Development of Approaches of Tumor Trapping Enhanced BB2R-Targeted Radiopharmaceuticals for Prostate Cancer

Wenting Zhang

University of Nebraska Medical Center

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DEVELOPMENT OF APPROACHES OF TUMOR TRAPPING ENHANCED BB2R-TARGETED RADIOPHARMACEUTICALS FOR PROSTATE CANCER

by

Wenting Zhang

A DISSERTATION

Presented to the Faculty of
the University of Nebraska Graduate College
In Partial Fulfillment of the Requirements
For the Degree of Doctor of Philosophy

Pharmaceutical Sciences
Graduate Program

Under the Supervision of Professor Jered C. Garrison

University of Nebraska Medical Center
Omaha, Nebraska

January, 2019

Supervisory Committee:

Surinder K. Batra, Ph.D.        Jonathan L. Vennerstrom, Ph.D.
Joseph A. Vetro, Ph.D.          Dong Wang, Ph.D.
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DEVELOPMENT OF TUMOR TRAPPING ENHANCED BB2R-TARGETED RADIOPHARMACEUTICALS FOR PROSTATE CANCER

Wenting Zhang

University of Nebraska, 2019

Supervisor: Jered C. Garrison, Ph.D.

ABSTRACT

The Gastrin-Releasing Peptide Receptor (BB2r) has been intensively investigated as a cancer target over the years. Numerous diagnostic and therapeutic BB2r-targeted agents have been developed for various solid tumors, including prostate cancers, due to the high expression level of BB2r on neoplastic relative to normal tissues. The development of those targeted agents have mainly utilized the modified c-terminal of bombesin(BBN), a peptide that has nanomolar binding affinity to human BB2r. However, a major issue that hinders the clinical translational potential of low-molecular weight, receptor-targeted agents, is their short residence time at tumor tissues due to the intrinsically high diffusion and clearance rates.

Detailed in this dissertation is a comparison study investigating important biological differences between two mouse models of prostate cancer and how these factors impact the delivery of BB2r-targeted agents. Specifically, we have evaluated the impact of differences in tumor vasculatur density, hypoxia burden and perfusion efficacy on BB2r-targeted agent uptake and distribution. Furthermore, herein, we proposed two
different approaches to increase the tumor residualization of BB2r-targeted radiopharmaceutical agents, by developing chemical approaches to “trap” BB2r-targeted agents in prostate cancer cells through adduct formation with macromolecules. First proposed, BB2r-targeted agents incorporating hydrophilic cysteine cathepsin (CC) inhibitors was developed. Due to the high concentration of CCs found in endolysosomal compartments, our agents have the ability to irreversibly bind to CCs after endocytosis. Two analogs, based on BB2r-targeted agonist and antagonist separately, demonstrated enhanced tumor retention and optimal tumor-to-non-target ratios compared to the matching controls. The second approach focused on the hypoxic nature of prostate cancer, which is due to the distorted architecture leading to the insufficient delivery of oxygen, and was examined as a mechanism to increase retention of BB2r-targeted agents. Specifically, we explored the hypoxic-selectivity of a 2-nitroimidazole phosphoramide nitrogen mustard (2-NIPAM), a potential tumor trapping agent which is able to irreversibly bind to intracellular nucleophiles in hypoxic tissues.

Overall, we seek to determine the most suitable mouse model in evaluating the BB2r-targeted agents. We are also exploring strategies to elongate the retention time of BB2r-targeted agents in the prostate tumor tissues, thereby increasing the clinical translational potential of these agents. Future works include: 1) synthesis and evaluation of 2-NIPAM incorporated BB2r-targeted peptides in vitro and in vivo; 2) synthesis and assessment of hydrophilic CC inhibitors integrated into other receptor-targeted analogs. Further work is needed to demonstrate the feasibility and wide applicability of our strategies in different receptor-targeted systems.
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<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>BB2r</td>
<td>Gastrin-Releasing Peptide Receptor</td>
</tr>
<tr>
<td>BBN</td>
<td>Bombesin</td>
</tr>
<tr>
<td>DOTA</td>
<td>1,4,7,10-tetraazacyclododecane-1,4,7,10-tetraacetic acid</td>
</tr>
<tr>
<td>PCa</td>
<td>Prostate cancer</td>
</tr>
<tr>
<td>DIEA</td>
<td>N,N-diisopropylethylamine</td>
</tr>
<tr>
<td>DMF</td>
<td>N,N-dimethylformamide</td>
</tr>
<tr>
<td>DCM</td>
<td>Dichloromethane</td>
</tr>
<tr>
<td>DCC</td>
<td>N,N’-dicyclohexylcarbodiimide</td>
</tr>
<tr>
<td>NMP</td>
<td>N-methylpyrrolidone</td>
</tr>
<tr>
<td>RPMI</td>
<td>Roswell Park Memorial Institute</td>
</tr>
<tr>
<td>F12K</td>
<td>Kaighn's Modification of Ham's F-12 Medium</td>
</tr>
<tr>
<td>HEPES</td>
<td>4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid</td>
</tr>
<tr>
<td>BSA</td>
<td>Bovine serum albumin</td>
</tr>
<tr>
<td>FBS</td>
<td>Fetal Bovine Serum</td>
</tr>
<tr>
<td>SDS</td>
<td>Sodium dodecyl sulfate</td>
</tr>
<tr>
<td>HBTU</td>
<td>O-benzotriazole-N,N,N’,N’-tetramethyl-uronium-hexafluoro-phosphate</td>
</tr>
<tr>
<td>PBS</td>
<td>Phosphate buffered saline</td>
</tr>
<tr>
<td>2-NIAA</td>
<td>2-nitroimidazole acetic acid</td>
</tr>
<tr>
<td>2-NIPAM</td>
<td>2-nitroimidazoles phosphoramidate nitrogen mustard</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Description</td>
</tr>
<tr>
<td>--------------</td>
<td>-------------</td>
</tr>
<tr>
<td>Se-Met</td>
<td>Selenomethionine</td>
</tr>
<tr>
<td>$K_m$</td>
<td>The Michaelis constant for the particular enzyme being investigated</td>
</tr>
<tr>
<td>$K_i$</td>
<td>The inhibitor constant</td>
</tr>
<tr>
<td>FACS</td>
<td>Fluorescence-activated cell sorting</td>
</tr>
<tr>
<td>HOBt</td>
<td>$N$-Hydroxybenzotriazole</td>
</tr>
<tr>
<td>RP-HPLC</td>
<td>Reverse-phase high-performance liquid chromatography</td>
</tr>
<tr>
<td>MS</td>
<td>Mass spectrometry</td>
</tr>
<tr>
<td>iv</td>
<td>Intravenous</td>
</tr>
<tr>
<td>ip</td>
<td>Intraperitoneal</td>
</tr>
<tr>
<td>NMR</td>
<td>Nuclear magnetic resonance</td>
</tr>
<tr>
<td>SPECT</td>
<td>Single-photon emission computed tomography</td>
</tr>
<tr>
<td>CT</td>
<td>Computed tomography</td>
</tr>
<tr>
<td>PEG</td>
<td>Polyethylene glycol</td>
</tr>
<tr>
<td>CCs</td>
<td>Cysteine cathepsins</td>
</tr>
<tr>
<td>mAb</td>
<td>Monoclonal antibody</td>
</tr>
<tr>
<td>HER2</td>
<td>Human epidermal growth factor receptor 2</td>
</tr>
<tr>
<td>RANKL</td>
<td>Receptor activator of nuclear factor kappa-B ligand</td>
</tr>
<tr>
<td>PSMA</td>
<td>Prostate-specific membrane antigen</td>
</tr>
<tr>
<td>PSA</td>
<td>Prostate-specific antigen</td>
</tr>
<tr>
<td>SCID</td>
<td>Severely combined immunodeficient</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Full Form</td>
</tr>
<tr>
<td>--------------</td>
<td>-----------</td>
</tr>
<tr>
<td>IC₅₀</td>
<td>half maximal inhibitory concentration</td>
</tr>
<tr>
<td>DCC</td>
<td>N,N’-Dicyclohexylcarbodiimide</td>
</tr>
<tr>
<td>EDC</td>
<td>1-Ethyl-3-(3-dimethylaminopropyl)carbodiimide</td>
</tr>
<tr>
<td>NHS</td>
<td>N-Hydroxysuccinimide</td>
</tr>
<tr>
<td>Lu</td>
<td>lutetium</td>
</tr>
<tr>
<td>I</td>
<td>Iodine</td>
</tr>
<tr>
<td>Tc</td>
<td>Technetium</td>
</tr>
</tbody>
</table>
CHAPTER 1. INTRODUCTION

1.1 Prostate Cancer and Gastrin-Release Peptide Receptor

It is estimated that prostate cancer was the second leading cause of death for men in the United State in 2018 and accounts for 19% of new cancer cases alone, according to the American Cancer Society. Prostate cancer is one of the most treatable malignancies with a 98.2% 5-year survival rate if diagnosed early and the cancer remains localized. The 5-year survival rate drops to 30% once metastasis begins. The Gastrin-releasing peptide receptor, also known as the BB2 receptor (BB2r), is a G-protein coupled receptor that upon activation works through the phospholipase C pathway to increase intracellular concentration of the signaling molecules inositol phosphates, diacyl glycerol and calcium. The BB2r is expressed in the human gastrointestinal tract and central nervous system, and plays a role in release of gastrointestinal hormones, epithelial cell proliferation, smooth muscle cell contraction, pancreatic enzyme secretion and other biological functions. Furthermore, BB2r is aberrantly overexpressed in several human cancers such as lung, breast, colon and prostate cancers, possibly due to constitutive receptor activation triggered by mutation. Elevated level of BB2r can assist the proliferation and invasion of human cancerous cells, via the activation of transcription factor Elk-1 and intermediate early gene c-fos and epidermal growth factor receptors (EGFR) pathway. In prostate cancer, the increased expression of BB2r activation can also stimulate the focal adhesion kinase (FAK) and upregulate
intracellular adhesion molecule (ICAM)-1, which facilitates the tumor differentiation and cancer cell-extracellular matrix attachment and tumor migration \textsuperscript{13-14}.

The BB2r has been intensively investigated as a clinical diagnostic and/or therapeutic target for prostate, pancreatic, breast, colorectal and small-cell lung cancers, due to this high expression of the receptor on tumor cells relative to normal tissues \textsuperscript{15-21}. To date, research have found that 38-72\% of breast cancer, 72-85\% of glioblastoma and 85-100\% of small-cell lung cancer have overexpressed BB2r \textsuperscript{22}. Specifically, for prostate cancer, it is estimated that 63-100\% of primary prostatic invasion carcinoma have high or very high level of BB2r \textsuperscript{23}. The prostate cancer progression often starts from early-stage, in which the growth and survival of the tumor is dependent on androgens. Androgen ablation therapy can cause the suspension of prostate cancer progression, but eventually the cancer is able to bypass its androgen dependence to become androgen-independent \textsuperscript{24-25}. Among the prostate cancer patients have underwent androgen ablation therapy, more than 50\% of the androgen-independent prostate cancers have been shown to be BB2r-positive \textsuperscript{26}. A study evaluating lymph node and bone metastasis of prostate cancer have also shown BB2r expression in 85.7\% and 52.9\% of cases, respectively \textsuperscript{26}. These finding greatly implicate the potential of developing diagnostic and therapeutic agents for BB2r-positive cancers.

Based on the research stated above, different approaches have been utilized to develop BB2r-targeted agents. Studies employing BB2r antagonists either alone or with other agents have exhibited anti-tumor growth effects. For instance, cytotoxic drugs (e.g.
doxorubicin and paclitaxel) has been conjugated to BB2r-targeted analog, leading to the specific inhibition of tumor cell proliferation \(^{14}\); also, the BB2r antagonist (PD176252) co-administrated with EGFR inhibitor (erlotinib) showed a synergistic anti-tumor effect \(^{27}\).

Another approach for the development of targeted diagnostics/therapeutics is to use BB2r-targeted peptides labeled with radioisotopes. Numerous analogs have been developed over the years, which are going to be discussed in the following contents in detail.

1.2 Targeted Radiopharmaceutical Agents

In the past few decades, the development of radiopharmaceuticals for diagnostic and therapeutic purposes has been widely explored. Targeted radiopharmaceutical agents comprising radionuclide(s) and a targeting vehicle, delivering the radioisotope specifically to tumor cells or other cells in the tumor microenvironment, have risen and became the center of the field. A variety of radionuclides such as \(^{68}\)Ga, \(^{64}\)Cu, \(^{99m}\)Tc, \(^{111}\)In, \(^{90}\)Y, \(^{177}\)Lu, \(^{123}\)I and \(^{18}\)F have been used for diagnosis and/or therapy \(^{28-33}\). The overall developmental strategy for targeted radiopharmaceuticals agents is mainly focused on the optimization of the in vivo pharmacokinetics, particularly regarding achieving high tumor-to-non-target (T/NT) ratios. The T/NT ratios are a good indicator of how efficient the in vivo delivery of the agent to the tumor vs the clearance of the analog, and its metabolites, from background, nontarget tissues. The higher the T/NT ratios for the radiopharmaceutical the better the imaging contrast for diagnosis and more tolerable the radiation damage to normal tissues during therapy \(^{34}\). In addition, the residence time of
the radiopharmaceuticals agents at the tumor sites is another important factor to be considered. This is particularly true for therapeutic purposes given that residence for a radiotherapeutic agents is going to directly correlate to the dose delivered to the tumors of patients. With those issues in mind, various delivery vehicles have been designed, synthesized and evaluated to date, including low- and high-molecular weight carriers. These platforms have unique pharmaceutical properties and different applications, as discussed below separately.

1.2.1 Antibodies-Based Radiopharmaceuticals

To date, there have been dozens of monoclonal antibody (mAb) (e.g. whole or fragments) based radiopharmaceuticals under investigated for clinical cancer therapy, including prostate cancer \(^{35-38}\). For instance, Zevalin\(^{®}\) and Bexxar\(^{®}\), both are mAb that targets the CD20 antigen, has been approved for the treatment of relapsed refractory B cell non-Hodgkin lymphoma. There are also several ongoing clinical trials that utilize high affinity mAb targeting prostate cancer biomarkers. To date, several of these trials have achieved promising results \(^{39-41}\), such as Lu-177 or Bi-213 labeled J591 with anti-prostate specific membrane antigen (PSMA) domain \(^{42-43}\) and \(^{131}\)I-labeled mAb CC49 against tumor-associated glycoprotein 72 (TAG72) for metastatic prostate cancer \(^{44}\). The development of tumor-specific antibodies-based radiopharmaceuticals have the advantage of long blood circulation time (long half-life), which requires lower doses upon administration \(^{36, 45}\). One major drawback with the production of monoclonal antibodies, which often relies on the bacteria or mammalian cells, is relatively complex
when compared to synthetic small molecules. Also, due to the high molecular weight of mAbs, there are other negative issues such as the sequestration by reticuloendothelial cells, slow tumor uptake and systematic clearance rates, which eventually cause adverse non-target toxicity in patients.

1.2.2 Small Molecule-Targeted Radiopharmaceuticals

Different from antibody-based radiopharmaceuticals, small molecular weight agents, including small molecules and peptides, have the advantage of rapid tumor targeting and blood clearance, which leads to higher T/NT ratios. They can be synthesized chemically and tolerate more harsh conditions (e.g. pH and temperature) upon modification and radiolabeling. These low molecular weight agents are also less likely to induce immunogenic response. For prostate cancer, the standouts in the field are small-molecule vehicles capable of targeting PSMA, which is highly expressed and well characterized in almost all prostate cancers regardless of cancer stage. For example, $^{99m}$Tc-MIP-1404, demonstrated a high probability (90%) to detect PSMA-positive lesions in patients with elevated prostate-specific antigen (PSA). In addition, $^{18}$F-DCFBC, another small molecular vehicle, has been evaluated and showed higher specificity for detecting high-grade and larger-volume tumors (Gleason score 8 and 9) compared to MRI. Besides imaging, PSMA-targeted agents have also underwent studies for therapeutic purposes and have yielded analogs capable of high radiotherapeutic delivery to prostate tumor (e.g. $^{124/131}$I-labeled MIP-1095 and $^{177}$Lu-PSMA).
1.2.3 Peptide-Targeted Radiopharmaceuticals

Another frequently discussed category of targeted radiopharmaceuticals is peptide-based agents, in which our laboratory is most interested in studying \(^{31,57}\). Just like other targeted radiopharmaceuticals, these peptide-based radiotracers also target the receptors that overexpressed on cancer cells or surrounding tissues. As mentioned before, the peptide-based radio-agents usually display favorable pharmacokinetics such as rapid targeting, fast plasma clearance and deep tumor penetration. But due to their intrinsic instability in protease-rich bioenvironments, the development of peptide-based agents often involved chemical modifications to enhance metabolic stability \(^{58}\). The first FDA-approved diagnostic radio-peptide, \(^{111}\)In-DTPA-octreotide (OctreoScan), targets somatostatin receptors in patients with neuroendocrine tumors and showed great targeting efficacy \(^{31}\). Additionally, gastrin release peptide receptors (i.e. BB2r) has also shown great potential for targeting tumors including prostate, breast and small-cell lung cancers \(^{31,59}\). Although there are a tremendous number of preclinical studies of BB2r-targeted agonist, there has been limited clinical investigation/development due to the lack of satisfactory outcomes, and undesired gastrointestinal side effects (e.g. abdominal cramps) of the agonistic analogs \(^{16,60-66}\). Only recently, the successful development of BB2r-antagonist (e.g. RM2 and RM26), which have shown better clinical translation potential, has revitalized the field and increased interest in BB2r-targeted agents for detection and staging.
Many promising BB2r-targeting radiotracers, listed in Table 1.1, are currently undergoing clinical evaluation. For instance, an ongoing Phase II clinical study uses $^{68}$Ga-NeoBOMB1 as a diagnostic agent targeting BB2r-overexpressing cancers and more than three Phase II clinical studies have been done by different groups evaluating the feasibility of $^{68}$Ga-RM2 as a diagnostic agent for patients with prostate cancer. These BB2r-targeted antagonists have shown higher tumor specific uptake, good sensitivity to detect both primary and metastatic prostate cancer and better T/NT contrast in patients compared to BB2r-agonist. Representative images of ongoing clinical studies using these new BB2r antagonists are shown in Figure 1.1.

Unfortunately, there are only few radiopharmaceuticals based on receptor-targeted peptides (e.g. $^{68}$Ga-DOTATATE) currently FDA approved for clinical usage, and a clinically useful BB2r-targeted agent is not among them. One of the major challenges preventing the clinical translation of peptide-based radiotracers is the short tumor residence time of these low-molecular weight agents, due to their rapid degradation and clearance by endogenous proteases and peptidases. Tumor retention is particularly crucial for radiotherapeutic applications since accumulation/retention is closely tied to deliverable therapeutic dose.
Table 1.1 Radiolabeled BB2r-targeted analogues that have been actively tested in clinical studies.

<table>
<thead>
<tr>
<th>Analogue</th>
<th>Sequence</th>
<th>BB2r IC₅₀ (nM)</th>
<th>Refs</th>
</tr>
</thead>
<tbody>
<tr>
<td>Antagonist</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>⁶⁸Ga-DOTA RM2</td>
<td>⁶⁸Ga-DOTA-4-amino-1-carboxymethyl-piperidine-D-Phe-Gln-Trp-Ala-Val-Gly-His-Sta-Leu-NH₂</td>
<td>7.7 ± 3.3</td>
<td>⁶⁷-⁶⁹</td>
</tr>
<tr>
<td>⁶⁸Ga/¹⁷⁷Lu-NeoBOMB1</td>
<td>⁶⁸Ga/¹⁷⁷Lu-DOTA-p-aminomethylaniline-diglycolic acid-D-Phe-Gln-Trp-Ala-Val-Gly-His-NH₂-CH₂-CH(CH₃)₂</td>
<td>2.5 ± 0.2</td>
<td>⁷⁰</td>
</tr>
<tr>
<td>⁶⁸Ga-SB3</td>
<td>⁶⁸Ga-DOTA-p-aminomethylaniline-diglycolic acid-D-Phe-Gln-Trp-Ala-Val-Gly-His-NHEt</td>
<td>1.5 ± 0.3</td>
<td>⁷¹</td>
</tr>
<tr>
<td>⁶⁴Cu-CB-TE2A-AR06</td>
<td>⁶⁴Cu-4,11-bis(carboxymethyl)-1,4,8,11-tetraazabicyclo(6.6.2)hexadecane)-PEG₄-D-Phe-Gln-Trp-Ala-Val-Gly-His-Sta-LeuNH₂</td>
<td>5.5 ± 1.3</td>
<td>⁶⁶</td>
</tr>
<tr>
<td>Agonist</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>⁶⁸Ga-BBN</td>
<td>⁶⁸Ga-NOTA-Aca-BBN₉₋₁₄</td>
<td>1.8 ± 0.7</td>
<td>⁷²-⁷³</td>
</tr>
</tbody>
</table>
Figure 1.1 PET/CT scan images of A) $^{68}$Ga-DOTA-RM$_2$ $^{67}$, B) $^{68}$Ga-NeoBOMB1 $^{70}$, C) $^{68}$Ga-SB$_3$ $^{71}$, D) $^{68}$Ga-NOTA-PEG$_3$-RM26, compared to BB2r-targeted agonist E) $^{68}$Ga-BBN $^{72}$ and F) $^{64}$Cu-CB-TE2A-AR06 $^{66}$ in prostate cancer patients. D) and E) are images of the same patient.
1.3 Structure of BB2r-Targeted Agonist and Antagonist Radiopharmaceuticals

The bombesin (BBN) peptide shares a C-terminal binding region sequence homology with gastrin releasing peptide (the endogenous ligand of BB2r), as illustrated in Figure 1.2. The developed BB2r-targeted agents are based on a variety of BBN-like peptides (i.e. intact, truncated and modified) 23,74-75. The typical structure of BB2r-targeted agonist and antagonist are also shown in Figure 1.2, which generally includes a radioisotope, a chelator, a linker and a peptide-based targeting vector. So far, the development of BB2r-targeted agents have largely utilized the BBN(7-14)NH₂ sequence (Gln-Trp-Ala-Val-Gly-His-Leu-Met-NH₂), which has nanomolar affinity to BB2r 76-79. A series of reports have identified alterations to the C-terminal fragment that result in antagonistic behavior while still achieving high BB2r-binding affinity 76,80-82. The BB2r antagonists often include the unnatural amino acid D-Phe⁶ inserted before Gln⁷, a modification between His¹²-Leu¹³, the elimination of Met¹⁴ and the substitution of Leu¹³-NH₂ (e.g. Leu-OCH₃ or Leu-(CH₂)₃-CH₃). To date, the potent BB2r-targeted antagonists (e.g. RM2 and RM26) having the structures with the presence of D-Phe⁶, insertion of Statin between His¹²-Leu¹³, des-Met¹⁴ and ended with NH₂ showed substantially promising results, which are currently under clinical evaluations 67-69,72. For example, small cohort clinical studies have showed that ⁶⁸Ga-RM₂ can detect 72% (13/18 patients) of the breast tumor tissues 83 and 63% (10/16 patients) of recurrent prostate cancers 69. Despite that BB2r-targeted antagonists have demonstrated lower BB2r binding affinities relative to agonists and the substantially lower rates of receptor-mediated endocytosis, which was originally thought to be crucial for tumor uptake/residualization, studies have clearly
demonstrated the superiority of BB2r-targeted antagonists compared to agonists\textsuperscript{84-88}. The superiority of the BB2r-targeted antagonist may possibly be due to a higher number of receptor binding sites compared to the agonist\textsuperscript{89}. Other factors such as lower disassociation constant ($k_{\text{off}}$) and an increase in metabolic stability of antagonist relative to agonists, also may possibly contribute to the unexpected higher retention in tumors\textsuperscript{90-92}.

Moreover, the chelators and linkers have also been demonstrated to impacts BB2r-targeting and in vivo biodistribution. The linker/spacer, as a connecter between the chelated radionuclide and the targeting peptide vector, is introduced to avoid the steric inhibition of the radiometal-chelator moiety to the binding affinity of the targeting vector. Moreover, it also greatly contributes to the alteration of the hydrophilicity and the optimization the pharmacokinetic profiles of radio-peptide analogs. Amino acids, carbohydrate groups, aliphatic hydrocarbons, aromatic derivatives, and polyethylene glycol (PEG) are the most commonly used linkers for BB2r-targeted agents\textsuperscript{93-95}. A conjugate containing no spacer, \textsuperscript{111}In-DOTA-BBN$_{7-14}$NH$_2$, demonstrated a 100-fold lower BB2r-binding affinity compared to an analog having 8-carbon aliphatic spacer\textsuperscript{96}. Analogs with five to eight carbon atoms as a spacer showed similar BB2r-binding affinity. Additionally, BB2r-targeted analogs having more hydrophilic linkers have also exhibited substantial lower liver uptake\textsuperscript{97-98}. Additionally, it has been demonstrated that the modification of charges on the peptide-pharmacophore can substantially impact the binding affinity of the analogs. However, the structure-and-function relationship of charges is not completely clear. Several studies has demonstrated that the negatively
charged linker showed more favorable pharmacokinetics and higher T/NT ratio than positively charged linkers \(^{99-101}\). For example, a significant increase of tumor uptake was observed by introducing single negative charged linker (\(\beta^3\text{hGlu}\)) to \(^{99m}\text{Tc(CO)}_3\)-BBN analogs compared to neutral (\(\beta^3\text{hSer}\)) and positively charged linker (\(\beta^3\text{hLys}\)) counterparts \(^{100}\). On the contrary, another attempt to utilize positively charged linker over neutral ones (glycine-4-aminobenzoyl) demonstrated that the positively charged linkers resulted in a 1.5-fold increase in BB2r-binding affinity. However, this increased didn’t significantly alter the tumor uptake, nor did it improve the T/NT ratios in vivo \(^87\).

In other cases, opposite phenomenon has been observed. The analogs that have more positive charges tend to have higher BB2r-binding affinity and more favorable in vivo pharmacokinetics \(^{102-103}\). A comparison study of \(^{68}\text{Ga-BBN}\) antagonists with various chelators (NOTA, DOTA, NODAGA and DOTAGA) incorporated was performed \(^{102}\). Examination of the local charge of Ga\(^{3+}\) complex with di-anionic NOTA, tri-anionic NODAGA and DOTA, and tetra-anionic DOTAGA found out to be +1, 0, 0 and −1, respectively. Consequently, the positively charged analogs demonstrated the highest BB2r-binding affinity, followed by neutral analogs and the negatively charged exhibiting the lowest affinity. The BB2r-affinity positively corelated with in vivo tumor uptake and T/NT ratios.
**Figure 1.2** Classic structure of BB2r-targeted peptide-based radiopharmaceuticals. The critical positions of the BB2r-targeted antagonists inducing antagonistic character are indicated by color.
1.4 Cystine Cathepsins and Its Inhibitors

Cystine cathepsins (CCs), a family of proteases that are largely contained in the endolysosomal environment, are predominantly known for the contribution to intracellular proteolysis and protein turnover. Meanwhile, these proteases are also involved in diverse normal physiological processes, including cell proliferation, migration and death \(^\text{104}\). Some of the CCs are constitutively expressed, for example, Cathepsin B and L, which has been estimated to have very high concentrations (~1mM) in endolysosomal compartments with each comprise up to 20% of the overall organelle content \(^\text{105}\). In a variety of cancers, however, cancerous cells have exhibited aberrant expression of CCs extracellularly, which has been identified to directly assist tumor progression, migration, invasion and in some cases to facilitate tumor angiogenesis \(^\text{104, 106}\).

It has been demonstrated that secreted Cathepsin B can degrade extracellular-matrix materials, such as type IV collagen and fibronectin, in both neutral and acidic environment \(^\text{107}\). Additionally, the upregulated expression of CCs has been positively correlated with poor prognosis \(^\text{108}\) and metastasis \(^\text{109}\) of cancer patients.

Thus, over the years, CCs have been investigated as a potential target in preclinical and clinical cancer research. Several strategies have been explored to block CCs activity, such as antibodies and small-molecule inhibitors, to name a few. Based on the binding mechanism, these small molecule CC inhibitors are generally classified as both reversible and irreversible inhibitors, which the latter holds the most interest for our laboratory. Representative structures of these inhibitors are shown in Figure 1.3. The
reversible inhibitor, with functionalities such as aldehydes, nitriles and cyclopropenones, can reversibly form non-covalent interactions with the enzyme. On the contrary, the irreversibly inhibitor, e.g. epoxysuccinates, vinyl sulfones and acyloxy methyl ketones (AOMK), can bind to the CCs through covalent bonds, which have presented promising clinical performance\textsuperscript{110-114}. Our preliminary work using an AOMK inhibitor as a trapping agent for neurotensin receptor-1 (NTR1) targeted analogs (Figure 1.4) has successfully proven this trapping principle. The results obtained from this work clearly demonstrated that the AOMK incorporated radio-peptides have a 2-fold enhancement in tumor retention at 24h post-injection compared to the matching inactive (non-trapping) control, without comprising the initial tumor uptake\textsuperscript{115}. Additionally, among other irreversible covalent inhibitors, another well-known and broad-targeting CC inhibitor is E-64, which can form thiol-ether bonds to CCs\textsuperscript{116-118}. Although studies utilizing CCs inhibitors as tumor imaging probe\textsuperscript{119-121} have been previously reported, in this dissertation for the very first time, we are employing it as trapping agent to increase the tumor residence time of our radioconjugates.
Figure 1.3 Representative structures of irreversible and reversible cysteine cathepsin protease inhibitors.
Figure 1.4 Representative structure of a cysteine cathepsins inhibitor AOMK incorporated NTR-1 targeted analogues. The AOMK derivative moiety is indicated in red color.
1.5 Tumor Hypoxia and Hypoxia Selective Trapping Agents (HSTA)

Tumor hypoxia results from the distorted vascular architecture found in tumors, which consequently causes irregular blood flow, reduces the efficacy of oxygen and other nutrients delivery to tissues. The partial pressure of oxygen ($pO_2$) at sea level is about 80-100 mmHg in human arterial blood and the median $pO_2$ ranges from 24-66 mmHg in normal tissues (e.g. skeletal muscle) \(^{122}\). The critical $pO_2$ value, which is the point at which tissues are considered hypoxic, is estimated to be 8-10 mmHg in tumors \(^{123}\). The oxygen measured in prostate carcinoma is significant lower (mean $pO_2 = 6$ mmHg) when compared to normal prostate tissue (mean $pO_2 = 26$ mm Hg) \(^{124-125}\). The median of 63% (n=247) of prostate tumors from patients presented the $pO_2$ value to be less than 10 mmHg \(^{125}\).

