Development of Hypoxia Trapping Enhanced BB2R-Targeted Radiopharmaceutics for Prostate Cancer

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DEVELOPMENT OF HYPOXIA TRAPPING ENHANCED BB2R-TARGETED RADIOPHARMACEUTICS FOR PROSTATE CANCER

by

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A DISSERTATION

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DEVELOPMENT OF HYPOXIA TRAPPING ENHANCED BB2R-TARGETED RADIOPHARMACEUTICS

FOR PROSTATE CANCER

Zhengyuan Zhou, Ph.D.

University of Nebraska, 2015

Supervisor: Jered C. Garrison, Ph.D.

ABSTRACT

The Gastrin-Releasing Peptide Receptor (BB2r) has been investigated as a diagnostic and therapeutic target for prostate and other cancers due to the high expression level on neoplastic relative to normal tissues. A variety of BB2r-targeted agents have been developed utilizing the bombesin(BBN) peptide, which has shown nanomolar binding affinity to human BB2r. However, as with most of the low-molecular weight, receptor-targeted drugs, a major challenge to clinical translation of BB2r-targeted agents is the low retention at the tumor site due to intrinsically high diffusion and efflux rates. Our laboratory seeks to address this deficiency by developing synthetic approaches to selectively increase retention of BB2r-targeted agents in prostate cancer. Hypoxic regions commonly exist in prostate tumors and many other cancers due to a chaotic vascular architecture which impedes delivery of oxygen. In this dissertation, we explore the incorporation of nitroimidazoles, a hypoxia-selective prodrug which irreversibly binds to intracellular nucleophiles in hypoxic tissues, into the BB2r-targeted agent paradigm. We seek to determine if these agents can increase the long-term retention in the tumor and thereby increase efficacy and clinical potential of BB2r-targeted agents.

To that end, we have developed several generations of hypoxia trapping enhanced BBN analogs. Our first in vitro investigation of hypoxia-enhanced $^{111}$In-labeled BBN conjugates demonstrated
significantly improved retention in hypoxic PC-3 human prostate cancer cells. However, it was determined that the proximity of the 2-nitroimidazole relative to the pharmacophore had a detrimental impact on BB2r binding affinity. To address the problem, our next generation of radioconjugates contained an extended linker to eliminate steric inhibition. The new design demonstrated substantially improved binding affinity and lower clearance rate of the 2-nitroimidazole containing radioconjugates under hypoxic conditions. In vivo biodistribution studies using a PC-3 xenograft mouse model revealed significant tumor retention enhancement. Further work is needed to clarify the mechanisms of cellular retention and to correlate the tumor hypoxia burden with the retention efficacy.
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<th>Description</th>
</tr>
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<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>HEPES</td>
<td>4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid</td>
</tr>
<tr>
<td>BSA</td>
<td>Bovine serum albumin</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-NIEA</td>
<td>2-nitroimidazole ethyl acetate</td>
</tr>
<tr>
<td>Se-Met</td>
<td>Selenomethionine</td>
</tr>
<tr>
<td>B\textsubscript{max}</td>
<td>Total receptor number</td>
</tr>
<tr>
<td>K\textsubscript{d}</td>
<td>Dissociation constant</td>
</tr>
<tr>
<td>HEF</td>
<td>Hypoxia enhancement factor</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Definition</td>
</tr>
<tr>
<td>--------------</td>
<td>------------</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>iv</td>
<td>Intravenous</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>PEG</td>
<td>Polyethylene glycol</td>
</tr>
<tr>
<td>ICAM-1</td>
<td>Intercellular adhesion molecule 1</td>
</tr>
<tr>
<td>SCLC</td>
<td>Small-cell lung cancer</td>
</tr>
<tr>
<td>FAK</td>
<td>Focal adhesion kinase</td>
</tr>
<tr>
<td>MAPK</td>
<td>Mitogen-activated protein kinase</td>
</tr>
<tr>
<td>NEP</td>
<td>Neutral endopeptidase</td>
</tr>
<tr>
<td>PA</td>
<td>Phosphoramidon</td>
</tr>
<tr>
<td>Ava</td>
<td>5-aminopentanoic acid</td>
</tr>
<tr>
<td>Azp</td>
<td>Azidoproline</td>
</tr>
<tr>
<td>PSMA</td>
<td>Prostate-specific membrane antigen</td>
</tr>
<tr>
<td>SCID</td>
<td>Severely combined immunodeficient</td>
</tr>
<tr>
<td>IC&lt;sub&gt;50&lt;/sub&gt;</td>
<td>half maximal inhibitory concentration</td>
</tr>
<tr>
<td>8-Aoc</td>
<td>8-Aminooctanoic acid</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>-----</td>
<td>----------</td>
</tr>
<tr>
<td>In</td>
<td>Indium</td>
</tr>
</tbody>
</table>
Chapter 1 INTRODUCTION: RADIOLABELED BOMBESIN ANALOGS FOR PROSTATE CANCER IMAGING

1.1 INTRODUCTION

The gastrin releasing peptide receptor (GRPr), also known as BB2 receptor (BB2r), has widespread distribution in the central nervous system (CNS) and gastrointestinal (GI) tract and mediates a variety of biological activities including modulation of memory [1], emotional arousal [2], stimulation of gastrin release and pancreatic secretion [3]. During the past 30 years, the BB2r has drawn great attention as a diagnostic and/or therapeutic target due to its overexpression in various cancers such as pancreatic, glioblastoma, prostate (PCa), breast, colorectal, and small-cell lung (SCLC). The growth of tumors and development of metastases depends on angiogenesis. The activation of overexpressed BB2r has been demonstrated to trans-activate epidermal growth factor receptor (EGFR) and induce mitogen-activated protein kinase (MAPK) phosphorylation, which results in proliferation of cancer cells [4, 5]. For both colon and prostate cancer, the BB2r activation can also up-regulate intracellular adhesion molecule (ICAM)-1 and stimulate focal adhesion kinase (FAK) thus facilitating cell attachment to extracellular matrix and promoting cell motility [6]. To date, overexpression of BB2r has been reported in 85 to 100% of SCLC, 38 to 72% of breast cancer, 72 to 85% of glioblastoma and 62 to 100% of prostate cancer samples [7]. These findings have given the impetus for the development of targeted theranostic agents for BB2r-positive cancers.

In 1989, peptide-based radiopharmaceuticals were first introduced into the clinic for localization of endocrine-related tumors using radioiodinated somatostatin analogs [8]. FDA-approved receptor-targeted radiopeptide $^{111}$In-DTPA$^6$-octreotide (OctreoScan) has been widely used for initial staging or re-evaluation of patients with neuroendocrine tumors. Due to their
small size and hydrophilic nature, radiolabeled peptides usually exhibit favorable pharmacokinetics including fast targeting, rapid plasma clearance and deep tumor penetrating properties. To date, a variety of radionuclides, such as $^{18}$F, $^{99m}$Tc, $^{111}$In, $^{188}$Re, $^{90}$Y, $^{64}$Cu, $^{177}$Lu, $^{44}$Sc and $^{68}$Ga, have been used in the design of diagnostic and therapeutic BB2r-targeted agents [9-17]. For those radioconjugates utilizing a radiometal, bifunctional chelating agents are used to obtain in vivo stable complexes. The bifunctional chelate design consists of four components: a radioisotope, a chelator, a linker and a targeting vector. To connect the chelated radioisotope and the targeting vector, the linker is introduced to not only prevent the radiometal-chelator complex from sterically inhibiting the binding affinity of the targeting vector, but also plays an important role in altering the hydrophilicity and optimizing the biodistribution characteristics of the radioconjugate. Amino acids, aromatic derivatives, aliphatic hydrocarbons, and polyethylene glycol (PEG) are the most utilized linking chemistries for BB2r-targeted agents. Bombesin (BN), a 14 amino acid amphibian peptide, shares the C-terminal binding sequence (Trp-Ala-Val-Gly-His-Leu-Met-NH$_2$) with the Gastrin Releasing Peptide (GRP, endogenous ligand of BB2r). It has been shown that these seven amino acids are responsible for the nanomolar BB2r-binding affinity for both BN and GRP (Figure 1.1). A diversity of BN- and GRP-like peptides (entire, truncated or modified) have been utilized for development of BB2r-targeted analogs [13, 18, 19]. To date, BN (7-14)NH$_2$ analogs have been the most frequently investigated BN agonist. Agonists not only bind to the receptor, but also activate the receptor to induce internalization thereby enhancing residence time in the BB2r-positive cell/tissue. Until recently, most BN radiotracers utilized for molecular imaging and therapy have mainly been agonists due to the belief that the internalization process provides higher tumor accumulation. However, recent findings suggest that some BN antagonists, which internalize poorly into BB2r-positive tissues, have superior in vivo tumor uptake and, surprisingly, substantially better retention relative to most, if not all,
reported agonists [20]. While the mechanism of BN antagonist retention is not yet fully understood, it is clear, based on the number of literature reports, that researchers have been devoting substantially more effort in the last few years to develop BN antagonists, which typically exhibit higher metabolic stability and low receptor dissociation rates [20-22].
Figure 1.1 Chemical structure of Gastrin-releasing peptide and bombesin peptide

<table>
<thead>
<tr>
<th>Peptide Type</th>
<th>Sequence</th>
<th>BB2 receptor binding region</th>
</tr>
</thead>
<tbody>
<tr>
<td>Gastrin-releasing</td>
<td>Lys-Ala-Leu-Val-Thr-Gly-Gly-Gly-Val-Ser-Val-Pro-Ala-His</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Met-Tyr-Pro-Arg-Gly-Asn-His</td>
<td>Trp-Ala-Val-Gly-His-Leu-Met-NH₂</td>
</tr>
<tr>
<td>Bombesin Peptide</td>
<td>Pyr-Gln-Arg-Leu-Gly-Asn-Gln</td>
<td>Trp-Ala-Val-Gly-His-Leu-Met-NH₂</td>
</tr>
</tbody>
</table>
An ideal peptide based radiotracer is characterized by rapid and specific targeting capability, high binding affinity, high metabolic stability and high target-to-background ratios. To meet these chemical, biological and pharmacokinetic characteristics; structure-activity investigations are often carried out to optimize binding affinity, stability, hydrophilicity, tumor uptake and retention and non-target tissue clearance. In this review, we focus on summarizing the literature of the past five years, particularly those studies that shed light on the structure-activity relationships that pertain to metabolic stability, N-terminal charges, and hydrophilicity. We also discuss multimerization/hybrid design and summarize results from clinical studies of BN agonist/antagonist that have been reported in the past five years.

1.2 METABOLIC STABILITY

Based on in vitro studies, BN analogs usually exhibit good human serum stability considering its fast targeting pharmacokinetics. However, it has been recently found that metabolism kinetics determined from in vitro serum studies of a radiolabeled peptide have poor correlation with the in vitro human blood stability or in vivo stability results [21, 23]. A recent clinical study conducted by Ananias et al. using $^{99m}$Tc-HYNIC(tricine/TPPTS)-Aca-Bombesin(7-14)NH$_2$ ($^{99m}$Tc-HABBN) failed to detect prostate cancer lesions in patients; this was mainly attributed to the unexpected low in vivo stability [24]. Less than 20% of the administered peptide was intact after 30 min, which is significantly lower than the in vitro human serum stability results (77% intact peptide after 24 h incubation). These findings strongly suggest the need to develop BN analogs with higher metabolic stability for successful clinical translation.

Replacement of L-amino acid with un-natural or modified amino acid has been a widely used strategy to prevent the proteolytic cleavage of radiolabeled peptides [25]. Recently, Marsouvanidis et al. reported the synthesis and comparative evaluation of five single-Gly$^{24}$ or
double-Gly\textsuperscript{24}/Met\textsuperscript{27} substituted GRP(18-27) radiotracers [13](Figure 2). Gly\textsuperscript{24} substitution by dAla (SARNC2), βAla(SARNC5), or Sar (SARNC6) were designed for higher metabolic stability and Met\textsuperscript{27} substitution with either Nle (SARNC3 dAla\textsuperscript{24}/Nle\textsuperscript{27}) or Leu (SARNC4 dAla\textsuperscript{24}/Leu\textsuperscript{27}) were designed to prevent the formation of methionine sulfoxide which has been proven to substantially reduce biological activity [26]. In vitro competitive binding studies revealed that \textsuperscript{99}Tc-SARNC5 had the highest binding affinity followed by \textsuperscript{99}Tc-SARNC6, \textsuperscript{99}Tc-SARNC2, \textsuperscript{99}Tc-SARNC3 and \textsuperscript{99}Tc-SARNC4. Unlike the IC\textsubscript{50} results, the least competitive \textsuperscript{99}Tc-SARNC4 showed the highest in vivo stability followed by \textsuperscript{99}Tc-SARNC6, \textsuperscript{99}Tc-SARNC2, \textsuperscript{99}Tc-SARNC5 and \textsuperscript{99}Tc-SARNC3. However, in vivo biodistribution studies demonstrated neither the analog with highest receptor affinity (SARNC5) or metabolic stability (SARNC4) alone lead to the most favorable in vivo profile. Instead, \textsuperscript{99}Tc-SARNC6, which had both high BB2r affinity and metabolic stability, exhibited the most desirable in vivo profile in terms of tumor uptake and tumor-to-non-target tissue ratio (Table 1.1).
Figure 1.2 Chemical structures of SARNCs
SARNC1-X^{24}/Y^{27} = Gly^{24}/Met^{27}; SARNC2-dAla^{24}/Met^{27}; SARNC3-dAla^{24}/Nle^{27}; SARNC4-Ala^{24}/Leu^{27}; SARNC5-βAla^{24}/Met^{27}; and SARNC6-Sar^{24}/Met^{27}
Table 1.1: In vitro and in vivo data of SARNC conjugate

<table>
<thead>
<tr>
<th></th>
<th>Blood</th>
<th>Muscle</th>
<th>Kidney</th>
<th>Tumor-to-non-target tissue ratio</th>
<th>Tumor uptake (5 min p.i.)</th>
<th>% of intact peptide (5 min p.i.)</th>
<th>IC50 (nM)</th>
<th>% of intact peptide</th>
<th>Tumor uptake (5 min p.i.)</th>
</tr>
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<tbody>
<tr>
<td>SARNC6</td>
<td>1.78</td>
<td>0.32</td>
<td>6.96</td>
<td>2.15</td>
<td>42%</td>
<td>6.96 ± 2.15</td>
<td>1.78</td>
<td>0.32</td>
<td>6.96 ± 2.15</td>
</tr>
<tr>
<td>SARNC5</td>
<td>42%</td>
<td>32%</td>
<td>2.73</td>
<td>0.78</td>
<td>45%</td>
<td>2.73 ± 0.78</td>
<td>42%</td>
<td>32%</td>
<td>2.73 ± 0.78</td>
</tr>
<tr>
<td>SARNC4</td>
<td>1.7%</td>
<td>35%</td>
<td>1.81</td>
<td>0.20</td>
<td>17%</td>
<td>1.81 ± 0.20</td>
<td>1.7%</td>
<td>35%</td>
<td>1.81 ± 0.20</td>
</tr>
<tr>
<td>SARNC3</td>
<td>2.96</td>
<td>1.33</td>
<td>164.75</td>
<td>25.86</td>
<td>17%</td>
<td>164.75 ± 25.86</td>
<td>2.96</td>
<td>1.33</td>
<td>164.75 ± 25.86</td>
</tr>
<tr>
<td>SARNC2</td>
<td>2.03</td>
<td>1.06</td>
<td>164.75</td>
<td>25.86</td>
<td>17%</td>
<td>164.75 ± 25.86</td>
<td>2.03</td>
<td>1.06</td>
<td>164.75 ± 25.86</td>
</tr>
<tr>
<td>SARNC1</td>
<td>1.66</td>
<td>0.12</td>
<td>1.17</td>
<td>0.69</td>
<td>17%</td>
<td>1.17 ± 0.12</td>
<td>1.66</td>
<td>0.12</td>
<td>1.17 ± 0.12</td>
</tr>
</tbody>
</table>

Table 1.1: In vitro and in vivo data of SARNC conjugate.
The amide bond in the backbone of peptides is susceptible to protease degradation. Studies have been designed to replace the trans-amide bond by a 1,2,3-triazole isostere due to their similar size, planarity, H-bonding capabilities, and dipole moment [27][Figure 1.3]. This replacement has been recently reported to be able to enhance proteolytic stability of radiolabeled peptides while maintaining receptor binding affinity and cell-internalization properties. Valverde et al. synthesized a series of $^{177}\text{Lu-DOTA-PEG}_4$-[Nle(14)]BBN(7-14)NH$_2$ based peptidomimetics with amide to triazole substitutions [28][Table 1.2]. In vitro blood serum studies revealed up to 20-fold higher stability of all backbone-modified radiotracers except for compound 2 and 10. For compound 3, 4, 6 and 8-10, the triazole substitutions completely ruined the BB2r binding affinity. Compound 5 and 7 exhibited identical BB2r avidity and internalization properties relative to the control compound 1, but superior metabolic stability (>3-fold half-life). In vivo studies using compound 5 confirmed enhancement of high proteolytic stability on improved tumor uptake. It’s interesting to note that the same strategy failed to apply on BN antagonist JMV594 (DPhe-Gln-Trp-Ala-Val-Gly-His-Sta-Leu-NH$_2$) which resulted in total loss of BB2r avidity [29]. These data suggest that the binding conformation and/or the binding site of the BN agonist and antagonist may be different.
Figure 1.3 Scheme of amide-to-triazole substitutions
<table>
<thead>
<tr>
<th>Compound Structure</th>
<th>Half-life/h</th>
<th>% Internalization after 4 h</th>
<th>KD/nM</th>
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<tr>
<td>[177Lu]DOTA-PEG$_4$-Gln-Trp-Val-Gly-His-Leu-Nle-NH$_2$</td>
<td>10</td>
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<td>7</td>
<td>2.0 ± 0.6</td>
<td>2.0 ± 0.6</td>
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<tr>
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<tr>
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<td>3</td>
<td>60</td>
<td>60</td>
</tr>
<tr>
<td>[177Lu]DOTA-PEG$_4$-Gln-Trp-Val-Gly-His-Leu-Nle-NH$_2$</td>
<td>2</td>
<td>60</td>
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<tr>
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<td>2</td>
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<tr>
<td>[177Lu]DOTA-PEG$_4$-Gln-Trp-Val-Gly-His-Leu-Nle-NH$_2$</td>
<td>2</td>
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<tr>
<td>[177Lu]DOTA-PEG$_4$-Gln-Trp-Val-Gly-His-Leu-Nle-NH$_2$</td>
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<tr>
<td>[177Lu]DOTA-PEG$_4$-Gln-Trp-Val-Gly-His-Leu-Nle-NH$_2$</td>
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<td>2</td>
<td>60</td>
<td>60</td>
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</table>

Table 1.2: Structures and biological properties of compounds 1–10.
It is well known that PEGylation can improve pharmacokinetics and pharmacodynamics of therapeutic molecules by increasing hydrophilicity and protection from enzymatic degradation [30]. Däpp et al. developed a PEGylated DOTA-[Cha13,Nle14]BN(7–14)NH₂ analogue by incorporating a 5 kDa PEG molecule to the ε-amino group of a β₃hLys-βAla-βAla spacer between the targeting peptide and the DOTA chelator [31]. (Figure 1.4). Compared to the non-PEGylated control, the PEGylated analogue exhibited a remarkable increase in protease stability with a 5 fold higher half-life in human plasma. However, the PEGylated analogue showed a significant lower internalization rate (internalized fraction: 3% versus 30%) and reduced binding affinity (16 nM versus 2 nM) relative to the non-PEGylated analogue. These results agree with, as reported earlier, not only protecting the targeting vector from enzymatic recognition, the PEGylation can also jeopardize the binding affinity and kinetics [30, 32]. Nonetheless, the PEGylated analogue exhibited 2-fold higher tumor uptake which might at least partially be attributed to its higher enzymatic stability.
Figure 1.4 Chemical structure of DOTA-Lys-BN and DOTA-PEG$_{5k}$-Lys-BN analogues
Besides direct chemical modification on the radiotracer itself, co-injection with a particular enzyme inhibitor provides an alternative way of reducing the in vivo metabolic rate of radiolabeled peptides. Neutral endopeptidase (NEP), a membrane-bound zinc-containing metallopeptidase, is expressed in a wide variety of tissues and cleaves peptides at the amino side of hydrophobic amino acid [33]. It has been demonstrated that the NEP acts as a tumor suppressor which can limit the proliferation of malignant pulmonary neuroendocrine cells by hydrolyzing bombesin-like peptides [34]. Phosphoramidon (PA) is a natural and potent inhibitor of NEP with Ki value of 2 nM. PA has been widely used to investigate the neuroprotective role of the NEP in Alzheimer’s disease with regard to the degradation of Aβ peptides [35]. To explore the potential of PA co-administration to enhance the proteolytic resistance of BN analogs, Nock et al. co-injected PA with radiolabeled somatostatin, gastrin, and bombesin analogues in a variety of tumor xenograft mouse models [36]. Surprisingly, the amount of intact radiopeptide in the mouse circulation after the injection of PA increased from <2% to 86%, 12% to 80%, and < 5% to 70% at 5 min post injection (p.i.). The resulting tumor uptake impressively escalated from < 1% to 14%, < 4% to 21%, and 2% to > 15% ID/g. Similarly, Marsouvanidis et al. co-injected [177Lu]DOTA-GRP(13-27) with PA in a PC3 xenograft mouse model [37]. In vivo stability studies revealed that with PA co-injection 63% of the initial dose in the blood remained intact at 5 min post-administration, whereas only ~ 30% remained intact without PA co-administration. The co-injection group also exhibited much higher in vivo tumor uptake (4.01% ID/g) than the radiotracer alone group (1.26% ID/g). Compared to strategies that involve the replacement of amino acids or the addition of side chains, which often results in lowered binding affinities or poorer biodistribution profiles, the strategy of co-injection of GRP/BN analogs with an enzyme inhibitor appears to be an attractive option to increase metabolic stability and in vivo targeting. As a consequence, the authors find it likely that this approach will gain more attention from
researchers as it continues to be exploited with GRP/BN analogs and other peptidic radiotracers. However, the safety of using endopeptidase inhibitors in humans needs to be further evaluated and scrutinized before investigators rely on this adjuvant as part of formulations for future clinical translation.

