the total fluorescence signals normalized by exposure time and ROI area (total fluorescent signal/(sec x pixels)) using a one-tail Student t-test with a level of significance set at p<0.05. Comparison between different organs in the orthotopic prostate group was calculated using a one-tail paired Student t-test with a level of significance set at p<0.05.
Figure 4.2. Fluorescence regions of interest (ROI) of (A) PC3 orthotopic prostate cancer model and (B) surgery-only control group. ROIs, in red, were drawn on the (i) monochrome images where the seminal vesicles, testes and prostate tumor were easily distinguishable. These ROIs were then registered onto the (ii) fluorescent composite images to evaluate the fluorescence from $^{64}\text{Cu}$-porphosome accumulation in selected tissues\textsuperscript{268}.

*Biodistribution studies:* Time-dependent biodistribution studies were carried out in orthotopic PC3 tumor-bearing animals weighing 25-30g. After an intravenous (tail vein) injection of $^{64}\text{Cu}$-porphosome solution (100-150\(\mu\)L, 16-23MBq, 150-200nmol Pyrolipid), animals were euthanized using 2% isoflurane and exsanguination, and the thoracic cavity was opened at 4h or 24h post injection. Blood samples were withdrawn from the heart through a syringe. Organs were excised, washed with saline, dried with absorbent tissue, weighed and counted on a gamma-counter. Organs of interest included the tumor, heart, spleen, lungs, liver, kidneys, adrenal, stomach, small intestine, large intestine, muscle, bone and brain. Organ uptake was calculated as a percentage of the injected dose per gram of tissue (%ID/g). The optimal 24h time point was established based on time-dependent imaging and biodistribution studies. Further biodistribution studies were carried out using animals weighing 25-30g that were separated into 4 different groups,
PC3 orthotopic prostate tumor, 22RV1 orthotopic prostate tumor, surgery control and healthy control. 24h post intravenous injection of $^{64}$Cu-porphysome solution (100-150µL, 16-23MBq, 150-200nmol Pyro-lipid), animals were euthanized and organs excised following the protocol above. However, organs of interest also included prostate tumor and healthy prostate.

*Microscopic tissue imaging and histology:* Tumor and healthy prostate samples from orthotopic prostate and control groups were placed in OCT media and frozen. These samples were allowed to radioactively decay at -80°C for at least 10 half-lives and then sectioned (10µm). Frozen tissue slices (10µm) were immersed in PBS for 5min, dried, and 10µL of mounting solution with DAPI, 40,6-diamidino-2-phenylindole (Vector Laboratories Inc., Burlingame, CA), was added as a nuclear stain. The sections were covered by a coverslip and fluorescently imaged (Olympus BX50, Olympus Corporation, Center Valley, PA; excitation 410±70nm, emission 685±40nm). The adjacent section following the imaged frozen section was H&E stained to confirm the presence of tumor. In the Ace-1 metastatic model, the femurs and tibias from the animals were excised and fixed in 10% buffered formalin for one week to allow for radioactive decay of at least 10 half-lives. These samples were then decalcified using ethylenediaminetetraacetic acid (EDTA, Sigma Aldrich, St. Louis, MO) for 2 weeks, sectioned (10µm) and H&E stained for histological analysis to confirm the presence of Ace-1-YFP-Luc metastases within the bone.
4.3. Results:

4.3.1. $^{64}$Cu-porphysome Uptake in the PC3 Orthotopic Tumor Model

The tumor uptake of $^{64}$Cu-porphysomes was evaluated in the PC3 orthotopic model at 4 and 24 h post injection, based upon the pharmacokinetics data. Time points beyond 2 half-lives $^{64}$Cu were not investigated as this would require both high radioactivity doses and long scan times to achieve measureable activity. At 4h the prostate tumor was not easily delineated in the PET/CT image (Fig. 4.3A) and the radioassay studies demonstrated a tumor %ID/g of only 3.16 ± 0.36 (Fig. 4.3C). The low tumor and high intestinal uptake (5.50 ± 2.09 %ID/g) gives a low tumor-to-tissue ratio (0.62 ± 0.18; Fig 4.3D), making it difficult to visualize the tumor at 4h, but it is clearly identifiable at 24h (Fig. 4.3B) due to a greater than 2-fold increase (p<0.001) tumor uptake, 6.83 ± 1.08 %ID/g (Fig. 4.3C), increasing the tumor-to-gut uptake to 1.53 ± 0.28 (Fig 4.3D). The retention of $^{64}$Cu-porphysomes within the tumor is evident from the tumor-to-muscle ratio increasing from 5.06 ± 0.49 to 12.7 ± 6.1 from 4h to 24h (Fig. 4.3D).
Figure 4.3. Representative MicroPET/CT images of (i) axial, (ii) coronal and (iii) sagittal single slices through the orthotopic PC3 tumor at (A) 4h and (B) 24h post i.v. injection of 500µCi $^{64}$Cu-porphysomes: white arrows - tumor, blue arrows - bladder; n = 4). (C) Biodistribution of $^{64}$Cu-porphysomes at 4h and 24h post i.v. injection, plotted as % injected dose/gram (%ID/g). (D) Tumor-to-tissue ratio in select tissues at 4h and 24h. Each point is mean ± 1 SEM of four determinations. (* = p<0.001)
Similar to many other nanoparticles, porphysomes are cleared through the hepatobiliary route, resulting in the high accumulation within the liver and spleen. Importantly, no accumulation was observed in the bladder at any time point, which has been the “Achilles heel” of many small molecule radiotracers used in prostate cancer imaging. 24h was chosen as the optimum imaging time point, as it provides the higher prostate tumor uptake, delineation and high tumor-to-background ratio.