Tumor hypoxia is a therapeutic concern due to it being strongly association with tumor aggressiveness, resistance to chemotherapy and radiotherapy, and a good indicator of poor prognosis of the patients \(^{126-127}\). Given this, tumor hypoxia is actively considered as a diagnostic and/or therapeutic target. Several approaches have been utilized to reduce/target tumor hypoxia including the administration of erythropoietin (EPO) to improve blood flow, RAS inhibitor to reduce local oxygen consumption in tumors, hypoxia inducible factor (HIF) pathway inhibitors to block hypoxia-induced downstream oncogenic pathways and the utilization of tumor-selective hypoxia-activated prodrugs \(^{128}\). Multiple classes of hypoxia-selective bioreductive drugs (e.g. nitroimidazole, aromatic N-oxides and quinones) have been synthesized and explored
preclinically and clinically and some examples are presented in Figure 1.5. Generally, these prodrugs can be selectively reduced under a hypoxic environment thereby generating reactive radicals and electrophiles. For example, the aromatic N-oxide tirapazamine (TPZ) can produce a DNA-reactive free radical; aliphatic N-oxides banozantrone (AQ4N) can transform into an aliphatic N-oxide DNA intercalator and nitrogen mustard prodrug PR-104 and TH-302 can generate activated nitrogen mustard as a cytotoxin. To date, one of the most promising hypoxia selective prodrugs is TH-302, which employs 2-nitroimidazole as the hypoxia-selective group and is the bromo analogue of the FDA approved DNA-alkylating agent, Ifosfamide. After reduction (triggered) in hypoxic cells, it will form a mustard moiety fragment serving as a DNA cross-linking agent. Additionally, the development of other hypoxia-triggered agents utilizing 2-nitroimidazole derivatives have been actively investigated. Pimonidazole HCl, a 2-nitroimidazole derivatives, is a hypoxia probe approved for intravenous use in the clinic since 1993. In radiopharmaceuticals, imaging agents employing 2-nitroimidazole derivatives as hypoxia-targeting agents have been actively explored. For example, \(^{18}\)F-fluoromisonidazole (\(^{18}\)F-FMISO), a conjugated-2-nitroimidazole derivative, is widely investigated under clinical trials to assess hypoxia levels by PET in various cancers.

Clinical studies have shown TH-302 has enhanced hypoxia selectivity, which makes it an ideal candidate for our studies as a hypoxia-selective trapping agent (HSTA) prodrug for conjugation with cellular nucleophiles upon hypoxia activation inside of cells. In detail, 2-nitroimidazole derivatives can be selectively reduced under hypoxic
conditions to form potent electrophiles. Once inside a hypoxic cell, these agent can be reduced and irreversibly bind to nucleophilic macromolecules, which leads to the agent being trapped and retained $^{146}$. Nitrogen mustard derivatives of 2-nitroimidazole have substantially longer lifetimes in biological solutions after activation than other 2-nitroimidazole derivative $^{147}$. Thus, we propose to design and develop radiolabeled BB2r-targeted peptides conjugated with 2-nitroimidazole phosphoramidate nitrogen mustard for cancer diagnosis/therapy. The rationale behind this approach is depicted in Figure 1.6.
Figure 1.5 Representative structures of hypoxia-selective bioreductive prodrugs.
Figure 1.6 Proposed mechanism of 2-NIPAM incorporated BB2r-targeted analogues in hypoxic BB2r-positive tumor cells.
1.6 Animal Models Used in Prostate Cancer Research

In the history of anticancer drug discovery, a critical component of preclinical stage research is the assessment of drug efficacy in clinically relevant tumor models. As the most widely utilized animal models, mouse models have been developed and evaluated in human cancer research, including prostate cancer, for many years. Mouse models provide valuable information concerning in vivo pharmacokinetics, pharmacodynamics and the mechanisms of drug action. Additionally, the closer the models can resemble the human tumor pathologically, the greater chance the preclinical data collected from those models can predicate the clinical outcomes and potentially guide clinical trials designs. To date, there are a variety of mouse models that are commonly used in preclinical cancer research, such as subcutaneous and orthotopic xenografts, genetically engineered mice (GEM) models and patient-derived xenograft (PDX) models. To summarize, highlighted features and comparisons for each mouse model are listed in Table 1.2.
Table 1.2 Comparison of animal models for the assessment of prostate cancers.

<table>
<thead>
<tr>
<th>Animal model</th>
<th>Advantages</th>
<th>Limitation</th>
</tr>
</thead>
<tbody>
<tr>
<td>Subcutaneous</td>
<td>• Low cost</td>
<td>• Mice are immunodeficient, lacking realistic tumor microenvironment information</td>
</tr>
<tr>
<td></td>
<td>• Lower skills required</td>
<td>• Hard to metastasis</td>
</tr>
<tr>
<td></td>
<td>• Short establish time</td>
<td>• Different progress to human cancer</td>
</tr>
<tr>
<td></td>
<td>• Commonly used</td>
<td></td>
</tr>
<tr>
<td>Orthotopic</td>
<td>• Have some similar human tumor microenvironment</td>
<td>• Higher cost and Higher skill required</td>
</tr>
<tr>
<td></td>
<td>• Possible to metastasis</td>
<td>• Long establish time (weeks)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>• Longer time to metastasis</td>
</tr>
<tr>
<td></td>
<td></td>
<td>• Mice still are immunodeficient</td>
</tr>
<tr>
<td>Genetical engineered mice (GEM)</td>
<td>• Tumor progress in a competent immune system with natural microenvironment</td>
<td>• Only limited number of genes can be targeted so far</td>
</tr>
<tr>
<td></td>
<td>• The mutations identified in human can directly induced in mice</td>
<td>• High cost and time consuming (months)</td>
</tr>
<tr>
<td></td>
<td>• The early stage of tumor development can be monitored</td>
<td>• Require lots of validation before successful</td>
</tr>
<tr>
<td>Patient cancer-Derived Xenografts (PDX)</td>
<td>• Have high human cancer relevance</td>
<td>• High cost</td>
</tr>
<tr>
<td></td>
<td>• Have high experimental robustness</td>
<td>• Need to confirm the exist of target biomarker in each patient samples</td>
</tr>
<tr>
<td></td>
<td>• Have preserved tumor histological and biological information</td>
<td>• Various tumor development time</td>
</tr>
</tbody>
</table>
For the past three decades, the human cancer cell line xenograft model has been the most common used mouse model. In this model, the human cancer cells lines are inoculated, either under the skin or directly into the organs, into immunodeficient mice to produce the subcutaneous or orthotopic xenograft mouse model, respectively. Studies have demonstrated that depending on the tumor cell types, the development of tumor ranges from days to weeks. Unfortunately, both subcutaneous and orthotopic cancer cell line generated mouse models rarely predict efficacy in human. Their rapid growth rate, opposite to the slowly progression of human cancers, exhibit a dramatically altered tumor microenvironment and are unable to reflect genetic changes during long-term tumor progression in patients. Specifically, the subcutaneous tumors lack an adequate representation for the primary tumor in patients due to lack of those local tumor microenvironment, which has a significant influence with regard to a patient’s response of chemotherapy. Based on the above reasons, biological variables, such as the degree of angiogenesis, found in patients are certainly better reflected in orthotopic models relative to subcutaneous models. Also, between those two models, it has been demonstrated that orthotopic models more commonly mimic the metastatic spread (e.g. lymph nodes) found in the clinic. With that said, due to the fast growth rate and low technical barrier in subcutaneous models, the subcutaneous model is still the predominate choice for the first-step screen and in vivo evaluation of cytotoxic agents. This model is an expedient choice for the first-time investigation of biological performance parameters, such as pharmacokinetic/pharmacodynamic, toxicity and drug resistance, for targeted drugs.
Another mouse model considered to be more advance is the genetically engineered mouse (GEM) model \cite{158-161}. In this model, one or more genes related to carcinogenesis are modified to promote tumor initiation and progression. One major advantage of this model is that the mice have a competent immune system and thus the mouse’s immune response to tumor development can be examined. For example, mice that are gene-engineered to express the simian virus 40 large tumor antigen-coding region, can progressively form prostatic disease that histologically resembles human prostate cancer \cite{162}. In this case, the prostate tumors can be detected in the mice as early as 10-weeks-of age. Despite GEM models having the ability to mimic molecular and histopathological of human tumors and to predict drug response and resistance, this model has major disadvantages, include having reduced clonal heterogeneity of human tumors, hard to generate tumor metastasis and the cost/time required to generate this model \cite{152-153, 163-164}.

Recently, another mouse model that has drawn considerable attention is the patient-derived xenograft (PDX) models \cite{165-167}. In this model, primary tumor tissues from patients are implanted into immunocompromised mice, which are considered to preserve the patient cancer biology in vivo. As consequence, it can provide valuable information regarding tumor histological and biological assessment, screening of tumor target and therapeutic response \cite{161, 168-170}. Studies has demonstrated a reliable correlation between the chemo-drug response in the PDX model and what is found for clinical outcomes in terms of tumor drug resistance (97%) and sensitivity (90%). As with all mouse models, PDX model also have their limitations. The successful engraftment rate varies depending on types/quality of human tumor samples and mouse strains.
Moreover, the initial presented human tumor stroma can be gradually replaced with mouse ones, causing the loss of the human-specific microenvironment. Most importantly, the generation of PDX models use immunodeficient mice, which limits the exploration of immune cell function in the tumor model.

For the development of new radiopharmaceuticals agents, xenograft mouse models (subcutaneous and orthotopic) using human cancer cell line are still the first choice, especially compared to PDX and GEM models, due to the relative ease of generation and availability. Furthermore, the fast growth rate and the standardized easy manipulated culture condition of immortalized cancer cell lines, compared to tumor cells from patients, make it more convenient to validation the in vitro pharmakinetic properties of the agents. Between these two models, the subcutaneous mouse model typically is more commonly utilized to evaluate the first-time pharmacokinetic profiles of agents due to its lower technical barriers. In reality, since there are no perfect animal models that 100% mimic human cancers, the choice of mouse models is generally dependent on the scientific purpose of the experiment, the technical difficulties, the time required to generate the model and the cost. The summary of mouse models commonly used to evaluate radiopharmaceuticals in prostate cancer research are shown in Table 1.3.

Lastly, the tumor blood vessels are generally abnormal in functionality with such properties as the lacking smooth-muscle layer, malfunctioning vascular architecture, inefficient lymphatic drainage and high interstitial fluid pressure (IFP), etc. Those factors cause variance in the distribution of blood throughout the tumor leading to a disparity
in tumor perfusion, eventually impacting the delivery of nutrients (e.g. oxygen) and
drugs. Specifically, the differences in the tumor microenvironment (TEM) between
subcutaneous and orthotopic xenograft models, such as blood vasculature density,
perfusion efficiency, oxygenation and interstitial fluid pressure (IFP) result in having a
significant impact on the delivery of intravenously distributed radiopharmaceuticals
agents and need to be taken into consideration when choosing a suitable model for
pharmaceutical evaluation. Studies have demonstrated that angiogenesis was generally
more extensive and overall perfusion was higher in the orthotopic relative to the
subcutaneous mouse model\textsuperscript{171-174}. Combining with the fact that the orthotopic tumors
generally show lower IFP and have more spatially distributed functional vasculature
than subcutaneous tumors, it is anticipated that the overall hypoxia burden is lower, and
the tumor uptake of agents is higher in orthotopic than subcutaneous tumors\textsuperscript{175-178}. 

Table 1.3 Animal models for the investigation of radiopharmaceuticals in prostate cancer.

<table>
<thead>
<tr>
<th>Animal</th>
<th>Cancer cell lines</th>
<th>Type of tumor cells inoculations</th>
<th>Refs</th>
</tr>
</thead>
<tbody>
<tr>
<td>Severe combined immunodeficient mice (SCID)</td>
<td>LNCa-P</td>
<td>Subcutaneous</td>
<td>179</td>
</tr>
<tr>
<td>SCID</td>
<td>PC-3</td>
<td>Subcutaneous</td>
<td>179-182</td>
</tr>
<tr>
<td>Athymic nu/nu mice</td>
<td>PC-3</td>
<td>Subcutaneous</td>
<td>92, 183-184</td>
</tr>
<tr>
<td>Athymic nu/nu mice</td>
<td>LNCa-P</td>
<td>Subcutaneous</td>
<td>185-186</td>
</tr>
<tr>
<td>Athymic nu/nu mice</td>
<td>PC-3</td>
<td>Orthotopic</td>
<td>187</td>
</tr>
<tr>
<td>Athymic nu/nu mice</td>
<td>CWR22rv</td>
<td>Subcutaneous</td>
<td>188-189</td>
</tr>
<tr>
<td>nu/nu Balb C</td>
<td>LNCa-P</td>
<td>Subcutaneous</td>
<td>190</td>
</tr>
</tbody>
</table>
1.7 Conclusion

For the past five years, the field of peptide-based radiopharmaceuticals targeting the BB2r has thrived, resulting in several promising agents that have been actively evaluated in clinical studies so far. In order to enhance the tumor retention of low molecule-weight radio-peptides, new concepts (e.g. utilizing enzyme inhibitors and homodimers/heterodimers) have been brought up and have shown to exhibit the potential to improve diagnosis and therapy. To better understand how the variance of biology in different mouse models impact BB2r-targeted agent delivery, we intend to first perform a direct comparison of perfusion and vascular density between subcutaneous and orthotopic tumors. As discussed in Chapter 2, the study will provide insight regarding the cause-effect relationship between these biological parameters and its impacts on drug delivery. By examining this, we hope to give researchers an informed choice when deciding the best in vivo model for their studies in prostate cancer. Moreover, the BB2r-targeted agents, like any other small molecule weight radiopharmaceuticals, demonstrates rapid targeting and uptake in receptor-positive tumors, yet relatively swift clearance from tumors due to intrinsically high diffusion and efflux rates leading to lower retention at the tumor site. Consequently, the diagnostic and therapeutic efficacy of the agent is reduced. Thus, in this dissertation, we propose to incorporate two different trapping agents in our tumor-targeted peptide backbone to address this issue. The comprehensive in vitro and in vivo evaluation of CC inhibitor incorporated BB2r-targeted agents was examined and discussed in Chapter 3. Moreover, the development and examination of hypoxia-selective 2-NIPAM for BB2r conjugation
was documented in Chapter 4. After receptor-mediated endocytosis in tumors, the modified agents can irreversibly bind to intracellular macromolecules and substantially elongate retention to further improve T/NT ratios. If successful, our strategies can potentially increase the clinical translational opportunities for BB2r-targeted agents and these outlined approaches may even be applied to other peptide-targeted radiopharmaceuticals in general.

2.1 INTRODUCTION

The gastrin-releasing peptide receptor (BB2r) is a G-protein coupled receptor that has been of significant interest to the field of cancer drug development due to its overexpression in a variety of cancers, including prostate cancer. Accordingly, numerous theranostic BB2r-targeted agents have been developed over the years with several agents going on to clinical trials. Generally, these agents have been based on synthetic derivatives of the C-terminal portions of the Bombesin (BBN) peptide, which retain nanomolar affinities for the BB2r. To evaluate newly developed BB2r-targeted agents, researchers have numerous tumor models (e.g., subcutaneous, orthotopic and genetically engineered mouse models) to choose. However, in many cases, how well these models mirror the pathology and clinical chemotherapeutic response of human cancers is roughly inversely related to the technical difficulties in generating the model. As such, researchers typically rely on relatively simple subcutaneous tumor xenografts to first study the biological performance of new agents. These models are invaluable for examining the pharmacokinetic profiles of agents, verifying in vivo BB2r-targeting and identifying lead compounds. Yet, it is well-known that the tumor biology of subcutaneous xenografts are not as reflective as other models (e.g., orthotopic) of human tumors. Arguably the most common xenograft model to
evaluate BB2r-targeted agents is the murine xenograft model utilizing the human prostate cancer PC-3 cell line, which has been shown to highly overexpress the BB2r.

The tumor microenvironment (TME) is a wide-ranging term utilized to describe the complex environment in which tumor exist. In regard to drug delivery, perhaps the most important TME variable is the density and functionality of the tumor vasculature. Tumor perfusion, essentially how well the tumor vasculature distributes blood throughout the tumor, directly influences the delivery of drugs and biologically important nutrients (e.g., oxygen). As a consequence, many solid tumor, including prostate cancer, contain significant regions of hypoxia due to their distorted and insufficiently developed vasculature. Hypoxia is also recognized as a crucial stimuli for tumor angiogenesis, metastasis and, in many cases, aggressiveness.

The purpose of this study is to elucidate the impact of tumor perfusion and vascular density on BB2r-targeted drug delivery and tumor hypoxia. Specifically, we undertake this investigation in subcutaneous and orthotopic PC-3 xenograft mouse models over a spectrum of tumor volumes to explore similarities and differences between the two models. By this study, we hope to give the research community utilizing these models a better understanding of the cause-effect relationship between these variables and their potential impact on drug delivery. To this end, sodium pertechnetate (Na99mTcO4), a freely diffusible blood perfusion agent, was employed to evaluate the PC-3 tumor perfusion efficiency. The functional vasculature density of the tumors was measured
using Hoechst staining. A novel BB2r-targeted analog, DOTA-(D)S-(D)S-(D)S-(D)S-(D)S-PEG3–BBN(7-14)NH2 (DOTA-SP714), was utilized to examine in vivo BB2r uptake and tumor distribution, as showed in Figure 2.1A. Lastly, the hypoxic burden of the PC-3 tumors was measured by pimonidazole staining.
Figure 2.1 A) Structure of $^{177}$Lu-DOTA-SP714. B) Experimental design and timeline.
2.2 MATERIALS AND METHODS

2.2.1 Reagents and Instruments

All solvents utilized for synthesis were ACS grade, purchased from Fisher Scientific and used without further purification, unless otherwise stated. Acetonitrile utilized for HPLC analysis and purification was HPLC grade and purchased from Fisher Scientific. Formic acid, N,N-diisopropylethylamine (DIEA), N,N-dimethylformamide (DMF), dichloromethane (DCM), N,N'-dicyclohexylcarbodiimide (DCC), N-methylpyrrolidone (NMP), thioanisol, sodium hydroxide, L-ascorbic acid, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES), bovine serum albumin (BSA), Hoechst 33342 and O.C.T. Compound were purchased from Fisher Scientific (U.S.). Water was deionized by Millipore® Milli Q Biocell Ultrapure Water System before use. D-Luciferin, O-Benzotriazole-N,N,N',N'-tetramethyl-uronium-hexafluoro-phosphate (HBTU), Fmoc-protected natural amino acids and the appropriate Rink Amide resins were purchased from Nova Biochem (U.S.). Ham's F-12K (Kaighn's) medium, phosphate buffered saline (PBS), and mammalian protein extraction reagent (M-PER) were purchased from Thermo Scientific (U.S.). Naturally abundant lutetium chloride (nat LuCl₃), triisopropyl silane, trimethylsilyl chloride, hydrochloric acid (HCl) and 3,6-dioxa-1,8-octanediethiol were purchased from Sigma-Aldrich (U.S.). Radioactive lutetium chloride (¹⁷⁷LuCl₃) was purchased from the National Isotope Development Center operated by the United States Department of Energy, Office of Science. Sodium Pertechnetate (Na⁹⁹mTcO₄⁻) was purchased from Cardinal Health™. The human prostate cancer PC-3 cell line was
obtained from American Type Culture Collection (U.S.) and cultured under vendor recommended conditions. TrypLE™ Express was purchased from Invitrogen (U.S.). TransduX™ was purchased from System Biosciences. Pre-made lentiviral expression particles was purchased from AMS Biotechnology Limited. Pimonidazole hydrochloride and FITC Mab were purchased from Hypoxyprobe™.

Four weeks-old NOD SCID (NOD.CB17-Prkdscid/NCrCrl) mice were obtained from Charles River Laboratories (Wilmington, MA). Food and water were given ad libitum. Five mice per group were kept in the same cage with an air filter cover and a light- (12 h light/dark cycle) and temperature-controlled (22 ± 1°C) environment prior to experiments. On the day of the experiment, each animal was isolated under the same housing conditions. The animals were treated in accordance to the Principles of Animal Care outlined by the National Institutes of Health and approved by the Institutional Animal Care and Use Committee of the University of Nebraska Medical Center.

Peptide synthesis was performed using an automated solid-phase peptide synthesizer employing traditional Fmoc chemistry and using a Rink Amide resin. Briefly, the resin (100 µmol of the resin substituted peptide anchors) was deprotected using piperidine, resulting in the formation of a primary amine from which the C-terminus of the growing peptide was anchored. microwave peptide synthesizer from CEM (U.S.). A Waters e2695 system equipped with a Waters 2489 absorption detector and a Waters Q-tof Micro electrospray ionization mass spectrometer was used to perform high performance liquid chromatography/mass spectrometry analyses. A Phenomenex
Jupiter C12 Proteo 250×10 mm semiprep column was used for the purification of bulk amounts of the BB2r-targeted peptide. The peptide was concentrated on a Centrivap (U.S.) Centrifugal Concentrator. Evaluation and purification of the radiolabeled conjugate was performed on a Waters 1515 binary pump equipped with a Waters 2489 absorption detector and a Bioscan (U.S.) Flow Count radiometric detector system, using a Phenomenex Jupiter C12 Proteo 250×4.6 mm column. Solid phase extraction was performed using Empore (U.S.) C18 10 mm high performance extraction disks. Gas anesthesia was administered at a vaporizer setting of 5% isoflurane (Halocarbon Corp, River Edge, NJ) with 0.5 L/min oxygen using an E-ZAnesthesia apparatus (EUTHANEX Corp, Palmer, PA). Cell sorting was accomplished by fluorescence activated cell sorting of living cells (FACS) using FACSArray. Biodistribution radiation measurements were made with a NaI (Tl) well detector constructed by AlphaSpectra, Inc. (U.S.). Bioluminescence images were acquired by IVIS® Spectrum. Animal tissue slides were generated by microtome cryostat HM 500 OM from MICROM. Confocal microscopy images were taken on a Leica LSM 510 META Microscope equipped with an argon laser and processed by ZEN blue software. The autoradiography studies were accomplished by GE Lifesciences Typhoon FLA 9500 laser scanner.

2.2.2 Solid-Phase Peptide Synthesis (SPPS)

Peptide synthesis was performed using an automated solid-phase peptide synthesizer employing traditional Fmoc chemistry and using a Rink Amide resin. Briefly, the resin (100 µmol of the resin substituted peptide anchors) was deprotected using
piperidine, resulting in the formation of a primary amine from which the C-terminus of the growing peptide was anchored. The Fmoc protected amino acids (300 µmol) with appropriate orthogonal protection were activated with HBTU and sequentially added to the resin. The resulting peptide was orthogonally deprotected and cleaved from the resin using a cocktail consisting of triisopropyl silane (0.1 mL), water (0.1 mL), 3,6-dioxaoctanedithiol (0.1 mL), trifluoroacetic acid (4.625 mL) and thioanisole (0.075 mL), respectively. The cleaved peptide was subsequently precipitated and washed using cold (0 °C) methyl-tert-butyl ether (10 mL x 3). The crude conjugate was dried by a centrifugation concentrator and weighed. ES-MS was used to determine the molecular mass of the prepared peptides. All conjugates were peak purified to ≥95% purity and quantified by RP-HPLC prior to in vitro/in vivo investigations.

2.2.3 HPLC Purification and Analysis Methodology

Sample analysis and radioconjugate purification was performed on an analytical Proteo column with a flow rate of 1.5 mL/min. HPLC solvents consisted of H₂O containing 0.1% formic acid (solvent A) and acetonitrile containing 0.1% formic acid (solvent B). Bulk peptide purification was performed using a semi-preparative Proteo column with a flow rate of 5.0 mL/min. For all unlabeled and ¹⁷⁷Lu-labeled conjugates, purification was achieved using an initial gradient of 80% A : 20% B which linearly decreased to 70% A : 30% B over a 15 minute time period. At the end of the run time, the column was flushed with the gradient 5% A : 95% B and re-equilibrated to the starting gradient.
2.2.4 Labeling with \textsuperscript{nat}LuCl$_3$

For the convenient characterization of the Lu-labeled peptide, naturally abundant natLu was used to substitute for $^{177}$Lu in the ES-MS and in vitro binding studies. A sample of conjugate (1 mg) was dissolved in ammonium acetate buffer (1 M, 200 µL, pH 5.5) and mixed with a solution of natLuCl$_3$ (5.5 mg). The solution was heated for 60 min at 50 °C. After cooling to room temperature, the natLu-conjugate was peak purified by RP-HPLC. natLu conjugates were $\geq 95\%$ purity before mass spectrometric characterization and in vitro binding studies were performed.

2.2.5 Radiolabeling BBN-analogs with $^{177}$LuCl$_3$

Radiolabeling was performed on BBN-analogs by mixing 100 µg samples with 37 MBq $^{177}$LuCl$_3$ in ammonium acetate buffer (1 M, 200 µL, pH 5.5). The solution was heated for 60 min at 90 °C. In order to separate radiolabeled peptides from unlabeled peptides on HPLC, 4-5 mg CoCl$_2$ was added to the crude peptide mixture and incubated for 5 min at 90 °C to increase the hydrophobicity of unlabeled conjugates. The resulting radioconjugate was allowed to cool to room temperature, peak purified using RP-HPLC ($\geq 95\%$) and concentrated using a C18 extraction disk. Elution of the extraction disk with ethanol/sterile saline solution (6 : 4, 150 µL × 2). The ethanol eluent was evaporated by purging with nitrogen gas.

2.2.6 Distribution Coefficient Studies

The partition coefficients were determined for the $^{177}$Lu-labeled conjugate. In a microcentrifuge tube, 0.5mL of 1-octanol was added to 0.5 mL phosphate-buffered saline
(pH 7.4) containing the radiolabeled peptide (100,000 cpm). The solution was vigorously stirred for 2 min at room temperature and subsequently centrifuged (100g, 5 min) to yield two immiscible layers. Aliquots of 100 µL were taken from each layer and the radioactivity of each was quantified by a γ-counter. Statistical analysis using the unpaired two-tailed Student’s t-test was performed in order to compare the logD values and p values <0.05 were considered statistically significant.

2.2.7 Radiochemical Stability Studies

In a vial, 25 µg samples of $^{177}$Lu-conjugate were mixed with 74 MBq of $^{177}$LuCl$_3$ in an ammonium acetate buffer (0.5 M, 120 µL, pH 5.5). The mixture was heated at 90 °C for 40 min and subsequently heated for an additional 5 min in the presence of 4-5 mg CoCl$_2$. The resulting radioconjugates were peak purified using RP-HPLC and concentrated using C18 extraction disks with ethanol/sterile PBS (6:4, 200 µL). The ethanol was evaporated using nitrogen gas and the resulting solutions were diluted with either 1 mL A: PBS, B: Ascorbic acid in 0.9% sodium chloride solution (final ascorbic acid concentration, 40mg/ml), or C: Selenomethionine (Se-Met) in buffer B (final Se-Met concentration, 0.2mg/ml). The purity of the radioconjugate solution was determined by HPLC at 24, 48 and 72 h.

2.2.8 In Vitro Competitive Cell-Binding Studies

The half maximum inhibitory concentration (IC$_{50}$) of the natLu conjugate was determined by competitive displacement cell-binding assays using PC-3 cells. The natLu-conjugate was used as substitutes for the corresponding $^{177}$Lu-radioconjugate.
Briefly, PC-3 cells (~3×10⁴) were suspended in RPMI 1640 media (pH 7.4, 4.8 mg/mL HEPES, and 2 mg/mL BSA) and incubated at 4°C for 40 min in the presence of radiolabeled [¹²⁵I-Tyr₄]-Bombesin and various concentrations of the ñLu-conjugate. At the end of the incubation period, the cells were centrifuged, aspirated and washed with media a total of three times. The cell associated activity was measured using a γ-counter and the IC₅₀ values determined by nonlinear regression using the one-binding site model of Graphpad PRISM 5 (U.S.).