1.3 IMPACT OF N-TERMINAL CHARGES ON BINDING AFFINITY

It has been previously observed that modification of the C-terminal portion of BN/GRP analogs profoundly impacts biological activity as well as the fact that amidation of the C-terminal end is required to achieve high avidity [38]; whereas the N-terminus of the peptide pharmacophore can typically be substantially altered to obtain favorable characteristics without significantly impacting binding affinity. However, one exception to that tenet appears to be N-terminal modifications that alter the charge state of the peptide. Studies have shown, for the most part, that incorporation of positive charge(s) at the N-terminus often led to analogs with higher BB2r affinity and increased in vivo tumor uptake [39]. Over the past few years, multiple reports have demonstrated this principle through utilization of standard and non-standard basic amino acid groups and use of charge offsets between the selected radioisotope and chelator.

Marsouvanidis et al. designed four $^{111}$In-labeled DOTA-GRP(13–27) \([\text{DOTA-Lys-Met-Tyr-ProArg-Gly-Asn-His-Trp-Ala-Val-Gly-His-Leu-Met-NH}_2]\) analogs with selected truncation site at position 13($^{111}$In-1), 14($^{111}$In-2), 17($^{111}$In-3), and 18($^{111}$In-4) of the human 27-mer GRP sequence [18]. $^{111}$In-1 contains both basic amino acid Lys$^{13}$ and Arg$^{17}$ while $^{111}$In-2 and $^{111}$In-3 contain only Arg$^{17}$, and $^{111}$In-4 contains neither of the positively charged amino acids (Figure 1.5). The in vitro competitive binding studies revealed that the truncation, which reduced the peptide chain length and reduced the N-terminal charge, had a significant impact on receptor affinity: $^{111}$In-1 ($IC_{50} = 2.3 \pm 0.2 \text{ nM}$) > $^{111}$In-2 ($IC_{50} = 6.6 \pm 0.9 \text{ nM}$) > $^{111}$In-3 ($IC_{50} = 19.2 \pm 2.9 \text{ nM}$) >> $^{111}$In-4 ($IC_{50} =
112 ± 16 nM. Highly correlated with in vitro results, the \(^{111}\text{In-1}\), which has the best binding affinity, showed the highest initial in vivo tumor uptake (7.74 ± 1.93% ID/g at 4 h p.i.) whereas \(^{111}\text{In-4}\) which has the poorest binding affinity exhibited the lowest tumor uptake (3.54 ± 1.09% ID/g at 4 h PI). However, the favorable in vivo profile of \(^{111}\text{In-1}\) is completely compromised by high kidney uptake (46.02 ± 3.73% ID/g at 4 h PI) and retention (29.00 ± 4.86% ID/g at 24 h PI) which is 8 to 16-fold higher than other analogs. Based on previous knowledge, the renal retention of \(^{111}\text{In-1}\) is at least partially due to the positively charged Lys\(^{13}\) which enhanced the binding to and re-uptake by the negatively charged, multi-ligand receptor megalin, located in the renal proximal tubules [40].
Figure 1.5 Molecular structures of $^{111}$In-1 and corresponding sequences of $^{111}$In-2, $^{111}$In-3, and $^{111}$In-4
In the bifunctional chelate design of BB2r-targeted analogs, the linking group is utilized not only to chemically connect the targeting vector and radiometal-chelator complex, but also serves as a convenient handle to fine tune the hydrophilicity and pharmacokinetics without structural alterations to the pharmacophore and/or radiometal-chelator complex. To increase the BB2r binding affinity and tumor uptake, Mansi et al. reported an $^{111}$In/$^{68}$Ga labeled BN antagonist RM2 ([D-F$_3$Phe$^6$, Ala$^{111}$]BN(6-13)OMe) utilizing a positively charged spacer (4-amino-1-carboxymethyl-piperidine) [41]. Compared with their previous analog $^{68}$Ga-RM1[20], which conjugated the same peptide but through a neutral glycine-4-aminobenzoyl spacer, the $^{68}$Ga-RM2 showed a 1.5-fold higher binding affinity towards the BB2r (Figure 1.6). However, the increased affinity did not substantially impact in vivo tumor uptake, little to no enhancement was observed in terms of tumor-to-non-target tissue ratio. On the contrary, the negatively charged linkers show a superior in vitro and in vivo profile than positively charged linkers in some cases. Mu et al. reported a series of $^{18}$F-labeled BN antagonists with different linking groups [42]. Compared to positively charged Arg-Ava(Arginine-5-aminopentanoic acid) linker, the negatively charged Ala(SO$_3$H)-Ava((R)2-amino-3-sulphopropanoic acid-5-aminopentanoic acid) not only improved the binding affinity by 6-fold, but also showed significantly better biodistribution characteristics in terms of tumor uptake and tumor-to-non-target tissue ratios.
Figure 1.6 Chemical structures of $^{111}$In-RM1 and $^{111}$In-RM2
To introduce an N-terminal positive charge, another option is to use differences in charge offset between the radiometal and the chelator. Several studies have been published in the past few years that were designed to investigate the potential of using bifunctional chelators to serve that purpose. Varasteh et al. synthesized four $^{68}$Ga-labeled BN antagonists (D-Phe-Gln-Trp-Ala-Val-Gly-His-Sta-Leu-NH$_2$, RM26) with different chelators (NOTA, DOTA, NODAGA, DOTAGA) [43]. The resulting local charge of the Ga$^{3+}$ complex with the di-anionic NOTA, tri-anionic NODAGA and DOTA, and tetra-anionic DOTAGA is +1, 0, 0 and −1, respectively. As predicted, the positively charged nat$^{68}$Ga-NOTA-PEG$_2$-RM26 exhibited the highest BB2r binding affinity (2.3 ± 0.2 nM), followed by two neutral analogs nat$^{68}$Ga-NODAGA-PEG$_2$-RM26(3.0 ± 0.2 nM) and nat$^{68}$Ga-DOTA-PEG$_2$-RM26(2.9 ± 0.2 nM), and the negatively charged nat$^{68}$Ga-DOTAGA-PEG$_2$-RM26 gave the lowest binding affinity (10 ± 0.6 nM). These in vitro BB2r-affinity studies correlated with higher in vivo tumor uptake and tumor-to-non-target organ ratios (Table 1.3). Similarly, Gourni reported a study of $^{68}$Ga/$^{64}$Cu-NOTA/NODAGA complex labeled BN antagonist MJ9 (D-Phe-Gln-Trp-Ala-Val-Gly-His-Sta-Leu-NH$_2$) [44]. The residual charge of Ga$^{3+}$ when complexed with di-anionic NOTA and tri-anionic NODAGA is +1 and 0, respectively. Correspondingly, the bivalent Cu$^{2+}$ forms a neutral (0) and a negative (−1) charge complexed with NOTA and NODAGA. With the +1 positive charge on the N-terminus, nat$^{68}$Ga-NOTA-MJ9 exhibited the highest affinity (0.5 ± 0.1 nM), followed by the two neutral analogs (2.1 ± 0.3 and 1.0 ± 0.1 nM), whereas the negatively charged nat$^{68}$Cu-NODAGA-MJ9 gave a 10-fold lower binding affinity (5.3 ± 2.5 nM). This performance trend extended into preclinical studies with nat$^{68}$Ga-NOTA-MJ9 demonstrating superior in vivo behavior with greater tumor uptake and better tumor-to-non-target organ ratios relative to the neutral and negatively charged radiometal-chelator systems (Table 1.2).
<table>
<thead>
<tr>
<th></th>
<th>Data Represent at 1 h p.i.</th>
<th>Data Represent at 2 h p.i.</th>
<th>Data Represent at 24 h p.i.</th>
</tr>
</thead>
<tbody>
<tr>
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<td>7.3 ± 1.6</td>
<td>3.0 ± 0.2</td>
</tr>
<tr>
<td>b</td>
<td>14.3 ± 1.5</td>
<td>11.9 ± 0.8</td>
<td>4.6 ± 1.7</td>
</tr>
<tr>
<td>c</td>
<td>6.5 ± 0.9</td>
<td>3.9 ± 0.7</td>
<td>2.3 ± 0.9</td>
</tr>
<tr>
<td>d</td>
<td>1.7 ± 0.5</td>
<td>2.0 ± 0.7</td>
<td>1.5 ± 0.3</td>
</tr>
<tr>
<td>e</td>
<td>5.5 ± 0.3</td>
<td>5.5 ± 0.2</td>
<td>2.5 ± 0.6</td>
</tr>
</tbody>
</table>

**Table 1.3** In vitro and in vivo data of different radiometal/chelator complexes.
1.4 INCREASING HYDROPHILICITY FOR IMPROVED NON-TARGET TISSUE CLEARANCE

For both diagnostic and therapeutic agents, high target-to-non-target organ ratios are needed to ensure appropriate imaging contrast and/or therapeutic index. High BB2r-avidity usually leads to favorable in vivo tumor uptake of BN/GRP analogs. However, tumor uptake is only part of the equation when developing a clinically viable agent. Clearance of the tracer from non-target tissues is equally important. As a consequence, the hydrophilicity of the agent plays a crucial role in balancing the blood circulation time needed for optimal tumor targeting with the rapid and efficient clearance from non-target organs. Generally, hydrophilic radiotracers are preferred due to their rapid washout from the abdominal cavity versus more hydrophobic radiotracers, which often show high liver uptake resulting in unwanted hepatobiliary clearance. Correlation of distribution coefficients (logD) and in vivo biodistribution characteristics indicates that logD values below -1.5 are required to avoid high hepatobiliary uptake [45]. Using the bifunctional chelate design, the water solubility of the BN/GRP analogs can be easily optimized by altering the composition of the linking group to include hydrophobic or hydrophilic constituents.

The impact of different lengths of aliphatic hydrocarbon linkers on pharmacokinetic effects of BN analogs has been thoroughly investigated previously [46, 47]. Recently, a series of $^{64}$Cu-NO2A-(X)-BN(7-14)NH$_2$ analogs with increasing lengths of aliphatic linkers (X=β-Ala, 5-Ava, 6-Ahx, 8-Aoc, 9-Anc) and rigid aromatic linker AMBA were synthesized by Lane et al. [48] (Figure 1.7). Roughly based on the HPLC retention time, the hydrophilicity of these BN analogs are ranked as follow: β-Ala > AMBA ≈ 5-Ava ≈ 6-Ahx > 8-Aoc > 9-Anc. With identical in vitro binding affinities to the BB2r, these analogs exhibited different in vivo kinetics which correlated with their hydrophilicity/hydrophobicity character. Higher hepatobiliary uptake was observed with increasing hydrophobicity on healthy CF-1 mice at 1 h p.i. Of the tested radioconjugates with
AMBA, 6-Ahx, 8-Aoc and 9-Anc linkers on a PC-3 tumor bearing mouse model, conjugates with the shorter and more hydrophilic AMBA linker showed the highest tumor-to-liver ratio followed by conjugates with the 6-Ahx linker. Conjugates with the most hydrophobic 9-Anc linker showed unfavorable significant amounts of abdominal accumulation.
Figure 1.7 Chemical structures of glycine-4-aminobenzoyl, β-Ala, 5-Ava, 6-Ahx, 8-Aoc, and 9-Anc linkers
In recent years, PEGs have been utilized more frequently as a linking group moiety. Advantageously, PEGs are able to reduce the hepatic uptake of radiotracers due to their hydrophilic nature [49]. The influence of short PEG spacers of various lengths on BN analogs has been investigated by Jamous et al. [50]. A series of $^{177}$Lu-labeled BN antagonists utilizing the JMV594 pharmacophore (MJ[1-4]) with PEG spacers of different lengths (n = 2, 4, 6, 12) were synthesized and evaluated both in vitro and in vivo. With increasing length of PEG linker, these conjugates showed slightly increased hydrophilicity (LogD range from -1.95 to -2.22). All conjugates demonstrated similar BB2r binding affinity with IC$_{50}$ values ranging from 6.2 to 9.9 nM except for the conjugate with the PEG$_{12}$ linker which exhibited significant lower binding affinity (IC$_{50}$ = 15.0 ± 5.3 nM). These results agree with a previously published study, using a 3500 Da PEG spacer, that showed the flexible long PEG chain might mask the receptor binding site and subsequently impair the binding affinity [51]. Of the tested conjugates, the conjugate with PEG$_6$ linker showed the highest clearance rate from pancreas, liver and kidney in PC-3 tumor-bearing mice.

1.5 MULTIMERIZATION

In nature, multimerization of biomolecules, such as DNA-DNA duplex formation and antibody recognition, are used to increase binding affinity and target specificity. It has been shown that the multimerization of therapeutic drugs and diagnostic agents are able to achieve this using two distinct mechanisms, enriching the local drug concentration and cooperative binding to two or more receptors simultaneously through the polyvalency effect [52, 53]. If the distance between different targeting ligands does not permit multiple binding events, the number of ligands in the vicinity of the neighboring binding sites would be correspondingly
enriched; if the distance between targeting ligands matches the receptors’ density on the cell surface, the ligands would bind to multiple receptors at the same time. Dimeric and tetrameric radiolabeled arginine-glycine-aspartic acid (RGD) analogs have been extensively investigated and proven to be an effective approach to enhance the $\alpha_v\beta_3$ integrin avidity, tumor uptake and tumor-to-background ratio [54-57]. From these studies and others, it has been shown that the linker length and flexibility are the key factors influencing the efficacy of the multimeric design. In order to bind to more than one receptor, the length of the linker should be able to accommodate the distance between targets. Therefore, the selection of the linker length should correlate to the cellular receptor density and the average distance between receptors on the plasma membrane. Meanwhile, certain rigidity of the linker is required to maintain the optimum distance but without disturbing the flexible binding of the targeting vectors.

1.5.1 HOMOMULTIMERIZATION

Several papers have been published recently regarding radiolabeled bombesin homodimers or homomultimers [58-61]. Fournier et al. designed and synthesized two $^{64}$Cu-labeled [D-Tyr6, bAla11, Thi13, Nle14] BBN(6-14)NH$_2$ based homodimers with one or two PEG$_1$ spacers (dimer 1 and 2, figure 1.8)[60]. No significant improvement of binding affinity was observed for both dimer 1 and 2 compare to the monomer (IC$_{50}$ value range from 1.76 to 2.51 nM). Using dimer 2 for further in vitro analysis, slower internalization rates and higher retention were found when compared to the monomer. Unfortunately, high liver (23.11 % ID/g) and kidney (26.37 % ID/g) uptake were observed for $^{64}$Cu-NOTA-dimer 2 which resulted in substantially lower tumor-to-kidney/liver ratios than the monomer. According to the author, the large molecular weight of dimer 2 might be the explanation of the high hepatobiliary excretion and low renal clearance.
Figure 1.8 Chemical structures of $^{64}$Cu/NOTA-dimer 1 and $^{64}$Cu/NOTA-dimer 2
Similarly, Lindner et al. reported a series of DOTA-PEG₄-BN(7–14)NH₂ (PESIN) based dimers, tetramers, and octamers using either no additional linker or an additional PEG₃ or PEG₇ linker [61]. Contrary to their expectations, especially for the tetramers and octamers with longer PEG spacers, significantly lower BB2r binding affinity was observed (IC₅₀ range from 149 to 419 nM). The dimer without an extended PEG linker showed a slightly increased binding affinity over the monomer control (7.77 versus 19.67 nM). In vivo microPET studies revealed identical performances for both the dimer and monomer in terms of tumor-to-organ ratios.

1.5.2 HETEROMULTIMERIZATION

Instead of using a homomultimer design and flexible aliphatic hydrocarbons or PEG linkers, Kroll et al. developed a bombesin heterodimer system with a defined distance between an agonist and an antagonist [62]. By using a conformationally well-defined azidoproline (Azp) containing oligoprolines as the backbone, three BN heterodimers were synthesized with distances between AMBA (agonist) and RM1 (antagonist) of 10(1), 20(2) and 30(3) Å, respectively (Figure 1.9). In vitro internalization studies revealed more than 50% of analog 2 internalized into the PC3 cells with 10% bound on the cell surface, which is among the highest total cellular uptake reported. The analysis of microPET/CT data showed 18.2% ID/g tumor uptake at 4 h post injection and up to 83.5% of initial uptake (15.2% ID/g) still retained after 21 h post injection. This heterodimer design as well as the rigid scaffold with a well-defined distance might be applicable to various targeting ligands.
Figure 1.9 Chemical structures of oligoproline-based hybrid BN ligands 1-3

1: n = 0 d = 10 Å  2: n = 1 d = 20 Å  3: n = 2 d = 30 Å
Covalently linked ligands for targeting multiple receptors have drawn great interest due to the fact that many cancer types overexpress not just one, but rather multiple valid molecular targets concurrently. RGD peptides have drawn great attention due to their ability to specifically target the αvβ3 integrin, which is highly expressed on the endothelial cells of tumor neovasculature as well as in many tumor cell types such as prostate, pancreatic and breast cancer [63]. In the past five years, dual targeting analogs consisting of BN and RGD peptides have been the most investigated heterodimeric BB2r-targeted systems [14, 15, 64-67].

Stott-Reynolds et al. designed an 111In/177Lu-labeled RGD-RM2 heterodimeric peptide for dual receptor targeting of prostate cancer [67]. In vitro competitive binding studies indicated high BB2r-affinity (≈ 5 nM) of the RGD-RM2 conjugate using the PC3 cell line, while only moderate αvβ(3) integrin receptor binding affinity (≈ 360 nM) was observed when tested on the αvβ3-expressing human glioblastoma U87-MG cell line. In vivo biodistribution studies revealed both high initial tumor uptake (average of 7.40 ± 0.53% ID/g at 1 h p.i.) and retention (average of 4.41 ± 0.91% ID/g at 24 h p.i.) in PC-3 tumor-bearing mice. Pre-injection of cold BN or cyclicRGD peptides is able to reduce the tumor uptake by ~42% or ~20%, respectively, which confirmed the dual-targeting efficacy of the designed heterodimeric peptide. It’s interesting to note from other literature reports of RGD-BN heterodimer designs, the affinity of either the αvβ3 or BB2r is usually substantially reduced [14, 15, 64, 65, 67]. Since dimeric RGD designs normally possess significantly higher αvβ3 affinity than RGD monomers, the reduced binding affinity of BN-RGD heterodimer is possibly due to the steric hindrance of the longer BN peptides.

Prostate-specific membrane antigen (PSMA) is a membrane-bound glycoprotein which is expressed at low levels in benign prostatic epithelium but highly expressed in prostate tumor epithelium [68]. This has given the impetus for the development of radiopharmaceuticals targeting PSMA for both diagnostic and therapeutic purpose for recurrent or metastatic
prostate cancer lesions [69-73]. Since both BB2r and PSMA are concurrently expressed on a large fraction of prostate cancer tissues, the potential of dual receptor-targeted radiotracers has been recently evaluated. The PSMA binding motif Glu-NH-CO-NH-Lys was conjugated to a previously reported BN agonist BZH3 (D-Tyr⁶, β-Ala¹¹, Thi¹³, Nle¹⁴) BN(6–14)NH₂ through a PEG₂/6-Ahx linking system by Eder et al. [74]. The resulting conjugates demonstrated similar BB2r binding affinity, compared with the monomeric reference, but 2.3-fold lower PSMA affinity. In vivo studies on both AR42J (BB2r positive) and LNCaP (PSMA positive) xenograft mice models show identical biodistribution profiles compared with corresponding monomeric targeting analogs, which suggests the potential to improve the sensitivity in clinical settings. In a similar design by Bandari et al., the PSMA-targeted agent DUPA(2-[3-(1,3-dicarboxypropyl)-ureido]pentanedioic acid) was conjugated to BN(7-14)NH₂[75] through a 5-Ava/6-Ahx linking system. The resulting hybrid targeting agent showed very good binding affinity to both BB2r (11.1 ± 0.64 nM) and PSMA (1.16 ± 1.35 nM) expressing cells. However, in vivo microPET/CT revealed unfavorable high liver, spleen, and small intestine retention which significantly limited the efficacy of imaging primary and metastatic tumor in the abdominal region. These researchers intend to pursue an alternative design using a BN antagonist, presumably due to the better clearance profiles of the antagonistic pharmacophores.