4.3.2. $^{64}$Cu-porphysome Selectivity in Orthotopic Prostate Tumors

To assess the selectivity of $^{64}$Cu-porphysomes for human prostate tumors, the PC3 model mimics focal prostate cancer, while the hypoxic 22RV1 orthotopic model mimics a more aggressive phenotype. Figure 4.4 displays representative PET/CT images comparing the PC3 and 22RV1 models with healthy male mice at 24h post $^{64}$Cu-porphysome injection. $^{64}$Cu-porphysomes clearly delineate the orthotopic tumors, while the non tumor-bearing mice displayed minimal signal in the prostate region. Encouragingly, $^{64}$Cu-porphysomes clearly demarcate not only the larger PC3 tumors, but also hypoxic tumors that were less than half their size (5 and 2 mm, respectively, as determined by MRI; Fig. 4.4I). Clear tumor delineation was also demonstrated by fluorescence imaging (Fig. 4.4F and G): PC3 tumors had approximately 9- and 4-fold higher fluorescence (total signal/(sec x area)), compared to the normal prostate tissue in both healthy mice and tumor-bearing animals (Fig 4.4H).
Figure 4.4. Representative MicroPET/CT images of (i) axial, (ii) coronal and (iii) sagittal single slices through: (A) orthotopic PC3 tumor (n=8), (B) orthotopic 22RV1 tumor (n=3), and (C) healthy male mice (n=3) at 24 h after i.v. injection of 500 chi $^{64}$Cu-porphysomes: PET image integration time 40 min. White arrows depict prostate tumor, blue arrows depict bladder. (D) Corresponding organ biodistribution measured ex vivo in orthotopic PC3 (n = 4), orthotopic 22RV1 (n = 3), orthotopic surgery control (n = 5) and healthy male mice (n = 3). (E) is a magnified view of the tumor and healthy prostate tissue uptake (p<0.05). Representative images of (F) PC3 prostate tumor-bearing animals and (G) surgery-control mice comparing i) monochrome and ii) composite fluorescence images: SV – seminal vesicles, T – testes, white arrow – orthotopic prostate tumor). (H) Fluorescent signal for orthotopic PC3 and surgery-control mice comparing tumor and seminal vesicles (* p<0.01, n=4). (I) MRI and size measurements of tumors in three representative 22RV1 orthotopic prostate cancer mice. (J) Tumor-to-tissue ratios in both orthotopic prostate tumor models at 24h post injection of $^{64}$Cu-porphysomes (22RV1 – n = 3, PC3 – n = 4).
The radioassay data confirmed the selectivity of $^{64}$Cu-porphysomes for cancerous tissue versus normal prostate tissue: PC3 tumor-to-prostate ratio of $5.75 \pm 1.53$, with $6.83 \pm 1.08$ %ID/g and $1.23 \pm 0.202$ %ID/g, respectively (Fig. 4.4D and E). The findings in the 22RV1 model were similar, with a tumor-to-prostate ratio of $7.24 \pm 2.66$ with $4.81 \pm 2.06$ %ID/g and $0.668 \pm 0.132$ %ID/g, respectively. At 24h post injection, both PC3 and 22RV1 had tumor-to-muscle ratios >12 (Fig. 4.4J). A second control group was used to evaluate whether the surgical tumor inoculation procedure affected the accumulation of $^{64}$Cu-porphysomes in the prostate gland. However, both the untreated control and surgery-only groups had similar accumulation: $1.45 \pm 0.167$ %ID/g and $1.10 \pm 0.500$ %ID/g (Fig. 4.4D and E). $^{64}$Cu-porphysome uptake in tumors for both models was significantly higher ($p<0.05$) than healthy prostate tissue in all groups. There was significant uptake in both the spleen and liver in all models, as would be expected for a nanoparticle which is too large to be cleared by the renal system.