2.2.9 Establishment of Stably Transfected PC-3-Luc Cells

To generate the PC-3-Luc cells, we utilized pre-made lentivirus particles encoding luciferase and GFP (EF1a-Luciferase (firefly)-2A-GFP (Puro), AMSBIO) for transfection. Briefly, normal PC-3 cells were seeded in a 24-well plate using 0.5 mL of medium containing 0.5 × 10⁵ cells/mL and incubated overnight. The medium was removed and replaced with 0.5 mL of fresh medium pre-mixed with 2.5 µL Transdux™. To each well was added 2 µL of the lentivirus particle solution and swirled to mix. The plate was returned to the incubator and cultured for 3 days. Once the cells grew to confluence, they were harvested and transferred to larger plates (from 6-well plates to 60 mm dishes). 1.5 mL of suspended cells in PBS at a concentration of 5 × 10⁶/mL was sorted by the FACS method to give the GFP positive (PC-3-Luc) cells that were returned to culture for in vitro and in vivo studies.

2.2.10 Cell Culture
The normal PC-3 cells and PC-3-Luc were cultured in our laboratory, as per ATCC protocols, in Ham’s F-12K medium containing 10% FBS, 2.5 mM L-glutamine, 15 mM HEPES and 0.5 mM sodium pyruvate. Cells were incubated at 37 °C at 5% CO₂.

2.2.11 Animal Studies

All animal experiments were performed in accordance with the NIH animal use guideline and protocol approved by the Institutional Animal Care and Use Committee (IACUC) at the University of Nebraska Medical Center (Omaha, NE).

2.2.11.1 Development of PC-3 Xenograft SCID Mice

All animal experiments were performed in accordance with the NIH animal use guideline and protocol approved by the Institutional Animal Care and Use Committee (IACUC) at the UNMC. NOD.CB17-PrkdcscidIJ (NOD SCID) mice were purchased from the Jackson Laboratory (Bar Harbor, ME). The mice were housed in groups of five in UNMC animal facility for the entire tumor generation period. All mice were under constant temperature (set up at 21 °C) and humidity on a 12-hour light/dark cycle, which lights on at 7:00 am. Standard food and filtered water were available ad libitum.

For the subcutaneous model, 5-week old female SCID mice were inoculated in the flanks with \(5 \times 10^6\) PC-3 cells in Matrigel®. The tumor size was monitored by caliper. For generation of the orthotopic model (31), the PC-3-Luc cells was selected by 0.5 µg/mL puromycin twice before inoculation. The male 6-weeks SCID mice were anesthetized, the muscles of abdomen area were cut after retracting the skin, and the prostate gland exposed. 50 µL of PC-3-Luc cell suspension at \(0.5 \times 10^6/\text{mL}\) in Matrigel® was injected
into a dorsal prostatic lobe. The wound was closed in two layers and the skin was clipped. Animals were given analgesic drugs for 3 days and the tumors were monitored by an IVIS optical imaging system.

2.2.11.2 In Vivo Biodistribution Studies

The body weight, tumor volume and tumor luminescence of the mice were recorded every three days. The mice were divided into three groups based on tumor volume (<300, 300-700 and >700 mm³). Each mouse (average weight: 20 g for female mice and 25g for male mice) was treated with pimonidazole solution (80mg/kg in PBS) via intraperitoneal injection. After 1h, the mice received an intravenously injection of 10 µCi (370 kBq) of the radio-RP-HPLC peak purified ¹⁷⁷Lu-labeled conjugate (¹⁷⁷Lu-C1) in 100 µL of PBS. After an additional 1h, the mice were injected intravenously with a PBS solution containing 10 µCi (370 kBq) of Na⁹⁹mTcO₄ and 15mg/kg Hoechst 33342. The animals were sacrificed 5 minutes later, and their tissues collected. The excised tissues were weighed, the radioactivity in each tissue was measured, and the %ID/g was calculated for each tissue. Due to the overlap of the energy spectra of Tc-99m and Lu-177, we first measured the total radioactivity in each organ at day 1 (A) and measured again at day 4 (B). Since the Tc-99m had completely decayed by day 4, the radioactivity observed at timepoint B was Lu-177. A decay correction was performed to calculate the amount of radioactivity associated with the Lu-177 component at day 1 (C). The difference of value between A and C was the radioactivity of Tc-99m at day 1.

2.2.12 Bioluminescent Imaging
Bioluminescent imaging was acquired according to standard protocol from the UNMC Small Animal Imaging Core Facility. Imaging and quantification of the signals were performed by Living Image® 4.5 software (PerkinElmer® Health Sciences). For in vitro imaging, PC-3-Luc cells were diluted and plated in a black, clear bottom 96-well plate. 50 µL D-luciferin (150 µg/mL) in media was added to each well 5 min prior to imaging (Figure 2.2). For in vivo imaging, mice were given 100 µL D-luciferin (150 mg/kg) 15-20 min prior to imaging and anesthetized by isoflurane. Mice were transferred to the imaging box and anesthesia nose cones attached. Images were acquired with auto exposure. Regions of interest from each image were selected and quantified.
Figure 2.2 (A) Representative images of in vitro bioluminescence of human prostate cancer cell line with or without luciferase expression gene (PC-3-Luc and PC-3). (B) Correlation between cell number and mean radiance (photons/sec) per well ($R^2=0.9992$).
2.2.13 Microscopy and Autoradiography

The tumors from mice were washed by deionized water, dried and embedded by O.C.T compound on dry ice. The frozen tumor sections were cut at 10 µm and stored at -80 °C for the following studies. The adjacent tumor slides were scanned for Hoechst 33342 and pimonidazole by a confocal microscopy and exposed to storage phosphor screens (BAS-IP SR 2025 E, GE Healthcare) for 3 days. The phosphor screens were subsequently scanned by Typhoon FLA 9500 (GE Healthcare) with 25 µm pixel size. The range of interest (ROI) was drawn to exclude the edge of each tumor slide. The fluorescent arithmetic mean intensity of images were measured by Zeiss ZEN (blue edition) software.

2.2.14 Statistical Analysis

Data was presented as Mean ± SD/SEM as noted. IC₅₀ values were determined by nonlinear regression using the one-binding-site model of GraphPad PRISM 5. Pearson correlation coefficients (r) were calculated to assess the relationship between tumor volume and tumor uptake of the radiotracer (¹⁷⁷Lu-DOTA-SP714 and Na⁹⁹mTcO₄). In vitro stability studies of the radioconjugates in various buffers were analyzed by the two-tailed Student’s t-test. For comparison between the two models, analysis of the biodistribution studies, hypoxic burden and blood vessel density of tumor was carried out by a two-tailed Student’s t-test. Comparisons of the means of the BB2r-uptake, blood perfusion, hypoxic burden and blood vessel density of the tumors among the three tumor volume groups for each animal model were analyzed by one-way ANOVA. If the
overall p-value was statistically significant, pairwise comparisons were adjusted using Tukey’s method. A p-value < 0.05 was considered statistically significant.

2.3 RESULTS

2.3.1 Synthesis and Radiolabeling of nat\(^{177}\)Lu-DOTA-SP714

DOTA-SP714 was synthesized with a yield of 26.6% as determined by HPLC-MS. The peptides were labeled with nat\(^{177}\)LuCl\(_3\) and subsequently purified by radio-RP-HPLC. Analysis of the chromatograms revealed the purity of the radiolabeled peptide was ≥ 95% with a radiolabeling yield of 90.5% (Table 2.1).

2.3.2 Distribution Coefficient and Competitive Binding Studies

The distribution coefficient (mean ± SD) at pH = 7.4 (LogD\(_{7.4}\)) for \(^{177}\)Lu-DOTA-SP714 was determined to be -2.2 ± 0.1 by radiometric analysis. The hydrophilic nature of the radioconjugate suggests that cellular internalization should occur only through BB2r-mediated endocytosis. Subsequently, competitive binding studies demonstrated that both peptides demonstrated low nanomolar binding affinity, with nat\(^{177}\)Lu-DOTA-SP714 (1.0 ± 1.7 nM) giving a significantly higher affinity relative to the unlabeled conjugate (9.8 ± 1.7 nM). Both the IC50 and the LogD7.4 values are provided in Table 2.1.
Table 2.1. Characterization of conjugates.

<table>
<thead>
<tr>
<th>Analogue</th>
<th>Molecular Formula</th>
<th>MW (m/z, [M+H])&lt;sup&gt;†&lt;/sup&gt;</th>
<th>RP-HPLC Retention Time/min</th>
<th>IC&lt;sub&gt;50&lt;/sub&gt;/nM&lt;sup&gt;‡&lt;/sup&gt;</th>
<th>LogD (pH 7.4)&lt;sup&gt;‡&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>DOTA-SP714</td>
<td>C&lt;sub&gt;85&lt;/sub&gt;H&lt;sub&gt;137&lt;/sub&gt;N&lt;sub&gt;23&lt;/sub&gt;O&lt;sub&gt;31&lt;/sub&gt;S</td>
<td>2008.0</td>
<td>8.67</td>
<td>9.8 ± 1.7</td>
<td>-</td>
</tr>
<tr>
<td>nat&lt;sup&gt;177&lt;/sup&gt;Lu-DOTA-SP714</td>
<td>C&lt;sub&gt;85&lt;/sub&gt;H&lt;sub&gt;134&lt;/sub&gt;LuN&lt;sub&gt;23&lt;/sub&gt;O&lt;sub&gt;31&lt;/sub&gt;S</td>
<td>2179.9</td>
<td>8.65</td>
<td>1.0 ± 1.7</td>
<td>-2.2 ± 0.1</td>
</tr>
</tbody>
</table>

<sup>†</sup> Values represent mean ± SEM (n=6)

<sup>‡</sup> Values represent mean ± SD (n=3)
2.3.3 Radiochemical Stability Studies

To investigate the susceptibility of $^{177}$Lu-DOTA-SP714 to radiolytic degradation and examine the efficacy of stabilizers, two stabilizing buffers formulated using ascorbic acid with/without selenomethionine were tested and compared to a control PBS buffer. The results from these studies are depicted in Figure 2.3. In PBS alone, 39.9% of a 0.37 MBq/mL (100µCi/mL) solution of $^{177}$Lu-DOTA-SP714 degraded over the course of 72 h. As expected, the addition of ascorbic acid (40 mg/mL) demonstrated substantial improvements in stability with only 17.3% radiolytic breakdown. However, the combination of ascorbic acid (40 mg/mL) and selenomethionine (0.2 mg/mL) gave superior results with only 5.3% breakdown observed by the 72 h timepoint.
2.3.4 Animal Model Establishment

In this study, subcutaneous and orthotopic xenograft models were generated. For the subcutaneous model, the diameters of tumor were determined by caliper measurements, while tumor growth in the orthotopic model was monitored weekly after implantation surgery using bioluminescence imaging (Figure 2.4A-B). The orthotopic model gave a gradual increase in estimated tumor volumes over the 6-weeks, while the subcutaneous model showed a sharp and rapid increase in tumor size starting at 4th-week (Figure 2.4C). While above measurements were used to non-invasively monitor tumor growth, the assignment of the tumor volume in our subsequent studies are based on caliper measurements of the excised tumors. For both measurements, tumor volumes were calculated by using the following formula: Volume=(Length×Width²)/2.
2.3.5 BB2r-Targeted Peptide Uptake and Perfusion in PC-3 Tumor Xenograft Models

To examine the in vivo BB2r-targeting efficacy and tumor perfusion of the two mouse models, we performed dual, simultaneous biodistribution studies utilizing the $^{177}$Lu-DOTA-SP714 and Na$^{99m}$TcO$_4$. The %ID/g in each organ and the total excretion (%ID) of the radioactivity from the mice are presented in Table 2.2. Overall, the scatter plots of the tumor uptake of $^{177}$Lu-DOTA-SP714 and $^{99m}$TcO$_4$ against tumor volume in both animal models are depicted in Figure 2.5A-2.5D. There was a significant moderate to strong positive correlation (Table 2.3), observed between tumor volume and BB2r-uptake (%ID/g) of the $^{177}$Lu-DOTA-SP714 ($r=0.55$, $p=0.0049$ and $r=0.73$, $p=0.0001$) as well as tumor perfusion ($^{99m}$TcO$_4$) ($r=0.47$, $p=0.021$ and $r=0.49$, $p=0.017$) in subcutaneous and orthotopic mice models, respectively. Interestingly, the correlation (Figure 2.6) between tumor perfusion (Na$^{99m}$TcO$_4$) and BB2r-uptake ($^{177}$Lu-DOTA-SP714) was weak to moderate for the subcutaneous ($r=0.28$, $p=0.19$) and orthotopic ($r=0.66$, $p=0.0006$) models, respectively, suggesting tumor size is a better indicator of BB2r-uptake in both models.

Grouping tumor volumes categorically, <300, 300-700 and >700 mm$^3$, revealed some interesting trends (Figure 2.5E&F). For the orthotopic model, the mean (mean ± SD) categorical tumor volumes were 215.62 ± 103.90, 574.14 ± 73.89 and 1052.78 ± 256.12 mm$^3$ for <300, 300-700 and >700 mm$^3$ groups respectively. With respect to the subcutaneous model, the corresponding mean categorical tumor volumes for <300, 300-700 and >700 mm$^3$ groups were 169.06 ± 74.94, 491.69 ± 114.89 and 1139.25 ± 378.68 mm$^3$. In the
orthotopic model, the average uptake (mean ± SD) of the $^{177}$Lu-DOTA-SP714 was 8.07 ± 3.23, 8.55 ± 2.65 and 11.13 ± 2.88 %ID/g for the <300, 300-700 and >700 mm$^3$ groups, respectively. By ANOVA test, while close (p=0.061), this trend of increased BB2r-uptake with increasing tumor volume did not meet statistical significance given our study size.

For the subcutaneous model, the mean uptake of the radioconjugate increased significantly (p=0.0014) with increasing tumor size from 4.16 ± 1.74 (<300 mm$^3$) to 5.35 ± 1.75 (300-700 mm$^3$) to 9.44 ± 3.66 (>700 mm$^3$) %ID/g. Uptake of the perfusion agent in subcutaneous tumors increased from 0.54 ± 0.32 (<300 mm$^3$) to 0.68 ± 0.49 (300-700 mm$^3$) to 0.86 ± 0.37 (>700 mm$^3$) %ID/g as tumor volume increased (p=0.15). Likewise, the average tumor uptake of the $^{99m}$TcO$_4^-$ for the orthotopic model increased when comparing the <300 mm$^3$ (2.23 ± 1.09 %ID/g), 300-700 mm$^3$ (2.94 ± 0.76 %ID/g) and >700 mm$^3$ (3.19 ± 0.66 %ID/g) groups (p=0.078).

The most striking differences were observed when comparing the BB2r-mediated uptake and perfusion between models at each tumor volume category. The average uptake of the $^{177}$Lu-DOTA-SP714 for the orthotopic model was 1.9, 1.6 and 1.2-fold higher compared to the respective tumor volumes of the subcutaneous model (<300 mm$^3$, p=0.0055; 300-700 mm$^3$, p=0.011; and >700 mm$^3$ group, p=0.34). Similarly, a substantial and statistically significant increase in tumor perfusion was observed for the orthotopic versus the subcutaneous model for each tumor volume category. The average uptake of perfusion tracer in orthotopic model was 4.1, 4.3 and 3.7-fold higher compared to the respective subcutaneous model (<300 mm$^3$, p=0.0008; 300-700 mm$^3$, p=0.0001; and >700 mm$^3$,p=0.0001).
Table 2.2. Biodistribution studies in tumor–bearing scid mice.

<table>
<thead>
<tr>
<th>Tissue (%ID/g)*</th>
<th>¹⁷⁷Lu-BB2r conjugates uptake</th>
<th>Perfusion (⁹⁹mTcO₄⁻)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Tumor volume (mm³)</td>
<td>&lt;300</td>
</tr>
<tr>
<td><strong>Subcutaneous</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>blood</td>
<td></td>
<td>0.49 ± 0.40</td>
</tr>
<tr>
<td>heart</td>
<td></td>
<td>0.16 ± 0.05</td>
</tr>
<tr>
<td>lung</td>
<td></td>
<td>1.08 ± 1.55</td>
</tr>
<tr>
<td>liver</td>
<td></td>
<td>0.29 ± 0.12</td>
</tr>
<tr>
<td>pancreas</td>
<td></td>
<td>19.38 ± 12.99</td>
</tr>
<tr>
<td>stomach</td>
<td></td>
<td>0.94 ± 0.53</td>
</tr>
<tr>
<td>spleen</td>
<td></td>
<td>1.46 ± 1.32</td>
</tr>
<tr>
<td>small intestine</td>
<td></td>
<td>2.53 ± 1.39</td>
</tr>
<tr>
<td>large intestine</td>
<td></td>
<td>1.38 ± 0.75</td>
</tr>
<tr>
<td>kidney</td>
<td></td>
<td>2.75 ± 1.29</td>
</tr>
<tr>
<td>Tissue (%ID/g)</td>
<td>Tumor volume (mm³)</td>
<td>Perfusion ((^{99m})TcO₄⁻)</td>
</tr>
<tr>
<td>---------------</td>
<td>---------------------</td>
<td>-----------------------------</td>
</tr>
<tr>
<td></td>
<td>&lt;300</td>
<td>300-700</td>
</tr>
<tr>
<td>tumor</td>
<td>4.16 ± 1.74</td>
<td>5.35 ± 1.75</td>
</tr>
<tr>
<td>muscle</td>
<td>0.17 ± 0.12</td>
<td>0.15 ± 0.07</td>
</tr>
<tr>
<td>bone</td>
<td>0.20 ± 0.12</td>
<td>0.26 ± 0.19</td>
</tr>
<tr>
<td>brain</td>
<td>0.03 ± 0.02</td>
<td>0.03 ± 0.01</td>
</tr>
<tr>
<td>excretion† (%ID)</td>
<td>68.51 ± 23.40</td>
<td>73.16 ± 7.00</td>
</tr>
</tbody>
</table>

**Orthotopic**

<table>
<thead>
<tr>
<th>Tissue</th>
<th>Tumor volume (mm³)</th>
<th>Perfusion ((^{99m})TcO₄⁻)</th>
</tr>
</thead>
<tbody>
<tr>
<td>blood</td>
<td>0.27 ± 0.19</td>
<td>0.33 ± 0.14</td>
</tr>
<tr>
<td>heart</td>
<td>0.13 ± 0.09</td>
<td>0.39 ± 0.49</td>
</tr>
<tr>
<td>lung</td>
<td>0.82 ± 0.50</td>
<td>0.92 ± 0.41</td>
</tr>
<tr>
<td>liver</td>
<td>0.26 ± 0.07</td>
<td>0.40 ± 0.18</td>
</tr>
<tr>
<td>pancreas</td>
<td>30.01 ± 12.48</td>
<td>40.12 ± 11.47</td>
</tr>
<tr>
<td>stomach</td>
<td>1.07 ± 0.53</td>
<td>1.59 ± 0.90</td>
</tr>
<tr>
<td>Tissue (%ID/g)*</td>
<td>Tumor volume (mm$^3$)</td>
<td>Perfusion ($^{99m}$TcO$_4^-$)</td>
</tr>
<tr>
<td>----------------</td>
<td>-----------------------</td>
<td>------------------------------</td>
</tr>
<tr>
<td></td>
<td>&lt;300</td>
<td>300-700</td>
</tr>
<tr>
<td>spleen</td>
<td>1.77 ± 1.19</td>
<td>2.02 ± 3.14</td>
</tr>
<tr>
<td>small intestine</td>
<td>2.97 ± 0.66</td>
<td>3.74 ± 1.15</td>
</tr>
<tr>
<td>large intestine</td>
<td>2.06 ± 1.02</td>
<td>4.52 ± 1.31</td>
</tr>
<tr>
<td>kidney</td>
<td>3.40 ± 0.92</td>
<td>4.80 ± 4.92</td>
</tr>
<tr>
<td>tumor</td>
<td>8.07 ± 3.23</td>
<td>8.55 ± 2.65</td>
</tr>
<tr>
<td>muscle</td>
<td>0.23 ± 0.12</td>
<td>0.22 ± 0.10</td>
</tr>
<tr>
<td>bone</td>
<td>0.29 ± 0.22</td>
<td>0.46 ± 0.22</td>
</tr>
<tr>
<td>brain</td>
<td>0.03 ± 0.01</td>
<td>0.06 ± 0.03</td>
</tr>
<tr>
<td>excretion† (%ID)</td>
<td>60.77 ± 29.28</td>
<td>67.22 ± 8.53</td>
</tr>
</tbody>
</table>

*, Organ uptake values expressed as %ID/g and values are n ≥ 8 unless otherwise noted.
†. Excretion values (%ID) were calculated using the activity values associated with the excreted urine and fecal material contents at the time of sacrifice.
Figure 2.5. Correlation of tumor uptake of $^{177}$Lu-DOTA-SP714 (A&C) and of $^{99m}$TcO$_4^-$ (B&D) in orthotopic (n=23) and subcutaneous (n=24) model respectively. E&F). Grouped box-whisker plot of tumor uptake of $^{177}$Lu-DOTA-SP714 and $^{99m}$TcO$_4^-$ in two animal models. (*p<0.05, **p<0.01, ***p<0.001, ns=no significance, +: mean, line at median.)
Table 2.3 Pearson correlation coefficient and p-value of tumor volume with uptake of radiotracer in mice models.

<table>
<thead>
<tr>
<th></th>
<th>Tumor uptake (%ID/g)</th>
<th>Tumor volume (mm³)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Pearson r</td>
</tr>
<tr>
<td>Orthotopic</td>
<td>¹⁷⁷Lu-DOTA-SP714</td>
<td>0.73</td>
</tr>
<tr>
<td></td>
<td>⁹⁹ᵐTcO₄⁻</td>
<td>0.49</td>
</tr>
<tr>
<td>Subcutaneous</td>
<td>¹⁷⁷Lu-DOTA-SP714</td>
<td>0.55</td>
</tr>
<tr>
<td></td>
<td>⁹⁹ᵐTcO₄⁻</td>
<td>0.47</td>
</tr>
</tbody>
</table>
Figure 2.3 Correlation of tumor uptake of $^{177}$Lu -DOTA-SP714 and blood perfusion agent $^{99m}$TcO$_4^-$ in A) orthotropic (n=23) and B) subcutaneous (n=24) mice model respectively.
2.3.6 Quantification of Hypoxic Burden and Blood Vessel Density in PC-3 Tumors

Prior to sacrifice of the mice, pimonidazole (hypoxia marker) and Hoechst (functional vasculature marker), were administered to examine the hypoxia burden and vascular density of the human PC-3 tumor xenografts. The excised human PC-3 tumors were sectioned and histologically evaluated for fluorescence signal using adjacent tumor slices. Both signals were calculated by mean intensity per \( \mu m^2 \) and grouped by tumor volume groups for each mouse model (Table 2.4).
Table 2.4. Mean intensity/µm² of hypoxic burden and blood vessel density in tumor xenograft.

<table>
<thead>
<tr>
<th>Tumor volume (mm³)</th>
<th>Mean Intensity/µm² (mean ± SEM)*</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Subcutaneous</td>
</tr>
<tr>
<td></td>
<td>Hypoxic burden</td>
</tr>
<tr>
<td>&lt;300</td>
<td>9.47e-6 ± 1.74e-6</td>
</tr>
<tr>
<td>300-700</td>
<td>3.84e-5 ± 6.98e-6</td>
</tr>
<tr>
<td>&gt;700</td>
<td>2.69e-5 ± 2.98e-6</td>
</tr>
</tbody>
</table>

* Values represent mean ± SEM (n≥6).
On average, subcutaneous tumors exhibited a 3.4-fold higher level of hypoxia burden (signal) than the orthotopic tumors (Figure 2.7A). For both xenograft models, the tumors demonstrated a trend toward maximal hypoxia burden for the 300-700 mm$^3$ group (Figure 2.7C). Overall, there was no significant difference (p=0.13) in hypoxia burden among the tumor volume groups in the orthotopic model (one-way ANOVA). However, a significant difference in hypoxia levels was observed within the tumor volume groups of the subcutaneous mouse model (p<0.001). Comparison of hypoxia burden for the <300 mm$^3$ tumor groups revealed no significant difference (p=0.053) between models. In contrast, significant differences (p=0.002 and p<0.0001, correspondingly) between xenograft models were observed for the 300-700 and >700 mm$^3$ groups, largely due to the substantial increase in hypoxia levels in the subcutaneous models for tumor volumes exceeding 300 mm$^3$. Specifically, the hypoxia burden increased 4.0-fold in the subcutaneous model for the 300-700 mm$^3$ group compared to the <300 mm$^3$ group (p<0.001). In both tumor models, the hypoxia levels decreased on average by 30-50% when the tumor size exceeded 700 mm$^3$ compared to the respective 300-700 mm$^3$ group (p>0.05).

The functional tumor blood vessel density of the excised PC-3 tumors are depicted in Figure 2.7B. On average, the subcutaneous tumors had a slightly higher functional vascular density compared to the orthotopic tumors. For both xenograft models, there was a trend toward higher vascular density as tumor volume increased (Figure 2.7D). For the subcutaneous model, there was a 2.4 and 5.2-fold increase of functional vascular
density in the 300-700 (p=0.013) and >700 mm$^3$ groups (p=0.0041) relative to the <300 mm$^3$ group. With respect to the orthotopic tumors, no increase in vascular density was observed for the 300-700 mm$^3$ (p=0.82) group compared to the <300 mm$^3$ group. Although, a 1.7-fold increase in vessel density was observed for the >700 mm$^3$ (p=0.084) tumor volume group relative to the smaller groups. For tumor group comparisons between models, the orthotopic tumors of the <300 mm$^3$ group had a significantly (p=0.0021) higher vascular density compared to the subcutaneous model, but no statistically significant differences were found between the 300-700 and >700 mm$^3$ groups (p=0.55 and p=0.11, correspondingly).
Figure 2.4 Summary of quantification of A) hypoxia burden B) blood vessel density of tumor slides in two mouse models. Line at grand mean. Box-whisker plots are indicating the variance of C) hypoxia burden and D) blood vessel density between different tumor volume groups in two animal models. (+: mean, line at median)
2.3.7 Qualitative Examination of the Distribution of the BB2r-Targeted Peptide in Tumor Xenografts

To better examine the distribution of $^{177}$Lu-DOTA-SP714 in the two xenografts models in relation to the hypoxic fractions and functional vasculature of tumors, sections of tumors were examined by autoradiography (BB2r-targeted peptide) and confocal microscopy to determine the hypoxic areas (Green) and functional vasculature (Blue). The representative images from the <300, 300-700 and >700 mm$^3$ group in subcutaneous and orthotopic mouse model are shown in Figure 2.8 and Figure 2.9. As expected, the predominate localization of the $^{177}$Lu-DOTA-SP714 was in areas that had significant functional vasculature. While in some areas that lacked vasculature were hypoxic and exhibited a significantly lower concentration of $^{177}$Lu-DOTA-SP714.
<table>
<thead>
<tr>
<th>Tumor volume (mm$^3$)</th>
<th>Hoechst</th>
<th>Pimonidazole</th>
<th>Autoradiography</th>
<th>Merge</th>
</tr>
</thead>
<tbody>
<tr>
<td>&lt;300</td>
<td><img src="image1.png" alt="Image" /></td>
<td><img src="image2.png" alt="Image" /></td>
<td><img src="image3.png" alt="Image" /></td>
<td><img src="image4.png" alt="Image" /></td>
</tr>
<tr>
<td>300-700</td>
<td><img src="image5.png" alt="Image" /></td>
<td><img src="image6.png" alt="Image" /></td>
<td><img src="image7.png" alt="Image" /></td>
<td><img src="image8.png" alt="Image" /></td>
</tr>
<tr>
<td>&gt;700</td>
<td><img src="image9.png" alt="Image" /></td>
<td><img src="image10.png" alt="Image" /></td>
<td><img src="image11.png" alt="Image" /></td>
<td><img src="image12.png" alt="Image" /></td>
</tr>
</tbody>
</table>

**Figure 2.5** Representative confocal microscopy images of adjacent subcutaneous tumor sections in different tumor volume group. The sections were scanned for Hoechst 33342 (blue), the fluorescent antibody of pimonidazole (green) and underwent autoradiography. The contrast of each image was optimized for clarity.
Figure 2.6 Representative confocal microscopy images of adjacent orthotopic tumor sections in different tumor volume group. The sections were scanned for Hoechst 33342 (blue), the fluorescent antibody of pimonidazole (green) and underwent autoradiography. The contrast of each image was optimized for clarity.
2.3.8 H&E Staining of Non-target Tissues

Upon dissection of the mice from the orthotopic mouse model with tumors exceeding 700 mm$^3$, gross anatomy abnormalities of the kidneys, pancreas and liver were perceived. The kidneys were often found to be pale and enlarged and the formation of fluid-filled cysts on the surface of the kidneys were observed, likely due to hydronephrosis. In several cases, enlargement and/or discoloration of the liver and pancreas were also noted. The tissues taken from orthotopic models along with analogous subcutaneous model (>700 mm$^3$ group) and normal control mouse were sectioned and underwent H&E staining, see Figure 2.10. All of the sectioned tissues associated with the orthotopic model were more diffuse than subcutaneous and normal controls probably due to the increased interstitial volume resulting from reduced urine output. For the orthotopic tumor group, micrometastasis were observed in the pancreas, but, surprisingly, were not found in the liver or kidney sections. No micrometastasis were observed in the sections obtained from the subcutaneous model.
Figure 2.7 Representative H&E image of the liver, pancreas and kidney tissue sections (10 x) of non-tumor bearing, subcutaneous and orthotopic xenograft mouse respectively. Scale bar: 100 µm.
2.4 DISCUSSION

The purpose of this work is to investigate how the vascular density and perfusion of tumors impact the hypoxia burden and drug delivery of BB2r-targeted agents. Specifically, we investigated these factors in subcutaneous and orthotopic xenografts across a range of tumor volumes. By understanding this, we hope to provide researchers a better understanding of these biological variables as well as the advantages and limitations of both subcutaneous and orthotopic models for BB2r-targeted agent development.