1.6 HYBRID BN ANALOGS

For most of the low-molecular weight, receptor-targeted drugs, one of the major challenges is low retention at tumor site due to intrinsically high diffusion and efflux rates. After receptor mediated uptake of radiotracers, a fast washout is often observed which substantially reduces the theranostic efficacy and the potential for clinical translation. To address this issue, one possible approach is to enhance the cellular retention time in cancer cells by adding another targeting agent which can be activated and bound to subcellular proteins or organelles. Santos-
Cuevas et al. have reported a $^{99m}$Tc-labeled lys$^{3}$-BN peptide hybrid with nuclear localization peptide sequence (NLS)-Tat(49-57, H-Arg-Lys-Lys-Arg-Arg-Arg-Gln-Arg-Arg-Arg-NH$_2$), which is derived from human immunodeficiency virus type 1 (HIV-1) Tat protein [76]. In vitro studies demonstrated that addition of Tat(49-57) efficiently delivered radiopharmaceuticals to the nuclei of both prostate and breast cancer cells. Fischer et al. designed a BN peptide hybrid with heat shock protein 90 (Hsp90) inhibitor [77]. Hsp90 is a ubiquitous protein that is overexpressed in most cancer cells and plays an important role in cell proliferation and survival [78]. Peptide sequence “shepherdin” (KHSSGCAFL) has been identified as a potent Hsp90 inhibitor with high affinity and specificity [79]. When investigating the combination of the extracellular BB2r-targeting peptide and the intracellular Hsp90 protein inhibitor, no increased retention of radioactivity was observed. A possible explanation is that the Hsp90-targeting peptide lost its specificity after enzymatic degradation in endosomes/lysosomes.

Our lab has been investigating the potential of utilizing the hypoxic characteristic of prostate cancer to increase retention time of BB2r-targeted agents [80, 81]. Tissue hypoxia is the result of an inadequate supply of oxygen. In most solid cancers, hypoxic regions commonly exist due to a chaotic vascular architecture which impedes delivery of oxygen and other nutrients. A recent clinical investigation found that 63% (median, n=247) of prostate tumors gave pO$_2$ measurements of less than 1.3 kPa (10 mm Hg, tissues less than this are generally defined as hypoxic)[82]. The extent of hypoxia in tumors appears to be strongly associated with the aggressiveness of the tumor phenotype, therapeutic resistance and patient prognosis [83, 84]. Since hypoxia is not present in most normal human tissues, a variety of bioreductive, hypoxia-selective prodrugs have been developed for the purpose of diagnostic and therapeutic applications for cancer [85-87]. Nitroimidazoles have been used extensively in basic and clinical investigation as diagnostic imaging agents [88, 89]. In hypoxic environments, nitroimidazoles
undergo a series of enzymatic reductions, mediated by nitroreductase enzymes, leading to the formation of strong electrophiles which can irreversibly bind to intracellular nucleophiles thereby trapping the agent in the hypoxic tissue. In our previously published studies [12], totally four $^{111}$In-labeled BN(7-14)NH$_2$ analogs were synthesized with increasing number (0-3) of the hypoxia trapping moiety 2-nitroimidazole incorporated on the linker ($^{111}$In-HE-1-4, Figure 1.10). Internalization and efflux studies demonstrated that the clearance rate of the radioconjugates containing 2-nitroimidazoles was substantially lower, relative to the control, under hypoxic conditions. Cellular protein binding analysis revealed up to 2 fold higher radioactive signal associated with macromolecules for 2-nitrimidazole containing conjugates compared with the control. Significant tumor retention improvement was observed at 72 h post injection for radioconjugates $^{111}$In-HE-2 and $^{111}$In-HE-4. Specifically, 6.7% and 21.0% of the initial 1h tumor uptake was retained for $^{111}$In-HE-2 and $^{111}$In-HE-4, whereas only 1.5% left for the control $^{111}$In-HE-1. Currently, we are working on correlating the hypoxia burden of PC3 xenograft tumors with the hypoxia trapping efficacy of our radioconjugates.
Figure 1.10 Hypoxia-enhanced $^{111}$In-BB2r–targeted conjugates
1.7 RECENT CLINICAL STUDIES

A total of eight clinical studies using BB2r-targeted radiotracer were performed in Europe during the past five years [24, 90-96]. Out of the eight studies, four radioisotopes ($^{99m}$Tc, $^{18}$F, $^{68}$Ga and $^{64}$Cu) and six BN analogs, including three antagonists and three agonists, have been investigated on recurrent glioma patients, prostate cancer patients or healthy men (Table 1.4). In this review, we focus on the six clinical studies of prostate cancer.
<table>
<thead>
<tr>
<th>Year</th>
<th>Disease</th>
<th>Number of Patients</th>
<th>Linking System</th>
<th>Chelator/Chelatable Antagonist</th>
<th>Radiolabeled Bombesin Analog</th>
<th>Antagonist</th>
<th>Antagonist-active Form</th>
<th>Prostate Cancer</th>
<th>Recurrence Prostate Cancer</th>
<th>Radical Prostatectomy</th>
<th>Recurrence Prostate Cancer</th>
<th>Radical Prostatectomy</th>
</tr>
</thead>
<tbody>
<tr>
<td>2015</td>
<td>Prostate Cancer</td>
<td>5 patients at early stage and 5 patients with radical prostatectomy</td>
<td>°-Cyano-4-[18F]fluorobenzoyl</td>
<td>HYNIC-Gly-His-3-2-Leu-4</td>
<td>Cu-CB-TE2A</td>
<td>Antagonist</td>
<td>Antagonist-active Form</td>
<td>Prostate Cancer</td>
<td>Recurrence Prostate Cancer</td>
<td>Radical Prostatectomy</td>
<td>Recurrence Prostate Cancer</td>
<td>Radical Prostatectomy</td>
</tr>
<tr>
<td>2014</td>
<td>Prostate Cancer</td>
<td>4 patients at early stage</td>
<td>°-Cyano-4-[18F]fluorobenzoyl</td>
<td>HYNIC-Gly-His-3-2-Leu-4</td>
<td>Cu-CB-TE2A</td>
<td>Antagonist</td>
<td>Antagonist-active Form</td>
<td>Prostate Cancer</td>
<td>Recurrence Prostate Cancer</td>
<td>Radical Prostatectomy</td>
<td>Recurrence Prostate Cancer</td>
<td>Radical Prostatectomy</td>
</tr>
<tr>
<td>2013</td>
<td>Prostate Cancer</td>
<td>6 patients at early stage and 2 patients with metastasis</td>
<td>°-Cyano-4-[18F]fluorobenzoyl</td>
<td>HYNIC-Gly-His-3-2-Leu-4</td>
<td>Cu-CB-TE2A</td>
<td>Antagonist</td>
<td>Antagonist-active Form</td>
<td>Prostate Cancer</td>
<td>Recurrence Prostate Cancer</td>
<td>Radical Prostatectomy</td>
<td>Recurrence Prostate Cancer</td>
<td>Radical Prostatectomy</td>
</tr>
<tr>
<td>2012</td>
<td>Prostate Cancer</td>
<td>5 healthy men</td>
<td>°-Cyano-4-[18F]fluorobenzoyl</td>
<td>HYNIC-Gly-His-3-2-Leu-4</td>
<td>Cu-CB-TE2A</td>
<td>Antagonist</td>
<td>Antagonist-active Form</td>
<td>Prostate Cancer</td>
<td>Recurrence Prostate Cancer</td>
<td>Radical Prostatectomy</td>
<td>Recurrence Prostate Cancer</td>
<td>Radical Prostatectomy</td>
</tr>
<tr>
<td>2011</td>
<td>Prostate</td>
<td>7 patients</td>
<td>°-Cyano-4-[18F]fluorobenzoyl</td>
<td>HYNIC-Gly-His-3-2-Leu-4</td>
<td>Cu-CB-TE2A</td>
<td>Antagonist</td>
<td>Antagonist-active Form</td>
<td>Prostate Cancer</td>
<td>Recurrence Prostate Cancer</td>
<td>Radical Prostatectomy</td>
<td>Recurrence Prostate Cancer</td>
<td>Radical Prostatectomy</td>
</tr>
<tr>
<td>2011</td>
<td>Prostate</td>
<td>15 patients</td>
<td>°-Cyano-4-[18F]fluorobenzoyl</td>
<td>HYNIC-Gly-His-3-2-Leu-4</td>
<td>Cu-CB-TE2A</td>
<td>Antagonist</td>
<td>Antagonist-active Form</td>
<td>Prostate Cancer</td>
<td>Recurrence Prostate Cancer</td>
<td>Radical Prostatectomy</td>
<td>Recurrence Prostate Cancer</td>
<td>Radical Prostatectomy</td>
</tr>
</tbody>
</table>

Table 1.4 Radiolabeled bombesin analogs under clinical evaluation
Ananias et al. evaluated the potential of $^{99m}$Tc-labeled BN(7-14)NH$_2$ agonist-HABBN for detection of prostate cancer in 8 patients [24]. Unfortunately, due to the poor metabolic stability, it failed to visualize both primary PCa and metastatic prostate cancer. A rapid metabolic degradation was observed with less than 20% intact peptide remaining after 30 min post injection. These findings provide further evidence that the in vivo stability in man does not correlate with in vitro human serum stability, suggesting the need to develop better protocols for predicting metabolic stability of peptidic radiotracers in humans.

Instead of using BN(7-14)NH$_2$ which has the oxidation-sensitive methionine at the C-terminus, Nock et al. synthesized $^{99m}$Tc-labeled BN agonist Demobesin 4 ($[^{99m}$Tc]$\text{DB4}$) which has methionine substituted by norleucine [97]. Both early stage prostate cancer patients and patients who have undergone hormone ablation therapy were recruited to evaluate the diagnostic capability of $[^{99m}$Tc]$\text{DB4}$ [94]. For the two patients who had cancer confined to the prostate and who have had no previous ablation therapy, $[^{99m}$Tc]$\text{DB4}$ successfully delineated the primary PCa. However, no $[^{99m}$Tc]$\text{DB4}$ uptake in the prostate gland was found for the six patients who had androgen ablation treatment. Moreover, the majority of the extensive bone metastatic disease was not visualized by the $[^{99m}$Tc]$\text{DB4}$. It has been previously shown that hormone treatment leads to the loss of GRPR expression [98]. Taking all of these studies into consideration, BB2r-targeted radiopharmaceuticals may only be useful for untreated, early-stage prostate cancer patients.

Encouraged by the superior imaging characteristics of somatostatin receptor antagonists, increasing number of BB2 receptor antagonists have been developed and tested in both animal models and human subjects. Two clinical studies of $^{68}$Ga-bombesin antagonist BAY 86-7548 were reported by Roivainen et al. [93] and Kähkönen et al. [92]. Five healthy men were studied first to investigate the safety, tolerability, metabolism, pharmacokinetics, biodistribution and radiation
dosimetry of BAY 86-7548. Fast plasma metabolism was observed (19% of total radioactivity in plasma was identified as intact BAY 86-7548 at 65 min post injection) with three metabolites being identified. The radioactivity mainly accumulated in the pancreas and liver and was excreted rapidly through renal clearance. The bladder wall and pancreas absorbed the highest dose at 0.61 and 0.51 mSv/MBq, respectively. The diagnostic potential of BAY 86-7548 was also investigated in 14 prostate cancer patients. Sensitivity, specificity and accuracy are widely used statistics to quantify how good and reliable of a diagnostic test [99]. Sensitivity assesses ability of a diagnostic test for detecting a positive disease. Specificity evaluates the chance of a diagnostic test to rule out patients without disease. Accuracy estimates the probability of a diagnostic test to correctly identify and exclude a given condition. In this study, a promising result with 88% sensitivity, 81% specificity, and 83% accuracy was found for the detection of primary PCa. Sensitivity of detecting ten histologically proven lymph node metastases was a little lower; around 70% of these lesions were detected. According to the authors, the possible explanations for the lower detection efficacy of metastatic relative to primary lesions might be: 1. reduction of BB2r expression, 2. the partial volume effects [100], or 3. small lesions were missed because the large intervals of whole-mount axial macrosections (8 mm).

Compared to $^{68}$Ga, $^{18}$F has superior physical characteristics for PET imaging, such as a lower positron range and a higher positron yield which lead to higher spatial resolution and sensitivity [101]. A very recent clinical study using $^{18}$F-labeled BN antagonist BAY 864367 has been carried out by Sah et al. [96]. Ten patients with biopsy proven PCa have been recruited for both BAY 864367 and $^{18}$F-Fluorocholine scan. The BAY 864367 displayed accumulation in three out of five patients with primary PCa and two out of five patients with recurrent PCa. The sensitivity of BAY 864367 is lower than an $^{18}$F-Fluorocholine scan, which delineated all suspicious lesions. Inverse correlation between BB2r expression and the Gleason score has been previously reported [102].
Compared with BAY 86-7548, the lower detection rate of BAY 865367 could be due to the fact that patients recruited in this study have higher Gleason scores than patients in Kahkonen et al.’s study (8.3 vs 3.7).

Copper 64 is another positron emitter which has been used in recent clinical studies for prostate cancer diagnosis. Compared with $^{68}$Ga and $^{18}$F, $^{64}$Cu has a relatively longer half-life (12.7 h versus 68 min and 110 min) which allows for better clearance of radioactivity from non-target tissues, which might improve imaging contrast. $^{64}$Cu-labeled BN antagonist AR06 was examined in four newly diagnosed PCa patients (T1c-T2b, Gleason 6-7) by Wieser et al. [95]. Rapid tumor accumulation was observed along with much slower clearance from other BB2r positive organs which leads to higher contrast tumor delineation in 3 out of 4 patients at all scanning time points. However, the ability of this radiotracer to detect metastatic lesions still needs to be evaluated.

1.8 CONCLUSION

In the past five years, great progress has been made in the field of radiolabeled bombesin analogs at both the preclinical and clinical level. New issues (e.g., non-correlated in vitro/in vivo metabolic stability and loss of BB2r expression after hormone ablation) have been discovered. Previously discovered mechanisms to increase in vivo efficacy (e.g., improved binding affinity with positive charge on N-terminal and increased non-target tissue clearance with elevated hydrophilicity) have been further verified. New concepts (e.g., co-injection with enzyme inhibitor and tumor specific cellular trapping) and new designs (e.g., homodimers and heterodimers) have been introduced and proven to have great potential for improving the diagnostic and therapeutic efficacy of BB2r-targeted agents.
Chapter 2 Development of Hypoxia Enhanced $^{111}$In-labeled Bombesin Conjugates: Design, Synthesis and In Vitro Evaluation in PC-3 Human Prostate Cancer

2.1 INTRODUCTION

As mentioned in Chapter 1, prostate cancer is among the most hypoxic of cancers found in the clinic. [103, 104] This state of hypoxia is a result of the often chaotic and poor vascular organization in the tumor thereby inhibiting the ability of the microvasculature to efficiently deliver oxygen. [104] This chaotic vascular architecture often leads to a high degree of oxygenation heterogeneity, with the tumor containing anoxic, hypoxic and normoxic regions, depending on the distance of the tissue from the nearest vascular vessel. Hypoxia has and continues to be a major obstacle in the treatment of numerous cancers, including prostate cancer, due to the increased chemotherapy and radiotherapy resistance of hypoxic tissue.[104, 105] While the average fraction of hypoxic tissue in prostate cancer tumors is not well known, it is expected that the fraction of hypoxic cells would be significant.[106, 107] Since hypoxia is a property that is not exhibited in most normal human tissues, several classes of bioreductive prodrugs (i.e. nitroimidazoles, aromatic N-oxides and quinones) are under development to exploit the hypoxic nature of tumors for diagnostic and therapeutic applications. One class of bioreductive hypoxia targeting agents that has undergone extensive basic and clinical investigation is nitroimidazole. [108-110] In hypoxic environments, this class of agent is selectively reduced ("triggered") thereby generating a reactive electrophile that becomes trapped in the hypoxic cell. In particular, 2-nitroimidazole derivatives have been actively

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1 The material presented in this Chapter was previously published: Wagh NK, Zhou Z, Ogbomo SM, Shi W, Brusnahan SK, Garrison JC. Development of hypoxia enhanced $^{111}$In-labeled Bombesin conjugates: design, synthesis, and in vitro evaluation in PC-3 human prostate cancer. *Bioconjug Chem*. Mar 21 2012;23(3):527-537
investigated for the development of hypoxia targeted agents capable of quantitating the hypoxic burden present in tumors using standard nuclear medicine imaging modalities. [111-115]

The BB2 receptor (BB2r), also known as the Gastrin-Releasing Peptide (GRP) receptor, is a subtype of the Bombesin receptor family.[116] The BB2r has been thoroughly investigated for the role the receptor plays in neoplastic tissue transformation and growth [117-119]. From these studies and subsequent work, it was demonstrated that the BB2r is expressed in significantly higher densities on a number of human tumors and cancer cell lines, including prostate cancer tissues, relative to normal tissues [117, 118, 120, 121]. These studies have provided the impetus for the development of BB2r-targeted agents for both diagnostic imaging and therapeutic applications. To date, most of these agents have revolved around synthetic analogs of Bombesin (BBN) - a fourteen amino acid, amphibian peptide that shares a C-terminal binding region sequence homology (Trp-Ala-Val-Gly-His-Leu-Met-NH₂, BBN(7-14)NH₂) with GRP (the native ligand for the BB2r)[122]. Analogs utilizing the BBN(7-14)NH₂ sequence are able to agonistically bind to the BB2r with high (typically nanomolar) affinity and facilitate endocytosis of the agent into the cell.

For BB2r-targeted drugs, as well as most small molecule based targeted ligands, the targeted conjugates typically demonstrate maximum tumor accumulation shortly after administration (~ 15-60 min)[122-124]. Unfortunately, one of the major challenges faced by many targeted peptides is the significant clearance of the drug relatively quickly (~ 1-4 hrs p.i.), after peak accumulation in the tumor, thereby reducing the diagnostic or therapeutic efficacy of the BB2r-targeted agent. In an attempt to increase the effectiveness of BB2r-targeted drugs, we and others have begun to evaluate various means of increasing the retention of the radiolabeled drug in prostate cancer tumors [125]. Herein, we report the evaluation of BB2r-targeted agents that include the use of 2-nitroimidazoles as hypoxia trapping moieties to increase the retention
of the drug in hypoxic tumor cells. Specifically, we synthesized four BB2r-targeted agents, that include (0 - 3) 2-nitroimidazole moieties, and evaluated the in vitro binding affinity and retention efficacy of the radioconjugates in normoxic and hypoxic PC-3 human prostate cancer cells. The structures of synthesized agents are depicted in Figure 2.1.
Figure 2.1 Hypoxia enhanced $^{111}$In-BB2r-targeted conjugates
2.2 MATERIALS AND METHODS

2.2.1. Chemicals and Equipment

Unless otherwise noted all solvents were used without further purification. Deionized water was purified by a Millipore (U.S.) Mili-Q-Biocel. Ethylbromoacetate was purchased from Acros Organics (U.S.). Silica and TLC plates were purchased from Sorbent Technologies (U.S.). Acetonitrile, formic acid, N, N-diisopropylethylamine (DIEA), N,N-dimethylformamide (DMF), dichloromethane (DCM), N,N'-dicyclohexylcarbodiimide (DCC), N-methylpyrrolidone (NMP), thioanisol, L-ascorbic acid, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES), bovine serum albumin (BSA) and sodium dodecyl sulfate (SDS) were purchased from Fisher Scientific (U.S.). 2-nitroimidazole was purchased from Amfinecom (U.S.). 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.). Fmoc-5-AOC and Fmoc-5-Ava were purchased from CreoSalus (U.S). Phosphate buffered saline (PBS) and Mammalian Protein Extraction Reagent (M-PER) were purchased from Thermo Scientific (U.S.). Indium-111 chloride (\(^{111}\text{InCl}_3\)) was purchased from MDS Nordon (Canada). Naturally abundant indium chloride (\(^{nat}\text{InCl}_3\)), triisopropyl silane and 3,6-dioxa-1,8-octanedithiol were purchased from Sigma-Aldrich (U.S.). The \(^{125}\text{I}-\text{Tyr}4\)-Bombesin was purchased from Perkin Elmer (U.S.). Cation exchange resin was purchased from Bio Rad (USA). Prostate cancer (PC-3) cell lines were obtained from American Type Culture Collection (U.S.) and cultured under vendor recommended conditions. Roswell Park Memorial Institute (RPMI) 1640 media and TrypLETM Express were purchased from Invitrogen/ GIBCO (U.S.). HypoxyprobeTM-1 Plus Kit was purchased from hypoxyprobe, Inc (U.S). Flow Cytometry Staining Buffer and Foxp3 permeabilization Buffer were purchased from eBioscience (U.S.). The peptides were synthesized on a Liberty microwave peptide synthesizer from CEM (U.S.). Nuclear magnetic resonance
spectra were recorded on Varian (U.S.) 500MHz INOVA spectrometer. 1H NMR chemical shifts are expressed as δ values (parts per million) and peaks are described as s for singlet and d for doublet. 13C NMR chemical shifts are expressed as δ values using acetone as an internal reference. HPLC/MS analyses were performed on a Waters (U.S.) e2695 system equipped with a Waters 2489 absorption detector and a Waters Qtof Micro electrospray ionization mass spectrometer. Evaluation and purification of radiolabeled conjugates 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. A Phenomenex (U.S.) Jupiter 10μ Proteo 250 × 10 mm semiprep column was used for the purification of bulk amounts of peptides. For the purification of conjugates, natIn-conjugates, and 111In-radioconjugates a Phenomenex Jupiter 10μ Proteo 250 × 4.60 mm analytical column was employed. Solid phase extraction was performed using Empore (U.S.) C18 10 mm high performance extraction disks. The separation of macromolecules and small molecules was performed using Amicon Ultra (Ireland) Ultracel-10K centrifugal filters in protein association analysis. Gamma decay detection of 111In and 125I for the in vitro binding, receptor saturation, efflux studies and protein binding fractionation studies was accomplished using a LTI (U.S.) Multi-Wiper nuclear medicine gamma counter. Flow cytometry studies were performed on BD FACSAria cell sorter (US).