The tumor selectivity of $^{64}$Cu-porphysomes was further demonstrated by fluorescent microscopy of tissue slices, comparing tumor and uninvolved prostate tissue from orthotopic PC3 and 22RV1 models with healthy prostate from surgery-only mice (Fig. 4.5). The regions that demonstrated high porphyrin fluorescence corresponded to areas with cancer cell morphology (oversized nuclei, disorganized structure), while uninvolved prostate tissue was characterized by organized glandular structures with small nuclei and showed minimal porphysome fluorescence. There was minimal fluorescence in the surgery-only prostate tissue sections. In summary, the PET/CT images, the biodistribution data, the in situ fluorescence and fluorescence microscopy all clearly demonstrate the selectivity of $^{64}$Cu-porphysomes for prostate tumors.
Figure 4.5. Microscopic confirmation of $^{64}$Cu-porphysome uptake and selectivity in orthotopic PC3, 22RV1 and surgery-only control groups. Frozen 10µm tumor and prostate tissue slices were DAPI stained (blue). Representative $^{64}$Cu-porphysome fluorescent (red) images were compared to sequential histology slices (H&E staining). Scale bars = 200µm, whole tissue slice in inset. (Pyro excitation 410±70 nm, detection 685±40 nm)\(^{268}\).

### 4.3.3. $^{64}$Cu-porphysomes for the Detection of Metastatic Prostate Tumors

The metastatic Ace-1-YFP-Luc prostate cancer cell line was used to mimic prostate metastases. This was simplest in the lower extremities that were well separated from the high uptake of $^{64}$Cu-porphysomes in the liver and spleen. Metastatic involvement was confirmed in four mice by BLI imaging at 14 d post Ace-1 injection, as illustrated in Figure 4.6A, and by histology post mortem (Fig 4.6C). $^{64}$Cu-porphysome uptake was seen in the 3D PET/CT images of the same mouse at 24h post injection (Fig. 4.6Bi), while the healthy animals showed minimal signal in the lower limbs (Fig. 4.6Bii). The
location of $^{64}$Cu-porphysome accumulation in the PET/CT images (Fig. 4.6 Bi) matched the localization of metastases in the histology slices; both demonstrate tumors in the distal femur (Fig. 4.6Bi – blue arrow, Ci) and proximal tibia (Fig. 4.6Bi – white arrow, Cii&iii). All 4 mice with confirmed metastases by BLI imaging and histology demonstrated $^{64}$Cu-porphysome accumulation in either the distal femur, proximal tibia or both. The development of spinal metastases is often associated with prostate cancer, so that we also evaluated PET/CT imaging of small metastases within the spinal column (Fig. 4.6D and E), confirmed by BLI and H&E histological analysis (Fig. 4.6A and F) in the lumbar vertebrae. There was no corresponding PET signal within the spine in control animals (Fig. 4.6E).
Figure 4.6. Prostate metastases imaging. (A) Representative bioluminescent images confirming the presence of bony metastases in the (i) supine and (ii) prone positions. (B) (i) Corresponding 3D MicroPET/CT images (blue arrow – distal femur metastases, white arrow – proximal tibia metastases) and (ii) 3D MicroPET/CT image of a healthy mouse. (C) Corresponding histology (H&E) of lower extremities confirming Ace-1 metastases in the (i) distal femur and (ii & iii) proximal tibia. Representative MicroPET/CT images of i) axial, ii) coronal and iii) sagittal single slices in (D) Ace-1 metastatic and (E) control animals 24h post i.v. injection of 500µCi $^{64}$Cu-porphysomes: inset shows zoomed views of a metastatic lesion. (F) Corresponding histology confirming Ace-1 metastases in the spinal column at (i) 1X and (ii) 4X magnification. T - metastases, BM - bone marrow, BC - cortex. 
4.4. Discussion

$^{64}$Cu-porphysomes may aid not only the current clinical practice for managing prostate cancer but also help translate new therapies into the clinic by addressing the three critical needs of prostate cancer imaging, as follows.