We first synthesized and characterized a new hydrophilic, BB2r-targeted radioconjugate (\(^{177}\text{Lu-DOTA-SP714}\)). In addition to examining it’s chemical and in vitro properties, we also accessed the solution stability of the radiocojugate. This investigation was prompted out of the need to perform in vivo experiments with large numbers of mice and the desire to reduce the need for constant synthesis of the radioconjugate. It is well established that ionizing radiation (e.g., \(\alpha\) and \(\beta\)-particles) can directly or indirectly, through the generation of solvated electrons and free radicals species, degrade peptides and proteins\(^{201}\). Ascorbic acid and selenomethionine have been demonstrated to have a protective effect against radiolytic degradation in both preclinical and clinical studies\(^{202-204}\). Indeed, Chen and co-workers examined these radioprotectants among others with Lu-AMBA, a clinically investigated BB2r-targeted agent\(^{205}\). Similar to their findings, our studies revealed that the combination of the two
radioprotectants, ascorbic acid (40mg/mL) and selenomethionine (0.2mg/mL), was better than ascorbic acid alone with approximately 95% of the radioconjugate intact by 72h.

The establishment of the subcutaneous and orthotopic PC-3 xenograft model was carried out over a four-eight week period depending on the model and the desired tumor size. The measured tumor size for orthotopic tumors initially outpaced subcutaneous, but the subcutaneous tumors demonstrated a more rapid growth at approximately 4-weeks post-inoculation. The disparity of growth rates between two models likely stem from the different measurement techniques employed and the initial cell numbers administered. Based on body weight measurements, the overall health of the mice appeared good across models and tumor size ranges investigated (Figure 2.11). However, for the largest orthotopic tumor volume group (>700 mm3), abnormalities were found upon gross examination of the kidneys, liver and pancreas. This prompted the histological examination of these tissues for both models. The tissues obtained from the orthotopic model were more diffuse and were consistent with a larger interstitial water content, likely due to urinary obstruction resulting from the PC-3 tumor in the prostate. This observed condition has been noted for other orthotopic prostate cancer mouse models 206.
Figure 2.8 Body weight changes of mice after the orthotopic (blue) and subcutaneous (red) xenograft implantation.
Examination of the perfusion characteristics of two models revealed some interesting findings. The average perfusion of the tumors increased as tumor size increased. However, orthotopic model showed the substantially higher perfusion (>3-fold) compared to subcutaneous ones for the smallest tumor group (<300 mm3). This is interesting given the similar amount of functional vasculature in both models based on Hoechst staining. This comparably higher perfusion rate has also been observed in other orthotopic/subcutaneous models. The most likely reason is that the subcutaneous tumors exhibit higher interstitial fluid pressure (IFP). In general, the functional vasculature of the orthotopic tumors are more spatially distributed throughout the tumors and have larger vessel diameters. This has been shown to reduce IFP in orthotopic over subcutaneous tumors, with the latter having a higher vasculature density on the periphery of the tumor. Another factor that might contribute to the observed perfusion differences is the efficacy of the lymphatic drainage of the tumors. If the lymphatic system present in the orthotopic tumors are more efficient thereby lowering IFP, this would lead to an enhancement in perfusion efficacy.

The BB2r-targeting efficacy of the $^{177}$Lu-DOTA-SP714 was examined in both PC-3 xenograft models. Generally, the average %ID/g uptake of the radioconjugate increased as the tumor size increased. Specifically, orthotopic tumors exhibited significantly higher uptake compared to subcutaneous tumors across tumor groups. This is almost certainly due to the increased perfusion of orthotopic over subcutaneous tumors. Thus, the substantial higher perfusion for PC-3 orthotopic tumors should be considered carefully when evaluating the targeting efficacy of BB2r- and other targeted agents.
Using pimonidazole, the hypoxia burden of the tumors was evaluated for both models. For the orthotopic tumors, no significant differences in hypoxia levels were observed across the tumor volume ranges. However, the subcutaneous tumors exhibited significant increases in hypoxia with tumor sizes that exceeded 300 mm³. The increased hypoxia levels for the 300-700 and >700 mm³ tumor groups may be attributable to the spatial distribution of the functional vascular on the periphery of tumors. For small tumors, the peripheral vasculature can adequately perfuse the whole tumor. Yet, as the tumor grows the vasculature becomes unable to perfuse/supply large segments of the tumor at the core thus resulting in significant increases in hypoxia burden. While hypoxia was observed for both models, medium to large subcutaneous tumors more reliably gave tumors with significant fractions of hypoxia, perhaps making this a more convenient model to investigate the efficacy of hypoxia-targeted or hypoxia-selective agents.

2.5 CONCLUSION

Orthotopic models are known to better simulate clinical prostate cancer, particularly with respect to the tumor microenvironment, compared to subcutaneous models. To better understand the how the biology of these tumor models impact BB2r-targeted agent delivery, we examined the tumor vascular perfusion, microvasculature density and hypoxia burden of orthotopic and subcutaneous PC-3 xenograft mouse models. Compared to the subcutaneous model, the results demonstrate that the orthotopic PC-3 tumors have a higher vascular perfusion that leads to higher BB2r targeting as well as
lower hypoxic burden. While the vessel density was slightly lower in orthotopic model compared to subcutaneous ones, no statistical significance was observed. In general, for both models, BB2r-targeting, perfusion and vascular density increased with increasing tumor volume. Immunofluorescence and autoradiography illustrated the microbiodistribution pattern of BB2r-targeted conjugate relative to functional vasculature and hypoxic regions. As expected, higher concentrations of the radiolabeled conjugate were observed near functional vasculature compared to hypoxic regions that were devoid of functional vasculature. Overall, this work demonstrates that the tumor microenvironments of orthotopic and subcutaneous PC-3 tumors are impactfully different in terms of drug delivery. Careful consideration should be taken when comparing the data of BB2r-targeted agents, as well as other targeted agents, in orthotopic and subcutaneous tumor models.
Chapter 3. Enhanced tumor retention of receptor-targeted agonist and antagonist: a comprehensive study of hydrophilic cysteine cathepsins inhibitor incorporated analogues in prostate cancer imaging

3.1 INTRODUCTION

Due to the elevated expression of the gastrin-releasing peptide receptor (BB2r) in numerous cancers (e.g., prostate, breast and lung), a number of BB2r-targeted diagnostic and therapeutic agents have undergone and continues to undergo clinical investigations. The vast majority of these agents are based on bombesin (BBN), a 14 amino acid amphibian peptide. In most instances, only the C-terminal fragment of the BBN peptide (e.g., BBN(7-14)NH₂: Glu-Trp-Ala-Val-Gly-His-Leu-Met-NH₂) is employed in BB2r-targeted agent design due to its ability to achieve nanomolar affinity for the targeted receptor. Over the years, examination of the structure-activity profile of this fragment has revealed numerous high BB2r-binding derivatives with different pharmacological profiles in terms of agonistic vs antagonistic character. However, despite the encouraging clinical and preclinical results of many of these studies, the relatively short tumor residence time of BB2r-targeted peptides still represents a translational hurdle, particularly with regard to radiotherapeutic approaches.

Cysteine cathepsins (CCs), a family of endolysosomal proteases, play key roles in numerous biological functions, but are primarily associated with intracellular protein turnover. In cancers, it has been demonstrated that these proteases contribute to
tumor progression. CCs become increasingly localized extracellularly in many cancers\textsuperscript{104, 213}, though it is important to recognize that the extracellular CC concentrations and activities in cancer are well below endolysosomal levels\textsuperscript{105, 214}. Due to its oncogenic role, over the years a variety of reversible and irreversible (e.g., E-64) CC inhibitors have been developed for therapeutic purposes\textsuperscript{22-23}. Specifically, several groups have reported efforts to develop fluorescent\textsuperscript{24-25} and nuclear medicine\textsuperscript{26} diagnostic probes targeting the upregulation of CCs in cancer cells.

The utilization of low-molecular weight targeted agents as diagnostics and therapeutics offer several advantages in terms of pharmacokinetic profile and clinical utility when compared to longer circulating macromolecules. However, one significant detriment to the use of low-molecular weight carriers is the relatively limited residence time at target (e.g., tumors) sites. This situation is particularly problematic for targeted radiotherapeutic applications in cancer where short residence times, in many cases, limits deliverable therapeutic doses. Our laboratory has recently begun exploring chemical approaches to enhance the retention (i.e., residualization) of low-molecular weight, receptor targeted agents\textsuperscript{115}. One of these approach uses irreversible CC inhibitors (i.e., CC trapping agents) to form high molecular weight adducts within the endolysosomal compartments of targeted cancer cells. These adducts have been shown, both in vitro and in vivo, to provide substantial enhancements in the retention of receptor-targeted drugs.
Here, for the first time, we explore our CC residualization approach using the well-validated, BB2r-targeted agonistic and antagonistic constructs BBN(7-14)NH₂ and RM2 (D-Phe-Gln-Trp-Ala-Val-Gly-His-Sta-Leu-NH₂). Our impetus for this line of investigation was to examine the efficacy of our methodology with two clinically validated pharmacophores. Additionally, by examining this agonistic and antagonistic pair, we hope to confirm mechanistically if, as we suspect, internalization is key to the efficient formation of highly retained adducts thereby resulting in increased tumor retention. Specifically, we synthesized an analog of E-64, a well-known CC inhibitor, and incorporated this into the structure of the agonist and antagonist BB2r-targeted constructs. The biological performance of these analogs was examined using in vitro and in vivo human prostate cancer PC-3 cell models. The results obtained herein reveal interesting mechanistic insights concerning the generality of our CC/endolysosomal trapping and leads us to question the extent of the antagonistic character of the RM2, and perhaps other, BB2r-targeted antagonists.

3.2 MATERIALS AND METHODS

3.2.1 Reagents and Instruments

All solvents used for reactions and silica gel purification were ACS grade and purchased from Fisher Scientific, unless otherwise stated. Acetonitrile utilized for HPLC analysis and purification was HPLC grade and purchased from Fisher Scientific. Water was deionized by Millipore® Milli Q Biocell Ultrapure Water System before use.
CDCl₃ was obtained from ACROS Organics™. Dimethyl sulfoxide was obtained from Cambridge Isotope Laboratories. Ethylenediaminetetraacetic acid (EDTA), Brij®35 Cu (II) chloride were obtained from Sigma-Aldrich (St Louis, MO). Fmoc-protected natural amino acids were purchased from Nova Biochem (Waltham, MA). Fmoc-NH-(PEG)₃-COOH were purchased from MilliporeSigma (St. Louis, MO). Fmoc-L-propargylglycine and N,N-diisopropylethylamine (DIEA) was obtained from Chem-Impex International (Wood Dale, IL). Sieber Resin (100-200 mesh) were purchased from ChemPep Inc (Wellington, FL). Deuterium oxide were purchased from Alfa Aesar® (Haverhill, MA). CA-074 was purchased from ApexBio (Houston, TX). (1-Cyano-2-ethoxy-2-oxoethylidenaminooxy)dimethylamino-morpholino-carbenium hexafluorophosphate (COMU) was purchased from AK Scientific (Union City, CA). Bovine serum albumin (BSA) was purchased from Atlas Biologicals, Inc. Human serum was obtained from MP Biomedicals (Santa Ana, CA). TrypLE Express was obtained from Invitrogen (Grand Island, NY). N,N-dimethylformamide (DMF), dichloromethane (DCM), petroleum ether (PE), methanol, ethyl acetate, acetonitrile, formic acid, trifluoroacetic acid (TFA), pyridine, piperidine and N-methylpyrrolidone (NMP), hydrochloric acid and L-glutamine were obtained from Fisher Scientific (Fair Lawn, NJ). Kaighn’s Modification of Ham’s F-12 Medium (F-12K Medium), 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES), Penicillin-Streptomycin, and phosphate buffered saline (PBS) were purchased from HyClone Laboratories, Inc. (Logan, UT). Cytofix Fixation buffer was obtained from BD Biosciences (San Jose, CA). Novex™ Tris-Glycine SDS sample buffer, Pierce™ RIPA buffer, PageRuler™ Prestained protein ladder,
HaltTM Protease inhibitor cocktail, LysoTracker® Green DND-26 and NuPAGE® sample reducing reagent (10×) were purchased from Thermo Fisher Scientific (Waltham, MA). Amicon Ultra-4 centrifugal filter (10 kDa) was purchased from Merck Millipore (Burlington, MA). [177Lu] LuCl3 was purchased from Oak Ridge National Laboratory (Oak Ridge, TN). The human prostate cancer PC-3 (CRL-1435™) was purchased from American Type Culture Collection.

Peptides were synthesized by solid phase peptide synthesis (SPPS) on a Liberty microwave peptide synthesizer from CEM Corporation (Matthews, NC). A Waters e2695 system equipped with a Waters 2489 absorption detector and a Waters Qtof Micro electrospray ionization mass spectrometer was used to perform high performance liquid chromatography/mass spectrometry (HPLC-MS) analyses. A Phenomenex Jupiter C12 Proteo 250×10 mm semiprep column was used for the purification of peptides. ¹H and ¹³C NMR spectrums were recorded on a Bruker Avance-III HD 500 MHz instrument. Residual protio-solvent signals (¹H NMR, CDCl₃ δ 7.26, DMSO-d₆ δ 2.50, ¹³C NMR, CDCl₃ δ 77.00, DMSO-d₆ δ 39.51) were used as internal references. Evaluation and purification of radiolabeled conjugates was performed on a Waters 1515 binary pump equipped with a Waters 2489 absorption detector and a Bioscan Flow Count radiometric detector system, using a Phenomenex Jupiter C12 Proteo 250×4.6 mm column. Solid phase extraction was performed using Empore (U.S.) C18 10 mm high performance extraction disks. Costar® Polystyrene TC-Treated Microplates (96-well plate and 6-well plate) were used for Cathepsin B inhibition kinetic studies and in vitro internalization-efflux study separately. Gas anesthesia was administered at a vaporizer setting of 5% isoflurane (Halocarbon...
Corp, River Edge, NJ) with 0.5 L/min oxygen using an E-Z Anesthesia apparatus (EUTHANEX Corp, Palmer, PA). Gamma decay detection of $^{177}$Lu for the in vitro cell studies and tissue homogenates was accomplished using an LTI (U.S.) Multi-Wiper™ nuclear medicine gamma counter. Gamma decay detection of $^{177}$Lu-labeled conjugates for biodistribution studies was accomplished using a NaI (TI) well detector constructed by AlphaSpectra, Inc (U.S.). Fluorescence intensities were measured by a SpectraMax® M5 multimode plate reader. Lab-Tek™ chambered borosilicate coverglass disks (8 well) were used for confocal cell imaging. Confocal microscopy images were taken on a Leica LSM710 META Microscope equipped with an argon laser. The fluorescent images were analyzed by the ZEISS ZEN blue software. Autoradiography was recorded via BAS storage phosphor screens and scanned by GE Lifesciences Typhoon FLA 9500 biomolecular imager. A hybrid single photon emission computed tomography system and x-ray computed tomography (SPECT/CT) (Flex Triumph, TriFoil Imaging, Northridge, CA, USA) fitted with a five pinhole (1.0 mm per pinhole) collimator was used for in vivo imaging study.

3.2.2 Animals

Four weeks-old NOD SCID (NOD.CB17-Prkdcsid/NCrCrl) mice were obtained from The Jackson Laboratory (Bar Harbor, ME). Food and water were given ad libitum. Five mice per group were kept in the same cage with an air filter cover under light- (12 h light/dark cycle) and temperature-controlled (22 ± 1°C) environment before experiment. At the day of experiment, each animal was kept in individual cage with same housing
condition. The animals were treated in accordance to the Principles of Animal Care outlined by National Institutes of Health and approved by the Institutional Animal Care and Use Committee of the University of Nebraska Medical Center.

3.2.3 Chemistry

3.2.3.1 Synthesis of Intermediate Compound of Cystine Cathepsin Inhibitor and Its Control

(9H-fluoren-9-yl)methyl (S)-(1-((2-azidoethyl)amino)-1-oxo-5-(3-((2,2,4,6,7-pentamethyl-2,3-dihydrobenzofuran-5-yl)sulfonyl)guanidino)pentan-2-yl)carbamate (I): To a solution of Fmoc-Arg(Pbf)-OH (500 mg, 0.77 mmol) in DMF (5 mL), EDCI (162 mg, 0.85 mmol) and NHS (116 mg, 1.00 mmol) were added dropwise at 0 °C. The solution was stirred at room temperature for 2h before adding to the solution containing 2-azidoethanamine (132 mg, 1.54 mmol) and (405 µL, 2.31 mmol) in DMF (3 mL). The mixture was kept stirring for overnight and partitioned in ethyl acetate (30 mL ×3) and brine (20 mL). The combined organic phase was washed with water and concentrated by rotary evaporation to yield a white foam (508 mg, 92%).

$^1$H-NMR (500 MHz, CDCl$_3$): δ 7.73 (d, J = 7.5 Hz, 2H), 7.55 (d, J = 7.0 Hz, 2H), 7.45 (br, 1H), 7.37 (d, J = 7.5 Hz, 2H), 7.24-7.22 (m, 2H), 6.27 (s, 2H), 6.05 (d, J = 7.0 Hz, 1H), 4.33-4.31 (m, 3H), 4.14 (m, 1H), 3.43-3.27 (m, 6H), 2.90 (s, 2H), 2.58 (s, 3H), 2.50 (s, 3H), 2.07 (s, 3H), 1.86-1.55 (m, 5H), 1.42 (s, 6H).

MS (m/z): [M+H]$^+$ calcd. for C$_{36}$H$_{51}$N$_{7}$O$_6$S H$: 717.3, found: 717.3.
(9H-fluoren-9-yl)methyl ((S)-1-(((S)-1-((2-azidoethyl)amino)-1-oxo-5-(3-(2,2,4,6,7-pentamethyl-2,3-dihydrobenzofuran-5-yl)sulfonyl)guanidino)pentan-2-yl)amino)-4-methyl-1-oxopentan-2-yl)carbamate (2): Compound 1 (500 mg, 0.70 mmol) was dissolved in 20% piperidine in DMF (10 mL) and stirred for 30 min. The solution was extracted with ethyl acetate (30 mL ×3) and the organic phase was concentrated by rotary evaporation. The residue was re-dissolved in DMF (5 mL) and added to the mixture of COMU (342 mg, 0.80 mmol) and Fmoc-Leu-OH (247mg, 0.70 mmol) in DMF (10 mL). The solution was stirred for 5 min before DIEA (250 µL, 1.4 mmol) was added in. The mixture was kept for stirring for another 2 h. Brine (100 ml) and ethyl acetate (30 mL ×3) were poured into the flask and the organic phase was separated, washed twice with aq. HCl (100 mL, 0.5N) saturated NaHCO₃ (100 mL), water (100 mL) and dried over Na₂SO₄. The organic layer was evaporated to dryness and was purified by flash column chromatography (silica gel, DCM/methanol = 15:1) to afford as a pale foam (424 g, 73%).

¹H-NMR (500 MHz, CDCl₃): δ 7.75 (d, J = 7.5 Hz, 2H), 7.57 (d, J = 7.5 Hz, 2H), 7.46 (br, 1H), 7.38 (t, J = 7.5 Hz, 2H), 7.8 (m, 2H), 6.08 (s, 2H), 5.47 (br, 1H), 4.72-4.33 (m, 5H), 4.14 (m, 1H), 3.46-3.21 (m, 6H), 2.92 (s, 2H), 2.57 (s, 3H), 2.50 (s, 3H), 2.01 (s, 3H), 1.65 (m, 2H), 1.43 (s, 6H), 1.26 (m, 2H), 1.11 (s, 2H), 0.96-0.92 (m, 8H).

MS (m/z): [M+H]+ calcd. for C₄₂H₅₅N₉O₇S H+: 830.4, found: 830.2.
(2R,3S)-3-((1-((1-((2-azidoethyl)amino)-1-oxo-5-(3-(2,2,4,6,7-pentamethyl-2,3-dihydrobenzofuran-5-yl)sulfonyl)guanidino)pentan-2-yl)amino)-4-methyl-1-oxopentan-2-yl)carbamoyl)oxirane-2-carboxylic acid (3). Compound 2 (200 mg, 0.24 mmol) was dissolved in 20% piperidine in DMF (5 mL) and stirred for 30 min. The solution was extracted with ethyl acetate (20 mL ×3) and the organic phase was concentrated by rotary evaporation. To the solution of the residue in DMF (5 mL), was added in the mixture of COMU (171 mg, 0.40 mmol) and (+/-)-trans-oxirane-2,3-dicarboxylic acid (158 mg, 1.20 mmol) in DMF (10 mL) and was stirred for 5 min before DIEA (500 µL, 2.8 mmol) was added in. The mixture was kept for stirring for another 2 h. Brine (100 ml) and ethyl acetate (30 mL ×3) were poured into the flask and the organic phase was separated, washed twice with aq. HCl (100 mL, 0.5N) and water (100 mL ×3), and dried over Na₂SO₄. The organic layer was evaporated to dryness and was purified by a semi-preparative Proteo C12 HPLC column with a 15 min gradient and a flow rate of 5.0 mL/min (40% - 90% ACN in water containing 0.1% formic acid) to give compound 5 as a yellow powder (99 mg, 57%).

¹H-NMR (500 MHz, DMSO-d₆): δ 8.59 (dd, J = 29.5, 8.0 Hz, 2H), 8.13 (m, 1H), 8.08 (m, 1H), 6.64 (br, 1H), 6.38 (br, 1H), 4.39 (m, 1H), 4.19 (m, 1H), 3.66 (s, 1H), 3.48 (d, J = 9.0 Hz, 1H), 3.26-3.16 (m, 4H), 3.02 (s, 2H), 2.96 (s, 2H), 2.54 (s, 1H), 2.47 (s, 3H), 2.42 (s, 3H), 2.01 (s, 3H), 1.59-1.23 (m, 13H), 0.88-0.84 (m, 6H).
$^{13}$C-NMR (125 MHz; DMSO-d$_6$): δ 172.0, 169.2, 163.5, 157.9, 156.5, 131.9, 124.8, 86.8, 53.1, 52.8, 51.9, 51.8, 51.7, 50.4, 42.9, 41.3, 28.8, 24.7, 23.5, 23.4, 22.0, 19.4, 18.1, 12.7.

MS (m/z): [M+H]$^+$ calcd. for C$_{31}$H$_{47}$N$_9$O$_9$S H$: 722.3, found: 722.2.

4-((1-((1-((2-azidoethyl)amino)-1-oxo-5-(3-((2,2,4,6,7-pentamethyl-2,3-dihydrobenzofuran-5-yl)sulfonyl)guanidino)pentan-2-yl)amino)-4-methyl-1-oxopentan-2-yl)amino)-4-oxobutanoic acid (4). This compound was synthesized by the similar method for compound 3, succinic acid was used instead of (+/-)-trans-oxirane-2,3-dicarboxylic acid. The compound was purified by a semi-preparative Proteo C12 HPLC column with a 15 min gradient and a flow rate of 5.0 mL/min (40% - 90% ACN in water containing 0.1% formic acid) to give a white powder (87 mg, 49%).

$^1$H-NMR (500 MHz, DMSO-d$_6$): δ 8.55-7.98 (m, 4H), 7.40 (br, 3H), 4.43 (dq, J = 51.5, 6.0 Hz, 1H), 4.24 (m, 1H), 3.51 (s, 1H), 3.26-3.17 (m, 4H), 3.10-3.06 (m, 3H), 1.66 (m, 1H), 1.54-1.44 (m, 6H), 0.88-0.83 (m, 6H).


MS (m/z): [M+H]$^+$ calcd. for C$_{31}$H$_{47}$N$_9$O$_9$S H$: 708.4, found: 708.2.
3.2.3.2 Synthesis of Cystine Cathepsin Inhibitor-BB2r Peptide Conjugates with Matching Controls

**General procedure for synthesis of compound 7-10.** To the mixture of compound 5 (2 µmol) and compound 3 (5 µmol) in water/2-butanol/DMF (200 µL, v/v/v=1:1:2) was added CuSO$_4$ (200 µg, 1.25 µmol) in water (50 µL). After stirring for 5 min at room temperature, a solution of ascorbic acid (6 µmol) in water (50 µL) was added to the mixture. The reaction mixture was continuously stirred for 1 h under nitrogen. The product was obtained by the purification via a semi-preparative Proteo C12 HPLC column with a 15 min gradient and a flow rate of 5.0 mL/min to give the product.

Compound 4 was clicked to BBN peptide in the similar way to yield control conjugates.

**General procedure for synthesis of compounds 11-14 (E-AG, C-AG, E-AN and C-AN, respectively).** Take E-AG for example, compound 3 clicked BB2r-targeted agonist peptide (compound 7) (1 µmol) and DOT-A-NHS ester (3 µmol) were dissolved in DMF (5 mL). The solution was basified with DIEA (0.081 mL, 0.47 mmol) and stirred at room temperature for overnight. The completion of the conjugation reaction was confirmed by HPLC before the removal of the solvent under nitrogen flow. A 90% TFA in DCM (500 µL) solution was added and the mixture was stirred at room temperature for 2 h under nitrogen. The solvent was removed by nitrogen flow and the residue was redissolved in DMF (300 µL) for the purification via a semi-preparative Proteo C12 HPLC column with a 15 min gradient and a flow rate of 5.0 mL/min to give the target compound.
3.2.4 Solid-Phase Peptide Synthesis (SPPS)

Peptide (compound 5 and 6) synthesis was performed using an automated solid-phase peptide synthesizer employing traditional Fmoc chemistry and using a Sieber resin (ChemPep Inc., U.S.). Briefly, the resin (0.7 meq/g) was deprotected using piperidine, resulting in the formation of a primary amine from which the C-terminus of the growing peptide was anchored. The Fmoc protected amino acids (300 µmol) with appropriate orthogonal protection were activated with COMU and sequentially added to the resin. The resulting peptide was cleaved from the resin using 1% trifluoroacetic acid (TFA) in dichloromethane (DCM) (5 × 3 mL) for 5 min. The filtrates were immediately neutralized with 2% pyridine in methanol (1 mL) and evaporated to dryness. The crude peptides were purified by HPLC with a 15 min gradient and a flow rate of 5.0 mL/min to give the target peptides, see Table 3.1 for chromatography and characterization details.

3.2.5 Radiolabeling of the Conjugates with $^{177}$LuCl₃

Radiolabeling was performed on all conjugates by mixing 25 µg samples with 37 MBq $^{177}$LuCl₃ (Department of Energy, U.S.) in sodium acetate buffer (0.5 M, 120 µL, pH 5.5) and incubating at 90 °C for 20 min. The mixture was purified by HPLC system and the radiolabeling efficiency (RE) was calculated based on the analysis of the chromatograms. To remove organic eluent and perform concentration, the radioactive conjugate was loaded onto an Empore (Eagan, MN) C18 high-performance extraction cartridge followed by washing with water (2 × 3 mL) and elution by ethanol/saline
solution (v/v = 6:4, 150 µL) to obtain the ^177^Lu-labeled radioconjugate for further biological experiments.

3.2.6 Distribution Coefficient (Log D\textsubscript{7.4}) of the Conjugates

In a microcentrifuge tube, 0.5 mL of 1-octanol was added to 0.5 mL phosphate-buffered saline (pH\textsubscript{7.4}) containing the radiolabeled peptide (200,000 cpm). The solution was vigorously stirred for 2 min at room temperature and subsequently centrifuged (100g, 5 min) to yield two immiscible layers. Aliquots of 100 µL were taken from each layer and the radioactivity of each was quantified by a γ-counter. Statistical analysis using the unpaired two-tailed Student’s t test was performed to compare the logD values and P values < 0.05 were considered statistically significant.

3.2.7 The Metabolic Stability of the Radioconjugates in Human Serum

The radioconjugates (11.1 MBq, 300 µCi) was added to 300 µL 10% human serum and incubated at 37 °C for 24 h. At predetermined time points (0, 4, and 24 h), acetonitrile (50 µL) was added to the mixture (50 µL) was centrifuged at 12,000g for 5 min. The supernatant was collected and dried with nitrogen flow. The sample was reconstituted in water (100 µL) and analyzed by radio-HPLC using the gradient described above.

3.2.8 In Vitro Competitive Cell-Binding Studies

The half maximum inhibitory concentration (IC\textsubscript{50}) of the unlabeled conjugates were determined by competitive displacement cell-binding assays using PC-3 cells. In these
studies, $^{177}$Lu-DOTA-SP714 ($^{177}$Lu-DOTA-dSer-PEG$_3$-BBN (7-14)), which was synthesized according to our previous work, served as the competitive radioligand for comparing the relative binding affinities of the conjugates. PC-3 cells (~1×10$^6$) were suspended in 100 µL RPMI 1640 media (pH 7.4, 4.8 mg/mL HEPES, and 2 mg/mL BSA) and incubated at 37°C for 40 min in the presence of radiolabeled $^{177}$Lu-DOTA-SP714 and various concentrations of the conjugates (0.1 nM – 1 µM) in 100 µL of medium. At the end of the incubation period, the cells were centrifuged, aspirated and washed with media a total of three times (5 × 500 µL). The cell associated activity was measured using a γ-counter and the IC$_{50}$ values determined by nonlinear regression using the one-binding site model of GraphPad PRISM 5 (U.S.).