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

The synthesis of 2-NIAA was carried out as reported in the literature by Gariépy and co-workers [126]. A brief description of the two-step procedure is reported below.

Synthesis of 2-Nitroimidazole Ethyl Acetate (2-NIEA): 2-bromoethyl acetate (1.2 mL, 7.18 mmol) was added to a solution of 2-nitroimidazole (1 g, 8.84 mmol) and potassium carbonate (2.0 g, 14.5mmol) in dry acetonitrile (15 mL). The mixture was stirred for 24 h at room
temperature, and the resulting precipitate was filtered and washed with acetone. Evaporation of the filtrate yielded a yellow oil (1.7 g). The pure product was isolated from the crude oil using flash chromatography (silica gel) eluted with CH₂Cl₂/MeOH/NH₃ (50:7:1) as yellowish oil: 0.9 g (52.9% yield); Analyses for 2-nitroimidazole ethyl acetate: ¹H NMR (CDCl₃): 7.260 (d, J = 3.7 Hz, 1H, H-imidazole ring), 7.089 (d, J = 1.5 Hz, 1H, H-imidazole ring), 5.10 (s, 2H, NCH₂COO), 4.29 (q, 2H, OCH₂CH₃), 1.30 (t, 3H, OCH₂CH₃); δc (CDCl₃): 166.0 (C=O), 128.5 (C₃H₃N₂O₂), 126.5 (CH-imidazole ring), 62.6 (NCH₂COO), 50.9 (OCH₂CH₃), 14.0 (OCH₂CH₃).

Preparation of 2-Nitroimidazole Acetic Acid (2-NIAA): The purified oil of 2-NIEA (0.9 g) was mixed into a solution of NaOH (4 N, 2.5 mL), water (10 mL), and MeOH (10 mL). The resulting solution was stirred at room temperature until no ester derivative was evident by TLC (30 min), at which point a cation-exchange resin (H⁺, Bio-Rad, 4 g), which had been protonated by washing with H₂SO₄ (1N; 30 mL; pH 2.5) and drying of the filtrate yielded a yellow paste (1.09 g). Chromatography of crude product on silica gel [mobile phase, CH₂Cl₂/CH₃CN/HCOOH (30:10:1)] yielded a white crystalline solid: 0.25 g. Analyses for 2-nitroimidazole acetic acid: ¹H NMR ¹H (D₂O): δH 7.28 (1H, s, H-imidazole ring), 7.06 (1H, s, H-imidazole ring), 4.93 (2H, s, NCH₂COO), 3.80 (1H, broad, COOH); ¹³C NMR(D₂O): 172.20 (C=O), 128.03 (CH-imidazole ring), 127.27 (CH-imidazole ring), 52.10 (NCH₂COOH).

X-ray Crystallographic Analysis. With respect to X-ray crystallography studies, single crystals were obtained by slow evaporation of a methanolic solution of 2-NIAA. Crystal data for 2-NIAA: C₅H₅N₃O₄, MW = 171.12 g/mol, monoclinic, space group P2₁/n, a = 7.3412(8) Å, b = 8.2046(9) Å, c = 11.5678(13) Å, α = γ = 90°, β = 103.796(2)°, V = 676.65(13) Å³, Z = 4, D calc = 1.639 Mg/m³, μ = 0.147 mm⁻¹, T = 100(2) K, Data was collected on a Bruker SMART 1K CCD, Refinement of data with I > 2σ(I)(1630 independent reflections, R int = 0.0217) gave a R₁(F) = 0.0349 and a wR₂(F²) = 0.0946 for all data with a GOF = 1.055. Crystallographic data for the structural analysis has been
deposited with the Cambridge Crystallographic Data Center (CCDC 838909). 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

2.2.3 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-dioxo-1,8-octanedithiol (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×3). The crude conjugate was dried by a centrivap concentrator and weighed. The purity of the crude 1, 2*, 3* and 4* conjugates ranged from 58-71 % by RP-HPLC. Isolated yields were 17.5, 11.9, 14.7 and 21.0% for 1, 2*, 3* and 4*, respectively. 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 investigations.

2.2.4 Coupling of 2-NIAA to Bombesin Conjugates

After purification of the conjugates, the 2-nitroimidazole acetic acid was manually coupled to ε- amino group of the lysine residue for each peptides 2*, 3* and 4* (15 mg each) using standard amidation chemistry. Briefly, the 2-NIAA (4.95, 9.40, 14.85 mg respectively) was
dissolved in M HBTU/DMF (0.45 M, 171 µL) solution followed by addition of DIEA/NMP solution (2.0 M, 96 µL). This solution was allowed to stand for 15 minutes before addition of the conjugate in DMF (200 µL). The reaction mixture was stirred for 3 hours at room temperature and subsequently evaporated to dryness. The residue was re-dissolved in water : acetonitrile (8:2), peak purified by RP-HPLC and characterized by mass spectrometry. Isolated conjugation yields were 34.2, 38.7 and 43.4 % for 2, 3 and 4, respectively.

2.2.5 Labeling with natInCl₃

For the convenient characterization of the ¹¹¹In-Bombesin conjugates, naturally abundant natIn was used to substitute for ¹¹¹In in the ES-MS and in vitro binding studies. A sample of conjugates (0.75 mg) was dissolved in ammonium acetate buffer (0.5 M, 200 µL, pH 5.5) and mixed with a solution of natInCl₃ (1.1 mg, 10 µmol). The solution was heated for 60 min at 90 °C. After cooling to room temperature, natIn-conjugates were then peak purified by RP-HPLC. Isolated yields were 36.7, 29.6, 33.5 and 41.9 % for natIn-1, natIn-2, natIn-3 and natIn-4, respectively. All natIn-conjugates were ≥ 95% purity before mass spectrometric characterization and in vitro binding studies were performed.

2.2.6 Radiolabeling with ¹¹¹InCl₃

A 250 µg sample of the conjugate was dissolved in ammonium acetate buffer (0.5 M, 250 µL, pH 5.5). ¹¹¹InCl₃ (1 mCi) was added to the vial containing the conjugate, and the solution was heated for 60 min at 90 °C and allowed to cool to room temperature. The resulting radioconjugates were peak purified using RP-HPLC (≥95%) and concentrated using C18 extraction disk. Elution of the extraction disk with ethanol/sterile saline solution (6:4, 600 µL) delivered the radioconjugate in high purity. L-ascorbic acid (~20 mg) was added to all
radioconjugates to reduce radiolysis. Radiolabeling yields for $^{111}$In-1, $^{111}$In-2, $^{111}$In-3 and $^{111}$In-4 were 32.3, 23.6, 26.9 and 36.1 %, correspondingly.

2.2.7 HPLC Purification and Analysis Methodology

When necessary, bulk sample purification was performed using a semi-preparative Proteo column with a flow rate of 5.0 mL/min. Sample purification for in vitro studies was performed on analytical Proteo column with a flow rate 1.5 mL/min. HPLC solvents consisted of H$_2$O containing 0.1% formic acid (solvent A) and acetonitrile containing 0.1% formic acid (solvent B). For unlabeled and $^{111}$/natIn-conjugates of 1-3, an initial gradient of 85 % A : 15 % B linearly decreased to 75 % A : 25 % B over a 15 minute time period. For unlabeled and $^{111}$/natIn-conjugates of 4, an initial gradient of 80 % A : 20 % B linearly decreased to 70 % A : 30 % B over a 15 minute time period. At the end of the run time for all HPLC experiments, the column was flushed with the gradient 5 % A : 95 % B and re-equilibrated to the starting gradient.

2.2.8 In Vitro Competitive Cell-Binding Studies

For in vitro binding studies, the inhibitory concentration (IC$_{50}$) for all conjugates and natIn-conjugates were determined using the PC-3 human prostate cancer cell line. $^{nat}$In-conjugates were used as substitutes for the corresponding $^{111}$In- radioconjugates. Briefly, the PC-3 cells ($\sim$3X10$^5$) were suspended in RPMI 1640 media (pH 7.4, 4.8 mg/mL HEPES, and 2 mg/mL BSA) and incubated at 4°C for 40 min in the presence of radiolabeled [$^{125}$I-Tyr$_4$]-Bombesin and various concentrations of the $^{nat}$In-conjugate. At the end of the incubation period, the cells were centrifuged, aspirated and washed with media a total of four times. The cell associated activity was measured using a gamma counter and the IC$_{50}$ values determined by nonlinear regression using the one-binding site model of Graphpad PRISM 5 (U.S.).
2.2.9 Flow Cytometric Analysis of Hypoxic Cell Induction

The PC-3 cells were plated at a concentration of $1 \times 10^6$ cells/well in 6 well plates, and incubated overnight in normoxic (95% air, 5% CO$_2$) and hypoxic (94.9% N$_2$, 0.1% O$_2$, 5% CO$_2$) environments. The cells were washed twice with PBS and incubated with 10 µM pimonidazole in 1 ml fresh medium (control well media did not contain pimonidazole) for 2 hours. Cells were again washed twice with PBS and detached from the well surface using TrypLE for 15 min at 37°C. As per manufacturer’s protocols, $10^6$ cells were treated with Foxp3 permeabilization solution at 4°C for 30 minutes, washed with buffer and resuspended. The PC-3 cells were subsequently incubated with the FITC-labeled Hypoxyprobe-1 monoclonal antibody (mAb1) (1:100 dilution in 0.5% BSA in PBS) in the dark at room temperature for 30 minutes. The stained cells were washed once, fixed in paraformaldehyde and subject to flow cytometric analysis. Cells were analyzed using a FACSArray instrument and FloJo software.

2.2.10 Receptor Saturation Binding Assays

Receptor saturation studies were performed on PC-3 cells under normoxic (95% air, 5% CO$_2$) and hypoxic (94.9% N$_2$, 0.1% O$_2$, 5% CO$_2$) conditions. PC-3 cells (3×10$^4$/well) were incubated overnight in 24-well plates at 37°C in RPMI 1640 media (pH 7.4, 4.8 mg/mL HEPES, and 2 mg/mL BSA) in hypoxic conditions. On the day of the experiment, the cells were seeded with fresh culture medium and incubated for 4 hour under normoxic or hypoxic conditions. Upon completion of the incubation, PC-3 cells were incubated with (30,000 cpm) [$^{125}$I-Tyr$_4$]-Bombesin and a series of [Tyr$_4$]-Bombesin concentrations ranging from 0.469 nM to 120 nM for 1 hour at 4°C. Non-specific binding was determined using 3 µM of the [Tyr$_4$]-Bombesin in the presence of the radioligand. At the end of the incubation time cells were aspirated and washed thrice with cold media and the remaining radioactivity was measured by solubilizing the cells with 10% SDS.
Non-linear regression analysis was then performed using Graphpad PRISM 5 (U.S.) to determine the $B_{\text{max}}$ and $K_d$ values for each experiment.

2.2.11 Efflux Studies

Efflux studies were performed using PC-3 cells under normoxic (95% air, 5% CO$_2$) and hypoxic (94.9% N$_2$, 0.1% O$_2$, 5% CO$_2$) conditions. PC-3 cells were incubated in six-well plates (0.5 x 10$^6$ / well) under hypoxic 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 removed, and the cells were washed with cold medium and incubated for 4 hour under normoxic and hypoxic condition. Cells were incubated for an additional 2 hour at 37°C in the presence of 100,000 cpm of each $^{111}$In-radioconjugate. Upon completion of the incubation at timepoints 0, 30, 60, 90 and 120 min, cells were washed thrice with media to discard the unbound peptide. Surface bound radioactivity was removed 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. The radioactivity of the effluxed, surface bound and internalized fractions for each radioconjugate was determined using a gamma counter. Statistical analyses were performed by two-way analysis of variance (ANOVA) using Graphpad PRISM 5 (U.S.).

2.2.12 Cellular Protein Analysis

For the cellular fractionation studies, the procedure for the preparation of normoxic and hypoxic PC-3 was carried out as outlined in the efflux studies above. On the day of the experiment, the medium was removed, and the cells were washed with warm medium and incubated for 4 hours under normoxic and hypoxic conditions. PC-3 cells were incubated for an additional 2 hours at 37°C in the presence of 30,000 cpm of each $^{111}$In-radioconjugate. At the end of this time, the cells were washed thrice with warm medium to remove the unbound,
extracellular radioconjugates and allowed to incubate for an additional hour at 37°C. Subsequently, PC-3 cells were washed thrice with PBS and lysed using 1ml of M-PER at 37°C. The cellular debris was centrifuged down and the supernatant was then transferred to an Amicon Ultracel 10k filter device with an extra PBS (1ml). The samples were centrifuged at 4000×g for 10 minutes and washed with PBS (1ml×2) per wash. The radioactivity associated with the molecular weight fractions was determined using a gamma counter.

2.3 RESULTS

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

In order to couple the 2-nitroimidazole to the BB2r-targeted peptide conjugates, the acetic acid derivative was synthesized as outlined in literature procedures.[126] Initially, the 2-nitroimidazole ethyl acetate was synthesized by formation of the potassium 2-nitroimidazole salt and subsequent reaction with 2-bromoethyl acetate. Conversion of the 2-nitroimidazole ethyl acetate to the acetic acid derivative was achieved using a cation exchange column pretreated with dilute H₂SO₄ prior to elution. The chemical constitution of 2-NIAA was confirmed by NMR and single crystal x-ray crystallography. All ¹H and ¹³C NMR data agreed with literature values. The molecular structure of 2-NIAA is depicted in Figure 2.2.
Figure 2.2 An ORTEP representation of the 2-aitroimidazole acetic acid

An ORTEP representation of the 2-NIAA with thermal ellipsoids drawn at 50% probability.

Selected bond lengths (Å) and angles (deg): O(1)-N(3) = 1.2223 (15), O(2)-N(3) = 1.2309 (14), N(3)-C(1) = 1.4387 (17), N(1)-C(1) = 1.3572 (16), N(2)-C(1) = 1.3161 (16), C(2)-C(3) = 1.372 (2), O(3)-C(5)= 1.2012 (16), O(4)-C(5) = 1.3206 (16), C(4)-C(5)= 1.5144 (17), O(1)-N(3)-O(2) = 125.13 (12), O(3)-N(3)-C(1) = 117.15 (11), O(2)-N(3)-C(1) = 117.71 (11), O(3)-C(5)-O(4) = 126.24 (12), O(3)-C(5)-C(4)=124.77 (12), O(4)-C(5)-C(4)=108.98(11).
2.3.2 Conjugate Synthesis and Radiolabeling

The conjugates were synthesized using standard solid-phase peptide synthesis. Specifically, four radioconjugates were synthesized using the DOTA-X-BBN(7-14)NH₂ paradigm (Figure 2.1). The conjugates were purified by RP-HPLC and isolated with yields of 17.5, 11.9, 14.7 and 21.0 % for conjugates 1, 2*, 3* and 4*, correspondingly. RP-HPLC retention times and mass spectrometric identification of the conjugates are listed in Table 2.1. Subsequently, 2-NIAA was coupled to the ε-amino group of lysine residue(s) of the conjugates using HBTU, DIEA and NMP in DMF. Analysis by RP-HPLC revealed that the coupling reactions were completed within 3 hours. The products obtained were consequently purified by RP-HPLC and characterized by ESI-MS (Table 2.1). The yields of conjugates 2, 3 and 4 were 34.2, 38.7 and 43.3 %, respectively.

Labeling and purification of the conjugates with natIn and ¹¹¹In were carried out under nearly identical conditions. In brief, ¹¹¹/natInCl₃ was incubated with the desired conjugate in ammonium acetate buffer (0.1M, pH 5.5) at 90°C for 1 hour. Radiolabeling yields for ¹¹¹In-1, ¹¹¹In-2, ¹¹¹In-3 and ¹¹¹In-4 were 32.3, 23.6, 26.9 and 36.1 %, correspondingly. Purification of the ¹¹¹/natIn-conjugates was accomplished by RP-HPLC. Concentration of RP-HPLC eluent was achieved by solid phase extraction on C₁₈ column with typically ≥ 90% recovery. Prior to in vitro analysis, ¹¹¹In-1-4 were found to have 99.0, 99.3, 97.7 and 95.3% radiochemical purity, correspondingly, as determined by RP-HPLC. Retention times and mass spectrometric characterization of the natIn-conjugates are given in Table 2.1.
For convenient mass spectra analysis, In was replaced by nat In. RP-HPLC methods described in Materials and Methods section. Values represent mean ± SEM. (n=6)

<table>
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<tr>
<th>Analog</th>
<th>IC₅₀/nM</th>
<th>Retention Time, min</th>
<th>Observed MS</th>
<th>Calculated MS</th>
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<td>In-DOTA (D)K-3-NIAA</td>
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<td>In-DOTA (D)K-5-AVA</td>
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Table 2.1 Mass spectrometric and RP-HPLC characterization of conjugates.
2.3.3 In Vitro Competitive Cell-Binding Studies

The BB2r binding affinity of the unlabeled and $^{nat}$In Bombesin conjugates were assessed by competitive binding assays performed at 4°C using the BB2r-positive human prostate cancer PC-3 cell line. All conjugates and $^{nat}$In-conjugates were able to displace $[^{125}]$-Tyr4-Bombesin from BB2r binding sites on the PC-3 cell membranes in a dose-dependent manner. For $^{nat}$In-labeled conjugates, conjugates 1 and 2 demonstrated the highest affinity binding (5.70 ± 1.31, 23.5 ± 15.9 nM) to the BB2r. Conjugates 3 and 4 exhibited substantially poorer binding affinity, relative to 1 and 2, with affinities of 93.0 ± 37.3 nM and 342 ± 99 nM, correspondingly. Some differences in binding affinity were observed between unlabeled and $^{nat}$In-labeled conjugates, but analysis of the data revealed no overall trend.

2.3.4 Hypoxia Staining and Fluorescence Analysis

Before beginning hypoxia experiments, the conditions used for the generation of hypoxic cells were validated by hypoxia staining. PC-3 cells were placed under hypoxic or normoxic conditions overnight and were subsequently exposed to pimonidazole for 2 hours. Pimonidazole, a 2-nitroimidazole based agent, has been widely used for the detection and quantification of hypoxia.[127-129] After exposure to pimonidazole, the cells were permeabilized and incubated with a FITC-conjugated primary antibody against pimonidazole adducts. The fluorescence analysis of the stained PC-3 cells by flow cytometry is depicted in Figure 2.3. Mean fluorescence of the normoxic PC-3 cells treated with pimonidazole was 56.8. Treatment of hypoxic PC-3 with the hypoxia staining agent yielded a significant increase in antibody binding with a mean fluorescence of 286. The increase in antibody binding for the hypoxic cells corresponds, as expected, with an increase in hypoxia staining due to a drop in the $O_2$ concentration. Based on previous literature reports, the values from this study are consistent
with hypoxia being generated in the PC-3 cells and therefore subsequent in vitro studies were carried out employing the hypoxia generating conditions of this experiment.[127]
Figure 2.3 Flow cytometric analysis of pimonidazole binding

Flow cytometric analysis of pimonidazole binding under control (no pimonidazole), normoxic (95% air, 5% CO$_2$) and hypoxic (94.9% N$_2$, 0.1% O$_2$, 5% CO$_2$) conditions in PC-3 cells.
2.3.5 Receptor Saturation Binding Assays

In order to determine if and to what extent the receptor expression changes under hypoxic conditions, receptor saturation studies were performed to quantify the receptor expression in PC-3 cells under normoxic and hypoxic conditions. The receptor saturation assays revealed that under normoxic and hypoxic conditions there were $0.35 \pm 0.08$ million and $0.30 \pm 0.06$ million binding sites, respectively, per PC-3 cell. The dissociation constant ($K_d$) values were found to be $1.0 \pm 0.05$ nM and $0.9 \pm 0.08$ nM, correspondingly, for normoxic and hypoxic PC-3 cells. Under the conditions of our experiments, these receptor saturation studies indicate that the BB2r expression at the cell surface does not significantly change with oxygen levels.