4.4.1. Accurate Intraprostatic Imaging

One current practice for men presenting with prostate cancer is radical localized treatment, despite the risk and regardless of tumor stage, resulting in significant overtreatment$^{251,252}$. Screening studies suggest that as many as 48 prostate cancer patients need to be treated in order to save one life$^{251,252}$. Radical treatments such as brachytherapy, external beam radiation therapy, and surgery remove or destroy the entire prostate gland in order to ensure complete eradication of the cancer. This approach is often successful at removing disease but drastically decreases patients’ quality of life$^{277,278}$. The associated morbidities of radical therapies, primarily impotence and incontinence, are devastating and occur at a high frequency$^{277,278}$. These side effects stem from damage or wholesale removal of neurovascular bundles necessary for normal function. The multifunctional nature of $^{64}$Cu-porphysomes may provide a means to avoid these side effects by acting as a PET treatment planning tool, delineating intraglandular foci of prostate tumor involvement and translating that onto the surgical table or radiotherapy planning through fluorescence image-guidance. Here, we mimic localized primary cancer development and its microenvironment using orthotopic prostate cancer models (PC3 and 22RV1). Similar to other nanoparticles, $^{64}$Cu-porphysomes are cleared through the hepatobiliary system, producing minimal background in the bladder and peri-prostatic tissues, and allowing clear visualization of the tumor. PET/CT imaging showed that $^{64}$Cu-porphysomes delineated hypoxic orthotopic tumors less than 2mm in size (Fig 4.3 and 4.4). The $^{64}$Cu-porphysome uptake demonstrated comparable, if not superior, %ID/g and tumor-to-muscle ratio to other preclinical prostate cancer imaging agents (amino acids and peptides 1.17-8.16 %ID/g, antibodies 3.0-13.2 %ID/g)$^{151,279-284}$. In situ fluorescence imaging after opening the peritoneal cavity clearly distinguished prostate tumors from the male reproductive organs (healthy prostate, seminal vesicles, and testes),
all of which showed minimal fluorescence (Fig. 4.4 and 4.5). Porphysome accumulation was heterogeneous in tumor tissue (seen in both PET/CT and fluorescence), possibly due to high interstitial pressures and the natural heterogeneity of the tumor microenvironment. The exact mechanism of $^{64}$Cu-porphysome uptake into cancer *in vivo* is not well understood and is the basis of ongoing studies. It is possible that nanoparticle extravasation drives the tumor accumulation, while the cell penetration capability of Pyropheophorbide-$\alpha^{285,286}$ itself drives the nanoparticle dissociation and cellular uptake. However, this “ringing” effect could be advantageous as it causes the highest uptake to be in the periphery of the tumor, giving a clear indication of the tumor boundaries. The power of $^{64}$Cu-porphysomes is that they can discriminate between prostate tumor and healthy prostate tissues using PET/CT and fluorescence imaging.

There are also a number of focal modalities currently under investigation including image-guided focal photothermal therapy, photodynamic therapy, high intensity focused ultrasound ablation, focal brachytherapy or image-guided radiotherapy and focal cryotherapy$^{251}$. Focal therapies have demonstrated their effectiveness to treat prostate cancer, from indolent to aggressive, with minimal side effects$^{250}$. While attractive due to their low side effects, focal therapies are currently constrained by inadequate tools for accurate intraprostatic imaging. For planning proper disease management, clinicians require a means that accurately depicts the extent of cancer within a diseased prostate. While MRI and trans-rectal ultrasound currently have extensive use in the clinic, they are hampered by limitations such as low sensitivity (particularly for small lesions), low specificity, and irreproducibility$^{250,259-265}$. The PET/CT imaging capability of $^{64}$Cu-porphysomes may provide a more accurate picture of tumor involvement within the prostate providing a potential effective treatment planning tool. Treatment planning with $^{64}$Cu-porphysome PET/CT imaging not only provides a full picture of the prostate but is non-invasive and would avert the need for inconvenient, inaccurate and painful repeated biopsies$^{260,287}$. Lastly, the fluorescence capability of $^{64}$Cu-porphysomes and its prostate tumor selectivity provides a means to guide and monitor the efficacy of focal treatment in real time ensuring complete eradication of compromised tissue.
4.4.2. Treatment Enhancement and Monitoring Recurrence

Assessing a patient’s response to treatment is a critical part of tailoring a treatment plan to their needs. Currently there is no method for monitoring response in real-time during a treatment procedure. Fluorescence imaging is an attractive modality for real-time monitoring of treatment response as it is highly sensitive and easily implemented. Using our orthotopic prostate cancer models, we demonstrated the ability of fluorescent imaging to macroscopically discriminate between diseased and healthy tissue (Fig. 4.4F-H). On the microscopic scale, fluorescence imaging of prostate and tumor tissue histology slices also demonstrated clear separation between tissue types (Fig. 4.5). Porphysome fluorescence only accumulated in malignant cells, confirmed by H&E staining, whereas healthy glandular prostate tissue demonstrated little to no fluorescence. The capability to visually identify malignant cells microscopically using $^{64}$Cu-porphysomes could make surgical procedures more successful as surgeons could monitor treatment response in real time, ensuring that the surgical bed is free of any residual tumor cells. This selectivity and the ability to directly visualize diseased tissue with fluorescence may give surgeons the information needed to make non-radical or nerve sparing surgeries more viable.

Currently, the recurrence rate for prostate cancer is as high as 30\%$^{288}$. Although, PSA is a valuable biochemical tool for monitoring recurrence after radical therapy$^{289}$, this assay does not give any information with regards to the progression or localization of disease. Patients with rising levels of PSA are again faced in the same unpleasant predicament when prostate cancer was first diagnosed suffering through painful biopsies and the uncertainty of poorly defined disease. There is currently no consensus on effective strategies to monitor and characterize recurrence$^{290}$. Furthermore, the translation of focal therapies into the clinic is constrained by our lack of tools to accurately detect recurrence as healthy prostate tissue spared by focal therapies can confound the PSA assay. The ability of $^{64}$Cu-porphysomes to detect prostate tumors less than 2mm in size by PET/CT (Fig 4.3 and 4.4) may present a viable option for identifying tumor foci and detecting the early stages of recurrence non-invasively. The added information demonstrating not only the presence but also the localization of the recurrent tumor may provide clinicians with
more information allowing them to better decide upon a treatment regime while giving them the tools needed to bring focal therapies to the forefront of patient care.