3.2.9 The Inhibition of Cathepsin B Activity by the Conjugates

The phosphate buffer (0.1 M, pH = 5.8) containing EDTA (1 mM), DTT (2.7 mM), and Brij®35 (0.03%) was prepared before the assay. The solution of Z-Arg-Arg-AMC (Bachem, Switzerland) in the assay buffer (50 µL, 1.3 mM) was mixed with the conjugate dissolved in assay buffer at predetermined concentrations (100 µL, 0.1 nM – 1 µM). The solution of cathepsin B (human Liver, Millipore, U.S.) in assay buffer (50 µL, 0.544 nM) was added to the mixture which was further incubated at 37 °C for 20 min. The fluorescence of the liberated aminomethylcoumarin at 460 nm using 355 nm excitation was measured and the IC$_{50}$ of the cathepsin B inhibition versus the samples without the inhibitor was determined by nonlinear regression using GraphPad Prism 5 (U.S.).

3.2.10 Determination of the Km and Vmax of Cathepsin B for Z-Arg-Arg-AMC
The solution of cathepsin B (human Liver) (50 µL, 2 nM) in assay buffer used above was mixed with 50 µL of solution of substrate Z-Arg-Arg-AMC in the assay buffer to get different final concentrations ([S]) (25 µM, 50 µM, 100 µM, 500 µM and 1mM). The mixture was incubated at 37 °C and the fluorescence of the liberated aminomethylcoumarin at 460 nm using 355 nm excitation was measured at predetermined time points (0, 2, 4, 6, 8, and 10 min). The fluorescence intensity was plotted versus time and the reaction rates (V0) were calculated as the slope of the trend lines obtained by linear regression. Km and maximum reaction rate (Vmax) were determined by nonlinear regression using GraphPad Prism 5 (U.S.).

3.2.11 Determination of the Inhibition Constant (Ki) of the Compounds to Cathepsin B

The cathepsin B (human Liver) in assay buffer (25 µL) was mixed with the conjugate (25 µL) in 96-well plate. After the solution was mixed, the Z-Arg-Arg-AMC in assay buffer (50 µL) was added to the well, yielding a final cathepsin B concentration of 1 nM, conjugate concentration ([C]) of 50 nM, and substrate concentration ([S]) of 25 µM, 50 µM, 100 µM, 500 µM and 1mM. The mixture was incubated at 37 °C and the fluorescence of the mixture at 460 nm using 355 nm excitation was measured at predetermined time points (0, 2, 4, 6, 8, and 10 min). The fluorescence intensity was plotted versus time with observed reaction rates (V0-obs) calculated as the slope of the trend lines obtained by linear regression. The observed rate constant (Kobs) and observed maximum reaction rate (Vmax-obs) was determined by nonlinear regression using
GraphPad Prism 5. The Ki was calculated from the equation $K_{obs} = K_m \times (1 + [C]_0/K_i)$. All measurements were in biological triplicate.

3.2.12 Cell Internalization Studies

PC-3 cells were incubated in six-well plates ($1 \times 10^6$/well) overnight. The cells were incubated with each $^{177}$Lu radioconjugate (100, 000 cpm) at 37 °C for 4 h. At 15, 30, 60, 120 and 240-min time points, the culture medium was removed, and the cells were washed with fresh medium (5 × 1 mL) to remove the unbound conjugates. The fraction of surface-bound radioactivity was removed by washing the cells twice with an acidic buffer (500 µL, 50 mM glycine–HCl/0.1 M NaCl buffer, pH 2.8). The amount of radioactivity remaining in cells was assigned as the internalized fraction. The radioactivity for each sample was measured by $\gamma$-counter. The cellular uptake of the radioconjugates were presented as a percentage of the surface-bound and internalized radioactivity relative to the total activity added to the tube.

3.2.13 Cellular Efflux Studies

Efflux studies were carried out under atmospheres that contain 5% CO$_2$ and 21% O$_2$. PC-3 cells were incubated in six-well plates ($1 \times 10^6$/well) overnight in RPMI 1640 media (pH 7.4, 4.8 mg/mL HEPES, and 2 mg/mL BSA). On the day of the experiment, the cells were incubated for 2 h at 37°C in the presence of 1 mL 100,000 cpm of each $^{177}$Lu-radioconjugates. Upon completion of the incubation at time points 0, 2, 4, 8 and 24 h, the media was collected to quantitate the amount of the effluxed ligand. Surface bound radioactivity was collected by washing the cells twice with an acid wash (1 mL, 50 mM...
glycine-HCl, 0.1 M NaCl buffer, pH 2.8). The cells were then lysed at 37°C using a 0.5% aqueous SDS solution and the lyses were collected as internalized ligand. The radioactivity of the effluxed, surface bound and internalized fractions for each radioconjugate at each oxygen concentration was determined using a γ-counter.

3.2.14 Competitive Cathepsin B Binding of the Radioconjugates with CA-074

The Cathepsin B (3 nM, 10 µL) in storing buffer (50 mM sodium acetate and 1 mM EDTA, pH 5.0) was pre-incubated with the commercial cysteine proteases inhibitor CA-074 (ApexBio, Houston, TX) (10 µM, 10 µL) for 30 min. The solution of radioconjugates (200,000 cpm) in 30 µL of binding assay buffer (5 mM Tris, 5 mM MgCl2, and 2 mM DTT, pH = 5.5) was added to the mixture and incubated on ice for 2 h. Aliquot (24 µL) of the solution was mixed with Novex Tris-Glycine SDS sample buffer (2x) (30 µL) and NuPAGE® Sample Reducing Agent (10X) (6 µL) and incubated for further 10 min. The mixtures (20 µL) was heated at 85°C for 2 min. The cool-downed solutions were loaded onto a Novex™ 16% tris-glycine gel and ran at 110 V for 90 min. After shaking in shrinking buffer (50 mL, 65% methanol, and 0.5% glycerol in DI water) at 4°C for overnight, the gel was dried for 6 h at room temperature and the ladders were painted with small amount of radioactivity. The gel was then exposed to a phosphor screen for 72 h which was subsequently scanned by Typhoon FLA 9500 imaging system at a 25 µm resolution to achieve autoradiograph image.

3.2.15 In vitro Cathepsin B Binding of the Radioconjugates
To the PC-3 cells (1 x 10⁶/well) seeded in 6-well plates was added the radioconjugates (0.74 MBq, 20 µCi) in 1 mL of cell culture medium. The cells were incubated at 37 °C for 24 h and the medium was removed followed by washing with PBS (3 x 1 mL). The cells (-3 x 10⁶) were trypsinized and combined in microcentrifuge. The RIPA buffer (80 µL) containing Halt™ protease inhibitor (Thermo Fisher Scientific, U.S.) (100 x, 0.8 µL) was added to the cell pellet and vigorously vortexed for 1 min. The suspension was incubated on ice for 15 min and centrifuged to remove the pellet. The supernatant (80 µL) was equally divided into two portions. The first portion (40 µL) was directly analyzed by centrifuged with Pierce™ protein concentrators (MWCO = 10kDa) to separate the low molecular weight radioactivity. The radioactivity in each fraction was quantified using a gamma counter to calculate the percentage of the cysteine proteases trapped radioconjugate in the total counts. On the other hand, 32 µL of the cell lysate was mixed with Tris-Glycine SDS sample buffer (2x) (40 µL) and NuPAGE® Sample Reducing Agent (10X) (8 µL). The mixtures were incubated on ice for another 15 min and then reduced at 85 °C for 2 min. 30 µL of the mixture was separated by Novex™ 16% tris-glycine gel. The autoradiograph of the SDS-PAGE gel was performed according to the same method as described above.

Similarly, the radioconjugates (0.74 MBq, 20 µCi) was added to PC-3 cells (1 x 10⁶/well) seeded in 6-well plates with 1 mL of cell culture medium. After 4h incubation at 37 °C, the medium was removed, and the plates were washed with PBS (3 x 1 mL). Fresh 1 mL cultural medium was added to each well and the plate were returned to incubate for another 2, 4 and 24h. In the end of incubation, the cells were wash with PBS
Cells lysate was prepared by using 80 µL RIPA buffer containing 1% protease inhibitor on ice for 15 min followed by vigorously vortex. The mixtures were analysis by the same method described above to generate autoradiography.

For blocking studies, the preparation of PC-3 cells seeded plate was the same as described above. The radioconjugates (0.74 MBq, 20 µCi) was added to each well with the presence of excessive unlabeled corresponding analogues (40 µM, 100 µL). The cells were incubated at 37 °C for 4 h and prepared by the same method as described above for autoradiography.

3.2.16 Confocal Microscopy of Europium (Eu)-Labelled Conjugates

PC-3 cells (3000/well) were seeded in Lab-Tek chambered borosilicate coverglass disks (8 well) for overnight. Meanwhile, EuCl₃.6H₂O (13.5 µg) was added to the 100 µL peptide (25 µg) aqueous solution and the mixture was stirred overnight at room temperature. The completion of reaction was monitored by HPLC-MS and purified by using 3M Empore™ C18 cartridge. The elution was dried with nitrogen flow and redissolved by growth medium of PC-3 cells. PC-3 cells were washed by PBS (3 × 0.5 mL), 100 µL of the Eu-labeled conjugate was added. Additionally, 100 µL LysoTracker® Green (150 nM) was added to the cells and the mixture was incubated for 2 h. The cells then left to efflux for 4 and 24 h, washed with PBS (500 µL) and fixed with formaldehyde (500 µL) prior to imaging. The imaging was obtained by collecting far red color wavelengths (405 nm excitation and 615 nm emission for Eu) and green color
wavelengths (488 nm excitation and 511 nm emission for LysoTracker® Green). ImageJ was used for the quantifying the fluorescence intensity of Eu and the co-localization efficiency. Mean pixel intensities were normalized by the total cell number per image.

### 3.2.17 Biodistribution Study

Male SCID mice (6 weeks of age) received subcutaneous injections of 100 µL PC-3 cells ($5 \times 10^6$) suspended in Matrigel® into the flanks. When the tumor diameter reached 50 mm, the mice were randomized into three groups and intravenously injected with 10 µCi (0.37 MBq) of the purified $^{177}$Lu labeled conjugates via tail vein. The mice were sacrificed and the tissues were excised at 4, 24, and 72h post-injection time points. The blood, tumor, and excised tissues were weighed. The radioactivity for each sample was measured using a γ-counter. The percentage injected dose per gram (%ID/g) and the radioactivity ratios between tumor and non-targeted tissues were calculated.

### 3.2.18 In vivo Adducts Formation of the Radioconjugates with Cathepsin B

The radioconjugates (800 µCi/mouse) were intravenously injected into PC-3 tumor bearing mice. The mice were sacrificed and the tumor, pancreas, liver, and kidney were excised at 24h post-injection time points. The tumor and organs were homogenized in RIPA buffer (50 mg /100 µL) containing Halt™ protease inhibitor (100 ×, 1 µL) on ice and centrifuged to remove the pellet. An aliquot (18 µL) of the supernatant of the sample at 24h was mixed with Novex Tris-Glycine SDS sample buffer (2×, 20 µL) and NuPAGE® Sample Reducing Agent (10X) (2 µL). The mixture was heated at 85 °C for 2min. The
cooled mixtures (20 µL) were loaded onto a Novex™ 16% tris-glycine gel and run at 110 V for 90 min. The autoradiograph of the SDS-PAGE was performed as described above.  

3.2.19 SPECT/CT Imaging Study  

1 mCi (37 MBq) of $^{177}$Lu labeled conjugates was intravenously injected into PC-3 tumor bearing mice. Upon predetermined time point (4h, 24h and 72h post-injection), mice were anesthetized with isoflurane and their body temperature was controlled using warm air during scanning. 360 projections over 360° with an x-ray tube current of 140 mA and voltage of 75 kV peak (kVp) at a magnification of 1.2 (field of view = 98.7 mm$^2$) was used to generate CT images. SPECT images was acquired by 64 projections at 10 s per projection over 360° using a radius of rotation (ROR) of 79 mm (field of view = 98.7 mm$^2$) and reconstructed using Triumph SPECT Reconstruction Application. Co-registration of anatomical CT images and functional SPECT was performed using a VivoQuant 3.5™ software package (Invicro Boston, MA, USA).  

3.3 RESULTS  

3.3.1 Synthesis of Endolysosomal Trapped BB2r-Targeted Agents  

The structures of the synthesized experimental and control BB2r-targeted analogs are depicted in Figure 3.1. Basing our CC inhibitor design on E-64, the initial challenge was to design an E-64 derivative analog, outlined in Scheme 3.1, that could be easily adapted to be conjugated to the BB2r-targeted constructs using copper(I)-mediated alkyne-azide cycloaddition. Starting from Fmoc- and Pbf-protected L-arginine, 2-azidoethanamine was coupled to the carboxylic acid of the amino acid through standard amidation
conditions. This was followed by standard peptide synthesis procedures to sequentially add L-leucine and (+/-) trans-oxirane-2,3-dicarboxylic acid derivatives to yield the desired Pbf-protected, epoxide-based inhibitor 3. Note that use of the ((+/-) trans-oxirane-2,3-dicarboxylic will result in two diastereomers. While the epoxide group (2S, 3S) in E-64 is stereochemically pure and has been demonstrated to be 7-fold more active than its enantiomeric analog (2R, 3R) in the E-64 construct, the overall differential in activities were not seen as significant enough to warrant separation for our intended application. Thus, both diastereomers of 3 were used in the studies outlined below. For our inactive control 4, succinic acid was utilized in place of the epoxide. With only the removal of the oxygen, the inactive control (i.e., no CC inhibition/adduct formation) has high structural similarity to the active inhibitor, 3, and will be utilized in our experiments as a negative control. The $^1$H NMR of compound 1-4, $^{13}$NMR of compound 3 and 4, and MS spectra of compound 1-4 were showed in Figure 3.2, 3.3, 3.4 and 3.5, respectively.
**Figure 3.1** Structure of BB2r-targeted peptide incorporated with CCs inhibitor and its matching control.

<table>
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<tr>
<th>Analogue</th>
<th>Peptide Backbone Sequence</th>
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<td>DOTA-E64-Agonist (E-AG)</td>
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<tr>
<td></td>
<td>DOTA-control-Agonist (C-AG)</td>
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<tr>
<td></td>
<td><em>DOTA L-Pra PEG&lt;sub&gt;3&lt;/sub&gt; Gln Trp Ala Val Gly His Leu Met NH&lt;sub&gt;2&lt;/sub&gt;</em></td>
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<tr>
<td>Antagonist (RM2)</td>
<td>DOTA-E64-Antagonist (E-AN)</td>
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<tr>
<td></td>
<td>DOTA-control-Antagonist (C-AN)</td>
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<tr>
<td></td>
<td><em>DOTA-L-Pra-PEG&lt;sub&gt;3&lt;/sub&gt;-D-Phe-Gln-Trp-Ala-Val-Gly-His-Sta-Leu-NH&lt;sub&gt;2&lt;/sub&gt;</em></td>
</tr>
</tbody>
</table>
Scheme 3.1 Synthesis of the trapping reagent and negative control.

Reagents and condition: (a) 2-azidoethanamine, EDCI, NHS, TEA, r.t., overnight, 92%; (b) 20% piperidine in DMF, 30 min, 73%; (c) Fmoc-Leu-OH, COMU, DIEA, 2h; (d) 20% piperidine in DMF, 30 min; (e) (+/-)-trans-oxirane-2,3-dicarboxylic acid, COMU, DIEA, 2h, 57%; (f) 20% piperidine in DMF, 30 min; (g) succinic acid, COMU, DIEA, 2h, 49%.
Figure 3.2 A-B) $^1$H-NMR spectra of compound 1-2.
Figure 3.3 A-B) $^1$H-NMR spectra of compound 3-4.
Figure 3.4 A-B) $^{13}$C-NMR spectra of compound 3-4.
Figure 3.5 A-D. Mass spectrometry of compound 1-4.
The alkyne containing agonist and antagonist BB2r-targed peptides were constructed using standard SPPS approaches to yield the protected peptides 5 and 6, respectively. Copper(I) mediated cycloaddition of the inhibitor (3) or control (4) was carried out with each of the peptides to form the four conjugates, see Figure 3.1 and Scheme 3.2. The DOTA-NHS ester was conjugated to the N-terminus of the peptide; followed by deprotection using 90% TFA to yield the desired conjugates. For clarity, we define the nomenclature of these conjugates as follows: BB2r-targeted agonist (AG), BB2r-targeted antagonist (AN), active inhibitor (E) and inactive inhibitor (C). Following this nomenclature yields the following BB2r-targeted agonists, E-AG and C-AG, and antagonists, E-AN and C-AN. These conjugates were readily labeled with $^{177}$LuCl$_3$ at 90°C for 20min in a 0.5 M sodium acetate (pH = 5.5) to achieve a radiolabeling efficiency that ranged from 71.5% to 84.0% (Figure 3.6). To establish the stability of the epoxide in the E-AG and E-AN conjugates, studies were conducted on the unlabeled conjugates under these labeling conditions and examined by LC-MS. No significant degradation of the epoxide was observed. Additionally, the serum stability of the $^{177}$Lu-labeled conjugates was examined over 24h using radio-HPLC, Figure 3.7, and all were found to be stable. HPLC purification condition, retention time and synthetic/radiochemical yields of the conjugates and radioconjugates are listed in Table 3.1.
**Schemes 3.2** Synthesis of cystine cathepsin inhibitor-BB2r peptide conjugates with matching controls

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\[ \text{Reagents and condition: (a) } \text{CuSO}_4, \text{ water/n-butanol/DMF (v/v/v=1:1:2), r.t., 5min; (b) ascorbic acid in water, N}_2\text{ (g), r.t., 1h; (c) DOTA-NHS ester, DMF, DIEA, r.t., overnight. (d) 90\% TFA in DCM, N}_2\text{ (g), r.t., 2h.} \]
Figure 3.6 Radio-HPLC profile of solution of $^{177}$Lu-labeling of BN conjugates with radiolabeling efficiency (RE). The purifications were carried by an analytical Proteo C12 HPLC column with a 20 min gradient (20%-35% ACN in water containing 0.1 formic acid) and a flow rate of 1.5 mL/min to give the target compounds.
Figure 3.7 Stability study of E-AG, C-AG, E-AN and C-AN in the presence of human serum.
Table 3.1 HPLC purification condition and mass information of compounds E-AG, C-AG, E-AN and C-AN.

<table>
<thead>
<tr>
<th>Entity</th>
<th>HPLC purification condition</th>
<th>MW (m/z,[M +H]+)</th>
<th>Retention time(min)</th>
<th>Yields (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Calcd(^d)</td>
<td>Found(^d)</td>
<td></td>
</tr>
<tr>
<td>DOTA-SP714</td>
<td>80%-70% ACN in water(^a)</td>
<td>2008.0</td>
<td>2008.6</td>
<td>8.8</td>
</tr>
<tr>
<td>E-AG</td>
<td>90%-65% ACN in water(^b)</td>
<td>2138.1</td>
<td>2138.0</td>
<td>11.0</td>
</tr>
<tr>
<td>C-AG</td>
<td>90%-65% ACN in water(^b)</td>
<td>2124.1</td>
<td>2124.4</td>
<td>11.8</td>
</tr>
<tr>
<td>E-AN</td>
<td>80%-70% ACN in water(^c)</td>
<td>2311.2</td>
<td>2131.2</td>
<td>9.3</td>
</tr>
<tr>
<td>C-AN</td>
<td>80%-70% ACN in water(^c)</td>
<td>2297.2</td>
<td>2297.3</td>
<td>8.5</td>
</tr>
</tbody>
</table>

\(^a\) The eluent contained 0.1% formic acid and was with 16 min gradient.
\(^b\) The eluent contained 0.1% formic acid and was with 15 min gradient.
\(^c\) The eluent contained 0.1% formic acid and was with 15 min gradient.
\(^d\) The mass was calculated and determined by low resolution mass spectrometry (LRMS)
\(^e\) Total yields of the SPPS for compounds.
3.3.2 Competitive Cell-Binding and Distribution Coefficient Studies

Using the BB2r-positive human prostate cancer PC-3 cell line, the BB2r binding affinity of the conjugates were evaluated. In these studies, SP714 (DOTA-(D-Ser)5-PEG3-Gln-Trp-Ala-Val-Gly-His-Leu-Met-NH$_2$), the compound used in Chapter 2, was used as the competitive ligand. All unlabeled analogues demonstrated nanomolar binding affinities, Table 3.2, with IC$_{50}$ values of 17.8 ± 3.8, 16.5 ± 3.3, 19.8 ± 3.0, and 23.5 ± 3.6 nM for E-AG, C-AG, E-AN and C-AN, respectively. These results suggest negligible impact of the active or inactive inhibitors on the BB2r binding efficacy of the conjugates. Using radiometric analysis, the distribution coefficient (LogD$_{7.4}$) of the radioconjugates were evaluated. All of the radiolabeled analogs demonstrated suitable hydrophilicity with values ranging from -3.4 ± 0.1 to -1.9 ± 0.1 (Table 3.2). As expected, the antagonistic analogs gave significantly higher logD$_{7.4}$ due to a relatively higher hydrophobic character.
Table 3.2 Characterization, BB2r binding affinity and Cathepsin B inhibition activity of conjugates.

<table>
<thead>
<tr>
<th>Analogue</th>
<th>Molecular Formula</th>
<th>LogD (pH=7.4)*</th>
<th>BB2r IC$_{50}$/nM$^b$</th>
<th>Cst B inhibition IC$_{50}$ (nM)$^c$</th>
<th>Cst B inhibition Ki (nM)$^c$</th>
</tr>
</thead>
<tbody>
<tr>
<td>E-AG</td>
<td>C$<em>{94}$H$</em>{148}$N$<em>{28}$O$</em>{28}$S</td>
<td>-3.13 ± 0.03</td>
<td>17.76 ± 3.79</td>
<td>25.85 ± 4.20</td>
<td>146.48 ± 7.98</td>
</tr>
<tr>
<td>C-AG</td>
<td>C$<em>{93}$H$</em>{150}$N$<em>{28}$O$</em>{27}$S</td>
<td>-3.35 ± 0.09</td>
<td>16.45 ± 3.28</td>
<td>&gt;1000$^e$</td>
<td>-</td>
</tr>
<tr>
<td>E-AN</td>
<td>C$<em>{103}$H$</em>{159}$N$<em>{29}$O$</em>{29}$S</td>
<td>-2.31 ± 0.18</td>
<td>19.79 ± 2.95</td>
<td>30.65 ± 5.92</td>
<td>140.99 ± 26.39</td>
</tr>
<tr>
<td>C-AN</td>
<td>C$<em>{103}$H$</em>{161}$N$<em>{29}$O$</em>{28}$S</td>
<td>-1.90 ± 0.14</td>
<td>23.52 ± 3.61</td>
<td>&gt;1000$^e$</td>
<td>-</td>
</tr>
</tbody>
</table>

*a. The Log D values were obtained as for the $^{177}$Lu-labelled forms of conjugates. Values represent mean ± SD (n=3)

*b. Values represent mean ± SD (n=3)

*c. No inhibition was observed in the test range
3.3.3 Cathepsin B Inhibition

To assess the activity of the CC inhibitors incorporated into the BB2r-targeted agents, the inhibitory potency of the conjugates against cysteine cathepsin B (CatB) was examined. The IC\textsubscript{50} values for all of the conjugates are provided in Table 3.2. The inhibition constants (Ki) for E-AG and E-AN, based on the generated rate curves shown in Figure 3.8, are also given in Table 3.2. Analogs containing the active inhibitor, E-AG and E-AN, gave nM IC\textsubscript{50} values for the inhibition of CatB, while those analogs containing the inactive control inhibitor had no inhibition of the protease over the concentrations investigated. The determined Ki values for E-AG and E-AN were statistically identical, but were approximately 9 fold higher than the 15 ± 1 nM inhibition value (data not shown) obtained for the active inhibitor 3 (no peptide attached, Pbf group removed). The results demonstrate that the attached peptides can negatively impact the potency of the inhibitor, but overall the influence of the peptide on the conjugates is relatively modest and should not significantly impact our intended application.
Figure 3.8 Determination of the $K_m$ and $K_i$ of cathepsin B (human Liver) to the substrate Z-Arg-Arg-AMC. A), C) and E) Time course of substrate hydrolysis of cathepsin B alone, and in the presence of E-AG and E-AN respectively. B), D) and F) The nonlinear regression of reaction rate ($t = 0 - 10$ min) versus the concentration of Z-Arg-Arg-AMC in the presence of cathepsin B alone, E-AG and E-AN respectively. The conjugate concentration was 25 nM. The $K_i$ was calculated from the equation $K_{obs} = K_m (1 + [C]/K_i)$. All measurements were in triplicate.
3.3.4 In Vitro Internalization and Efflux Studies

The internalization and efflux profiles of the four $^{177}$Lu-labeled conjugates were examined using the PC-3 cell line, Figure 2. As expected, the internalization rate of the two agonistic conjugates, 13.5% to 13.2% for $^{177}$Lu-E-AG and $^{177}$Lu-C-AG, correspondingly, far outpaced the antagonistic analogs, 1.7% to 1.8% for $^{177}$Lu-E-AN and $^{177}$Lu-C-AN, by the 4h time point. The percentage of surface bound radioactivity for the two antagonists was nearly two-fold higher than the corresponding internalized signal demonstrating that the RM2 based antagonists do not efficiently, relative to the agonists, induce receptor-mediated internalization. Efflux of the internalized radioactivity over 24h demonstrated that analogs containing the active inhibitor exhibited a higher retention efficacy compared to the radioconjugates containing the inactive control inhibitor. By the 24h time point, 38.8% of $^{177}$Lu-E-AG has effluxed comparing to 54.3% of C-AG in vitro (p<0.01).
Figure 3.9 Internalization assay depicted as percentage of internalized and surface bond activity of $^{177}$Lu-labeled conjugates in PC-3 cells. Values are mean ± SD (n=3).
3.3.5 *In Vitro* Adduct Studies

Using autoradiographic SDS-PAGE, the ability of the radioconjugates to form adducts with CatB and in live PC-3 cells was established, Figure 3.10. Incubation of the $^{177}$Lu-labeled conjugates containing active inhibitors, $^{177}$Lu-E-AG and $^{177}$Lu-E-AN, with CatB (human liver) for 2h, Figure 3.10A, produced bands with molecular weight of ~27 kDa, which correspond to the heavy chain of CatB. Co-incubation with CA-074, a well-known, commercial CC inhibitor that inhibits the active site of CC completely abolishes the ability of $^{177}$Lu-E-AG and $^{177}$Lu-E-AN to form adducts, indicating that these adducts bind to the active site of CatB.

After confirmation of CatB adduct formation, all four radioconjugates were incubated with PC-3 cells for 24h. At the end of that time, the cells were washed with fresh media, lysed and evaluated by centrifugal filtration and SDS-PAGE. In Figure 3.10B, the SDS-PAGE of the cell lysate demonstrated that those analogs capable of CC adduct formation, $^{177}$Lu-E-AG and $^{177}$Lu-E-AN, gave adduct bands, while those radioconjugates with the inactive control inhibitor, $^{177}$Lu-C-AG and $^{177}$Lu-C-AN, showed no signs of adduct formation.