2.3.6 Efflux Studies

To evaluate the ability of the 2-nitroimidazole hypoxia trapping moieties to enhance the retention of the BB2r-targeted drugs, efflux studies were performed under normoxic and hypoxic environments to determine the rate at which the $^{111}$In-labeled radioconjugates 1 - 4 were effluxed from the PC-3 cells. In these studies, $^{111}$In-1, which does not have a 2-nitroimidazole incorporated into the structure of the radioconjugate, is the control and will be used to compare the relative effectiveness of the hypoxia trapping conjugates ($^{111}$In-2-4) in both hypoxic and normoxic conditions.

The radioconjugates were incubated in the presence of the PC-3 cells for 2 hours prior to the start of the efflux studies. During this incubation time period, $^{111}$In-1 and $^{111}$In-2 demonstrated significant accumulation of the radioactivity with 22.6 and 20.5 %, respectively, of the added radioconjugates internalized under normoxic conditions. The same radioconjugates demonstrated a marked decline in overall uptake under hypoxic conditions with 17.8 and 15.0 % internalized, respectively, in the same time span. In comparison, $^{111}$In-3 and $^{111}$In-4
demonstrated internalization of only 1.4 and 0.8 %, correspondingly, of the added radioactivity under normoxic conditions. Under hypoxic conditions, the $^{111}$In-3 and $^{111}$In-4 radioconjugates exhibited uptakes of 1.3 and 1.2 %, respectively, during the incubation period. Comparison of the internalization of the radioconjugates revealed that internalization corresponded well with the binding affinities observed in the competitive binding assays. Also, internalization under hypoxic conditions yielded significantly lower uptake for $^{111}$In-1 and $^{111}$In-2 relative to $^{111}$In-3 and $^{111}$In-4.

The efflux of the radioconjugates over time viewed as a percentage of the initial internalized activity is depicted in Figure 2. At all timepoints investigated, the radioconjugates demonstrated significantly higher retention of the radioconjugates under hypoxic conditions relative to normoxic. In the normoxic studies, the $^{111}$In radioconjugates 1-4 demonstrated substantial efflux with 32, 55, 31 and 29 %, respectively, of the radioactivity material being externalized within the first 30 minutes of the experiment. Under hypoxic conditions radioconjugates 1-4 exhibited significantly lower clearance of the radioactivity with 5, 14, 11 and 13 % of the intracellular radioactivity being externalized from the cell. After the initial 30 minute timepoint, the rate of clearance of the radioactivity substantially declined under both normoxic and hypoxic conditions. With the exception of $^{111}$In-2, the radioconjugates demonstrated an increased retention of 20-30 % of the initial internalized activity under hypoxic relative to normoxic conditions. Interestingly, $^{111}$In-2 exhibited a 50% increase in retention under hypoxic conditions which is significantly higher than the other radioconjugates.
Figure 2.4 Efflux assays for the $^{111}$In-radioconjugates in PC-3 cells

Efflux assays depicted as percentage of initial internalized activity for the $^{111}$In-radioconjugates in PC-3 cells. Values are mean ± SEM (n=3).
An alternative way to view the effect hypoxia has upon the clearance of the radioconjugates is by directly comparing the internalized activity of the radioconjugate for each timepoint under normoxic and hypoxic conditions. The hypoxia enhancement factor (HEF) is the ratio of the internalized activity of the radioconjugate under hypoxic conditions over the internalized activity observed under a normoxic environment. The HEF ratios for each radioconjugate at each timepoint are depicted in Figure 2.5. With the exception of $^{111}$In-4, all of the radioconjugates at the initial timepoint demonstrated favored retention of the radioconjugates in normoxic conditions. Although, by the 30 minute timepoint, all of the radioconjugates displayed significantly higher retention in hypoxic relative to normoxic cells (i.e. HEF > 1). This observation corresponds with the substantially higher clearance of the radioconjugates under normoxic relative to hypoxic conditions noted during the first 30 minutes of the efflux experiment. From 30 to 120 minutes, the $^{111}$In-labeled radioconjugates 1–4 demonstrated an average HEF ratio of 1.08 ± 0.03, 1.58 ± 0.15, 1.27 ± 0.12 and 1.83 ± 0.17, respectively. The radioconjugates $^{111}$In-2-4 exhibited significantly ($p < 0.001 – 0.05$) higher average HEF ratios compared to control (i.e. $^{111}$In-1). However, the HEF ratios of $^{111}$In-3 were substantially lower than that of either $^{111}$In-2 or $^{111}$In-4. For $^{111}$In-1, the control for this experiment, the radioconjugate exhibited no higher than a 10% increase in retention under hypoxic conditions from time points 30 to 120 minutes.
Figure 2.5 HEF ratios for the efflux studies of $^{111}$In-BB2r-targeted radioconjugates

HEF ratios for the efflux studies of $^{111}$In-BB2r-targeted radioconjugates under normoxic (95% air, 5% CO$_2$) and hypoxic conditions (94.9% N$_2$, 0.1% O$_2$, 5% CO$_2$). Values are mean ± SEM (n=3).
2.3.7 Cellular Protein Analysis

In a hypoxic environment, 2-nitroimidazoles undergo reduction and eventual irreversible conjugation with intracellular nucleophiles. It has been estimated that approximately 20% of such adduct formations are with nucleophilic groups (e.g. thiols) on proteins.\[130, 131\] In order to better evaluate the protein binding capabilities of the 2-nitroimidazole containing BB2r-targeted agents, the protein association properties of $^{111}$In-2 was evaluated against the controls, $^{111}$In-1 and $[^{125}$I-Tyr$_4$]BBN, under hypoxic and normoxic environments. The radioconjugates $^{111}$In-3 and $^{111}$In-4 were not evaluated because of the poor internalization of these analogs. After 2 hours of incubation with the radioconjugates under hypoxic or normoxic conditions, the PC-3 cells were lysed using a protein extraction reagent, centrifuged and the supernatant filtered using a 10 kDa centrifugal filter. Analysis of the protein associated radioactivity as a percentage of total intracellular activity is depicted in Figure 2.6. The radioconjugates $^{111}$In-1 and $[^{125}$I-Tyr$_4$]BBN both demonstrated similar levels, approximately 3.15 to 5.60 %, of protein association during the timespan of the experiment. Statistical analysis of the $^{111}$In-1 and $[^{125}$I-Tyr$_4$]BBN data revealed no significant impact on protein association under hypoxic or normoxic conditions. For the $^{111}$In-2 radioconjugate, the protein association under normoxic conditions was statistically the same as those found in controls. However, $^{111}$In-2 demonstrated a protein association under hypoxic conditions of approximately 13 % which was three fold higher than that observed under a normoxic environment. These findings strongly imply that the mechanism of protein retention of $^{111}$In-2 is associated with the 2-nitroimidazole moiety and is oxygen dependent.
Figure 2.6 Protein association studies for $^{111}$In-1, $^{111}$In-2 and $[^{125}$I-Tyr$_4$]-BBN

Protein association studies for $^{111}$In-1, $^{111}$In-2 and $[^{125}$I-Tyr$_4$]-BBN under normoxic (95% air, 5% CO$_2$) and hypoxic (94.9% N$_2$, 0.1% O$_2$, 5% CO$_2$) conditions in PC-3 cells. Values are mean ± SEM (n=3).
2.4 DISCUSSION

In the area of cancer diagnostics and therapeutics, targeted, low molecular weight agents, such as those based on small peptides, sugars and steroids, generally offer the advantage of rapid tumor targeting and swift clearance from non-target tissues relative to the analogous large molecular weight counterparts (e.g. antibodies, nanoparticles, etc.).[132-135] However, one significant challenge with low molecular weight, targeted agents has been obtaining optimal retention in the target tissue due to significant clearance of the radiotracer from the tumor. Since most of the targeted agents develop maximal accumulation in the target site within a short time (i.e. 15 min to 1 hour) after administration, it would be advantageous to develop techniques to selectively increase retention in the tumor thereby increasing the diagnostic and therapeutic efficacy of the agents.

Tumor hypoxia is the result of the inefficiency of the vascular architecture to adequately deliver nutrients, such as oxygen, to areas within the tumor. Since it has been demonstrated that human cancers, including prostate cancer, have significant levels of hypoxia, the development of hypoxia targeted drugs for both diagnostic and therapeutic applications in cancer has been an active area of research.[104, 115, 136-140] In particular, 2-nitroimidazole derivatives, which selectively reduce and become trapped within hypoxic cells, have been among the most thoroughly investigated class of hypoxia selective agents. This study seeks to design BB2r-targeted agents that incorporate the hypoxia selective trapping agent 2-nitroimidazole into the linking group of the radioconjugate. It is estimated that upon reduction and activation of the 2-nitroimidazole approximately 20% of the hypoxia selective agent become irreversibly conjugated to proteins within the cell.[131] In order to determine if one or more 2-nitroimidazoles would increase the retention of the radioconjugate in hypoxic cells, $^{111}$In-2-4, were synthesized with one, two or three 2-nitroimidazoles, respectively, incorporated into the
linking group of the radioconjugate. Radioconjugate $^{111}$In-1 is a thoroughly investigated BB2r-targeted peptide that does not contain 2-nitroimidazole moieties and thus was used as the control in our experiments.[124]

Synthesis of the $^{111}$In-labeled radioconjugates 1-4 began with the synthesis of the 2-NIAA according to literature procedures.[126] The 2-NIAA was thoroughly characterized by NMR and the structural constitution of the moiety confirmed by x-ray crystallography. Conjugates 1 and 2-4* were synthesized and purified by RP-HPLC in fairly low yields (12-22 %). Coupling of the 2-NIAA to the conjugates 2*-4* was accomplished using standard amidation chemistry. Further purification of 2-4 by RP-HPLC provided the conjugates in modest yields (34 - 44 %). Radiolabeling of conjugates 1-4 proceeded easily and with high purity ($\geq$ 95%) using standard radiolabeling conditions.[124]

In vitro competitive BB2r binding assays of $^{111}$In-1-4 were performed using PC-3 cells with [$^{125}$I-Tyr$_4$]-Bombesin as the competitive radioligand. For the natural indium labeled conjugates, these studies demonstrated the following trend: $^{111}$In-2 > $^{111}$In-1 > $^{111}$In-3 > $^{111}$In-4. Analyzing the structure-activity relationship with respect to binding affinity, it was determined that inclusion of more than one 2-nitroimidazole in the linking group had an unfavorable effect on the binding affinity of the radioconjugate. This strongly indicates that the 2-nitroimidazoles are interfering, through charge or sterics, with the ability of the pharmacophore (i.e. BBN(7-14)NH$_2$) to bind strongly to the BB2r. Since the pKa of 1-substituted-2-nitroimidazoles, such as misonidazole, are generally significantly below physiological pH, the 2-nitroimidazoles are likely not charged.[141, 142] Given this, it is probable that the loss in binding affinity in $^{111}$In-3 and $^{111}$In-4 are primarily a result of steric interference of the 2-nitroimidazole trapping moieties with the BB2r-targeting vector. Based on the $^{111}$In-2 radioconjugate, the inclusion of a linker between the 2-nitroimidazole-amino acid residue and the pharmacophore of at least five carbon lengths is
needed to achieve more optimal binding. This need for adequate spacers will be considered when designing future 2-nitroimidazole containing radioconjugates.

In order to examine the effect hypoxia has on the uptake and retention of the designed $^{111}$In labeled radioconjugates, the conditions for the generation of hypoxic PC-3 cells was first validated. For this purpose pimonidazole, a well-known and commonly used agent for the assessment of cellular hypoxia, was used for the confirmation of hypoxia generation in PC-3 cells.[127-129] The targeting mechanism of the pimonidazole utilizes the same 2-nitroimidazole moiety employed in the design of the $^{111}$In-2-4 radioconjugates. Exposure of PC-3 cells incubated under the hypoxic conditions of our studies revealed a fivefold increase in pimonidazole binding compared to PC-3 cells incubated under normoxic conditions as determined by flow cytometry. These results are consistent with similar hypoxia analysis utilizing pimonidazole.[127] Since pimonidazole utilizes the same 2-nitroimidazole moiety for hypoxia targeting as the $^{111}$In labeled radioconjugates in our study, the cellular hypoxia generated utilizing our conditions were determined to be sufficient for the evaluation of the BB2r expression and the efficacy of the radioconjugates in PC-3 cells.

The design of targeted agents that are selectively enhanced toward hypoxic cells requires an understanding of the expression of the target under normoxic as well as hypoxic conditions. Since these agents gain entry into the cell through receptor-mediated endocytosis, if expression of the receptor target is significantly down-regulated under hypoxic conditions, any enhancement in hypoxic retention is likely to be substantially inhibited. Conversely, if under hypoxic conditions the target increases in expression; both the targeting and hypoxia enhancement of the drug would be expected to be improved. With this in mind, receptor saturation studies were undertaken to evaluate the expression of the BB2r on the surface of the PC-3 cells under hypoxic and normoxic conditions. From these studies, it was determined that
the BB2r expression was 0.35 ± 0.08 and 0.30 ± 0.06 million receptors per PC-3 cell under normoxic and hypoxic conditions, correspondingly. During this study, oxygen levels, at least throughout the course of our investigation, did not play a significant role in the surface receptor expression of the BB2r. These findings are in accordance with a report from Martínez and colleagues of the BB2r expression in the H209 human lung cancer cell line under normoxic and hypoxic conditions.[143] With respect to overall BB2r expression of the PC-3 cells, the values obtained agree well with other reports.[144]

Efflux studies were performed under normoxic and hypoxic conditions to evaluate the efficacy of the hypoxia trapping moieties to enhance retention in hypoxic cells. The initial 2 hour incubation of the radioconjugates with the PC-3 cells yielded a bimodal uptake pattern. $^{111}$In-1, the control for our experiment, and $^{111}$In-2 demonstrate significant (22.6 % and 20.5 % of the dose, respectively) uptake, while $^{111}$In-3 and $^{111}$In-4 exhibited significantly lower internalization (1.4 % and 0.8 %, respectively). Based on the in vitro binding data, the significant decline in internalization with respect to $^{111}$In-3 and $^{111}$In-4 is likely due to the poor receptor affinity of these radioconjugates relative to $^{111}$In-1 and $^{111}$In-2. Interestingly, for some of the radioconjugates, oxygen levels seemed to have a significant effect on the initial uptake values of the radioconjugates. This is particularly true for $^{111}$In-1 and $^{111}$In-2 in which a significant reduction of 25 % was observed under hypoxic conditions for both radioconjugates. The cause for this decline is unknown; however, it was interesting to note that within the first 30 minutes of the efflux study at normoxia, all radioconjugates undergo substantial decline (29 – 55 %) to levels that are significantly below that of the hypoxic levels. Contrarily, for the hypoxic studies, the amount of internalized activity declined relatively little (5 - 15 %) during the same time period. Further investigations are needed to elucidate the exact cause for these contrasting
observations. After the initial 30 minute timepoint, there was relatively little decline in internalized activity among the radioconjugates.

As stated earlier, normoxic levels of internalized activity initially were greater, excepting $^{111}\text{In-4}$, in normoxic rather than the hypoxic studies. Although by 30 minutes, the situation had reversed itself with all radioconjugates demonstrating hypoxic/normoxic ratios greater than one. The radioconjugate $^{111}\text{In-4}$ demonstrated the highest average hypoxia enhancement (1.83 ± 0.17) followed closely by $^{111}\text{In-2}$ (1.58 ± 0.15). Interestingly, $^{111}\text{In-3}$ has a fairly low average enhancement of 1.27 ± 0.12 compared to $^{111}\text{In-2}$. Given that $^{111}\text{In-3}$ has two 2-nitroimidazole moieties in comparison to one incorporated into $^{111}\text{In-2}$, the lower hypoxia enhancement is unexpected. The reason for this circumstance is not clear. Remarkably, even the control in our experiment, $^{111}\text{In-1}$, demonstrated a small (≤ 10 %) increase in retention under hypoxic conditions. Speculatively, the retention in the control under hypoxic conditions may be attributed to slower metabolism kinetics or other hypoxia-impaired cellular functions needed to efflux the radioconjugates. Ultimately, more studies, which are in progress, are needed to obtain a clearer understanding of this process. Nevertheless, all of the experimental radioconjugates (i.e. $^{111}\text{In-2-4}$) demonstrated significantly higher hypoxia enhancement than observed in the control, strongly suggesting that 2-nitroimidazoles are at least partially responsible for this enhancement.

It is well known that 2-nitroimidazole derivatives based on small molecules bind irreversibly to proteins under hypoxic conditions. In order to elucidate whether or not BB2r-targeted agents employing 2-nitroimidazoles can conjugate to proteins, the cellular protein of the PC-3 cells were isolated after incubation with $^{111}\text{In-1}$, $^{111}\text{In-2}$ and $^{125}\text{I}$-Tyr$_4$BBN under hypoxic and normoxic conditions. The controls, $^{111}\text{In-1}$ and $^{125}\text{I}$-Tyr$_4$BBN, demonstrated 3.15 to 5.60 % association with the proteins under both normoxic and hypoxic conditions. Statistical analysis of
the difference between the hypoxic and normoxic protein association for the controls revealed no statistically significant difference ($p > 0.05$). Since the controls do not contain reactive moieties that would be expected to irreversibly bind to macromolecules, the protein association for the controls are most likely due to reversible, non-specific binding. Under normoxic conditions the $^{111}$In-2 radioconjugate demonstrated similar protein association as controls. However, under hypoxic conditions the $^{111}$In-2 radioconjugate demonstrated a threefold increase in protein association ($p < 0.001$). These results indicate that the increase in protein association of $^{111}$In-2 is oxygen dependent and is consistent with the trapping mechanism of the 2-nitroimidazole. While the exact binding mode of the $^{111}$In-2 with proteins is not known beyond doubt, the findings from a superfluity of 2-nitroimidazole literature and the results of this study lead us to the conclusion that the increased protein association of the $^{111}$In-2 radioconjugate is likely irreversibly bound to the proteins. Assuming the $^{111}$In-2 protein association under normoxic conditions is reversible, non-specific binding, the increased protein association of the radioconjugate under hypoxic conditions signifies that $\sim 10\%$ of the internalized cellular activity is likely irreversibly bound to cellular proteins.

2.5 CONCLUSION

In conclusion, we have synthesized and evaluated three BB2r-targeted radioconjugates that have 2-nitroimidazole hypoxia trapping moieties conjugated to the linking group of the peptide for the purpose of enhancing retention in hypoxic cancers. Our studies indicate that placement of the 2-nitroimidazole moieties close to the pharmacophore has a detrimental effect on the ability of the radioconjugate to adequately bind to the BB2r. The BB2r-targeted agents that include 2-nitroimidazole moieties demonstrated improved longitudinal retention in hypoxic relative to normoxic PC-3 cells. Additionally, we found that the inclusion of 2-nitroimidazole moieties in the BB2r-targeted agent design significantly increases protein association, by a likely
irreversible conjugation reaction, under hypoxic conditions. Also, evaluation of the PC-3 human prostate cancer cell line reveals that the BB2r expression is not dependent on oxygen levels and suggests that the BB2r would therefore still be a valid target on the hypoxic fraction of BB2r-positive prostate cancer cells. While further work is still needed, this work suggests a potential new avenue to significantly increase the cellular retention of many targeted agents in hypoxic cancers. Our future work will focus on elucidating the mechanisms of retention of the hypoxia enhanced BB2r-targeted agents and evaluating the efficacy of these agents in *in vivo* cancer models.
3.1 INTRODUCTION

As mentioned in Chapter 2, the disadvantage of many small molecules based, peptide-targeted radiotracer is low retention at the tumor site due to intrinsically high diffusion and efflux rates. This can substantially reduce the diagnostic and therapeutic efficacy of the agent as well as its potential for clinical translation.

We have previously demonstrated that these hypoxia-enhanced BB2r-targeted peptides significantly increase retention in hypoxic PC-3 human prostate cancer cells [145]. From these studies, it was determined that the proximity of the 2-nitroimidazole relative to the pharmacophore had a substantial impact on BB2r binding affinity. Herein, we present the synthesis and in vitro properties of hypoxia-enhanced BB2r-targeted radioconjugates with extended linking groups to improve BB2r binding affinity. Additionally, utilizing biodistribution and microSPECT/CT imaging studies, we report the first in vivo investigation of these agents in a PC-3 xenograft mouse model.