4.4.3. Imaging Prostate Cancer Bone Metastases

Castrate-resistant metastatic spread is associated with death within 18-36 months\textsuperscript{291-294}. Currently, there is no ideal strategy for detecting prostate cancer metastases\textsuperscript{295}, the primary cause of morbidity and mortality in these patients. A critical clinical need in prostate cancer imaging is the detection of occult metastatic involvement such that systemic agents can be given earlier in the disease spectrum for a more curative approach. Here, we have demonstrated that \textsuperscript{64}Cu-porphysomes are able to detect \textgreater{}2mm metastases in the bones of the lower extremities and spinal column in our Ace-1 metastatic cancer model (Fig. 4.6). Metastases were clearly visualized with PET/CT and confirmed with histological staining. Detection of small metastases has proven difficult for contemporary bone scans which have a detection limit of approximately 1cm\textsuperscript{296-298}. The whole-body imaging capability of PET/CT, combined with the sensitivity of \textsuperscript{64}Cu-porphysomes to detect small metastatic lesions, 2mm or smaller, potentially provides a novel and potent means to detect micrometastases, localize metastatic spread and to monitor local or systemic treatment responses.

4.4.4. Future Potentials

We have demonstrated the imaging potential of \textsuperscript{64}Cu-porphysomes in multiple imaging applications; they show excellent selectivity to image local disease and detecting metastatic bony spread. These preliminary findings are extremely encouraging but there is still room for improvement (Fig. 4.7).
Figure 4.7. Future potential of porphysomes: (A) targeting the nanoparticles by functionalizing with receptor ligands, antibodies, etc., (B) chelating paramagnetic metal ions with the porphyrin building-blocks for MRI contrast, (C) exploiting porphysomes high-payload chelating abilities to deliver radiotherapeutics, (D) entrapping soluble drugs or contrast agents within the aqueous core and exploiting the strong absorbance of porphyrins for (E) photothermal therapy with highly quenched, intact porphysomes and (F) photodynamic therapy with porphyrin-lipid monomers following dissociation of the nanostructure\textsuperscript{268}.

There is flexibility in the formulation, in particular, the amounts of stabilizing cholesterol and shielding PEG can be varied to fine tune the biodistribution, pharmacokinetics and modify uptake in target and non-target tissues. An in depth study to evaluate the relationship between porphysome composition and their \textit{in vivo} behavior is necessary to improve upon our current formulation. The fraction of $^{64}$Cu labeling of the porphyrin building blocks can be fine tuned to the specific imaging application. For example, a highly-labeled formulation would be best suited for whole body detection of metastatic disease with PET/CT alone, while a low or intermediate fractional labeling would allow
the full exploitation of porphysomes’ multimodal nature for treatment planning and fluorescence-guided resection. As a PEG-coated nanoparticle, the porphysome platform can easily be functionalized with targeting ligands\textsuperscript{143}, for targets such as PSMA\textsuperscript{279,280,299,300}, EpCAM\textsuperscript{301}, or VEGFR\textsuperscript{282}, thereby further enhancing the tumor uptake and minimizing off-target accumulation and specificity. This functionalization may provide a means to better stratify patients based upon molecular markers, providing a means to tailor the aggressiveness and extent of therapy to the aggressiveness of the disease. Thus, they serve as a means to achieve personalized cancer treatments. As we seek to improve the performance of \textsuperscript{64}Cu-porphysomes, we are also investigating ways to expand their applications. Due to the high specific activity that can be achieved, labeling with \textsuperscript{67}Cu would convert porphysomes into a platform agent for radio(immuno)therapy. Indeed, porphyrin-lipid building blocks of porphysomes can be tailor-made with a range of porphyrinoids, such as bacteriochlorophyll-\textsuperscript{a}\textsuperscript{131,143} or texaphyrin, introducing further unique photonic properties but also the capability to chelate a variety of metal ions. For example, a texaphyrin could stably chelate Lutetium-177, a radionuclide better suited for radiation therapy, or by chelating paramagnetic gadolinium with texaphyrin, MRI imaging capabilities can also be incorporated into porphysomes.