To examine the role of BB2r-mediated internalization on adduct formation, a parallel study to the one above was conducted by incubating PC-3 cells with $^{177}$Lu-E-AG and $^{177}$Lu-E-AN in the presence or absence of an appropriate BB2r blocking agent. The results, Figure 3.10C, clearly demonstrate that adduct formation of the radioconjugates is abolished when a blocking agent is added. This strongly implies that receptor-mediated
internalization is primarily responsible for cellular adduct formation for both the agonistic and antagonistic radioconjugates. Lastly, in Figure 3.10D, the time-dependent cellular retention of $^{177}$Lu-E-AG and $^{177}$Lu-E-AN was investigated by incubation with PC-3 cells for 2h, followed by washing and lysis at the desired timepoint. $^{177}$Lu-E-AG demonstrated strong and similar adduct formation bands at 2 and 4h modestly decreased by 24h. Interestingly, $^{177}$Lu-E-AN gave its weakest bands at 2h; however, these bands progressively strengthened over the 24h time course. This finding may be attributed to surface bound antagonist slowly internalizing and trapping in the endolysosomal compartments over time.
Figure 3.10 The autoradiography of the SDS-PAGE demonstrated that A) the binding of conjugates with cathepsin B can be completely inhibited by cysteine proteases inhibitor CA-074; B) the intracellular adducts formation can be observed when E-64-conjugated BBN-peptide incubated with PC-3 cells, while control compounds cannot. ($^{177}$Lu-E-AG/E-AN was incubated with purified CatB serving as reference); C) the intracellular adducts formation can be blocked after co-incubation with excessive unlabeled E-AG and E-AN, respectively; D) there was a time-dependent retention of cathepsin B-conjugates adducts in PC-3 cells after pre-incubation with radiolabeled compounds for 4h.
3.3.6 Cellular Trafficking Using Confocal Microscopy

To further evaluate the cellular trafficking of the conjugates, E-AG and C-AG were labeled with EuCl₂•6H₂O, incubated with PC-3 cells and imaged by confocal microscopy, Figure 3.11A. The Eu-labeled conjugates demonstrated robust signal (red) after 2h of incubation. Examination of the efflux profiles at the 4 and 24h time points, Figure 3.11A and 3.11B, showed that the mean fluorescent intensity per cell for Eu-E-AG remained 1.3- and 1.8-fold higher, respectively, relative to the control Eu-C-AG. Using Lysotracker™ (green) to identify endolysosomal compartments, the residence/colocalization of the Eu-labeled conjugates in these compartments was quantified, Figure 3.11A and 3.11C. As expected, co-registration of Eu-E-AG and Eu-C-AG signals with the Lysotracker™ was similar at the 0 and 4h time points, indicating that roughly equal and increasing amounts of the two Eu-labeled conjugates are being trafficked to the endolysosomal compartments. However, by 24h, the signal corresponding to Eu-E-AG was at a maximum, with 93% of the signal corresponding to the CC-rich, endolysosomal compartments, while the Eu-C-AG reduced significantly from 91% at 4h to 70% at 24h suggesting that the conjugate/metabolites are leaving the endolysosomal compartments and being trafficked elsewhere in the cell.
Figure 3.11 A) The representative confocal microscopy images of PC-3 cells pre-incubated for 4h with Europium-labeled compounds with BBN agonist backbone. Scale bar: 20 µm. B) Time-dependent fluorescence intensity of Eu per cell as quantified from images. C) Co-localization efficiency of Eu(red) and Lysotracker(green). All analysis was performed in more than 5 random images and were demonstrated as mean ± SD. *p < 0.05, **p < 0.01,
3.3.7 Biodistribution Studies

The in vivo biodistribution studies of $^{177}$Lu-E-AG, $^{177}$Lu-C-AG, $^{177}$Lu-E-AN and $^{177}$Lu-C-AN were investigated in PC-3 tumor-bearing SCID mice, Table 3.3. All of the conjugates demonstrated rapid blood and muscle clearance by 4h, primarily through the renal clearance pathway. As a consequence, the kidneys were the site of the highest non-target, non-BB2r-mediated uptake. The initial renal uptake for $^{177}$Lu-E-AG and $^{177}$Lu-E-AN was 10.44 ± 3.25 and 7.37 ± 0.92 %ID/g, respectively. This was higher than the matching control analogs, $^{177}$Lu-C-AG and $^{177}$Lu-C-AN, which had corresponding initial uptake values of 5.93 ± 2.05 (p<0.05) and 5.43 ± 1.74 (p<0.05) %ID/g. The renal clearance profile of $^{177}$Lu-E-AG and $^{177}$Lu-E-AN at 24 and 72h time points was substantially slower than the control analogs. At the 72h time point, $^{177}$Lu-E-AG showed significant higher retention, 68.0% of the 4h uptake value (p=0.05), compared to $^{177}$Lu-C-AG with 18.4% of the 4h uptake (p<0.01). Similar findings were seen with the antagonists. $^{177}$Lu-E-AN and $^{177}$Lu-C-AN retained 49.9 (p=0.01) and 13.1% (p<0.01), respectively, of the initial uptake at 72h. This renal retention is almost certainly due to the well-known uptake mechanisms of the kidney, which are leading to endolysosomal trapping, and thus higher retention, in the renal proximal tubule.

It is well-established that both the mouse and human pancreas are BB2r-positive tissue. Thus, in our biodistribution the BB2r-targeting capabilities of our analogs can be evaluated using both the PC-3 tumor xenograft and the endogenous mouse pancreas. At the 4h time point, the tumor uptake of $^{177}$Lu-E-AG and $^{177}$Lu-C-AG were
not significant different with values of 3.28 ± 1.10 and 2.86 ± 0.39 %ID/g, respectively. However, by the 24h time point, retention of the initial uptake for $^{177}$Lu-E-AG was 89.9% (P>0.05), while the $^{177}$Lu-C-AG decreased significantly to 64.3% (P<0.01). At 72h post-injection, 62.8% (2.06 ± 0.16 %ID/g) of the original uptake for $^{177}$Lu-E-AG was retained (P>0.05), whereas $^{177}$Lu-C-AG exhibited only 32.9% (0.94 ± 0.14 %ID/g) retention (p<0.0001). Similar retention profiles for these two radioconjugates were observed in the BB2r-positive mouse pancreas. Initial pancreas uptake values were 36.46 ± 11.66 %ID/g and 41.20 ± 18.39 %ID/g for $^{177}$Lu-E-AG and $^{177}$Lu-C-AG, respectively. By 72h, the retention of the initial uptake was 72.2% (26.32 ± 1.53 %ID/g) (P>0.05) and 42.7% (17.60 ± 0.43) (p=0.04).

For the antagonists, initial PC-3 tumor uptake was substantially higher than the agonists with statistically identical uptake values of 11.88 ± 1.68 and 13.13 ± 2.93 %ID/g for $^{177}$Lu-E-AN and $^{177}$Lu-C-AN, respectively. By 24 h post-injection, the tumor retention of $^{177}$Lu-E-AN was 53.2% (6.32 ± 0.55%ID/g) (p=0.0008), while $^{177}$Lu-C-AN was 28.9% (3.79 ± 0.48%ID/g) (p=0.0008). At 72h, $^{177}$Lu-E-AN and $^{177}$Lu-C-AN had retention values of 31.9% (3.79 ± 0.73 %ID/g) (p<0.0001) and 14.7% (1.94 ± 0.46 %ID/g) of the initial uptake (p=0.0003). The significantly higher retention of the active endolysosomal trapping antagonist, $^{177}$Lu-E-AN, clearly demonstrates that this approach can improve the retention of RM2 based BB2r-antagonistic targeting vectors. However, the retention values for the antagonists were substantially lower than the agonists at similar time points, suggesting that reduced internalization rate of the antagonists reduces overall trapping efficiency. Pancreatic uptake of the BB2r-targeted antagonists was minimal at
the 4h time point due to the well-known clearance of these targeting vectors from the pancreas.\(^{86, 217}\).
Table 3.3 Biodistribution Studies in PC-3 Tumor–Bearing SCID Mice.

<table>
<thead>
<tr>
<th>Tissue (%ID/g)</th>
<th>4h</th>
<th>24h</th>
<th>72h</th>
<th>4h</th>
<th>24h</th>
<th>72h</th>
</tr>
</thead>
<tbody>
<tr>
<td>177Lu-E-AG</td>
<td></td>
<td></td>
<td></td>
<td>177Lu-C-AG</td>
<td></td>
<td></td>
</tr>
<tr>
<td>blood</td>
<td>0.07 ± 0.07</td>
<td>0.02 ± 0.02</td>
<td>0.01 ± 0.02</td>
<td>0.02 ± 0.02</td>
<td>0.03 ± 0.04</td>
<td>0.01 ± 0.01</td>
</tr>
<tr>
<td>heart</td>
<td>0.03 ± 0.03</td>
<td>0.12 ± 0.16</td>
<td>0.06 ± 0.06</td>
<td>0.16 ± 0.17</td>
<td>0.12 ± 0.17</td>
<td>0.06 ± 0.10</td>
</tr>
<tr>
<td>lung</td>
<td>0.10 ± 0.01</td>
<td>0.08 ± 0.14</td>
<td>0.15 ± 0.11</td>
<td>0.04 ± 0.03</td>
<td>0.20 ± 0.04</td>
<td>0.03 ± 0.04</td>
</tr>
<tr>
<td>liver</td>
<td>0.44 ± 0.16</td>
<td>0.46 ± 0.13</td>
<td>0.42 ± 0.09</td>
<td>0.22 ± 0.05</td>
<td>0.18 ± 0.06</td>
<td>0.10 ± 0.04</td>
</tr>
<tr>
<td>pancreas</td>
<td>41 ± 12</td>
<td>34 ± 5</td>
<td>26.3 ± 1.5</td>
<td>41 ± 18</td>
<td>31 ± 6</td>
<td>17.6 ± 0.4</td>
</tr>
<tr>
<td>stomach</td>
<td>0.9 ± 0.5</td>
<td>0.4 ± 0.2</td>
<td>0.40 ± 0.06</td>
<td>0.47 ± 0.11</td>
<td>0.33 ± 0.02</td>
<td>0.10 ± 0.03</td>
</tr>
<tr>
<td>spleen†</td>
<td>0.017 ± 0.003</td>
<td>0.04 ± 0.04</td>
<td>0.03 ± 0.01</td>
<td>0.05 ± 0.05</td>
<td>0.03 ± 0.03</td>
<td>0.10 ± 0.03</td>
</tr>
<tr>
<td>small intestine</td>
<td>1.9 ± 0.7</td>
<td>1.73 ± 0.08</td>
<td>1.13 ± 0.17</td>
<td>2.1 ± 0.4</td>
<td>0.86 ± 0.18</td>
<td>0.40 ± 0.05</td>
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<tr>
<td>large intestine</td>
<td>5.0 ± 1.1</td>
<td>1.8 ± 0.2</td>
<td>0.6 ± 0.2</td>
<td>5 ± 3</td>
<td>1.2 ± 0.4</td>
<td>0.49 ± 0.15</td>
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<td>kidney</td>
<td>10 ± 3</td>
<td>10 ± 3</td>
<td>7.1 ± 0.9</td>
<td>6 ± 2</td>
<td>3.8 ± 1.4</td>
<td>1.09 ± 0.10</td>
</tr>
<tr>
<td>tumor</td>
<td>3.3 ± 1.1</td>
<td>3.0 ± 0.4</td>
<td>2.06 ± 0.16</td>
<td>2.9 ± 0.4</td>
<td>1.8 ± 0.3</td>
<td>0.94 ± 0.14</td>
</tr>
<tr>
<td>muscle</td>
<td>0.04 ± 0.06</td>
<td>0.02 ± 0.02</td>
<td>0.06 ± 0.06</td>
<td>0.06 ± 0.06</td>
<td>0.03 ± 0.05</td>
<td>0.02 ± 0.02</td>
</tr>
<tr>
<td>bone</td>
<td>0.4 ± 0.3</td>
<td>0.5 ± 0.2</td>
<td>0.28 ± 0.09</td>
<td>0.01 ± 0.01</td>
<td>0.03 ± 0.05</td>
<td>0.08 ± 0.08</td>
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<tr>
<td>brain</td>
<td>0.03 ± 0.01</td>
<td>0.03 ± 0.04</td>
<td>0.01 ± 0.01</td>
<td>0.01 ± 0.01</td>
<td>0.13 ± 0.09</td>
<td>0.0009 ± 0.0002</td>
</tr>
<tr>
<td>excretion†</td>
<td>78 ± 4</td>
<td>84 ± 2</td>
<td>87.6 ± 1.2</td>
<td>81 ± 4</td>
<td>90.4 ± 1.7</td>
<td>93.6 ± 0.6</td>
</tr>
<tr>
<td>177Lu-E-AN</td>
<td></td>
<td></td>
<td></td>
<td>177Lu-C-AN</td>
<td></td>
<td></td>
</tr>
<tr>
<td>blood</td>
<td>0.01 ± 0.01</td>
<td>0.04 ± 0.03</td>
<td>0.02 ± 0.02</td>
<td>0.2 ± 0.2</td>
<td>0.02 ± 0.03</td>
<td>0.007 ± 0.004</td>
</tr>
<tr>
<td>heart</td>
<td>0.2 ± 0.2</td>
<td>0.08 ± 0.14</td>
<td>0.01 ± 0.01</td>
<td>0.6 ± 0.6</td>
<td>0.23 ± 0.07</td>
<td>0.23 ± 0.18</td>
</tr>
<tr>
<td>lung</td>
<td>0.5 ± 0.3</td>
<td>0.8 ± 0.4</td>
<td>0.4 ± 0.5</td>
<td>0.6 ± 0.3</td>
<td>0.9 ± 1.6</td>
<td>0.3 ± 0.3</td>
</tr>
<tr>
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<td>0.4 ± 0.2</td>
<td>0.63 ± 0.18</td>
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<td>0.22 ± 0.05</td>
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<td>0.7 ± 0.5</td>
<td>0.5 ± 0.2</td>
<td>1.5 ± 1.0</td>
<td>0.4 ± 0.4</td>
<td>0.10 ± 0.09</td>
</tr>
<tr>
<td>stomach</td>
<td>0.41 ± 0.19</td>
<td>0.11 ± 0.06</td>
<td>0.02 ± 0.03</td>
<td>0.5 ± 0.4</td>
<td>0.03 ± 0.03</td>
<td>0.02 ± 0.03</td>
</tr>
<tr>
<td>spleen†</td>
<td>0.01 ± 0.02</td>
<td>0.02 ± 0.02</td>
<td>0.02 ± 0.01</td>
<td>0.02 ± 0.01</td>
<td>0.02 ± 0.02</td>
<td>0.02 ± 0.02</td>
</tr>
<tr>
<td>small intestine</td>
<td>0.26 ± 0.13</td>
<td>0.13 ± 0.02</td>
<td>0.05 ± 0.03</td>
<td>0.26 ± 0.09</td>
<td>0.08 ± 0.03</td>
<td>0.02 ± 0.02</td>
</tr>
<tr>
<td>large intestine</td>
<td>0.62 ± 0.14</td>
<td>0.09 ± 0.03</td>
<td>0.06 ± 0.01</td>
<td>0.78 ± 0.19</td>
<td>0.15 ± 0.08</td>
<td>0.04 ± 0.04</td>
</tr>
<tr>
<td>kidney</td>
<td>7.4 ± 0.9</td>
<td>6.7 ± 1.4</td>
<td>3.7 ± 1.8</td>
<td>5.4 ± 1.7</td>
<td>1.6 ± 0.4</td>
<td>0.7 ± 0.2</td>
</tr>
<tr>
<td>tumor</td>
<td>11.9 ± 1.7</td>
<td>6.3 ± 0.6</td>
<td>3.8 ± 0.7</td>
<td>13 ± 3</td>
<td>3.8 ± 0.5</td>
<td>1.9 ± 0.5</td>
</tr>
<tr>
<td>muscle</td>
<td>0.15 ± 0.14</td>
<td>0.21 ± 0.18</td>
<td>0.15 ± 0.17</td>
<td>0.13 ± 0.13</td>
<td>0.2 ± 0.4</td>
<td>0.03 ± 0.03</td>
</tr>
<tr>
<td>bone</td>
<td>0.21 ± 0.16</td>
<td>0.6 ± 0.2</td>
<td>0.2 ± 0.3</td>
<td>0.6 ± 0.3</td>
<td>0.5 ± 0.9</td>
<td>0.07 ± 0.07</td>
</tr>
<tr>
<td>brain</td>
<td>0.09 ± 0.05</td>
<td>0.05 ± 0.06</td>
<td>0.01 ± 0.01</td>
<td>0.06 ± 0.05</td>
<td>0.01 ± 0.02</td>
<td>0.2 ± 0.3</td>
</tr>
<tr>
<td>excretion†</td>
<td>86 ± 2</td>
<td>92.4 ± 1.5</td>
<td>93 ± 4</td>
<td>83 ± 4</td>
<td>95.5 ± 1.5</td>
<td>98.0 ± 0.7</td>
</tr>
</tbody>
</table>

* Organ uptake values expressed as %ID/g and values are n≥4 unless otherwise noted.

† Excretion values (%ID) were calculated using the activity values associated with the spleen, excreted urine and fecal material contents at the time of sacrifice.
3.3.8 SPECT/CT Imaging

Based on the biodistribution studies, the antagonistic analogs, $^{177}\text{Lu-E-AN}$ and $^{177}\text{Lu-C-AN}$, with their higher tumor uptake were chosen to be evaluated by SPECT/CT imaging. These longitudinal imaging studies, Figure 3.12, were conducted in PC-3 bearing mice at 4, 24 and 72h time points. For each time point, the SPECT/CT images for both conjugates were set on the same intensity scale. At 4h post-administration, significant uptake was observed in the PC-3 tumors and kidney uptake for both radioconjugates. After 24h, reductions were seen in both kidney and tumor retention. By 72h, $^{177}\text{Lu-E-AN}$ demonstrated substantially higher retention in the PC-3 tumors compared to $^{177}\text{Lu-C-AN}$. However, the $^{177}\text{Lu-C-AN}$ exhibited, as expected, noticeably lower renal retention compared to $^{177}\text{Lu-E-AN}$. Overall, the SPECT/CT imaging studies correlated well with the previous biodistribution studies.
Figure 3.12 Fused micro-SPECT/CT images of $^{177}$Lu-C-AN and $^{177}$Lu-E-AN in PC-3 tumor-bearing mice at 4, 24 and 72 h after injection. Tumors and kidneys are indicated by red and green arrows, respectively.
3.3.9 Ex Vivo Adduct Studies

To confirm the in vivo formation of high molecular weight adducts, all four radioconjugates were administered to PC-3 tumor bearing mice and the tumor, pancreas, liver and kidney were excised at 24h post-injection. These tissues were lysed and subjected to SDS-PAGE, see Figure 3.13. Unsurprisingly, the controls analogs, $^{177}\text{Lu-C-AG}$ and $^{177}\text{Lu-C-AN}$, demonstrated no bands associated with adduct formation. However, both of the active endolysosomal trapping analogs, $^{177}\text{Lu-E-AG}$ and $^{177}\text{Lu-E-AN}$, revealed adduct formation that ranged roughly from 20-32 kDa in molecular weight, but, interestingly, the intensities of these bands were tissues-dependent. For the $^{177}\text{Lu-E-AG}$, strong signal was observed in the tumor, pancreas and kidneys. The tumor and kidney bands were similar with the highest intensity observed at approximately 30 kDa, whereas the highest band for the pancreas was around 25 kDa. In the case of $^{177}\text{Lu-E-AN}$, no bands were seen in the pancreas, but were readily visualized in the tumor and kidney lysates and weakly in lysates of the liver. The overall profile of the bands observed in the tumor and kidney for $^{177}\text{Lu-E-AN}$ were similar to the agonist.
Figure 3.13 The autoradiography of SDS-PAGE of the lysate from tumor, liver, pancreas and kidney at A) 24h post-injection of $^{177}$Lu-E-AG and -C-AG; B) 24h post-injection of $^{177}$Lu-E-AN and -C-AN in PC-3 tumor bearing mice.
3.4 DISCUSSION

The development of diagnostic and therapeutic BB2r-targeted radiopharmaceuticals continues to be explored for a variety of cancer \(^\text{23, 218}\). Initial development focused on agonistic analogs due to the belief that internalization was key to long-term tumor retention. However, this perception has been challenged with the success of recently developed antagonists \(^\text{84, 219}\). Exceptional work by the groups of Maecke and others have identified antagonistic analogs that have substantially higher tumor uptake and similar retention profiles compared to agonistic analogs \(^\text{86-87, 90-91, 220-221}\). Speculatively, the higher tumor uptake may be attributable to the ability of antagonists to bind to BB2 receptors that are G-protein bound and unbound, whereas agonists are limited to G-protein bound receptors \(^\text{89, 220}\). If so, the BB2r-targeted antagonists have a much larger receptor population to target accounting for their higher initial tumor uptake. Even though many BB2r-antagonists have demonstrated some level of internalization, the higher than expected retention profiles for the antagonists has been attributed to factors such as lower disassociation constant \((k_{\text{off}})\) and an increase in metabolic stability relative to agonists \(^\text{90-92}\).

Despite the success of BB2r- and other receptor-targeted agents, the inherently higher diffusion and metabolic rates of low-molecular weight agents leads to rapid tumor clearance, which diminishes the effectiveness of the diagnostic and/or therapeutic agent. To address this challenge, our laboratory has recently begun exploring the use of CC inhibitors to enhance the long-term retention of receptor-targeted agents \(^\text{115}\).
Mechanistically, we have demonstrated that these inhibitors are able to form adducts within the CC-rich, endolysosomal compartments after receptor-mediated internalization. To continue studying this trapping technique, we synthesized agonistic and antagonistic BB2r-targeted analogs with the expectation of demonstrating that the agonist preferentially benefited from our endolysosomal trapping approach given the efficient internalization of this targeting vector. To our surprise, this turned out not to be the case with both agonistic and antagonistic analogs demonstrating substantially higher long-term tumor retention. Below, we discuss how our results shed light not only on the general applicability of our endolysosomal trapping approach with BB2r-targeted agents, but also question previous assumptions that BB2r-targeted antagonists are not, eventually, internalized. These findings may help explain the longer than expected retention profile of this class of agent.

Incorporation of the CC inhibitor and inactive control into the structure of the BB2r-targeted agonist and antagonist was carried out without difficulty. Labeling ($^{177}$Lu or Eu) of the epoxide containing conjugates was accomplished, even at elevated temperatures, without degradation. Though, at elevated temperatures utilization of an ammonium acetate buffer instead of a sodium acetate buffer did result in degradation (data not shown), likely due to ring opening from nucleophilic attack by ammonia. Serum stability studies demonstrated that the analogs were stable over a 24h period with no significant degradation observed. The CatB inhibition and the competitive BB2r-binding studies indicated that the peptides do not substantially impact the irreversible inhibition of CatB and, vice versa, the inhibitor does not negatively impact BB2r-affinity.
As expected, internalization of the agonists, $^{177}\text{Lu-E-AG}$ and $^{177}\text{Lu-C-AG}$, proceeded rapidly and was more efficient relative to the antagonists, $^{177}\text{Lu-E-AN}$ and $^{177}\text{Lu-C-AN}$. The surface-bound activity for the antagonists were approximately 2-fold higher than the internalization, which is consistent with literature 86-87, 217. Both the agonistic and antagonistic radioconjugates containing the active CC inhibitor showed significantly higher retention in the efflux studies, speculatively due to endolysosomal trapping upon internalization. Using confocal microscopy, both the agonistic and antagonistic Eu-labeled conjugates demonstrated receptor-mediated internalization into the endolysosomal compartments of the cell. Over the course of 24h, Eu-E-AG revealed significantly higher retention in the cells relative to Eu-C-AG. In addition, examination of the colocalization with Lysotracker™ revealed a substantially higher tendency of Eu-E-AG to be retained in the CC-rich, endolysosomal compartments of the cell relative to the control conjugate. These finding are consistent with our premise of adduct formation with CCs in the endolysosomal compartments of the cell upon receptor-mediated internalization.

Adduct formation studies were conducted using SDS-PAGE and centrifugal filtration techniques. The ability of active endolysosomal trapping conjugates to form adducts with CatB was confirmed by SDS-PAGE, whereas the inactive control conjugates, as expected, showed no adduct formation. Examination with cell lysates revealed multiple adduct bands for $^{177}\text{Lu-E-AG}$ and $^{177}\text{Lu-E-AN}$, with the agonistic demonstrating a higher adduct signal due to a higher rate of internalization over the 24h timeframe. One of the adduct bands (~27 kDa) from the cell lysate studies corresponds
to CatB (heavy chain only), which is one of, if not the most, abundant CC expressed in endolysosomal compartments of cells. The band at ~33 kDa may be the immature CatB (single chain form). Previously, applying the same approach, but under different experimental conditions (i.e., different CC inhibitor, peptide and cell line), we confirmed by Western blot that the CatB-adducts was the predominant species found in the cellular lysates. For this particular study; however, we did not unambiguously confirm the identity of the adducts.

Blocking the BB2r with excess unlabeled agonist and antagonist prevented the active endolysosomal trapping conjugates from forming CC adducts. This confirms that adduct formation was dependent upon BB2r-mediated internalization. To examine the time-dependence of adduct formation (Figure 3.10D), the PC-3 cells were exposed to \(^{177}\text{Lu-E-AG}\) and \(^{177}\text{Lu-E-AN}\) for 4h, washed to remove extracellular activity and lysed at 2, 4 and 24h. The agonist demonstrated the largest amount of adducts early on (2 and 4h), but the adduct signal diminished significantly by 24h. This results are similar to results obtained from our previously work related to utilization of endolysosomal trapping agents with NTR1-targeted agonist. We attributed this decline for the agonist to the protein turnover (degradation) of CCs and the resulting adducts, in the endolysosomal compartments. Interestingly, the concentration of adducts for \(^{177}\text{Lu-E-AN}\) increased over the 24h time period. This is almost certainly due to a steady rate of internalization of the surface bound radioactivity. Maecke and co-workers hypothesized that this antagonistic targeting vector may undergo slow internalization. Our results lend further credence to this postulation and suggests that this particular...
antagonistic targeting vector may be better thought of as slow internalizing instead of non-internalizing. How far these observations may be extended to other BB2r-targeted peptides, and in general, to other receptor-targeted constructs, remains uncertain. Though, utilization of this endolysosomal trapping approach may serve as an ideal and convenient technique to better examine the internalization profile of a wide variety of receptor-targeted agents.

The biodistribution studies verified that all radioconjugates, including those containing the active CC inhibitor, provided good blood and muscle clearance data demonstrating that the utilized CC is stable in vivo, at least over the timeframe employed here. Initial PC-3 tumor uptake for the RM2 based antagonists were approximately 3-4-fold higher than the agonists, which is consistent with other reports. For both the agonist and antagonistic targeting vectors, employing active endolysosomal trapping agents led to significantly higher levels of retention relative to analogous controls. \(^{177}\text{Lu-E-AG}\) demonstrated a 1.4- and 2.0-fold increase in tumor retention at 24 and 72h, relative to \(^{177}\text{Lu-C-AG}\). It could be argued that endolysosomal trapping approach had the most significant benefit for the antagonist. Relative to \(^{177}\text{Lu-C-AN}\), \(^{177}\text{Lu-E-AN}\) had PC-3 tumor retention value that were 1.8- and 2.2-fold greater at 24 and 72h, respectively. Ex vivo examination of the PC-3 tumors by centrifugal centrifugation at 24 h post-administration revealed that for both \(^{177}\text{Lu-E-AG}\) and \(^{177}\text{Lu-E-AN}\) greater than 70% of the radioactivity was associated with macromolecules (data not shown). SDS-PAGE demonstrated that in vivo tumor adducts formation profile for both active endolysosomal trapping radioconjugates were identical. It should be pointed out,
that many cancers have an increase concentration of CC in their extracellular environment. Indeed, this increased CC expression has been the impetus for the development of targeted diagnostic agents. While the contribution of the extracellular CC to adduct formation cannot be discounted, our in vitro data and the similar ex vivo SDS-PAGE adduct profiles for the agonist and antagonist would, in our opinion, argue that internalization was the primary route of in vivo adduct formation in the tumor.

Non-target uptakes of greater that 40 %ID/g in the BB2r-positive mouse pancreas was observed for the agonistic analogs $^{177}$Lu-C-AG and $^{177}$Lu-E-AG. As expected, adduct formation in the pancreas for $^{177}$Lu-E-AG was confirmed by SDS-PAGE leading to a significant increase in retention relative to $^{177}$Lu-C-AG by 24h. For the antagonists $^{177}$Lu-C-AN and $^{177}$Lu-E-AN, pancreas uptake was comparatively negligible by 4h, likely due to the rapid clearance of this construct by this timepoint. Strikingly, unlike the corresponding agonist, $^{177}$Lu-E-AN demonstrated no signs of adduct formation by SDS-PAGE. This suggests that, unlike the tumors, the antagonists do not induce internalization in the mouse pancreas. Speculatively, this contrast with the human cancer cells may be due to structural/homology differences between the mouse and human BB2r with regard to binding/activation and/or differences in the basal endocytic activity between these cell types.

For both the agonist and antagonists, the endolysosomal trapping approach increased renal retention over the long term. This is almost certainly due to the well-
known endocytic, reuptake mechanism that is present in the normal kidney. Adduct formation was observed for the radioconjugates with the active trapping agent, implying that this reuptake mechanism leads to adduct formation in the endolysosomal compartments of renal cells, in all probability the proximal tubule cells.

3.5 CONCLUSION

In this study, the potential of a CC inhibitor to act as an endolysosomal trapping agent to improve the tumor retention of BB2r-targeted agents was examined. Specifically, a hydrophilic CC inhibitor, based on the well-known E-64, was incorporated into both agonistic (BBN7-14) and antagonistic (RM2) BB2r-targeted constructs. It was found that incorporation of the CC inhibitor into the pharmacophores did not substantially impact either the CC inhibition potency of the inhibitor or the BB2r-affinity of the peptide. For both the agonistic and antagonistic analogs, in vitro studies demonstrated that the CC inhibitors prompted the formation of CC adducts when internalized into BB2r-positive PC-3 prostate cancer cells. In vivo studies clearly demonstrated macromolecular adduct formation and a substantial increase (approximately 2-fold) in tumor residualization for the CC trapping, BB2r-targeted agents. Overall, this data demonstrates the potential of the CC endolysosomal trapping approach to improve both agonistic and antagonistic BB2r-targeted agent development. When appraised in context with our previously published work concerning CC trapping, NTSR1-targeted agents, this technique, in our opinion, could have broad applicability to enhance targeted drug development.
Chapter 4. Synthesis and Evaluation of A Radiolabeled Phosphoramidemustard with Selectivity for Hypoxic Cancer Cells

4.1 INTRODUCTION

Hypoxia is a well-known characteristic of many solid cancers. This phenomenon is attributable to the chaotic nature of tumor vasculature, leading to the inefficient delivery of oxygen and other nutrients and resulting in a heterogeneous distribution of these materials in the tumor. Tumor hypoxia has been implicated in both drug and radiation resistance, which has caused treatment failure in numerous cancers. Given this, it is not surprising that many researchers over the years have focused on the design of prodrugs that aim to exploit tumor hypoxia for diagnostic and therapeutic applications.

Up to now, various hypoxia based diagnostic (e.g., $^{18}$F-FMISO) and therapeutic (e.g., AQ4N, PR-104 and TH-302) prodrugs have been under development. The hypoxia-selectivity of many of these agents is based on bioreductive mechanisms, leading to the transformation of these drugs into reactive species in hypoxic tissues.