3.2 MATERIALS AND METHODS

3.2.1 Chemical and Supplies

Unless otherwise noted all solvents were used without further purification. Deionized water was purified by a Millipore (U.S.) Milli-Q-Biocel. Acetonitrile, formic acid, N, N-diisopropylethylamine (DIEA), N,N-dimethylformamide (DMF), dichloromethane (DCM), N,N'-dicyclohexylcarbodiimide (DCC), N-methylpyrrolidone (NMP), thioanisol, sodium hydroxide, L-

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ascorbic acid, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES), bovine serum albumin (BSA) and sodium dodecyl sulfate (SDS) were purchased from Fisher Scientific (U.S.). 2-nitroimidazole was purchased from Amfinecom (U.S.). O-Benzotriazole-N,N,N’,N’-tetramethyluronium-hexafluoro-phosphate (HBTU), Fmoc-protected natural amino acids and the appropriate Rink Amide resins were purchased from Nova Biochem (U.S.). Fmoc-8-AOC was purchased from CreoSalus (U.S). Roswell Park Memorial Institute (RPMI) 1640 media, phosphate buffered saline (PBS), and mammalian protein extraction reagent (M-PER) were purchased from Thermo Scientific (U.S.). Indium-111 chloride (\(^{111}\text{InCl}_3\)) was purchased from MDS Nordon (Canada). Naturally abundant indium chloride (\(^{nat}\text{InCl}_3\)), triisopropyl silane and 3, 6-dioxa-1, 8-octanedithiol were purchased from Sigma-Aldrich (U.S.). The \(^{125}\text{I}-\text{Tyr}^4\)-Bombesin was purchased from Perkin Elmer (U.S.). Prostate cancer (PC-3) cell lines were obtained from American Type Culture Collection (U.S.) and cultured under vendor recommended conditions. TrypLE™ Express was purchased from Invitrogen (U.S.). The separation of macromolecules and small molecules was performed using Amicon Ultra (Ireland) Ultracel-30K centrifugal filters in protein association analysis. Four week-old Institute of Cancer Research severely combined immunodeficient (ICR SCID) mice were obtained from Charles River Laboratories (Wilmington, MA). Food and water were given ad libitum. Each animal was kept in individual cage with an air filter cover under light- (12 h light/dark cycle) and temperature-controlled (22±1°C) environment. 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.

3.2.2 Equipment

The peptides were synthesized on a Liberty microwave peptide synthesizer from CEM (U.S.). HPLC/MS analyses were performed on a Waters (U.S.) e2695 system equipped with a Waters
2489 absorption detector and a Waters Qtof Micro electrospray ionization mass spectrometer. Evaluation and purification of radiolabeled conjugates 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. A Phenomenex (U.S.) Jupiter 10µ Proteo 250 × 10 mm semiprep column was used for the purification of bulk amounts of peptides. The peptides were concentrated on a Centrivap (U.S.) Centrifugal Concentrator. For the purification of conjugates, natIn-conjugates, and 111In-radioconjugates a Phenomenex Jupiter 10µ Proteo 250 × 4.60 mm analytical column was employed. Solid phase extraction was performed using Empore (U.S.) C18 10 mm high performance extraction disks. Hypoxic PC-3 cells were incubated in hypoxic glove box with temperature, CO2 and humidity controller (Coy Laboratory Products INC, Grass Lake, MI). Gamma decay detection of 111In and 125I for the in vitro binding, receptor saturation, efflux studies and protein binding fractionation studies was accomplished using a LTI (U.S.) Multi-Wiper nuclear medicine gamma counter. 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). Biodistribution radiation measurements were made with a NaI (TI) well detector constructed by AlphaSpectra, Inc. (U.S.). Small animal SPECT/CT data acquisition is achieved with the dual Flex Triumph CT/SPECT instrument (GE Healthcare, Gamma-Medica Ideas, Northridge, CA).

3.2.3 Cell Lines and Xenograft Models

Prostate cancer (PC-3) cell lines were obtained from American Type Culture Collection (U.S.) and cultured under vendor recommended conditions. All animal experiments were conducted 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. Four week-old Institute of Cancer Research severely combined immunodeficient
(ICR SCID) mice were obtained from Charles River Laboratories (Wilmington, MA). Food and water were given ad libitum. Each animal was kept in individual cages equipped with a HEPA air filter cover in a light- and temperature-controlled environment. Bilateral PC-3 tumors were induced by subcutaneous injection of \(5.0 \times 10^6\) cells in Matrigel (BD Biosciences). The tumors were allowed to grow ranging from 0.1 to 1 g (4-6 weeks post-inoculation), before the mice were utilized in pharmacokinetic studies.

### 3.2.4 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-dioxa-1,8-octanediethiol (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×3). The crude conjugate was dried by a centrivap 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.

### 3.2.5 Coupling of 2-NIAA to Bombesin Conjugates

The 2-NIAA was manually coupled to ε- amino group of the lysine residue for peptides 2*, 3* and 4* (9.2, 29.3 and 16.4 mg respectively) using standard amidation chemistry. Briefly, for
peptides 2* and 4*, the 2-NIAA (3.6 and 12.1 mg, respectively)/NHS (3.6 and 6.1 mg, respectively) mixtures were dissolved in HBTU/DMF (0.5 M, 83.4 and 142.4 µl) solution followed by addition of DIEA/NMP solution (5.7 M, 18.1 and 30.9 µl). For peptide 3*, the 2-NIAA (16.6 mg)/NHS (11.2 mg) mixtures were dissolved in DCC/DMF (0.9 M, 141.2 µl) solution followed by addition of DIEA/NMP solution (5.7 M, 56.3 µl). These solutions were allowed to stand for 2 h in an ice bath before addition of the conjugate in DMF (200 µL). The reaction mixture was stirred overnight at room temperature and subsequently evaporated to dryness. The residue was re-dissolved in water: acetonitrile: formic acid (8:2:0.05), peak purified by RP-HPLC and characterized by mass spectrometry.

3.2.6 Labeling with natInCl₃

For the convenient characterization of the ¹¹¹In-Bombesin conjugates, naturally abundant natIn was used to substitute for ¹¹¹In in the ES-MS and in vitro binding studies. A sample of conjugates (0.5 mg) was dissolved in ammonium acetate buffer (1 M, 200 µL, pH 5.5) and mixed with a solution of natInCl₃ (5.5 mg, 50 µmol). The solution was heated for 60 min at 50 °C. After cooling to room temperature, natIn-conjugates were then peak purified by RP-HPLC. All natIn-conjugates were ≥ 95% purity before mass spectrometric characterization and in vitro binding studies were performed.

3.2.7 Radiolabeling with ¹¹¹InCl₃

Radiolabeling was performed on all conjugates by mixing 100 µg samples with 37 MBq ¹¹¹InCl₃ in ammonium acetate buffer (1 M, 200 µL, pH 5.5). The solution was heated for 60 min at 90 °C. The resulting specific radioactivities were 0.64, 0.71, 0.78 and 0.86 MBq/nmol for ¹¹¹In-1, ¹¹¹In-2, ¹¹¹In-3 and ¹¹¹In-4. In order to separate radiolabeled peptides from unlabeled peptides on HPLC, 4-5 mg CoCl₂ were then added and incubated for 5 min at 90 °C to increase the
hydrophobicity of unlabeled conjugates. The resulting radioconjugates were allowed to cool to room temperature and peak purified using RP-HPLC (≥95%) and concentrated using C_{18} extraction disk. Elution of the extraction disk with ethanol/sterile saline solution (6 : 4, 150 μL × 2) delivered the radioconjugate in high purity. L-ascorbic acid (~20 mg) was added to all radioconjugates to reduce radiolysis. The specific activities for all peak-purified $^{111}$In-conjugates are essentially the theoretical maximum of 1725 MBq/nmol.

### 3.2.8 HPLC Purification and Analysis Methodology

When necessary, bulk sample purification was performed using a semi-preparative Proteo column with a flow rate of 5.0 mL/min. Sample purification for in vitro/in vivo studies was performed on analytical Proteo column with a flow rate 1.5 mL/min. HPLC solvents consisted of H$_2$O containing 0.1% formic acid (solvent A) and acetonitrile containing 0.1% formic acid (solvent B). For all unlabeled and $^{111}$/natIn-conjugates, purification was achieved using an initial gradient of 85 % A : 15 % B which linearly decreased to 75 % A : 25 % 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.

### 3.2.9 In Vitro Competitive Cell-Binding Studies

Briefly, the PC-3 cells (~$3\times10^7$) 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 45 min in the presence of radiolabeled [$^{125}$I-Tyr$_4$]-Bombesin and various concentrations of the natIn-conjugate. At the end of the incubation period, the cells were centrifuged, aspirated and washed with media a total of four times. The cell associated activity was measured using a gamma counter and the IC$_{50}$ values determined by nonlinear regression using the one-binding site model of Graphpad PRISM 5 (U.S.).
3.2.10 In Vitro Internalization and Efflux Studies

Efflux studies were performed using PC-3 cells under normoxic (95% air, 5% CO₂) and hypoxic (94.9% N₂, 0.1% O₂, 5% CO₂) conditions. PC-3 cells were incubated in six-well plates (0.5 × 10⁶ / well) under hypoxic conditions overnight in RPMI 1640 media (pH 7.4, 4.8 mg/mL HEPES, and 2 mg/mL BSA). On the day of experiment, the cells medium were changed and incubated for 2 h under normoxic and hypoxic conditions, respectively. The cells were then pre-incubated for 2 h at 37°C in the presence of 100,000 cpm of each ¹¹¹In-radioconjugate. Upon completion of the incubation, cells were washed thrice with media to discard the unbound peptide. At time points 0, 2, 4 and 8 h, the media 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 lysates 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.).

3.2.11 Cellular Protein Analysis

For the cellular fractionation studies, the procedure for the preparation of normoxic and hypoxic PC-3 cells (2.5×10⁵ / well) was carried out as outlined in the efflux studies above. On the day of the experiment, the medium was removed, and the cells were washed with medium and incubated at 37°C in the presence of 100,000 cpm of each ¹¹¹In-radioconjugate. At time points 2, 4 and 8 h, the cells were washed thrice with medium to remove the extracellular radioconjugates and then lysed using 1mL of M-PER at 37°C. The cellular debris was centrifuged down at 13300xg and the supernatant was then transferred to an Amicon Ultracel 30kDa filter
device with extra PBS (1mL). The samples were centrifuged at 4000×g for 10 minutes and washed with PBS (1mL×2). The radioactivity associated with the molecular weight fractions was collected and determined using a gamma counter.

3.2.12 Pharmacokinetic Studies of $^{111}$In-Radioconjugates in PC-3 Xenograft SCID Mice.

Pharmacokinetic studies were carried out using PC-3 tumor bearing SCID mice. The mice were inoculated with PC-3 cells, and the tumors were allowed to grow ranging from 0.1 to 1 g (4-6 weeks post-inoculation), before the mice were utilized in pharmacokinetic studies. Each mouse (average weight, 20 g) received an intravenous bolus via the tail vein of 7.5 μCi (277.5 kBq) of the radio-RP-HPLC peak purified $^{111}$In-radioconjugate ($^{111}$In-1, $^{111}$In-2 or $^{111}$In-4) in 100 μL of saline. The mice were sacrificed and their tissues were excised at 1, 4, 24, 48 and 72 h time points post injection. The excised tissues were weighed, the radioactivity in each tissue was measured, and the %ID or %ID/g was calculated for each tissue. Blocking Studies were also investigated on $^{111}$In-4 by co-injection with 300 µg of unlabeled conjugate 4 (n=3).

3.2.13 Small Animal SPECT/CT Imaging Studies.

The SPECT g-camera incorporates an array of 5-by-5 solid state cadmium/zinc/telluride (CZT) modules, each with 1.5-mm (0.06-inch) pixel array of 16 x 16, providing a total of 80 x 80 pixel array within a field of view of 12.7-cm by 12.7-cm (5-inch by 5-inch). The X-ray detector is complementary metal oxide semiconductor (CMOS)-based device, having a 2240 X 2368 pixel matrix with a 50 mm pitch to yield a 12 mm x 12 mm (4.72 inch x 4.72 inch) field of view. The mice were administered 4~11MBq (0.108~0.300 mCi) of the desired BB2r-targeted peptide in 100 - 200 μL of saline via tail vein injection. At 1, 24, 48 and 72 h post injection, mice were anesthetized with 1~1.5% isoflurane delivered in a 2:1 mixture of nitrous oxide/oxygen. Image acquisition was accomplished using a FLEX Triumph X-ray computed tomography/single photon
emission computed tomography system (CT/SPECT) and software (Gamma Medica, Inc., Northridge, CA) fitted with a 5-pinhole (1.0 mm/pinhole) N5F75A10 collimator. 64 SPECT projections (30 to 90-s acquisition time per projection based on the actual counts) for each image were acquired using Triumph_SPECT and reconstructed using SpectReconstructionApp. 512 CT projections for each image were acquired and reconstructed using Triumph X-O 4.1. Co-registration of anatomical CT images and functional SPECT was performed using 3D image visualization and analysis software VIVID, which is based on Amira 4.1.

3.2.14 Statistical Analysis

IC\textsubscript{50} values were determined by nonlinear regression using the one-binding site model of Graphpad PRISM 5. Comparisons of each two groups for efflux studies, cellular protein analysis studies and biodistribution studies were analyzed by the 2-tailed Student t test, and P value of less than 0.05 were considered statistically significant.

3.3 RESULTS

3.3.1 Conjugate Synthesis and Radiolabeling

Four radioconjugates were synthesized using the DOTA-X-8-AOC-BBN(7-14)NH\textsubscript{2} paradigm (Figure 3.1). The yields of conjugates 1, 2*, 3* and 4* ranged from 16.67 to 20.44 % as determined by RP-HPLC. The 2-NIAA coupling of conjugate 2* and 4* were performed by O-benzotriazole-N, N, N’, N’-tetramethyl-uronium-hexafluoro-phosphate (HBTU) conjugation, whereas conjugate 3* was coupled by using N, N’-Dicyclohexylcarbodiimide (DCC). All attempts to conjugate 3* with 2-NIAA using HBTU resulted in poor yields (<1%). The products were purified by RP-HPLC and isolated with yields of 17.4, 26.7 and 19.0 % for conjugates 2, 3 and 4. RP-HPLC retention time and mass spectrometric identification of the conjugates are listed in Table 3.1.
Figure 3.1 Hypoxia enhanced $^{111}$In-BB2r-targeted conjugates
For convenient mass spectra analysis, In was replaced by nat In.

Values represent mean ± SEM (n=6).

<table>
<thead>
<tr>
<th>Analogue</th>
<th>RP-HPLC Retention Time / min</th>
<th>IC50 / nM</th>
<th>Observed MS</th>
<th>Calculated MS</th>
<th>Molecular Formula</th>
</tr>
</thead>
<tbody>
<tr>
<td>4</td>
<td>6.9 ± 1.2</td>
<td>10.55</td>
<td>2423.3</td>
<td>2423.3</td>
<td>C10H14InN33Os2O8</td>
</tr>
<tr>
<td>3</td>
<td>5.8 ± 1.1</td>
<td>2229.5</td>
<td>2230.5</td>
<td>2230.5</td>
<td>C10H14InN33Os2O8</td>
</tr>
<tr>
<td>2</td>
<td>7.3 ± 1.1</td>
<td>2035.0</td>
<td>2034.9</td>
<td>2034.9</td>
<td>C10H14InN33Os2O8</td>
</tr>
<tr>
<td>1</td>
<td>7.1 ± 1.1</td>
<td>1840.2</td>
<td>1839.7</td>
<td>1839.7</td>
<td>C10H14InN33Os2O8</td>
</tr>
<tr>
<td>4</td>
<td>20.1 ± 1.3</td>
<td>2311.5</td>
<td>2312.0</td>
<td>2311.5</td>
<td>C10H14InN33Os2O8</td>
</tr>
<tr>
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<td>17.1 ± 1.2</td>
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<td>C10H14InN33Os2O8</td>
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<td>1852.5</td>
<td>1852.5</td>
<td>C10H14InN33Os2O8</td>
</tr>
</tbody>
</table>

Table 3: Mass spectrometric and RP-HPLC characterization of conjugates.
3.3.2 In Vitro Competitive Cell-binding Studies

The BB2r binding affinity of the conjugates and \(^{nat}\)In-conjugates were investigated by competitive binding studies using the BB2r-positive, PC-3 cell line. All conjugates and \(^{nat}\)In-conjugates demonstrated nanomolar binding affinities. The \(^{nat}\)In-labeled conjugates had IC\(_{50}\) values of 7.1 ± 1.1, 7.3 ± 1.1, 5.8 ± 1.1 and 6.9 ± 1.2 nM for \(^{nat}\)In-1-4, respectively. Slightly lower binding affinities were observed for unlabeled conjugates compared with \(^{nat}\)In-labeled, but statistical analysis revealed no overall trend.

3.3.3 In Vitro Internalization and Efflux Studies

We have previously reported that the surface expression of the BB2r remains essentially unchanged under the hypoxic conditions employed in our studies [145]. In these studies, \(^{111}\)In-1, which does not have a 2-nitroimidazole incorporated, is the control to compare the relative effectiveness of the hypoxia trapping conjugates (\(^{111}\)In-2-4). The PC-3 cells were first incubated in the presence of the radioconjugates for 2 h prior to the start of the efflux studies. During this incubation period, all of the radioconjugates investigated under both normoxic and hypoxic conditions demonstrated similar levels of internalization, ranging from 18 to 22% of the total radioactivity added.

The efflux of the radioconjugates over time given as a percentage of the initial internalized activity is depicted in Figure 3.2. Within the first 2 h of the experiment, the \(^{111}\)In radioconjugates 1-4 under hypoxic conditions demonstrated a lower efflux rate relative to normoxic conditions. The maximum differences of percentage effluxed radioactivity were observed at 8 h time point for \(^{111}\)In-1-4. Specifically, 41.4, 60.7, 69.1 and 69.4 % of initially internalized radioactivity was retained under hypoxic conditions compared with only 34.8, 35.3, 33.2 and 29.7 % retained under normoxic conditions, respectively. \(^{111}\)In-1 also exhibited a significant decrease in
clearance rate after 2 hours under hypoxic conditions. However, the increased retention showed by $^{111}\text{In-1}$ is limited relative to $^{111}\text{In-2-4}$ which have 2-nitroimidazoles incorporated into the structure of the radioconjugate. The radioconjugates $^{111}\text{In-2-4}$ demonstrated significantly higher retention under hypoxic conditions relative to normoxic conditions ($P<0.0001$).
Figure 3.2 Efflux assays for the $^{111}$In-radioconjugates in PC-3 cells

Efflux assays depicted as percentage of initial internalized activity for the $^{111}$In-radioconjugates in PC-3 cells. Values are mean ± SD (n=5).
The internalized activity of the radioconjugate was compared as an additional means of evaluating the retention effect under normoxic and hypoxic conditions. Hypoxia enhancement factor (HEF) is defined as the ratio of the amount of activity remaining in the hypoxic cells versus the normoxic PC-3 cells of radioconjugates internalized. The HEF for each radioconjugate at each time point is depicted in Figure 3.3. At the initial time point, all of the radioconjugates demonstrated a similar accumulation under both normoxic and hypoxic conditions where the HEF is approximately equal to 1. At the 2 h time point, all of the radioconjugates incorporated with 2-nitroimidazole started to display significantly higher retention in hypoxic relative to normoxic cells (i.e., HEF > 1). By the 8 h time point, the $^{111}$In-1-4 demonstrated an average HEF of $1.17 \pm 0.12$, $1.95 \pm 0.28$, $2.72 \pm 0.35$ and $3.29 \pm 0.25$, respectively. The radioconjugates $^{111}$In-2-4 exhibited significantly higher average HEF ratios than the control. The strong positive linear relationship between the HEF and the number of 2-nitroimidazoles incorporated was confirmed by linear regression analysis (R > 0.96).
Figure 3.3 HEF ratios for the efflux studies of 111In-BB2r-targeted radioconjugates

HEF ratios for the efflux studies of 111In-BB2r-targeted radioconjugates under normoxic (95% air, 5% CO₂) and hypoxic conditions (94.9% N₂, 0.1% O₂, 5% CO₂). Values are mean ± SD (n=5).
3.3.4 Cellular Protein Analysis