\textbf{4.4.5. Beyond Imaging}

The capabilities of porphysomes are not only limited to imaging (Fig. 4.7), they have been shown to have therapeutic applications in photodynamic therapy (PDT), photothermal therapy (PTT), and as drug delivery vehicles\textsuperscript{143}. The high concentration of Pyro molecules in porphysomes means they can act as a high payload photosensitizer delivery vehicle. Currently, interstitial PDT is in phase I clinical trials for the treatment of prostate cancer; however the non-specific uptake of photosensitizers and inability to measure photosensitizer concentration at target sites have been limitations\textsuperscript{251,302-313}. With the demonstrated prostate cancer selectivity of \textsuperscript{64}Cu-porphysomes by both PET and fluorescence imaging, porphysomes afford the capability of selective high payload delivery of an efficient photosensitizer, Pyro. This may provide a highly effective treatment planning tool (PET imaging), dosimetry aid (photosensitizer quantification) and fluorescence image-guided PDT agent for the treatment of prostate cancer, not currently
achievable by any other photosensitizer. Image-guided focal PTT is another emerging localized treatment modality and is in phase I clinical trials for the specific treatment of prostate tumors\cite{314-316}. However, the long photothermal treatment times are unsatisfactory in the clinic, driving an interest in the development of photothermal enhancement agents. Porphysomes are well suited to this task as their highly quenched nature leads to efficient transformation of laser energy into heat. Again, the selective accumulation of porphysomes in prostate cancer makes them candidate photothermal agents. Not only could they decrease treatment times, but would also limit collateral damage to surrounding healthy tissue by lowering the overall light energy dose delivered. $^{64}$Cu-porphysomes are well suited as PTT agents that also intrinsically provide a means to plan, guide, and monitor treatment throughout PTT using its multimodal imaging characteristics. Porphysomes are also being explored as drug delivery vehicles. With their selectivity to metastases, porphysomes are attractive candidates to deliver chemotherapeutics to advanced stage disease.

4.5 Conclusions

We believe that the porphysome platform has the potential to address several unmet clinical needs in prostate cancer imaging and treatment. The plethora of therapeutic potentials of porphysomes will be enriched by the multimodal imaging properties of $^{64}$Cu-porphysomes enabling treatment planning, image-guided therapy, and follow-up monitoring in prostate cancer patients. In conclusion, we have validated the \textit{in vivo} sensitivity and selectivity of $^{64}$Cu-porphysomes in a number of clinically relevant prostate cancer models using PET and fluorescence imaging. Although prostate cancer cell lines were used, the \textit{in vivo} models themselves better mimic the microenvironment, location and progression of prostate cancer as seen in patients. The unique combination of properties of porphysomes offers a promising all-in-one agent that spans tumor detection, treatment, interventional guidance, treatment response assessment and monitoring of recurrence, using both radionuclide- and photonic-based strategies.
Chapter 5

Conclusions

Currently the only clinically approved use for porphyrins is as photosensitizers\textsuperscript{3-5,7}. From this body of work, it becomes apparent that the role of porphyrins may have a larger impact in the field of cancer imaging and therapy, including but not limited to photodynamic therapy: the photoactivity of porphyrins can be enzymatically controlled by incorporating them into photodynamic molecular beacons; the uptake of porphyrins can be improved by conjugating them to targeting moieties; the imaging capabilities of porphyrins can be extended to other modalities by the chelation of metal ions; and porphyrin-conjugates can self assemble into nanovesicles, allowing for high payload delivery in an organic multifunctional liposome-like nanoparticle. With the shift towards personalized cancer care, the uses of these porphyrin-based probes represent potential strategies to achieve specific targeting to an individual’s disease.

Here, we evaluated the application of multimodal porphyrin-based probes in different potential clinical scenarios. Photodynamic molecular beacons are uniquely suited as a treatment aid in the management of patients suffering from spinal metastases; targeted porphyrin probes, PPF, demonstrated promise for multimodal imaging in serous ovarian cancer patients; \textsuperscript{64}Cu-porphysomes possess the capability to address the current clinical needs in prostate cancer imaging. Each of these probes has their own application, filling a critical need in a particular niche within the field of cancer diagnostics and therapy. For example, the promising effects of PDT on vertebral metastases has led to a Phase I clinical trial. However, the therapeutic window of PDT is limited by the use of Visudyne due to its non-specific uptake in the spinal cord, which reduces the aggressiveness with which this treatment can be used safely\textsuperscript{179,180}. By utilizing the activation specificity of photodynamic molecular beacons, the tumor-to-spinal cord selectivity of PDT may be improved, resulting in an increased therapeutic window. The beauty of photodynamic molecular beacons is that they remain optically and photodynamically silent unless in the
presence of the target enzyme, which should only be expressed by target cells, thus making the preservation of healthy tissue possible. Using a local administration of photodynamic molecular beacons combined with the self-delivery capabilities of porphyrins, the selectivity of beacons can be further enhanced and activation kinetics increased. However, this particular clinical indication is not limited solely to the use of photodynamic molecular beacons, since an argument could also be made for using targeted porphyrin probes. Because the ultimate goal is complete destruction within the vertebrae in order to make room for the bone strengthening cement during vertebroplasty, this may also be achieved using PPF. In addition to targeting specificity, these targeted probes do not require any wait time for activation. Thus, similar PDT damage may be achievable, albeit using a local injection; because these targeted probes are always “on”, any uptake in the spinal cord would be detrimental when using a systemic injection. In contrast, although porphysomes would allow for high payload delivery of porphyrins which may result in increased tumor damage, only when porphysomes are unquenched will they be photoactive, which appears to be on the order of hours, i.e. they have considerably slower activation kinetics than photodynamic molecular beacons.