These reactive species are typically electrophilic metabolites which exhibit increased retention in hypoxic cells by forming intracellular adducts with opportunistic nucleophilic biomolecules. For hypoxia-selective diagnostic agents, the increased formation and retention of these adducts in hypoxic tissues allows the generation of...

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target-to-non-target ratios benefiting to in vivo imaging of hypoxic tissues. From a therapeutic perspective, hypoxia activated chemo-therapeutic agents typically generate radicals (e.g., TPZ) or electrophilic mustard agents (e.g., PR-104 and TH-302) that can induce DNA damage, which ultimately prompts cancer cell death. The advantage of these prodrugs is the increased selectivity and cytotoxicity for hypoxic tumors relative to normoxic non-target tissues.

Our laboratory is interested in the development of diagnostic and radiotherapeutic agents that can utilize the hypoxic nature of cancers. Previously, we have found that when 2-nitroimidazoles, a class of hypoxia-selective pro-drugs, are conjugated to receptor-targeted peptides, these bioconjugates exhibit increased long-term retention in hypoxic cells, presumably through adducts formed with intracellular biomolecules. This initial work has given us the impetus for the continued investigation and development of other prodrugs that may serve as hypoxia-selective trapping agents (HSTAs). One of the potential candidates is the 2-nitroimidazole-5-yl-methyl based compound evofosfamide (i.e., TH-302), which is depicted as 2 in Figure 4.1. Evofosfamide is a hypoxia-activated prodrug that has undergone several phase I/II/III clinical trials for the treatment of advanced solid cancers, including sarco-mas, and oesophageal adenocarcinomas. This prodrug is activated through reduction facilitated by cellular reductases (e.g., cytochrome P450) to generate a radical anion. Under hypoxic conditions, the radical anion is exposed to further reduction, leading to the fragmentation and generation of an active nitrogen mustard. The goal of our work is to develop a derivative of evosfosfamide that can be easily conjugated to imaging agents.
(e.g., fluorophores or radioisotopes) for the in vitro and in vivo detection of hypoxia or to targeting vectors to enhance the retention of these agents in hypoxic tissues. Herein, we describe the synthesis of a novel 2-nitroimidazole phosphoramidate nitrogen mustard (2-NIPAM, 3) that is capable of conjugating to other moieties through the well-known azide-alkyne cycloaddition reaction. Utilizing human prostate cancer PC-3 and DU145 cell lines, we have investigated the cytotoxicity and hypoxia selectivity of this analog relative to evososfamide (2) and a negative control (i.e., non-activatable, 1). In addition, we optimized the azide-alkyne cycloaddition reaction conditions for 2-NIPAM, radio-labeled the analog with $^{125}$I and investigated the hypoxia trapping capability of this radiolabeled agent under normoxic and hypoxic conditions. The results obtained from our investigations have suggested that 2-NIPAM has the potential to serve as a hypoxia-selective imaging agent and/or trapping agent for receptor-targeted constructs.
Figure 4.1 Chemical structure of phosphamide mustard negative control 1, positive control 2 and 2-NIPAM 3.
4.2 MATERIALS AND METHODS

4.2.1. Reagents and Instruments

All solvents used for reactions and silica gel purification were ACS grade and purchased from Fisher Scientific, unless otherwise stated. Acetonitrile utilized for HPLC analysis and purification was HPLC grade and purchased from Fisher Scientific. Water was deionized by Millipore® Milli Q Biocell Ultrapure Water System before use. TLC silica gel 60 plate was purchased from EMD Millipore. CDCl₃, sodium hydride was obtained from ACROS Organics™. Dimethyl sulfoxide was obtained from Cambridge Isotope Laboratories. 4-(Dimethylamino) pyridine (DMAP), Cu (II) chloride, CuI, phosphorus (V) oxychloride, sodium sulfate and tetrabromomethane were obtained from Sigma-Aldrich. Boc anhydride was obtained from Chem-Impex International. Deuterium oxide and triphenylphosphine were purchased from Alfa Aesar®. 2-Bromoethylamine hydrobromide, alamarBlue®, ammonium chloride, ethanolamine, imidazole, L-glutamine, propargyl bromide, sodium chloride, tetrabutylammonium fluoride (TBAF) and triethylamine (Et₃N) were obtained from Thermo Fisher. (1-Methyl-1H-imidazol-5-yl) was purchased from AstaTech. Tert-Butyldimethylchlorosilane (TBSCI) was purchased from Oakwood Chemical. (1-methyl-2-nitro-1H-imidazol-5-yl) methanol was purchased from ChiralStar.), Dulbecco’s Modified Eagle Medium (DMEM), bovine serum albumin (BSA), hydrochloric acid and sulfuric acid, Kaighn's Modification of Ham’s F-12 Medium (F-12K Medium), 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES), Penicillin-Streptomycin, phosphate buffered
saline (PBS) and silica gel (230-400 mesh) were obtained from Fisher Scientific. $^{125}$I NaI was purchased from American Radiolabeled Chemicals, Inc. The human prostate cancer PC-3 (CRL-1435™) and DU145 (HTB-81™) was purchased from American Type Culture Collection.

A Waters e2695 system equipped with a Waters 2489 absorption detector and a Waters Qtof Micro electrospray ionization mass spectrometer was used to perform high performance liquid chromatography/mass spectrometry analyses. $^1$H, $^{13}$C and $^{31}$P NMR spectrums were recorded on a Bruker Avance-III HD 500 MHz instrument using as the solvent. Triphenylphospjate was used as $^{31}$P standard. A Phenomenex Jupiter 5u C18 300A 250×10 mm semiprep column was used for the purification of 2-nitroimidazole phosphoramidate mustard and its controls. A Phenomenex Jupiter C12 Proteo 250×10 mm semiprep column was used for the purification of model compounds of 5-$[^{25}$I]iodo-1,2,3-triazoles. Waters 1515 binary pump equipped with a Waters 2489 absorption detector and a Bioscan Flow Count radiometric detector system using a Phenomenex Jupiter C12 Proteo 250×4.6 mm column. Falcon™ Polystyrene Microplates (96-well plate and 6-well plate) were used for in vitro cytotoxicity assay and efflux study separately. SpectraMax® M5 Multi-Mode Microplate Readers was used to quantify fluorescence intensities. EVOS FL Cell Imaging System was used for time-dependent cytotoxicity assay. Hypoxic PC-3 and DU145 cells were incubated in hypoxic glove box with temperature, CO$_2$ and humidity controller (Coy Laboratory Products INC, Grass Lake, MI). Gamma decay detection of $^{125}$I for the efflux studies was accomplished using a LTI (U.S.) Multi-Wiper nuclear medicine gamma counter.
4.2.2 Synthesis of 2-nitroimidazole Phosphoramidate Mustard (2-NIPAM)

Compounds 1 through 9 were synthesized on the basis of reported methods. Similar synthetic procedures were applied to the synthesis of both the negative control, imidazole phosphoramidate mustard 1 and the positive control, 2-nitroimidazole phosphoramidate mustard, 2. Details of synthesis can be found in the following content. 

$^1$H spectra are reported as chemical shifts (δ) in ppm relative to TMS (δ = 0) and $^{31}$P NMR are referenced to H$_3$PO$_4$ (δ = 0).

**tert-butyl(2-hydroxyethyl)carbamate (4).** To a stirred solution of ethanolamine (6.16 g, 100.8 mmol) in 200 mL dry CH$_2$Cl$_2$ was added Et$_3$N (19.2 mL, 137.46 mmol) at rt for 30 min and then the solution was cooled to 0 °C. To this, a solution of Boc$_2$O (20.0 g, 91.64 mmol) in 60 mL of dry CH$_2$Cl$_2$ was added dropwise and stirred at rt overnight. The mixture was quenched with saturated aqueous NH$_4$Cl, and extracted with CH$_2$Cl$_2$ three times. The combined organic extracts were dried over anhydrous Na$_2$SO$_4$, filtered and concentrated by rotary evaporation to yield 4 as a colorless oil (15.6 g, 96.8 mmol, 96%).

Rf=0.31(12.5% Et$_2$O/petroleum ether)

$^1$H NMR (500 MHz, CDCl$_3$): δ 5.10 (s, 1H), 3.66-3.65 (m, 2H), 3.25-3.25 (m, 2H), 3.02 (s, 1H), 1.42 (s, 9H).

MS (ESI+) m/z calcd for C$_7$H$_{15}$NO$_3$ [M+H]$^+$ 162.11, found 162.09.

**tert-butyl(2-((tert-butyldimethylsilyl)oxy)ethyl)carbamate (5).**
To a stirred solution of 4 (10 g, 62.03 mmol) and imidazole (12.67 g, 186.1 mmol) in 200 mL dry CH₂Cl₂ with DMAP (0.4 g, 3.27 mmol), TBSCl (11.22 g, 74.44 mmol) was slowly added at 0 °C. The solution was warmed to rt and stirred overnight, then quenched with saturated aqueous NH₄Cl solution. The mixture was extracted three times with CH₂Cl₂ and the combined organic layers were dried over anhydrous Na₂SO₄, filtered and the solvent removed by rotary evaporation. The product was purified via flash column chromatography on silica gel using 20% Et₂O in petroleum ether to elute 5 (16.8 g, 98%) as a clear colorless oil.

\[ R_f=0.47(20\%\text{ Et}_2\text{O/petroleum\ ether}). \]

\[ ^1\text{H NMR}\ (500\text{ MHz, CDCl}_3): \delta 4.84\ (br, 1H), 3.65\ (m, 2H), 3.22\ (m, 2H), 1.44\ (s, 9H), 0.89\ (s, 9H), 0.054\ (s, 6H). \]

\[ ^{13}\text{C NMR}\ (125\text{ MHz, CDCl}_3) \delta 155.96, 79.12, 62.26, 42.88, 28.41, 25.89, 18.30, -2.96, -5.37. \]

\[ \text{MS (ESI+) } m/ z \text{ calcd for C}_{13}\text{H}_{29}\text{NO}_3\text{Si [M+H]}^+ 276.19, \text{ found 276.05.} \]

**tert-butyl(2-((tert-butyldimethylsilyl)oxy)ethyl)(prop-2-yn-1-yl)carbamate (6).** To a suspension of NaH (2.9 g, 72.61 mmol) in 100mL dry THF was added a solution of 5 (10 g, 36.3 mmol) in 30 mL dry THF at 0 °C. The mixture was stirred at rt for 1.5 h and subsequently cooled down to 0 °C. To this reaction mixture propargyl bromide (13.7 mL of an 80% wt solution in toluene, 90.76 mmol) was added dropwise. Allowing to warm to rt, the reaction mixture was stirred overnight and quenched with 50mL MeOH followed by 100 mL water. The aqueous layer was extracted three times with CH₂Cl₂ and the combined organic layers were dried
over anhydrous Na$_2$SO$_4$, filtered and the solvent removed by rotary evaporation. The product was purified via flash column chromatography on silica gel using (2% Et$_2$O in petroleum ether) to provide 6 (6.6 g, 58%) as clear colorless oil.

R$_f$=0.38(20% Et$_2$O/acetone)

$^1$H NMR (500 MHz, CDCl$_3$): $\delta$ 4.16 (d, 2H), 3.74 (s, 2H), 3.42 (s, 2H), 2.18 (s, 1H), 1.47 (s, 9H), 0.89 (s, 9H), 0.049 (s, 6H).

$^{13}$C NMR (125 MHz, CDCl$_3$): $\delta$ 155.93, 80.09, 79.05, 62.24, 48.47, ,43.08, 38.08, 32.25, 28.39, 25.87, 18.28, -5.39.

HRMS (ESI+) m/z calcd for C$_{16}$H$_{31}$NO$_3$Si [M+H]$^+$ 314.2151, found 314.2155.

$\text{tert}-\text{butyl}(2$-$\text{hydroxyethyl})$(\text{prop-2-yn-1-yl})\text{carbamate (7). To a solution}$

of 6 (5 g, 15.95 mmol) in 100 mL CH$_2$Cl$_2$ was added TBAF (1M in THF) (22.33 mL, 22.33 mmol) at 0 °C. The reaction mixture was stirred overnight at rt and quenched with saturated aqueous NH$_4$Cl solution, extracted three times with CH$_2$Cl$_2$. The organic layers were dried over anhydrous Na$_2$SO$_4$, filtered and the solvent was removed by rotary evaporation. The product was purified via flash column chromatography on silica gel using 20% acetone in petroleum ether as the eluent to provide 7 (2.7 g, 85%) as a clear light yellow oil.

R$_f$=0.54(50% Et$_2$O/acetone).

$^1$H NMR (500 MHz, CDCl$_3$): $\delta$ 4.09 (br, 2H), 3.82 (m, 2H), 3.52 (m, 2H), 2.27 (s, 1H), 1.50 (s, 9H).
\(^{13}\text{C} \text{NMR}\) (125 MHz, CDCl\(_3\)): \(\delta\) 207.11, 156.82, 79.54, 71.60, 62.45, 43.13, 30.88, 28.35.

\textbf{MS} (ESI+) \(m/z\) calcd for C\(_{10}\)H\(_{17}\)NO\(_3\) [M+H]\(^+\) 200.12, found 200.14.

\textit{tert}-butyl(2-bromoethyl)(prop-2-yn-1-yl)carbamate (8). To a solution of 7 (2 g, 10.04 mmol) and of triphenylphosphine (3.42 g, 13.05 mmol) in 50 mL dry THF a solution of carbon tetrabromide (4.33 g, 13.05 mmol) in 30 mL acetonitrile was added dropwise while the reaction temperature was slightly above room temperature. The reaction was stirred for 2 h, and the solvent was removed by rotor evaporation. The residue was purified via flash column chromatography on silica gel using 20% acetone in petroleum ether as the eluent to provide 8 (2.04 g, 77.8\%) as clear light yellow oil. The product 8 was immediately used in next step synthesis.

\(^1\text{H} \text{NMR}\) (500 MHz, CDCl\(_3\)): \(\delta\) 4.15 (dd, 2H), 3.69 (m, 2H), 3.51 (br, 2H), 2.24 (s, 1H), 1.48 (s, 9H).

\textbf{MS} (ESI+) \(m/z\) calcd for C\(_{10}\)H\(_{16}\)BrNO\(_2\) [M+H]\(^+\) 262.04, found 261.91.

\textit{N-(2-bromoethyl)prop-2-yn-1-amine hydrochloride} (9). To a solution of 8 (0.5 g, 1.92 mmol) in 50 mL Et\(_2\)O was added anhydrous hydrogen chloride gas at 0 °C continuously for 3 h. 9 (0.3 g, 80\%) gradually precipitate out of solution as a white solid. The product was filtrated to remove solvent and used for the next reaction without further purification.

\(^1\text{H} \text{NMR}\) (500 MHz, DMSO-\(d_6\)): 9.3245 (s, 2H), 3.99-3.98 (d, 2H), 3.90-3.88 (t, 2H), 3.77 (s, 1H).
13C NMR (125 MHz, DMSO-d6): δ 80.229, 75.331, 47.622, 36.193, 27.045.

HRMS (ESI+) m/z calcd for free base C₅H₈NBr [M+H]+161.9918, found 161.9921.

(2-bromoethyl)(dichlorophosphoryl)phosphoramidic dichloride

(11). To a suspension of 2-Bromoethylamine hydrobromide (3 g, 14.6 mmol) in 50 mL Et₂O was added POCl₃ (1.4 mL, 14.64 mmol) slowly at -5°C with stirring. Et₃N (4.08 mL, 97.61 mmol) was added and the reaction mixture was allowed to stir overnight. The solution was filtered and the filtrate was concentrated down. The residue was purified by distillation to provide 8 (2.7 g, 85%) as white crystal.

1H NMR (500 MHz, CDCl₃): δ 4.13-4.03 (m, 2H), 3.73-3.70 (t, 2H).

13C NMR (125 MHz, CDCl₃): δ 50.41, 26.25.

31P NMR (202 MHz, CDCl₃): δ 9.44.

1-Methyl-1H-imidazol-5-yl)methyl N,N-bis(2-bromoethyl)phosphordiamidate (1). To a suspension of 2-Bromoethylamine hydrobromide (10 g, 48.8 mmol) in 100 mL CH₂Cl₂ was added POCl₃ (2.28 mL, 24.4 mmol) slowly at -15°C with vigorous stirring. To this reaction mixture a solution of Et₃N (10.96 mL, 97.6 mmol) in 50 mL CH₂Cl₂ was added dropwise, followed by stirring at -10 °C for 2h. The solid residue was filtered and washed with small amount 5mL of cold CH₂Cl₂ and the filtrate was concentrated by rotor evaporation to about 20 mL. The residue was filtered and washed with cold CH₂Cl₂ again. The resulting filtrate was concentrated down by rotor evaporation. To a
suspension of (1-Methyl-1H-imidazol-5-yl)methanol (27.5 mg, 0.25 mmol) in 5 mL CH₂Cl₂ was added solution of the residue (20 mg in 5 mL CH₂Cl₂) from the precious step at 0 °C. To the reaction mixture Et₃N (16.9 mg, 0.167 mmol) in 2 mL CH₂Cl₂ was added at 0 °C and stirred overnight in room temperature. The solvent was removed by rotor evaporation. The residue was purified by a semi-preparative Jupiter 5u C18 HPLC column with a flow rate of 5.0 mL/min. The final yields of 1 was 13.8% (3.4 mg) as colorless oil.

³¹H NMR (500 MHz, DMSO-d₆): δ 7.63 (s, 1H), 6.938 (s, 1H), 4.91-4.84 (m, 4H), 3.63 (m, 3H), 3.40 (m, 4H), 3.09 (m, 4H).

³¹P NMR (202 MHz, DMSO-d₆): δ 14.82.

MS (ESI⁺) m/z calcd for C₉H₁₇Br₂N₄O₂P [M+H]⁺ 402.95, found 402.95.

1-Methyl-2-nitro-1H-imidazol-5-yl)methyl N,N-bis(2-bromo-ethyl)phosphordiamidate (2). Compound 2 was synthesized by the same synthetic procedure as 1 and obtained as a yellow oil in 11.7% yield.

³¹H NMR (500 MHz, DMSO-d₆): δ 7.24 (s, 1H), 4.98-4.96 (m, 4H), 3.93 (m, 3H), 3.41 (m, 4H), 2.88 (m, 4H).

³¹P NMR (202 MHz, DMSO-d₆): δ 15.30.

HRMS (ESI⁺) m/z calcd for C₉H₁₆Br₂N₅O₄P [M+H]⁺ 447.9385,found 447.9382.
1-Methyl-2-nitro-1H-imidazol-5-yl)methyl N,N-bis(2-bromoethyl)prop-2-ynephosphordiamidate (3). To a suspension of Compound 9 (94.6 mg, 0.484 mmol) in 0 mL CH₂Cl₂ was added phosphorus trichloride (30 mg, 0.22 mmol) at room temperature, followed by the addition of TEA (397.9 mg, 3.93 mmol) in 10 mL CH₂Cl₂ dropwise. The reaction mixture was stirred at room temperature for 1h and then (1-Methyl-2-nitro-1H-imidazol-5-yl)methanol (41.2 mg, 0.26 mmol) in 5 mL CH₂Cl₂ was added. After stirring for another 2h, the reaction mixture was cooled to -20°C and the tert-butyl hydroperoxide (48 µL, 0.26 mmol, 5M in decane) was added. The solution was warmed to room temperature and stirred for 1h. The reaction mixture was quenched with saturated aqueous NH₄Cl solution, extracted three times with CH₂Cl₂. The organic layers were dried over anhydrous Na₂SO₄, filtered and the solvent was removed by rotary evaporation. The residue was purified by a semi-preparative Jupiter 5u C18 HPLC column with a flow rate of 5.0 mL/min. The final yields of 3 was 7.4% (8.4 mg) as yellow oil.

¹H NMR (500 MHz, CDCl₃): δ 5.15-5.14(d, 2H), 4.10 (s, 3H), 3.93-3.82 (m, 8H), 3.52 (m, 4H), 2.35 (m, 2H).

³¹P NMR (202 MHz, CDCl₃): δ 15.62.

¹³C NMR (125 MHz, CDCl₃): δ 129.44, 78.95, 73.39, 56.58, 47.81, 36.28, 34.52, 29.41.


4.2.3 X-ray Crystallographic Analysis
The single crystal generated from synthesis was stored in -80°C before characterization. Crystal data for compound 9: C₆H₉NBr₆, MW = 574.60 g/mol, orthorhombic, space group P2₁2₁2₁, a = 7.30499(16) Å, b = 11.3505(2) Å, c = 16.9283(3) Å, α = β = γ = 90°, V = 1403.62(5) Å³, Z = 4, \(D_{calc} = 2.719 \text{ g/cm}^3\), μ = 20.381 mm⁻¹, T = 100 (2) K, Data was collected on a Bruker SMART 1K CCD. Refinement of data with \(I > 2σ(I)\) (2909 independent reflections, \(R_{int} = 0.0758\)) gave a \(R_1(F) = 0.0346\) and a \(wR_2(F^2) = 0.0925\) for all data with a GOF = 1.061. Crystallographic data for the structural analysis has been deposited with the Cambridge Crystallographic Data Center (CCDC 1566695). Crystal data for compound 11: C₆H₅BrC₆H₄NiO₂P₂, MW = 354.77 g/mol, orthorhombic, space group P2₁/c, a = 11.2875(5) Å, b = 7.8601(4) Å, c = 12.5373(6) Å, α = γ = 90°, β = 102.528(5), V = 1085.84(9) Å³, Z = 8, \(D_{calc} = 2.188 \text{ g/cm}^3\), μ = 16.756 mm⁻¹, Data was collected on a Bruker SMART 1K CCD. Refinement of data with \(I > 2σ(I)\) (2215 unique reflections, \(R_{int} = 0.0473\)) gave a \(R_1(F) = 0.0477\) and a \(wR_2(F^2) = 0.1527\) for all data with a GOF = 0.996. Crystallographic data for the structural analysis has been deposited with the Cambridge Crystallographic Data Center (CCDC 1566696). Copies of this information may be obtained free of charge from the CCDC (www: http://www.ccdc.cam.ac.uk) or from e-mail jcgarrison@unmc.edu.

4.2.4 In Vitro Cytotoxicity Study

PC-3 and DU145 cells were separately cultured in F-12K and DMEM medium supplemented with 10% fetal bovine serum, 2.5 mM L-glutamine, 15 mM HEPES and 1% penicillin/streptomycin. Cells were seeded 24h before the experiment in 96-well
plates at a density of 15,000/mL with 200µL medium of PC-3 cells and 12,500/mL with 200µL medium. On the day of the experiment, serial dilutions of the test compound with medium were administrated to cells. The plates were incubated for 2h, or longer if indicated, under either normoxic (95% air, 5% CO₂) or hypoxic (94.9% N₂, 0.1% O₂, 5% CO₂) conditions. After washing with fresh medium, cells were cultured for 72h in 200µL complete medium under normoxic conditions. Cell viability was determined by AlamarBlue. The 50% inhibitory concentration (IC₅₀) of the test compound relative to the untreated control were calculated by Graphpad PRISM 5.

4.2.5 Radiolabeling of 2-NIPAM with [¹²⁵I]-NaI

The general procedure for the preparation of Nat/¹²⁵I-labeled Azide-Alkyne Cycloaddition conjugates were adapted from previous reported synthetic strategy. To 1.2 mL anhydrous acetonitrile, copper(II) chloride (4.02 mg, 30 µmol) was added to make CuCl₂/TEA solution. To this mixture, anhydrous triethylamine (4.53 mg, 45 µmol) was added and sonicated until a clear burgundy solution was formed. The solution was gently mixed before adding slowly over 5 min a solution of 2-NIPAM (0.1 µmol) in 20 µL acetonitrile. To this mixture, Na¹²⁵I (7.4 MBq) in 6.5 µL H₂O was added followed by the addition of another 0.5 µL benzyle azide in 20 µL of anhydrous acetonitrile. The resultant solution was heated at 60 °C for 90 min. The purification of the radiolabeled azide-alkyne cycloaddition conjugates was performed by using RP-HPLC (≥10%).

4.2.6 Distribution Coefficient
The distribution coefficient was determined (n = 5) for each $^{125}$I-labeled radioconjugate. In a 1.5 mL centrifuge tube, 0.5 mL of 1-octanol was added to 0.5 mL of phosphate-buffered saline (pH 7.4) containing the radiolabeled peptide (400,000 cpm). The solution was vigorously stirred for 2 min at room temperature and subsequently centrifuged (8,000g, 5 min) to yield two immiscible layers. Aliquots of 100 µL were taken from each layer, and the radioactivity of each was quantified by an LTI (Elburn, IL) Multi-Wiper nuclear medicine gamma counter.

4.2.7 In vitro Efflux Study

PC-3 and DU145 cells were incubated in 6-well plates (0.5 × 10^6/well) under normoxic (95% air, 5% CO$_2$) or hypoxic (94.9 % N$_2$, 0.1 % O$_2$, 5 % CO$_2$) conditions overnight in RPMI 1640 media (pH 7.4, 4.8 mg/mL HEPES, and 2 mg/mL BSA). On the day of the experiment, the medium was replaced with fresh medium and incubated for 2 h under normoxic or hypoxic conditions, respectively. The cells were pre-incubated for 2h at 37 °C in the presence of 100,000 cpm of $^{125}$I-radioconjugates. Upon completion of the incubation, cells were washed thrice with medium to discard the unbound peptide. At time points 1, 2, 4, 6 and 8 h, the medium was harvested for quantitative analysis as the effluxed ligand. Surface bound radioactivity was collected by washing the cells twice with an acid wash (50 mM glycine-HCl/0.1 M NaCl buffer, pH 2.8). The cells were then lysed at 37 °C using a 10% aqueous SDS solution and the lyses were collected as internalized ligand. The radioactivity of the effluxed, surface bounded and internalized fractions for each radioconjugate was determined using a Multi Viper gamma counter.
Statistical analyses were performed by two-way analysis of variance (ANOVA) using Graphpad PRISM 5 (U.S.).

4.2.8 Statistical Analysis

IC\textsubscript{50} values were determined by nonlinear regression using the 1-binding-site model of GraphPad PRISM 5 (GraphPad Software, Inc). Comparisons of the 2 groups for efflux studies were analyzed by the 2-tailed Student t test, and P values of less than 0.05 were considered statistically significant.

4.3 RESULTS

4.3.1 Synthesis of 2-nitroimidazole Acetic Acid (2-NIAA)

Our first synthetic goal in the synthesis of 2-NIPAM, 3, was to produce the alkyne containing secondary amine, 9. The synthesis of compound 9 (N-(2-bromoethyl)prop-2-yn-1-amine hydrochloride), as outlined in Scheme 4.1, was initiated with the dual protection of 2-aminoethan-1-ol.

Reagents, conditions, and yields: (a)Et₃N, CH₂Cl₂, 96.0%; (b) Imidazole, 4-DAMP, TBDMSCl, CH₂Cl₂, 98.3%; (c) propargyl bromide, NaH, THF, 58.0%; (d) TBAF, THF, 85.0%; (e) CBr₄, PPh₃, ACN, 77.8%; (f) HCl (g), Et₂O, 79.6%.
The amine and alcohol functional groups of the starting material were protected with Boc and tert-butyldimethylsilyl groups, respectively. Deprotonation of the protected amine using sodium hydride and subsequent reaction with propargyl bromide yielded the protected secondary amine. Deprotection of the silyl protecting groups employing a fluoride source (i.e., TBAF) provided compound 7. To this point, compound 7 was obtained in three steps with an overall yield of 85%. An Appel reaction, utilizing triphenylphosphine and CBr₄, was employed to convert the alcohol into a bromide, giving compound 8. After work-up, column chromatography was utilized to purify the compound and remove byproducts (e.g., PO(PH₃) and starting reagents (e.g., CBr₄). Purified 8 was dissolved in Et₂O and the Boc group removed by the introduction of HCl gas yielding intermediate 9 in quantitative yield. The structure of 9 was confirmed by x-ray crystallography (Figure 4.2 and Table 4.1), but it is noteworthy that a molecule of CBr₄ also co-crystalized with the desired compound. After further investigation, it was found that additional chromatographic purifications of 8 were able to remove residual CBr₄, resulting in the isolation of white crystalline 9. Overall, the synthetic route in Scheme 4.1 produced compound 9 in 80% yield. The structures of the compound synthesized in this scheme were confirmed by mass spectrometry, ¹H and ¹³C NMR spectra. (Figure 4.3-8, correspondingly).
Figure 4.2 Crystal structure of compound 9. Selected bond lengths (Å) and angles (deg): Br(1)-C(1) =1.954(7), N(1)-C(2)=1.497(9), N(1)-C(3)=1.502(8), C(2)- C(1)=1.498(10), C(3)-C(4)=1.450(10), C(4)-C(5) =1.181(11), C(2)-N(1)-C(3)=1 14.5(5), N(1)-C(2)-C(1) = 107.6(6), C(4)-C(3)-N(1)= 109.3(6), C(5)-C(4)-C(3)= 179.1(9), C(2)-C(1)-Br(3)= 108.9(5).
### Table 4.1 Crystal data and structure refinement for compound 9.