It is well established that 2-nitroimidazoles are reductively-activated in a hypoxic environment. This activation leads to the irreversible conjugation of the reduced 2-nitroimidazole moiety with intracellular nucleophiles (e.g., thiols), including those contained in proteins, to form adducts [131, 146, 147]. In order to better elucidate the mechanism of the observed increase in retention of the 2-nitroimidazole containing BB2r-targeted agents under hypoxic conditions, the protein association properties of the conjugates were evaluated under hypoxic and normoxic environments. At the 2, 4 and 8 h post incubation time points, the PC-3 cells were lysed and centrifuged. The supernatant was then filtered using a 30 kDa centrifugal filter. The ratio of protein associated radioactivity as a percentage of total intracellular radioactivities under hypoxic conditions over the percentage of protein associated radioactivity under a normoxic environment is depicted in Figure 3.4. The control radioconjugates $^{111}\text{In-1}$ demonstrated similar ratios, from 1.08 to 1.38, during the timespan of the experiment. The 2-nitroimidazole containing BB2r-targeted conjugates demonstrated at least one fold higher protein association under hypoxic conditions than that observed under normoxic conditions. For $^{111}\text{In-4}$, at 4 and 8 h time points, up to three fold higher hypoxic/normoxic protein association ratios were observed relative to the control. These results strongly suggest that 2-nitroimidazoles are partially responsible for this enhancement.
Figure 3.4 Protein association studies

Protein association studies under normoxic (95% air, 5% CO₂) and hypoxic (94.9% N₂, 0.1% O₂, 5% CO₂) conditions in PC-3 cells. Values are mean ± SD (n=5).
3.3.5 Biodistribution studies

The in vivo biodistribution of the $^{111}$In-1, $^{111}$In-2 and $^{111}$In-4 radioconjugates were investigated in PC-3 tumor bearing SCID mice. Due to the similar efflux and protein association properties of $^{111}$In-3 and $^{111}$In-4, $^{111}$In-3 was not investigated in vivo. The results obtained from pharmacokinetic studies of $^{111}$In-1, $^{111}$In-2 and $^{111}$In-4 at 1, 4, 24, 48 and 72 h p.i. are summarized in Table 3.2. All of the investigated $^{111}$In-radioconjugates demonstrated rapid blood clearance at 1 h post injection. Clearance of the radioconjugates proceeded largely through the renal/urinary system. At 1 h post injection, the highest accumulation was found in the pancreas for all three radioconjugates with $70.96 \pm 15.88$, $33.70 \pm 27.11$ and $33.04 \pm 19.50 \%$ID/g, respectively. These results are due to the high expression of BB2r in rodent pancreas and are consistent with previous reports [148, 149]. The tumor retention of radioconjugate $^{111}$In-4 ($2.80 \pm 1.18 \%$ID/g) at 1 h p.i. is substantially lower than $^{111}$In-1 ($5.82 \pm 2.63 \%$ID/g) and $^{111}$In-2 ($6.06 \pm 3.35 \%$ID/g). However, by the 72 h post injection time point, 1.5%, 6.7% and 21.0% of the initial 1 h uptake was retained in the tumor tissue corresponding to radioconjugates $^{111}$In-1, $^{111}$In-2 and $^{111}$In-4 (Figure 3.5). The tumor retention observed for both $^{111}$In-2 ($0.41 \pm 0.07 \%$ID/g, P<0.01) and $^{111}$In-4 ($0.60 \pm 0.40 \%$ID/g, P<0.05) was found to be significantly higher as compare with the control $^{111}$In-1 ($0.09 \pm 0.10 \%$ID/g). With the exception of the kidneys, the addition of the 2-nitroimidazoles did not increase the non-target retention of the BB2r-targeted agents. The initial kidney uptake for all of the radioconjugates investigated was approximately 15 %ID/g at the 1 h time point. By 72 h post injection the conjugates $^{111}$In-2 ($2.66 \pm 0.73 \%$ID/g) and $^{111}$In-4 ($8.83 \pm 5.69 \%$ID/g) demonstrated significant retention (P<0.05) in the kidneys as compared to $^{111}$In-1 (0.76 $\pm 0.67 \%$ID/g). In this study, the kidney retention correlated with an increase in the 2-nitroimidazole moieties of the BB2r-targeted agent. The co-injection of an excess of unlabeled conjugate 4 along with $^{111}$In-4 resulted in significantly reduced radioactivity in the pancreas (1.16
\[ \pm 0.66 \%\text{ID/g} \), kidney \( (12.37 \pm 7.92 \%\text{ID/g}) \) and tumor \( (0.44 \pm 0.34 \%\text{ID/g}) \) at 4 h post injection

\( (P<0.05, \text{one-tailed}) \).
Table 3.2 Biodistribution Studies in PC-3 Tumor-Bearing SCID mice

<table>
<thead>
<tr>
<th>Tissue (%ID/g)</th>
<th>1 h p.i.</th>
<th>4 h p.i.</th>
<th>24 h p.i.</th>
<th>48 h p.i.</th>
<th>72 h p.i.</th>
</tr>
</thead>
<tbody>
<tr>
<td>blood</td>
<td>0.11 ± 0.22</td>
<td>0.00 ± 0.00</td>
<td>0.00 ± 0.00</td>
<td>0.00 ± 0.00</td>
<td>0.00 ± 0.00</td>
</tr>
<tr>
<td>heart</td>
<td>0.00 ± 0.00</td>
<td>0.00 ± 0.00</td>
<td>0.00 ± 0.00</td>
<td>0.00 ± 0.00</td>
<td>0.00 ± 0.00</td>
</tr>
<tr>
<td>lung</td>
<td>0.21 ± 0.40</td>
<td>0.02 ± 0.03</td>
<td>0.00 ± 0.00</td>
<td>0.00 ± 0.00</td>
<td>0.00 ± 0.00</td>
</tr>
<tr>
<td>liver</td>
<td>0.40 ± 0.32</td>
<td>0.09 ± 0.11</td>
<td>0.03 ± 0.03</td>
<td>0.00 ± 0.00</td>
<td>0.00 ± 0.00</td>
</tr>
<tr>
<td>pancreas</td>
<td>70.96 ± 15.88</td>
<td>37.17 ± 10.52</td>
<td>12.07 ± 3.05</td>
<td>6.51 ± 2.26</td>
<td>0.00 ± 0.00</td>
</tr>
<tr>
<td>stomach</td>
<td>5.87 ± 3.63</td>
<td>1.02 ± 0.72</td>
<td>0.34 ± 0.26</td>
<td>0.11 ± 0.23</td>
<td>0.00 ± 0.00</td>
</tr>
<tr>
<td>small intestine (%ID)</td>
<td>8.15 ± 2.93</td>
<td>2.04 ± 0.59</td>
<td>0.96 ± 0.52</td>
<td>0.66 ± 0.18</td>
<td>0.02 ± 0.04</td>
</tr>
<tr>
<td>large intestine (%ID)</td>
<td>5.07 ± 1.26</td>
<td>4.74 ± 2.08</td>
<td>1.62 ± 0.54</td>
<td>1.71 ± 0.87</td>
<td>0.46 ± 0.28</td>
</tr>
<tr>
<td>kidney</td>
<td>13.41 ± 5.84</td>
<td>4.82 ± 1.60</td>
<td>2.18 ± 0.67</td>
<td>1.38 ± 1.01</td>
<td>0.76 ± 0.67</td>
</tr>
<tr>
<td>tumor</td>
<td>5.82 ± 2.63</td>
<td>2.16 ± 1.01</td>
<td>1.32 ± 0.62</td>
<td>0.83 ± 0.48</td>
<td>0.09 ± 0.10</td>
</tr>
<tr>
<td>muscle</td>
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<td>0.00 ± 0.00</td>
<td>0.00 ± 0.00</td>
<td>0.00 ± 0.00</td>
<td>0.00 ± 0.00</td>
</tr>
<tr>
<td>bone</td>
<td>0.00 ± 0.00</td>
<td>0.00 ± 0.00</td>
<td>0.00 ± 0.00</td>
<td>0.00 ± 0.00</td>
<td>0.00 ± 0.00</td>
</tr>
<tr>
<td>brain</td>
<td>0.00 ± 0.00</td>
<td>0.00 ± 0.00</td>
<td>0.00 ± 0.00</td>
<td>0.00 ± 0.00</td>
<td>0.00 ± 0.00</td>
</tr>
<tr>
<td>excretion (%)</td>
<td>35.88 ± 14.38</td>
<td>82.06 ± 4.65</td>
<td>91.07 ± 1.75</td>
<td>93.42 ± 3.26</td>
<td>98.44 ± 0.48</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Tissue (%ID/g)</th>
<th>1 h p.i.</th>
<th>4 h p.i.</th>
<th>24 h p.i.</th>
<th>48 h p.i.</th>
<th>72 h p.i.</th>
</tr>
</thead>
<tbody>
<tr>
<td>blood</td>
<td>1.21 ± 0.61</td>
<td>0.00 ± 0.00</td>
<td>0.02 ± 0.02</td>
<td>0.00 ± 0.00</td>
<td>0.00 ± 0.00</td>
</tr>
<tr>
<td>heart</td>
<td>0.00 ± 0.00</td>
<td>0.00 ± 0.00</td>
<td>0.00 ± 0.00</td>
<td>0.00 ± 0.00</td>
<td>0.00 ± 0.00</td>
</tr>
<tr>
<td>lung</td>
<td>1.21 ± 0.98</td>
<td>0.00 ± 0.00</td>
<td>0.00 ± 0.00</td>
<td>0.00 ± 0.00</td>
<td>0.00 ± 0.00</td>
</tr>
<tr>
<td>liver</td>
<td>0.87 ± 0.38</td>
<td>0.15 ± 0.05</td>
<td>0.13 ± 0.05</td>
<td>0.08 ± 0.01</td>
<td>0.06 ± 0.02</td>
</tr>
<tr>
<td>pancreas</td>
<td>33.70 ± 27.11</td>
<td>14.01 ± 13.97</td>
<td>1.98 ± 1.35</td>
<td>0.25 ± 0.17</td>
<td>0.22 ± 0.12</td>
</tr>
<tr>
<td>stomach</td>
<td>1.51 ± 1.69</td>
<td>0.49 ± 0.20</td>
<td>0.06 ± 0.08</td>
<td>0.07 ± 0.09</td>
<td>0.04 ± 0.08</td>
</tr>
<tr>
<td>small intestine (%ID)</td>
<td>4.62 ± 1.42</td>
<td>1.44 ± 0.61</td>
<td>0.23 ± 0.10</td>
<td>0.12 ± 0.06</td>
<td>0.02 ± 0.02</td>
</tr>
<tr>
<td>large intestine (%ID)</td>
<td>2.02 ± 0.7</td>
<td>3.82 ± 1.11</td>
<td>0.57 ± 0.18</td>
<td>0.35 ± 0.14</td>
<td>0.15 ± 0.04</td>
</tr>
<tr>
<td>kidney</td>
<td>14.88 ± 4.55</td>
<td>5.09 ± 1.70</td>
<td>3.74 ± 1.65</td>
<td>2.03 ± 0.42</td>
<td>2.66 ± 0.73</td>
</tr>
<tr>
<td>tumor</td>
<td>6.06 ± 3.35</td>
<td>1.89 ± 0.92</td>
<td>1.08 ± 0.49</td>
<td>0.58 ± 0.24</td>
<td>0.41 ± 0.07</td>
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<tr>
<td>muscle</td>
<td>1.69 ± 1.72</td>
<td>0.12 ± 0.25</td>
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<td>0.00 ± 0.00</td>
<td>0.00 ± 0.00</td>
</tr>
<tr>
<td>bone</td>
<td>0.71 ± 0.42</td>
<td>0.14 ± 0.15</td>
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<td>0.00 ± 0.00</td>
<td>0.00 ± 0.00</td>
</tr>
<tr>
<td>brain</td>
<td>0.02 ± 0.05</td>
<td>0.00 ± 0.00</td>
<td>0.00 ± 0.00</td>
<td>0.00 ± 0.00</td>
<td>0.00 ± 0.00</td>
</tr>
<tr>
<td>excretion (%)</td>
<td>17.26 ± 15.53</td>
<td>81.24 ± 8.28</td>
<td>94.89 ± 0.67</td>
<td>96.10 ± 0.61</td>
<td>95.90 ± 0.95</td>
</tr>
</tbody>
</table>
Table 3.2 (continued) Biodistribution Studies in PC-3 Tumor-Bearing SCID mice

<table>
<thead>
<tr>
<th>Tissue (%ID/g)</th>
<th>1 h p.i.</th>
<th>4 h p.i.</th>
<th>24 h p.i.</th>
<th>48 h p.i.</th>
<th>72 h p.i.</th>
</tr>
</thead>
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<tr>
<td>blood</td>
<td>0.75 ± 0.54</td>
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<td>0.00 ± 0.00</td>
<td>0.00 ± 0.00</td>
<td>0.00 ± 0.00</td>
</tr>
<tr>
<td>heart</td>
<td>0.73 ± 1.46</td>
<td>0.00 ± 0.00</td>
<td>0.00 ± 0.00</td>
<td>0.00 ± 0.00</td>
<td>0.00 ± 0.00</td>
</tr>
<tr>
<td>lung</td>
<td>0.61 ± 1.04</td>
<td>0.41 ± 0.82</td>
<td>0.00 ± 0.00</td>
<td>0.00 ± 0.00</td>
<td>0.00 ± 0.00</td>
</tr>
<tr>
<td>liver</td>
<td>0.38 ± 0.36</td>
<td>0.30 ± 0.24</td>
<td>0.20 ± 0.45</td>
<td>0.45 ± 0.33</td>
<td>0.01 ± 0.02</td>
</tr>
<tr>
<td>pancreas</td>
<td>33.04 ± 19.50</td>
<td>7.73 ± 2.70</td>
<td>0.62 ± 1.25</td>
<td>0.06 ± 0.12</td>
<td>0.00 ± 0.00</td>
</tr>
<tr>
<td>stomach</td>
<td>0.50 ± 0.75</td>
<td>0.00 ± 0.00</td>
<td>0.00 ± 0.00</td>
<td>0.00 ± 0.00</td>
<td>0.00 ± 0.00</td>
</tr>
<tr>
<td>small intestine (%ID)</td>
<td>3.18 ± 1.77</td>
<td>1.00 ± 0.59</td>
<td>0.25 ± 0.18</td>
<td>0.01 ± 0.01</td>
<td>0.09 ± 0.14</td>
</tr>
<tr>
<td>large intestine (%ID)</td>
<td>2.16 ± 0.94</td>
<td>2.38 ± 1.05</td>
<td>0.83 ± 0.16</td>
<td>0.57 ± 0.33</td>
<td>0.24 ± 0.27</td>
</tr>
<tr>
<td>kidney</td>
<td>17.90 ± 10.88</td>
<td>25.79 ± 4.66</td>
<td>23.40 ± 12.33</td>
<td>12.87 ± 2.76</td>
<td>8.83 ± 5.69</td>
</tr>
<tr>
<td>tumor</td>
<td>2.80 ± 1.18</td>
<td>1.43 ± 0.62</td>
<td>0.92 ± 0.46</td>
<td>0.69 ± 0.53</td>
<td>0.60 ± 0.40</td>
</tr>
<tr>
<td>muscle</td>
<td>0.00 ± 0.00</td>
<td>0.00 ± 0.00</td>
<td>0.00 ± 0.00</td>
<td>0.00 ± 0.00</td>
<td>0.00 ± 0.00</td>
</tr>
<tr>
<td>bone</td>
<td>0.00 ± 0.00</td>
<td>0.00 ± 0.00</td>
<td>0.00 ± 0.00</td>
<td>0.00 ± 0.00</td>
<td>0.00 ± 0.00</td>
</tr>
<tr>
<td>brain</td>
<td>0.00 ± 0.00</td>
<td>0.00 ± 0.00</td>
<td>0.00 ± 0.00</td>
<td>0.00 ± 0.00</td>
<td>0.00 ± 0.00</td>
</tr>
<tr>
<td>excretion (%ID)</td>
<td>61.95 ± 23.27</td>
<td>80.86 ± 2.63</td>
<td>87.21 ± 2.75</td>
<td>91.55 ± 1.73</td>
<td>93.85 ± 2.78</td>
</tr>
</tbody>
</table>

<sup>a</sup> Organ uptake values expressed as %ID/g and values are mean ± SD (n=4) unless otherwise noted.

<sup>b</sup> Excretion values were calculated using the activity values associated with the excreted urine, bladder, and fecal material contents at the time of sacrifice.
Figure 3.5 Percentage tumor retention of $^{111}$In-radioconjugates

Percentage tumor retention of $^{111}$In-1, $^{111}$In-2 and $^{111}$In-4 in PC-3 tumor-bearing SCID mice.

Values are mean ± SEM (n=4).
3.3.6 Small Animal SPECT/CT Imaging Studies

Small animal SPECT/CT Imaging studies were performed in PC-3 tumor bearing SCID mice using the $^{111}$In-1, $^{111}$In-2, and $^{111}$In-4 radioconjugates. The whole body images and the respective axial slices of the PC-3 tumors at 1, 24, 48 and 72 h p.i. are depicted in Figure 3.6. At 1 h post injection, significant abdominal uptake was observed in all cases due to the accumulation of radioactivity in the G.I. tract and pancreas as previously demonstrated in the biodistribution studies. Axial slices of the PC-3 tumor for all the radioconjugates investigated exhibited substantial accumulation of radioactivity in the tumor tissue after the rapid clearance of the radioconjugates through the renal/urinary system. For $^{111}$In-4 radioconjugates, conspicuous kidney retention is observed, echoing the biodistribution studies.
Figure 3.6 Fused micro SPECT/CT and axial images

Fused micro SPECT/CT and axial images of $^{111}$In-1, $^{111}$In-2 and $^{111}$In-4 in PC-3 tumor-bearing mice at 1, 24, 48 and 72 h after injection. Tumors and kidneys are indicated by red and green arrows respectively.
3.4 DISCUSSION

To determine if the incorporation of 2-nitroimidazoles would increase the retention of the radioconjugate in hypoxic PC-3 cells, we have previously synthesized four BB2r-targeted agents, that included 2-nitroimidazole moieties [145]. In vitro studies showed improved longitudinal retention of the 2-nitroimidazole containing BB2r-targeted agents in hypoxic relative to normoxic PC-3 cells. However, it was determined that the steric interference of the 2-nitroimidazole with the BB2r-targeting vector resulted in poor binding affinities which severely impeded internalization of these conjugates. In this study, an extended linker (8-AOC) was incorporated between the 2-nitroimidazole-amino acid residue and the pharmacophore. The BB2r affinities of (1-4) natural indium labeled and unlabeled conjugates versus $^{[125]}$I-Tyr$_4$ BBN were performed for the GRP receptor using the PC-3 cell line. All natIn-BBN conjugates demonstrated nanomolar binding affinity. Based on these results, the incorporation of the 8-AOC linker has eliminated the detrimental impact of 2-NIAA side chain on pharmacophore binding.

Internalization and efflux studies demonstrated that the clearance rate of the radioconjugates containing 2-nitroimidazole was substantially lower relative to the control under hypoxic conditions. Specifically, 6.6, 25.4, 35.9 and 39.7% more retention were observed at the 8 h time point for $^{111}$In-1-4 under hypoxic conditions relative to normoxic conditions. $^{111}$In-3 and $^{111}$In-4 which have more than one 2-nitroimidazoles (2 and 3, respectively) exhibited a higher retention effect than $^{111}$In-2 which has only one 2-nitroimidazole. Inclusion of more than one hypoxia trapping moiety may increase the chances for the 2-nitroimidazole containing radioconjugates to form protein adducts thus enhancing the long-term retention of the radioconjugate in the cell. Ultimately, further investigation into the identification and quantification of the protein-adduct is needed to obtain a clearer understanding of the
mechanism involved in this process. It was also interesting to note that, $^{111}$In-1 exhibited a slightly lower clearance rate under hypoxic conditions, which could be due to the decreased metabolic rate under hypoxic conditions [105]. With respect to the hypoxia enhancement factor, significantly higher retention in hypoxic relative to normoxic cells was observed for $^{111}$In-2-4 at the 2 h time point. The HEF continued to increase for $^{111}$In-1 and $^{111}$In-2 throughout the experiments, but remained constant for $^{111}$In-3 and $^{111}$In-4 after the 4 h time point. A strong positive linear relationship between the HEF and the number of 2-nitroimidazoles incorporated was confirmed for the 8 h time points.

Cellular protein analysis of the control radioconjugate $^{111}$In-1 demonstrated minimal hypoxic/normoxic cellular protein association ratios which are likely due to reversible, nonspecific binding. For the 2-nitroimidazole containing BB2r-targeted conjugates, up to a 2 fold increase was observed under hypoxic conditions. These results suggest that the significantly higher protein association ratio of 2-nitroimidazole containing radioconjugates is due, at least in part, to the irreversible binding to intracellular proteins which is consistent with the known trapping mechanism of 2-nitroimidazole.