In the case of ovarian cancer detection and treatment, the role of PDT as a potential treatment modality was evaluated and has led to Phase II clinical trials using Photofrin. Unfortunately, these trials did not demonstrate significant complete response or long-term tumor control, owing to the heterogeneity and lack of tumor specificity of the photosensitizer. Targeted porphyrin probes may address these limitations, as we have clearly demonstrated a high degree of tumor specificity by folate-targeted porphyrins (PPF) using primary human ovarian cancer in vivo models. The increased selectivity afforded by conjugating targeting moieties to porphyrins markedly reduces the potential risk of collateral damage to normal tissues. However, the applications of targeted porphyrin probes can be extended beyond PDT, since the intrinsic NIR fluorescent properties of porphyrins make these probes suitable for photodetection. Small metastatic studding was clearly visible on the peritoneum in the primary human ovarian ascites in vivo models often as is the case in patients. Due to the depth penetration limitation of optical imaging, fluorescence imaging must occur intraoperatively or laprascopically. By exploiting the natural stable ability of
porphyrins to chelate metal ions, PET imaging capabilities can be introduced, providing non-invasive “unlimited” depth penetration visualization. In this clinical scenario, photodynamic molecular beacons may also have a role in better preservation of normal tissue during PDT, by using a local or intraperitoneal injection. However, because the activation of beacons relies upon extracellularly-secreted enzymes, with the large area of the abdomen, activation away from the target site as well as diffusion of the activated beacon from target tissue are concerns. Preliminary results indicate that untargeted porphysomes do not preferentially accumulate in primary human ovarian cancer mammary fat pad xenografts (Fig. 5.1). Although, again, they have the advantage of high payload delivery of a large number of porphyrins as well as the ability to be core-loaded with drugs, unless their in vivo biodistribution can be altered to increase their uptake at targeted sites while simultaneously decreasing the normal tissue accumulation, their role in ovarian cancer may be limited.

Figure 5.1. $^{64}$Cu-porphysome uptake in primary human ovarian cancer mammary fat pad xenograft model. A) Representative MicroPET/CT images (coronal images (top) and single transverse slices passing through the tumors (bottom), (n = 3)) at 24 h after intravenous injection of 500 µCi $^{64}$Cu-porphysomes. B) Tissue uptake of $^{64}$Cu-porphysomes in selected organs at 24h after intravenous injection, where primary human ovarian tumor uptake was 4.710 % ID/g.
We demonstrated the clinical potential of multimodal PET/optical $^{64}$Cu-porphysomes to address current clinical needs in prostate cancer imaging. $^{64}$Cu-porphysomes clearly delineated localized prostate tumors, with low non-specific accumulation in surrounding normal tissues (testes, seminal vesicles) and high-tumor uptake. They also accurately imaged prostate cancer bone metastases, which demonstrates potential for treatment planning and monitoring treatment response and recurrence. Since small molecules are cleared through the renal system, photodynamic molecular beacons and targeted porphyrin probes are limited in their use as prostate imaging agents due to their high accumulation in the adjacent bladder. $^{64}$Cu-porphysomes are uniquely suited as potential imaging agents for prostate cancer due to its high vascularity\textsuperscript{274} and thus, we expect increased $^{64}$Cu-porphysome uptake and retention in prostate tumors owning to the nanoparticles’ ability to extravasate through this chaotic vasculature\textsuperscript{275}.

Due to the limited studies to date of porphysomes, it is difficult to define the potential impact they may have in the field of cancer research. These organic porphyrin nanovesicles have potential widespread applications, due to their several intrinsic functionalities. Similar to the small molecular porphyrin probes, porphysomes possess fluorescent and photodynamic capabilities and could also aid in personalized medicinal approaches to cancer therapy by functionalizing them. In contrast to small-molecule porphyrin probes, porphysomes deliver a high payload of porphyrins, 80 000 porphyrins per nanoparticle, which allows these nanovesicles to possess other unique photonic properties, including photothermal and photoacoustic affects. Furthermore, porphysomes have liposome-like properties and, therefore, can be actively loaded with dyes or drugs. Finally, as demonstrated in this work, porphysomes’ capability can also be extended beyond photonics by their intrinsic ability to chelate metals. Uniquely, this direct radiolabeling procedure, where the single building block of the nanovesicle, porphyrin-lipid, is used as the chelator obviates the need for exogenous chelators and is the most reliable and accurate method for quantitatively tracking the distribution of these nanoparticles \textit{in vivo}. The fraction of labeled porphyrin building blocks can be fine tuned to specific applications and, thereby, the properties of porphysomes can be distinctively controlled. For this reason, porphysomes have the potential for a wide range of applications in cancer imaging and therapy. Depending on the amount of radioisotope
chelated, porphysomes can be designed for imaging (PET/SPECT) or used for radiation therapy while still maintaining their optical properties. By also chelating other metal ions such as paramagnetic manganese, MRI imaging capabilities can be incorporated into porphysomes. Thus, these porphyrin-lipid nanovesicles can truly be tailored for multiple applications with natural multifunctional capabilities, all obtained simply due to the inherent multimodality nature of porphyrins.