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Figure 4.3 A-C) Mass spectrometry of compound 1-3.
Figure 4.4 A-C) Mass spectrometry of compound 4-6.
Figure 4.5 A-C) $^1$H-NMR spectra of compound 4-6.
Figure 4.6 A-C) $^1$H-NMR spectra of compound 7-9.
Figure 4.7 A-C) $^{13}$C-NMR spectra of compound 7-9.
Figure 4.8 $^{13}$C-NMR spectra of compound 9.
Initial attempts to synthesize the phosphordiamidate mustards centered on utilizing an asymmetric approach using a phosphoramic dichloride precursor (Scheme 4.2)\textsuperscript{251-252}. Unfortunately, this approach yielded multiple products with one of the largest byproducts being a bisphosphoramidate \textbf{11}. The structure of \textbf{11} was confirmed by X-ray crystallography, Figure 4.9 and Table 4.2. Attempts to obtain the desired compounds by modifying the reaction conditions (i.e. substitution of different amines, varying reaction times and temperatures) were unsuccessful.
Scheme 4.2 Attempt to synthesize asymmetric phosphoramidate mustard.

Reagents, conditions, and yields: (a)propargyl bromide, Et₂O, Et₃N, -5°C; (b)Bis(2-chloroethyl)amine hydrochloride, ACN, Et₃N, pyridine, 40°C; (c) H₂O.
Figure 4.9 Crystal structure of compound 11. Selected bond lengths (Å) and angles (deg): Br(1)-C(1) =1.952(5), P(1)-C(11)=1.979(17), P(1)-C(12)=1.996(18), P(1)-O(1)=1.450(4), P(1)-N(1)=1.670(4), C(2)-C(1)=1.514(7), C(2)-N(1)=1.502(6), C(11)-P(1)-C(12)= 102.95(8), O(1)-P(1)-C(11)= 115.55(17), O(1)-P(1)-C(12)= 113.10(19), O(1)-P(1)-N(1)= 113.0(2), N(1)-C(2)-C(1) = 114.4(4), P(2)-N(1)-P(1) = 121.3(3), C(2)-N(1)-P(1)= 118.2(3), C(2)-C(1)-Br(1)= 113.1(3).
Table 4.2 Crystal data and structure refinement for compound 11.

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Figure 4.10 A-C) $^1$H, $^{13}$C and $^{31}$P-NMR spectra of compound 11.
With these setbacks, we switched our approach to focus on a symmetric phosphoramidate mustard synthesis, outlined in Scheme 4.3, to obtain 3 along with our positive (2) and negative (1) controls. The negative control, 1, is an analog of 2 that utilizes an imidazole instead of a nitroimidazole, thereby eliminating its ability to fragment and form an active phosphoramidate mustard. The controls were synthesized by reaction of two equivalents of bromoethylamine with POCl₃ to yield the phosphoramidate chloride intermediate. This intermediate was subsequently reacted under basic conditions with (1-methyl-1H-imidazol-5-yl)methanol or its 2-nitroimidazole derivative to produce compound 1 in 13.8% yield and 2 in 11.7% yield, respectively. The synthesis of compound 3 was accomplished by the sequential addition of 2.2 equivalents of 9 with PCl₃, followed by reaction with 1-methyl-2-nitro-1H-imidazol-5-yl)methanol and oxidation via tert-butyl hydroperoxide. The structures of compound 1, 2 and 3 were confirmed by mass spectrometric, \(^1\)H, \(^{13}\)C and/or \(^{31}\)P NMR spectra analysis (Figure 4.11-4.14).
Scheme 4.3. Synthesis of imidazole and its nitro-derivative conjugated phosphoramidate mustard (1-3)

\[ \text{Reagents, conditions, and yields: (a) propargyl bromide, Et}_3\text{N, CH}_2\text{Cl}_2 \; \text{b) Et}_3\text{N, CH}_2\text{Cl}_2, \; 13.8\% \; (1), \; 11.7\% \; (2); \; (c) \text{compound 9, Et}_3\text{N, CH}_2\text{Cl}_2; \; (d) \text{tert-butyl hydroperoxide, Et}_3\text{N, CH}_2\text{Cl}_2, \; 7.4\% \; (3).} \]
Figure 4.11 A-C). Mass spectrometry of compound 1-3.
Figure 4.12 A-C) $^1$H-NMR spectra of compound 1-3.
Figure 4.13 $^{13}$C NMR spectra of compound 3.
Figure 4.14 $^{31}$P NMR spectra of compound 1-3.
4.3.2 Time-Dependent *In Vitro* Cytotoxicity Study

The cytotoxicity of compounds 1, 2 and 3 were evaluated in PC-3 and DU145 human prostate cancer cell lines under both normoxic and hypoxic conditions using a viability assay, results shown in Table 4.3. As expected, the negative control, 1, demonstrated an IC\textsubscript{50} that was > 0.85 mM for both cell lines. Due to its inability to form an active phosphoramide mustard under hypoxic conditions, the cytotoxicity of 1 was >100-fold lower than either 2 or 3. Additionally, the negative control demonstrated no hypoxia selectivity (i.e., hypoxia cytotoxicity ratio (HCR) = 1). The positive control, 2, demonstrated increased cytotoxicity (IC\textsubscript{50} = 4.14 ± 0.66 – 6.49 ± 0.67 µM) in both cell lines under hypoxic conditions relative to normoxic conditions, (IC\textsubscript{50} = 687 ± 32.6 – 842 ± 52.5 µM). The hypoxia selectivity for 2 was found to be approximately 2-fold higher in DU145 cells (HCR=203) compared to PC-3 cells (HCR=106). These results agree with previous reports in which evofosfamide (2) demonstrated lower hypoxia selectivity in PC-3 cells (HCR=190) relative to DU145 cells (HCR=240). Compound 3, 2-NIPAM, demonstrated slightly higher cytotoxicity (IC\textsubscript{50} = 1.65 ± 0.09 – 4.70 ± 0.01 µM) compared to the positive control under hypoxic conditions. Interestingly, relative to the positive control, the hypoxia selectivity of 3 was 2-fold lower (HCR = 62) for PC-3 cells, but had similar values (HCR = 225) for DU145 cells. There is no significant difference in the HCR between compound 2 and compound 3 (P>0.05) in the DU145 cell line, but a significant difference in PC-3 cells (P<0.05) was observed.
Table 4.3. Cytotoxicity of prostate cancer cell lines with tested compounds

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<th>IC₅₀(µmol/L; N₂)</th>
<th>IC₅₀(µmol/L; air)</th>
<th>HCR</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>PC-3</td>
<td>~1,000</td>
<td>~1,000</td>
<td>ND</td>
</tr>
<tr>
<td></td>
<td>DU145</td>
<td>851 ± 52.9</td>
<td>856 ± 55.2</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td>PC-3</td>
<td>6.49 ± 0.67</td>
<td>687 ± 32.6</td>
<td>106</td>
</tr>
<tr>
<td></td>
<td>DU145</td>
<td>4.14 ± 0.66</td>
<td>842 ± 52.5</td>
<td>203</td>
</tr>
<tr>
<td>2</td>
<td>PC-3</td>
<td>4.70 ± 0.01</td>
<td>293 ± 39.6</td>
<td>62</td>
</tr>
<tr>
<td></td>
<td>DU145</td>
<td>1.65 ± 0.09</td>
<td>372 ± 41.6</td>
<td>225</td>
</tr>
</tbody>
</table>

Result (mean ± SEM) are from 2 or more independent experiments carried out in quintuplicate. HCR: hypoxia cytotoxicity ratio. HCR = IC₅₀(air)/IC₅₀(N₂).
To evaluate the timed-dependent cytotoxicity of compound 3, IC_{50} assays were performed using both cell lines under different incubation times. As shown in Figure 4.15A and 4.15B, the cytotoxicity of 3 was time-dependent in both cell lines with increased cytotoxicity as incubation time increased. As previously noted, higher cytotoxicity for 3 was observed in DU145 cells compared to PC-3 under hypoxic conditions. Representative images depicting the cytotoxicity for both cell lines incubated for 2, 4 and 6h with 1 x 10^{-8} - 1 x 10^{-3} M 2-NIPAM under hypoxic and normoxic conditions are shown in Figure 4.16 and 4.17. The IC_{50} of 3 after 6h incubation was determined to be 0.78 µM in DU145 cells, and 1.69 µM in PC-3 cells, as shown in Table 4.4. At any rate, these studies demonstrate that incorporation of the alkyne groups into the phosphoramidate structure did not substantially impact the cytotoxicity or hypoxia selectivity of 3. This strongly suggests that compound 3 is able to undergo the same activation mechanism that has already been established for 2 (i.e., TH-302) \textsuperscript{254}. 
Figure 4.15 A & B. Time-dependent cytotoxicity of compound 3 (2-NIPAM) in DU145 and PC-3 cell lines, values are mean ± SEM (n=5).
Figure 4.16: Microscopy images of hypoxia selective toxicity of $1 \times 10^{-8}$ M 2-NIPAM with 2h, 4h and 6h administration in PC-3 cells (inverted microscope, ×20). Scale bar: 200µm.
Figure 4.17: Microscopy images of hypoxia-selective toxicity of 1 x 10^{-8} M 2-NIPAM with 2h, 4h and 6h administration in DU145 cells (inverted microscope, x 20). Scale bar: 200µm.
Table 4.4 Summary of time-dependent cytotoxicity of compound 3.

<table>
<thead>
<tr>
<th>Compound exposure time</th>
<th>IC₅₀(µmol/L; N₂)</th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>PC-3</td>
<td>DU145</td>
<td></td>
</tr>
<tr>
<td>2h</td>
<td>4.70 ± 0.01</td>
<td>1.65 ± 0.09</td>
<td></td>
</tr>
<tr>
<td>4h</td>
<td>3.00 ± 0.22</td>
<td>1.17 ± 0.15</td>
<td></td>
</tr>
<tr>
<td>6h</td>
<td>1.69 ± 0.41</td>
<td>0.78 ± 0.10</td>
<td></td>
</tr>
</tbody>
</table>

Result (mean ± SEM) are from 3 or more independent experiments carried out in quintuplicate.
4.3.3 Radiolabeling of 2-NIPAM with $^{[125]}$I-NaI

In order to investigate the conjugation and in vitro trapping efficacy of 2-NIPAM (3), we radiolabeled 3 with $^{125}$I utilizing a Cu(I)-mediated cycloaddition reaction \(^{250}\). However, before proceeding we sought to optimize the cycloaddition reaction conditions. Our previous unpublished work demonstrated that 2-nitroimidazoles can undergo rapid degradation when exposed to certain metals (e.g., Cu(II) salts). With that in mind, the stability of the 2-nitroimidazole reactants and the conjugation efficacy of the ruthenium-based and Cu(I) catalysts under various experimental conditions (i.e., temperature and reaction time) were examined. The coupling of 3 with benzyl azide utilizing two common ruthenium-based catalysts (Cp*RuCl (PPh\(_3\))^2 and Cp*RuCl (COD)) did not result in the desired product as evaluated by LC-MS, but instead lead to multiple byproducts at both room and elevated temperatures. Our initial belief was that the ruthenium catalysts, under the conditions employed, were leading to the degradation of the 2-nitroimidazole functionality of 3. To further investigate this, we incubated the ruthenium catalyst in the presence of 2-nitroimidazole-1-acetic acid. LC-MS demonstrated the reaction led to a gradual transformation of the analog (data not shown), presumably by reduction of the nitro group to the amine \(^{255,256}\). Fortunately, the 2-nitroimidazole functionality of 3 remained stable using Cu(I)-mediated cycloaddition conditions at both room temperature and 60°C with yields of 81.2 % and 82.7 %, respectively.

Following the cycloaddition optimization, we employed a reported procedure by Årstad and colleagues to incorporate $^{125}$I into the resulting triazole ring of the
cycloaddition products. Briefly, the procedure involves a one-pot reaction that utilizes copper (II) chloride combined with Et₃N to generate the cycloaddition product with the ¹²⁵I incorporated on the 5-position of the triazole ring. While some mechanistic studies were performed, the precise mechanism of the ¹²⁵I-incorporation remains unclear. However, some findings appear to be certain, 1) the Et₃N is able to reduce a portion of the Cu(II) species in solution to Cu(I), thereby generating the needed species to carry out the cycloaddition reaction and 2) the incorporation of the ¹²⁵I is carried out by a Cu(I) species. At any rate, we explored the utilization of this technique with 2-NIPAM (3), as depicted in Scheme 4.4. Utilizing the CuCl₂/Et₃N system in a water-acetonitrile mixture, we first examined the incorporation of non-I (non-radioactive) into 2-NIPAM utilizing benzyl azide. Upon completion of the 90min reaction at 60°C, the reaction progress was evaluated by LC-MS, of which the LC spectra was shown in Figure 4.18 And the mass spectrometric spectra of were showed in Figure 4.19. The cycloaddition product 13, without the incorporation of the iodide, was observed at 5.0 min, while the single iodinated product 14 was observed at 7.6 min. As can be seen from the chromatograms, the incorporation efficiency of the iodide into the triazole was modest. In order to aid separation and purification of the iodinated species (i.e., 14 and 15) the mobile phase was changed from water:acetonitrile to water:methanol. The chromatogram of 14 using the water:methanol eluent is depicted in Figure 4.20. Utilizing analogous reaction conditions, the reaction of 3, benzyl azide and ¹²⁵I was carried out and evaluated by radio-HPLC using the same gradient. As expected, 15 had an identical retention times
compared to non-radioactive 14. While the overall radiochemical yield for 15 was poor (10%), the yield was more than sufficient for further in vitro characterization.
Scheme 4.4 Synthesis of 5-[¹²⁵⁴I]Iodo-1,2,3-trizoles (13-15)

\[
\text{N} = \text{Br} \quad \text{N} = \text{O} \\
\text{N} = \text{Br} \quad \text{N} = \text{O} \\
\text{N} = \text{H}(13), \text{N} = \text{I}(14), \text{I}^{125}(15)
\]
Fig 4.18 Mass spectrometry of compound 13 and 14.
Fig 4.19 LC-MS chromatogram profile of products of azide-2-NIPAM cycloaddition reaction. (Eluent: ACN/H₂O).
Fig 4.20 LC-MS chromatogram profile of compound 14 aligned with Radio-labeled HPLC profile of compound 15. (Eluent: Methanol/H$_2$O).
4.3.4 Determination of Distribution Coefficient and \textit{In Vitro} Efflux Study

Studies were performed to determine the distribution coefficient (LogD\textsubscript{7.4}) of 15. The \textsuperscript{125}I-labeled conjugate was found to have a distribution coefficient of 0.55 ± 0.06 which demonstrated that the compound is hydrophobic enough to readily enter cells through passive diffusion. In order to evaluate the cellular trapping/retention efficacy of compound 15, we investigated the biological performance of this radiolabeled agent using \textit{in vitro} efflux studies. The PC-3 and DU145 cells were initially treated with 15 for 1h at 37\textdegree C. Uptake of the \textsuperscript{125}I-labeled conjugate in both cell lines ranged from 2\% to 4\% of the total radioactivity added under both normoxic and hypoxic conditions, respectively. The efflux results for 15 over an 8 h period in both PC-3 and DU145 cells under normoxic and hypoxic conditions are given in \textbf{Figure 4.21}. For all cell lines and oxygen conditions, more than 50\% of 15 was effluxed from the cells within the first 2h. However, the \textsuperscript{125}I-labeled conjugate demonstrated lower efflux rates under hypoxic conditions in both cell lines relative to normoxic conditions. At 8h after efflux began, 85.8\% and 88.3\% of the initial internalized radioactivity of 15 was externalized in normoxic condition in DU145 and PC-3 cells, respectively, compared to 72.2\% and 70.5\% under hypoxic conditions. The increased retention of 15 under hypoxic conditions was found to be statistically significant (P < 0.05) in both cell lines when compared to normoxic data.
Figure 4.21 Efflux assays depicted as percentage of initial internalized activity for $^{125}$I-radioconjugates compound 15 in PC-3 and DU145 cells. Values are mean ± SD (n=5).
4.4 DISCUSSION

Our laboratory continuously pursuing the usage of hypoxia-selective trapping agents to increase the retention time of receptor-targeted radiotracer in hypoxic cancers. Following our initial works with 2-nitroimidazole conjugated hypoxia-selective prodrugs, we start to explore the properties of 2-nitroimidazole-5-yl methyl based phosphordiamidate mustards. The prototype compound evofosfamide can undergo series of reduction in hypoxic environment and release bromo-isophosphoramidate mustard (Br-IPM). Instead of taking advantage of DNA crosslink properties of evofosfamide, we are more interested in employing its hypoxic-selectivity and conjugating it to receptor-targeted radiotracers in the future. Thus, we designed compound 3 to fulfill this purpose.

The key intermediate compound 9 was successfully synthesized in 80% yield by a series of reactions as shown above. During the process, we tried to purified intermediates compound 5 and 6 by distillation, which worked well for large scale (gram level) collection. However, considering the time-cost, column chromatography would be a better choice with high consistence from batch to batch. Meanwhile, to deprotect Boc groups of compound 8, instead of introducing HCl gas into solutions, we also used hydrochloric acid-ethanol solutions as solvent directly. The mixture was stirred in room temperature for 3h and concentrated by rotary evaporation. The product 9 was precipitate out in cold Et2O as white crystal. This improvement has been used in all our following experiments.
In the attempt to synthesize asymmetric phosphordiamidate mustards, several modifications had been done but the results were disappointed. Major difficulties included complicated reaction products showed by LC-MS and incompatibility of products with column chromatography. Attempted purification by HPLC showed that there were no target products characterized by NMR. Thus, it encouraged us to switch to synthesize symmetric phosphordiamidate mustard. Compared to (1-Methyl-1H-imidazol-5-yl)methanol, its 2-nitroimidazole derivatives was less reactive in the synthesis of phosphordiamidate mustards. The changes from primary amine to N,N-dialkyl phosphoramidate mustards further decreased yield. During the synthesis, the major side product that had been characterized was phosphordiamidate mustards containing one molar of bis-bromoethylamine and two molar of 1-Methyl-1H-imidazol-5-yl)methanol. Since the polarity of this side product was close to the product, carefully modification of the gradient of column chromatography was necessary.

Considering the future application of conjugate 2-NIPAM to receptor-targeted radiotracer, thus we choose PC-3 and DU145 prostate cancer cell lines that are commonly used for in vitro studies based on their high receptor expression level (e.g. gastrin-releasing peptide receptor). In cytotoxicity studies, 2-NIPAM demonstrated similar hypoxia cytotoxicity ratio compared to TH-302 in DU145 cells. While, it showed 3-4 fold higher cytotoxicity under normoxic conditions relative to TH-302. This suggests that either 2-NIPAM is more easily activated than TH-302 under normoxic conditions or that the “inactive” form of 2-NIPAM is more cytotoxic than TH-302. Overall, the comparison studies of 2-NIPAM vs controls demonstrate that incorporation of the
alkyne groups into the phosphoramidate structure did not substantially impact the cytotoxicity or hypoxia selectivity of 2-NIPAM. This strongly suggests that 2-NIPAM is able to undergo the same activation mechanism that has already been established for TH-302 (i.e., evofosfamide).\textsuperscript{257} This hypoxia-selective cytotoxicity was also showed to be time-dependent. As expected, the elongated incubation time of cells with hypoxia trapping agents would lower IC\textsubscript{50} values of compounds. Most likely, the enhanced cytotoxicity is a result of the increase in cellular adducts formation.

In the selection of cycloaddition reaction conditions of 2-NIPAM, we carefully take the activity and instability of bromo- and 2-nitroimidazole- groups into consideration. With the success of using Cu(I) to mediate cycloaddition reactions, we decided to use one-pot radiolabeling of 2-NIPAM with I-125 resulting in cycloaddition products with trizole rings. The dilemma of characterizing compound 15 directly was that the MS equipment we have is not sensitive enough to perform MS analysis of radiotracer quantities of it. Also, the labs with high sensitivity MS would not allow us to test radio-labeled compound in their equipment. Because of that, we decided to use HPLC to characterize compound 15 and perform retention time comparison to the non-radioactive analog (compound 14). The hydrophobicity of compound 15 allowed it passive diffused into cells. At the end of 8h efflux studies, it had been showed that 13.6\% and 17.8\% increased retention of compound 15 in DU145 and PC-3 cells under hypoxic conditions. This is likely due to hypoxia activation and adduct formation with intracellular biomolecules.
4.5 CONCLUSION

In conclusion, we have designed and successfully synthesized a novel 2-nitroimidazole phosphoramidate mustard (2-NIPAM), modeled after evofosfamide, that allows easy conjugation of the hypoxia-selective drug to imaging moieties and other constructs through an azide-alkyne cycloaddition reaction. Our interest in 2-NIPAM, and other hypoxia-selective drugs, lies in their potential for selective activation and retention in hypoxic tissues, such as observed with many cancers. We envision that this selectivity can be exploited for the purpose of selectively increasing the retention of diagnostic and therapeutic agents in hypoxic tumor tissues. Our studies demonstrated that 2-NIPAM had similar cytotoxicity and hypoxia-selectivity compared to evofosfamide, establishing that the incorporation of the alkyne functionality did not substantially impact the activity of the agent. Using a synthesized $^{125}$I-labeled 2-NIPAM conjugate, we observed that the analog demonstrated greater cellular retention under hypoxic conditions, suggesting that retention is due to activation and adduct formation with intracellular macromolecules. Given these results, we plan on further investigating the potential of 2-NIPAM alone as a hypoxia diagnostic agent and in conjugation with receptor-targeted agents for cancer imaging and targeted radiotherapeutic applications.
Chapter 5. DISCUSSION AND FUTURE DIRECTION

Over the past several decades, the development of targeted radiopharmaceuticals for diagnostic and therapeutic purposes has thrived. These agents contain a radionuclide(s) and a targeting vehicle, such as a small molecule, peptide or antibody. Thus, these agents are capable of delivering the radioisotope specifically to tumor cells or to the tumor microenvironment. Compared to the conventional systematic chemotherapeutic agents, this approach has the advantage of higher specificity to enhance the tumor-specific targeted delivery and lower non-target tissue accumulation. By now, many targeted radiopharmaceuticals have been successfully used in clinic for different types of cancers, for example, 1-amino-3-$^{18}$F-fluorocyclobutyl-1-carboxylic acid ($^{18}$F-FACBC) is used for diagnosis of prostate cancer and yttrium-90 ibritumomab tiuxetan is utilized for treatment of B-cell lymphoma.

Among those receptors used for the development of targeted radiopharmaceuticals, the gastrin-releasing peptide receptor (BB2r) has been intensively investigated in the past two decades for various solid tumors including prostate cancer. It is estimated that 63-100% of primary prostatic invasion carcinomas has high to very high levels of BB2r. The current standard method to treat early-stage localized prostate cancer since 1904 is prostatectomy. For patients with prostate cancer that has metastasized beyond the prostate capsule surgery is no longer a viable option. Initially, the growth and survival of prostate tumors are dependent on androgens. Patients suffering from advanced, metastatic prostate cancer undergoing androgen ablation therapy can experience the
suspension of prostate cancer progression. However, the cancer eventually is able to bypass its androgen dependence to become androgen-independent allowing the cancer to progress\textsuperscript{24-25, 260}. To date, there are no chemotherapy agents available to successfully treat patients with advanced, androgen-independent prostate tumors. However, more than 50\% of this population of androgen-independent tumors have been shown to be BB2r-positive\textsuperscript{24-26}. Studies have shown that the expression of BB2r in lymph node and bone metastasis of prostate cancer was in 85.7\% and 52.9\% of cases, respectively\textsuperscript{26}. These finding are the impetus for developing BB2r-targeted diagnostic to detect metastatic cancer lesions and therapeutic agents for advanced prostate cancers.

Recently, several radiolabeled (e.g. Ga-68) BB2r-targeted antagonists have shown great potential in ongoing clinical studies with small-cohort of patients for the detection of recurrent prostate tumors\textsuperscript{66-68, 71-72}. These results demonstrated their favorable tumor uptake and image contrast, and feasibility of identifying smaller tumors when compared to standard prostate cancer imaging (CT, MRI, \textsuperscript{18}F-NaF PET/CT, and \textsuperscript{99m}Tc-methylene diphosphonate bone scanning) tracers. Thus, these agents are promising candidates for prostate cancer detection, staging and progression monitor, etc. Nevertheless, further studies in larger patient population are necessary to evaluate the sensitivity of those BB2r-targeted agents in PET/CT and correlation regarding factors like the tumor aggressiveness and tumor size. Meanwhile, the major challenge of radiopharmaceutical based on receptor-avid peptides, low-molecular weight agents, is the rapid plasma clearance and intrinsic instability in protease-rich bioenvironments. As a consequence, their diminished residence time in tumors often limits their clinical translational...
potential, especially for therapeutic purposes.

In chapter 2 of this dissertation, we first compared several biologically variables (e.g., receptor expression, perfusions, etc.) in two popular mouse models, subcutaneous and orthotopic xenograft mouse models. These factors are known to influence drug delivery, thus we tried to evaluate the impact of them on the delivery of BB2r-trageted analogs in vivo. This data, not only provide researchers with possible explanation of the results obtained from mouse model studies, but also reminds them to cautiously chose which model to employ, considering the variance of parameters between the two mouse models.

In chapter 3 and 4, we aimed to address the low tumor retention profile of peptide-based radiopharmaceuticals by incorporating different trapping agents into the receptor-targeted, peptide-based radiotracers. Upon receptor-mediated endocytosis of radio-agents, we expected the trapping agents forming covalent bond with intracellular macromolecules, which leads to significantly higher retention in tumor. This strategy is expected to selectively increase the residence time in tumor tissues along with optimizing tumor-to-non-target ratios. In chapter 3, we selected BB2r-targeted peptide as our targeting vector and used the CC inhibitor, E-64, as a trapping agent for our study. After the radiotracers were internalized through receptor-mediated endocytosis, the conjugated CC inhibitor irreversibly bind to the enriched CCs in endolysosomal compartments through forming thiol-ether linkages. Our studies have demonstrated a substantially increase in the tumor retention and favorable T/NT ratios of radiolabeled
BB2r-targeted peptides in vitro and in vivo. We believe this strategy can grant BB2r-targeted agents a better chance to be translated into the clinic for diagnosis and especially the treatment of advanced prostate cancers.

The second approach examined, as discussed in chapter 4, is to employ the 2-nitroimidazole trapping moiety (2-NIPAM) as trapping agents incorporated into the peptide radiotracer. Theoretically, after the radiotracer undergoes a similar endocytosis process in hypoxic tumor cells, the 2-nitroaimidazole group is activated and irreversibly bind to intracellular nucleophilic macromolecules present in the cell. In the first stage of this study, we examined the cytotoxicity and hypoxia-selectivity of 2-NIPAM. Incorporation of the alkyne functionality into the mustard did not substantially impact the activity of 2-NIPAM. The synthesized $^{125}$I-labeled 2-NIPAM conjugate demonstrated greater cellular retention under hypoxic conditions, suggesting that retention is due to activation and adduct formation with intracellular macromolecules. Future studies including incorporation of 2-NIPAM into BB2r-targeted analogs and in vitro/in vivo evaluation of radioconjugates are needed.

**Future Directions**

1. **Synthesis of 2-NIPAM incorporated BB2r-targeted analogues.**

   Recently, the activated prodrug of 2-NIPAM, TH-302 has been utilized in several clinical studies targeting solid tumor 261. These studies encourage us to further use 2-NIPAM as hypoxia trapping agents, considering the fact that they share the same intracellular reduction mechanism. As we already demonstrated the hypoxia selectivity
of 2-NIPAM, and its ability to participate in click-chemistry to a compound with azide moiety, we plan to incorporate it with a BB2r-targeted peptide backbone. This strategy aims at enhancing the BB2r-positive tumor uptake of the radiotracer. Thus, matching controls will be synthesized and evaluated with 2-NIPAM conjugated analogs side-by-side in our following studies.


We previous used hydrophobic cysteine cathepsin inhibitors as our trapping agent to improve the tumor uptake of receptor-targeted peptide analogues. As a result, the analogues demonstrated high renal and liver uptake of the tracer, which persuaded us to investigate the impact of total hydrophilicity of the radiotracer on the uptake of non-target tissues. Based on our unpublished data, the increased hydrophilicity dramatically decreases liver and kidney uptake of the tracer. Among all the hydrophilic candidates, we choose to use E-64 as our model CC inhibitor as discussed earlier. On top of eliminating liver uptake, we successfully demonstrated the strategy of using E-64 to enhance tumor retention and attenuate the kidney uptake in BB2r-positive prostate cancer mouse model.

In the future, we intend to demonstrate the applicable potential of this strategy in the development of other receptor/small molecule-based tumor-targeted radiotracers. 1) We’ve chosen the somatostatin receptor as our next target and the in vitro evaluation is currently underway. 2) Further investigation of the impact of linker and chelator on the
tumor uptake and T/NT ratio will also be examined. 3) Meanwhile, since our conjugated E-64 derivative BB2r-antagonist showed considerable tumor retention with the most ideal T/NT ratios, especially the tumor-to-kidney ratio, we plan to explore further its therapeutic translation potential. To do so, the human dosimetry estimates based on our mouse biodistribution data will be calculated first. Furthermore, the maximum tolerated dose (MTD) of BB2r-targeted antagonist will be generated in a non-tumor bearing mouse model.


Subcutaneous mouse model is commonly used in preclinical studies, having advantages due to low cost and faster tumor generation time. On the other hand, this model also has limitation, such as providing insufficient reliably clinically relevant data, for example, lack of a clinically relevant oncological microenvironment and less power in predicting drug responses in humans. Thus, we plan to first use subcutaneous model for our initial pro-drug screen. Meanwhile, with the need to overcome the above drawbacks with this model, we have the intention to use orthotopic and patient derived tumor xenografts mouse models to test our leading compounds. The purpose of this is to further elucidate the in vivo behavior of our radio-tracers, thereby essentially increasing the clinical translation potential of these agents.
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