Although all 3 porphyrin-based probes demonstrate promise in specifically localizing within the tumor, we must also discuss the limitations these strategies face, both as imaging and therapy agents as well as with their implementation in the clinic. First, off-target effects are a concern. Because photodynamic molecular beacons are extracellularly activated, critical tissues may be PDT damaged if activated beacons are allowed sufficient time to diffuse from the target site to normal tissues; the selectivity of photodynamic molecular beacon activation is dependent upon the cleavage specificity of the linker peptide sequence for the target enzyme. Normal tissue accumulation of targeted porphyrin probes are dependent upon the specificity and binding affinity of the homing molecule (folate) and the receptor (folate receptor), and the overexpression of the targeted receptor at tumor sites. Thus, the molecular target must be carefully considered for both photodynamic molecular beacons and targeted porphyrin probes. The selective accumulation of porphysomes, in the studies discussed within this thesis, solely rely upon the enhanced permeability and retention effect, the clinical significance of which is contentious as it does not enable uniform delivery of nanoparticles to all regions of tumors in sufficient quantities. However, as already discussed, we demonstrate that the use of local administration of photodynamic molecular beacons and targeted porphyrin probes may reduce off-target accumulation. Further work is necessary to evaluate whether targeted porphysomes may enhance their specificity for diseased tissue.

The clinical translation of these porphyrin-based probes may also be a challenge. In all 3 strategies, only Pyro and Bchl are used. Unfortunately, neither Pyro nor Bchl are currently FDA approved. Approval is a lengthy and costly process with a number of regulatory hurdles, the details of which are beyond the scope of this discussion. However, it should be noted that Pyro is an analog of 2-(1-Hexyloxyethyl)-2-devinyl pyropheophorbide-a (HPPH, Photochlor) which has seen success in phase I and II clinical
trials\textsuperscript{318}. Another concern regarding the clinical approval of photodynamic molecular beacons is the associated clinical toxicity from the quencher moiety, Black Hole Quencher 3\textsuperscript{319}. However, it should be mentioned that no toxicity was observed in any of our preclinical studies. Good manufacturing practices, GMP, are underway for porphysomes, but have not yet been implemented for either photodynamic molecular beacons or targeted porphyrin probes. However, the local administration of small-molecule porphyrin probes and their fast activation/uptake kinetics (less than an hour) may allow these agents to be evaluated as medical devices rather than drugs, which may allow quicker translation into the clinic. Regardless, these porphyrin-based approaches are powerful as they can, in principle, destroy cells based upon their specific molecular characteristics.

Over the course of this work, it becomes clear the importance of a simplified composition from a regulatory viewpoint. With the use of porphyrins, their natural multimodal nature provides a plethora of possibilities for both imaging and therapy with all this versatility built into a single molecule. This may make the clinical possibilities of porphyrin-based multimodal imaging and therapy more viable. Regardless, before the successful clinical translation of these probes, it is imperative that we not only demonstrate high tumor uptake, but that the signal-to-background ratio of tumor to healthy tissue uptake of these probes is several fold thereby increasing our ability accurately delineate tumor tissue and better preserve healthy tissue during therapy. Since the discovery of porphyrins, a considerable body of work has evaluated the potential role of porphyrins in photodetection, photodynamic therapy and as metal chelators. Even with the numerous derivatives and analogs of porphyrins that have been synthesized and investigated, little success has occurred in improving these molecules’ tumor selectivity and specificity. Hopefully by utilizing the strategies outlined in this work, photodynamic molecular beacons, targeted porphyrin probes and porphysomes, we may see successful implementation of these molecules in the clinic.
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159 Zeng, D. et al. (64)Cu Core-Labeled Nanoparticles with High Specific Activity via Metal-Free Click Chemistry. ACS Nano, (2012).


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List of Publications

1. **Liu TW†, MacDonald TD†, Jin CS, Gold JM, Bristow RG, Wilson BC, Zheng G.** Inherently Multimodal Nanoparticle-Driven Tracking and Real-Time Delineation of Orthotopic Prostate Tumors and Micrometastases. ACS Nano (Submitted)


(†authors contributed equally to the work)

Publications in Progress