The in vivo biodistribution of each radioconjugate was investigated in PC-3 tumor bearing SCID mice, except for $^{111}$In-3 due to the similar efflux and protein association properties as $^{111}$In-4. At 1 h post injection, $^{111}$In-1 and $^{111}$In-2 share comparable tumor uptake, 5.82 ± 2.63 %ID/g and 6.06 ± 3.35 %ID/g, respectively. However, a substantially lower tumor uptake (2.80 ± 1.18 %ID/g) was observed for $^{111}$In-4 which has three 2-nitroimidazoles incorporated. Given the similar uptake of $^{111}$In-2 and $^{111}$In-4 in the BB2r-positive pancreas, the reason behind the reduced tumor uptake is unclear. At 4 h post injection, most of the radioconjugates were cleared through the renal/urinary system (80 - 82 %ID) which is consistent with other investigations of BB2r- targeted radioconjugates [149]. By the 72 h post injection time point, the
radioconjugates were largely cleared from most tissues, including the pancreas [150]. Significant tumor retention enhancement was observed at 72 h post injection for radioconjugates $^{111}$In-2 and $^{111}$In-4. Specifically, 6.7% and 21.0% of the initial 1h uptake in tumor was retained for $^{111}$In-2 and $^{111}$In-4, compared with only 1.5% remaining for the control $^{111}$In-1. However, the hypoxia burden of each tumor is unknown which limits the ability to fully interpret the relationship between tumor retention effect and incorporation of the hypoxia trapping moiety. The hypoxia burden in tumor xenograft mouse models has been shown to increase (modest correlation) with an increase in tumor size, but this trend is highly dependent on the cell-line [151]. Linear regression analysis of the %ID/g retention of our radioconjugates in PC-3 tumors versus tumor weight revealed no correlation between retention and tumor size. Interestingly, in the clinic, the extent of hypoxia is independent of tumor size in a variety of human cancer including head and neck, cervix and lung cancer [152-154]. Further study to correlate the tumor hypoxia burden with radioconjugate retention is ongoing. Significantly lower tumor and pancreas uptake caused by co-injection of an excess of unlabeled conjugate 4 indicates that accumulation of $^{111}$In-4 is largely mediated by the BB2r.

It is interesting to note the unusually high kidney retention for 2-nitroimidazole containing conjugates relative to the control conjugate. Especially for $^{111}$In-4, up to 8.83 ± 5.69 % ID/g in kidney was observed at 72 h post injection, information which has not been reported in any research related to BBN or 2-nitroimidazole based radioconjugates [148, 149, 155, 156]. Renal retention of radiolabeled targeting peptide has long been addressed as one of the dose-limiting factors in radionuclide therapy and various mechanisms have been demonstrated to be involved in high renal uptake [157]. Cationic peptides are preferentially reabsorbed by the proximal tubules due to the anionic binding sites on the brush border membrane [158, 159]. Megalin and cubilin are known to be associated with the proximal tubular reabsorption of structurally
different proteins, peptides, and drugs [160]. Moreover, low or very low tissue oxygen tensions exist under physiologic conditions in kidneys, which facilitates urine concentration [161]. Co-infusion of competitive inhibitors such as lysine, arginine and succinylated gelatin can reduce the reabsorption by endocytosis or transporters [162-164]. In preliminary co-injection blocking studies (data not shown), both EF5 (a 2-nitroimidazole based hypoxia marker) and lysine co-injection with $^{111}$In-4 can reduce the retention of radioconjugates in kidney. Further studies are needed to fully understand the mechanism of kidney retention of hypoxia trapping enhanced BBN conjugates in order to develop specific methods to reduce the renal toxicity.

MicroSPET/CT images of all radioconjugates using PC-3 tumor-bearing mice at 1 h post injection (Fig. 6), showing significant abdominal uptake, are consistent with the data obtained from biodistribution studies. The PC-3 tumor xenografts in all mice are easily visualized. For the $^{111}$In-4 radioconjugates, containing three 2-nitromidazoles, significant activity in kidneys was observed which strongly agrees with previously established biodistribution data.

3.5 CONCLUSION

We have synthesized three BB2r-targeted radioconjugates with 2-nitroimidazole hypoxia trapping moieties incorporated to enhance the retention in hypoxic cancer cells. In vitro competitive binding studies indicate that inclusion of extended linker 8-AOC eliminated the detrimental effect on binding affinity that was determined in our previous report. The 2-nitroimidazole trapping moieties containing BB2r-targeted agents demonstrated significant higher retention and protein association properties in hypoxic relative to normoxic PC-3 cells. In vivo biodistribution studies revealed great potential of incorporated trapping moieties to increase the residence time of BB2r-targeted agents in PC-3 xenograft tumor. Further works are
needed to clarify the mechanisms of increased retention effects at the molecular level and to correlate the tumor hypoxia burden with the retention efficacy.
Chapter 4 Synthesis, Radiochemical Stability and In Vitro Investigation of a New Series of Nitroimidazole Incorporated 177Lu-labeled Bombesin Analogs

4.1 INTRODUCTION

As mentioned in previous chapters, our observations to date seem to be consistent with this proposed mechanism of action with the 2-nitroimidazole incorporated BB2r-targeted agents exhibiting a prolonged retention in hypoxic prostate cancer cells and a resultant increase in the association with intracellular proteins [165].

In this report, we continue to explore the development of hypoxia-enhanced BB2r-targeted agents. Specifically, we synthesize a new series of hypoxia-enhanced BB2r-targeted agents, depicted in Figure 4.1, with 0, 1, 3 and 5 2-nitroimidazoles. In addition, the design includes a polyethylene glycol linker to increase the hydrophilicity of the resulting BB2r-targeted agents. With these analogs in hand, we further explore the effect oxygen concentrations have on the retention of the hypoxia-enhanced BB2r-targeted agents in human prostate cancer cells. Furthermore, with previously analoged series (not reported), we observed significantly increased rates of radiolysis with 2-nitroimidazole incorporated BB2r-targeted agents relative to controls (absence of 2-nitroimidazoles)[165]. Herein, we additionally describe the use of radioprotectants to increase the longevity of the prepared hypoxia-enhanced BB2r-targeted agents for future use.
Figure 4.1 Hypoxia enhanced 177Lu-BB2r-targeted conjugates
4.2 MATERIALS AND METHODS

4.2.1 Chemical and Supplies

Unless otherwise noted all solvents were used without further purification. Deionized water was purified by a Millipore (U.S.) Mili-Q-Biocel. Acetonitrile, formic acid, N, N-diisopropylethylamine (DIEA), N,N-dimethylformamide (DMF), dichloromethane (DCM), N-methylpyrrolidone (NMP), N-Hydroxysuccinimide (NHS), 1-Ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride (EDC), thioanisol, sodium hydroxide, L-ascorbic acid, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES), bovine serum albumin (BSA) and sodium dodecyl sulfate (SDS) were purchased from Fisher Scientific (U.S.). 2-nitroimidazole was purchased from Amfinecom (U.S.). 2-nitroimidazole acetic acid (2-NIAA) was synthesized as reported earlier. O-Benzotriazole-N,N,N′,N′-tetramethyl-uronium-hexafluorophosphate (HBTU), Fmoc-NH-PEG₃-COOH, Fmoc-protected natural amino acids and the appropriate Rink Amide resins were purchased from Nova Biochem (U.S.). Fmoc-D-lys-OH hydrochloride was purchased from CreoSalus (U.S). Roswell Park Memorial Institute (RPMI) 1640 media and phosphate buffered saline (PBS) were purchased from Thermo Scientific (U.S.). Naturally abundant lutetium chloride (⁹⁹⁹¹LuCl₃), triisopropyl silane, trimethylsilyl chloride, hydrochloric acid (HCl) and 3, 6-dioxa-1, 8-octanediethyl were purchased from Sigma-Aldrich (U.S.). The [¹²⁵I-Tyr₄]-bombesin and lutetium-177 chloride (¹⁷⁷¹LuCl₃) were purchased from Perkin Elmer (U.S.). The human prostate cancer cell line, PC-3, was obtained from American Type Culture Collection (U.S.) and cultured under vendor recommended conditions. TrypLE™ Express was purchased from Invitrogen (U.S.).
4.2.2 Equipment

The peptides were synthesized on a Liberty microwave peptide synthesizer from CEM (U.S.). HPLC/MS analyses were performed on a Waters (U.S.) e2695 system equipped with a Waters 2489 absorption detector and a Waters Qtof Micro electrospray ionization mass spectrometer. Evaluation and purification of radiolabeled conjugates 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. A Phenomenex (U.S.) Jupiter 10µ Proteo 250 × 10 mm semiprep column was used for the purification of bulk amounts of peptides. The peptides were concentrated on a Centrivap (U.S.) Centrifugal Concentrator. For the purification of conjugates, nat-Lu-conjugates, and 177Lu-radioconjugates a Phenomenex Jupiter 10µ Proteo 250 × 4.60 mm analytical column was employed. Solid phase extraction was performed using 3M Empore (U.S.) C18 10 mm high performance extraction disks. Nuclear magnetic resonance spectra were recorded on Varian (U.S.) 500MHz INOVA spectrometer. 1H NMR chemical shifts are expressed as δ values (parts per million) and peaks are described as s for singlet and d for doublet. Hypoxic PC-3 cells were incubated in a hypoxic glove box with temperature, CO2 and humidity controller (Coy Laboratory, U.S.). Gamma decay detection of 177Lu and 125I for the in vitro binding, efflux studies was accomplished using a LTI (U.S.) Multi-Wiper nuclear medicine gamma counter.

4.2.3 Synthesis of Methyl ([(9H-fluoren-9-yl)methoxy]carbonyl)-D-lysinate Hydrochloride, Compound I

The synthesis of compound I followed the procedure described earlier. Briefly, Fmoc-D-lysine hydrochloride (500 mg, 1.2 mmol) was placed in a 25ml round bottomed flask with a magnetic stirrer and dissolved in anhydrous methanol (10 ml). To this solution, trimethylsilyl chloride (0.6 ml, 6 mmol) was added and the reaction was stirred at room temperature for 36 h.
The solvent was evaporated to dryness to yield a yellow oil. Anhydrous diethyl ether (20 ml) was added to the oil which precipitated a white solid (compound I) after 15 min of stirring. The resulting mixture was filtered and washed with ether (2×5 ml) to give Fmoc-D-lys methyl ester hydrochloride with 98% yield (506 mg). $^1$H NMR 1H (DMSO): $\delta$ 8.16 (s, 2H, NH$_2$)7.87 (d, A FMOC)7.83 (t, B FMOC) 7.32 (t, C FMOC) 4.27 (s, 1H, NH), 4.29 (d, F CH$_2$ FMOC) ,4.25 (t, E CH FMOC ), 3.60 (s, 3H,CH3),3.43 (s, 2H ,CH2),1.65-156 (m, 4H CH2- CH2), 2.72 (s, 2H ,CH2), 1.36 (d, 1H ,CH). $^{13}$C NMR (DMSO): 172.89 (C=O Ester ), 156.18 (C=O NH), 143.78 (G, C benzene)140.75 (H,C benzene),127.67 (C-C benzene) 127.09 (B, C benzene),125.27 (A, C benzene),120.15 (D, C benzene),65.63 (F,C aliphatic), 53.75(M, C aliphatic) , 51.92 (N, C aliphatic), 46.66 (E, C benzene ), 38.33 (I, C aliphatic), 30.54( K, C aliphatic) 26.38 (J, C aliphatic) 22.47 (L, C aliphatic). MS (ESI): m/z calculated for C$_{22}$H$_{26}$N$_2$O$_4$:382.189. Found (m/z, [M + H$^+$]):383.092.

4.2.4 Synthesis of Methyl N2-(((9H-fluoren-9-yl)methoxy)carbonyl)-N6-(2-(2-nitro-1H-imidazol-1-yl)acetyl)-D-lysinate, Compound II

Compound II was synthesized using established amidation chemistry. Briefly, 2-NIAA (58 mg, 0.31 mmol), NHS (110 mg, 0.93 mmol) and EDC (178 mg, 0.93mmol) were added to a 25 ml round bottom flask with stir bar and dissolved in DMF (20 ml). The solution was allowed to stand for 2 h in an ice bath. Compound I (130 mg. 0.31mmol) was added to the solution and stirred overnight at room temperature. The volume of the DMF solution was reduced to 10 mL and the remaining solution was purified by semi-preparative RP-HPLC. HPLC solvents consisted of H$_2$O containing 0.1% formic acid (solvent A) and acetonitrile containing 0.1% formic acid (solvent B). Solvent A and B were used for all RP-HPLC method described in this paper unless mentioned elsewhere. For purification of compound II, an initial gradient of 70 % A : 30 % B linearly decreased to 75 % A : 25 % B over a 15 minute time period. At the end of the run time
for all HPLC experiments, the column was flushed with the gradient 5 % A : 95 % B and re-equilibrated to the starting gradient. **\(^1\)H NMR 1H (DMSO):** δ 8.31 (s, 2H ,NH2) 7.89 (d, A FMOC) 7.71 (1H, d, imidazole ring) ,7.53 (1H, d, imidazole ring) ,7.41 (t, B FMOC) 7.31 (t, C FMOC),5.05 (2H, s, NCH₂COO), 4.30( s,1H ,NH) A.29 ( d , F, CH₂, FMOC) ,4.22( t E, CH FMOC ), 3.61 (s, 3H,CH3),3.39 (s, 2H ,CH₂), 3.07 (s, 2H ,CH₂), 1.69-1.57 (m, 4H CH2- CH2),1.41-1.31 (d, 1H ,CH). **\(^{13}\)C NMR (DMSO):** 172.98(C=O Ester ),165.39 (NO2-C imidazole ring ) 156.18 (C=O NH), 143.82 ( G, C benzene)140.77 ( H, C benzene),128.80 (CH-imidazaole ring), 127.69 (CH-imidazole ring), 127.45 (C-C benzene) 127.11 ( B, C benzene),125.26 ( A, C benzene),120.16 ( D, C benzene),65.65 (F,C aliphatic), 53.82M, C aliphatic) , 51.89 (N, C aliphatic), 46.68 (NCH₂), 40.0 (E, C benzene ), 30.32 (I, C aliphatic), 30.54( K, C aliphatic) 28.51 ( J, C aliphatic) 22.83 (L, C aliphatic).

**4.2.5 Synthesis of N2-(((9H-fluoren-9-yl)methoxy)carbonyl)-N6-(2-(2-nitro-1H-imidazol-1-yl)acetyl)-D-lysine, Compound III**

The methyl ester on compound II was taken off by hydrolysis with HCl. The hydrolysis process was monitored by HPLC. Briefly, compound II (100 mg, 0.2 mmol) was dissolved in 50 ml acetonitrile and excess amount of concentrated HCl was added. The conversion of methyl ester to carboxylic acid was monitored by RP-HPLC using the same gradient for compound II. When 100% conversion ratio (UV-314nm) was achieved, the solutions were neutralized by sodium carbonate and the final product compound III was extracted by methanol. The resulting solutions were concentrated and peak purified by HPLC. **\(^1\)H NMR 1H (DMSO):** δ 8.32 (s, 2H ,NH2) 7.88 (d, A FMOC )7.86 (d, D FMOC) 7.72(1H, d, imidazole ring) ,7.53 (1H, d, imidazole ring) ,7.41 (t, B FMOC) 7.33 (t, C FMOC),5.05 (2H, s, NCH₂COO), 4.27( s,1H ,NH) A.25 ( d , F CH₂, FMOC) ,4.22( t E CH FMOC ), 3.43 (s, 2H ,CH₂),1.69-1.57 (m, 4H CH2- CH2), 3.07 (s, 2H ,CH₂), 1.39-1.33 (d, 1H ,CH). **\(^{13}\)C NMR (DMSO):** 174.17(C=O Ester ),165.34 (NO2-C imidazole ring ) 156.18 (C=O NH),
143.83 (G, C benzene) 140.73 (H, C benzene), 128.78 (CH-imidazole ring), 127.65 (CH-imidazole ring), 127.42 (C benzene), 127.09 (B, C benzene), 125.31 (A, C benzene), 120.13 (D, C benzene), 65.57 (F, C aliphatic), 53.92 (M, C aliphatic), 51.57 (N, C aliphatic), 46.68 (NCH₂), 40.12 (E, C benzene), 38.65 (L, C aliphatic), 22.99 (L, C aliphatic). MS (ESI): m/z calculated for C₂₆H₂₇N₅O₇: 521.191. Found (m/z, [M + H⁺]): 522.121.

4.2.6 X-ray Crystallization of Compound II

X-ray Crystallographic Analysis: Single crystals of the compound II were grown from a mixture of water : methylene dichloride : hexane (1 : 1 : 0.5) solution by slow evaporation. A suitable crystal was selected and mounted on a Cryoloop and placed on an Xcalibur, Onyx, Ultra diffractometer. The crystal was kept at 100 K during data collection. Using Olex2, the structure was solved with the XS structure solution program using Direct Methods and refined with the XL refinement package using Least Squares minimization.

Crystal data for compound II C₂₇H₂₉N₅O₇ (M = 535.55): monoclinic, space group P2₁ (no. 4), \(a = 4.88855(9) \, \text{Å}, \, b = 15.3693(3) \, \text{Å}, \, c = 17.7026(4) \, \text{Å}, \, \beta = 96.630(2)°, \, V = 1321.16(5) \, \text{Å}^3, \, Z = 2, \, T = 100 \, \text{K}, \, \mu(\text{Cu Kα}) = 0.823 \, \text{mm}^{-1}, \, D_{calc} = 1.346 \, \text{g/mm}^3, \, 11238 \, \text{reflections measured} (7.64 \leq 2\Theta \leq 153.58), \, 5302 \, \text{unique} (R_{int} = 0.0474) \, \text{which were used in all calculations}. \, \text{The final} \, R_1 \, \text{was} \, 0.0403 \, (>2\sigma(I)) \, \text{and} \, wR_2 \, \text{was} \, 0.1119 \, \text{(all data) with} \, \text{GOF=} \, 1.082. \, \text{Crystallographic data for the structural analysis has been deposited with the Cambridge Crystallographic Data Center (CCDC 838909). Copies of this information may be obtained free of charge from the CCDC (www:} \, \text{http://www.ccdc.cam.ac.uk}) \, \text{or from e-mail} \, jcgarrison@unmc.edu

4.2.7 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 0, 1*, 2* and 3 was orthogonally deprotected and cleaved from the resin using a cocktail consisting of triisopropylsilane (0.1 mL), water (0.1 mL), 3,6-dioxa-1,8-octanedithiol (0.1 mL), trifluoroacetic acid (4.625 mL) and thioanisole (0.075 mL). The cleaved peptide was subsequently precipitated and washed using cold (0 °C) methyl-tert-butyl ether (40 mL×3). The crude conjugate was dried by a centrivap 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 investigations. For conjugates 0 and 1*, an initial gradient of 85 % A : 15 % B linearly decreased to 75 % A : 25 % B over a 15 minute time period. For conjugates 2*, an initial gradient of 80 % A : 20 % B linearly decreased to 70 % A : 30 % B over a 15 minute time period. For conjugates 3, an initial gradient of 75 % A : 25 % B linearly decreased to 65 % A : 35 % B over a 15 minute time period. At the end of the run time for all HPLC experiments, the column was flushed with the gradient 5 % A : 95 % B and re-equilibrated to the starting gradient.

4.2.8 Coupling of 2-NIAA to Bombesin Conjugates

The 2-NIAA was manually coupled to ε-amino group of the lysine residue for peptides 1* (20 mg, 9.8 µmol) and 2* (20 mg, 9.4 µmol) using established amidation chemistry. Briefly, for peptides 1* and 2*, the 2-NIAA (1.8 mg, 10.7 µmol and 5.3 mg, 30.9 µmol, respectively), NHS (3.4 mg, 29.2 µmol and 10.8 mg, 93.8 µmol, respectively) and EDC (5.6 mg, 29.2 µmol and 17.9 mg, 93.9 µmol) mixtures were dissolved in DMF (200 µL). These solutions were allowed to stand for 2 h in an ice bath before addition of the conjugate 1* and 2* in DMF (200 µL). The reaction mixture was stirred overnight at room temperature and subsequently evaporated to dryness.
The residue was re-dissolved in water : acetonitrile (8 : 2), peak purified by semi-preparative RP-HPLC and characterized by mass spectrometry.

4.2.9 Labeling with $^{nat}$LuCl$_3$

For the convenient characterization of the $^{177}$Lu-Bombesin conjugates, naturally abundant $^{nat}$Lu was substituted for $^{177}$Lu in the ES-MS and in vitro binding studies. A sample of conjugates (1 mg) was dissolved in ammonium acetate buffer (0.5 M, 200 μL, pH 5.5) and mixed with a solution of $^{nat}$LuCl$_3$ (4-5 mg). The solution was heated for 30 min at 50 °C. After cooling to room temperature, $^{nat}$Lu-conjugates were then peak purified by RP-HPLC. All $^{nat}$Lu-conjugates were ≥ 95% purity before mass spectrometric characterization and in vitro binding studies were performed.

4.2.10 Radiolabeling with $^{177}$LuCl$_3$

Radiolabeling was performed on all conjugates by mixing 25 μg samples with 37 MBq $^{177}$LuCl$_3$ in ammonium acetate buffer (0.5 M, 120 μL, pH 5.5). The solution was heated for 60 min at 90 °C and allowed to cool to room temperature. The resulting specific activities were 3.23, 3.52, 4.09 and 4.67 MBq/nmol for $^{177}$Lu-0, $^{177}$Lu-1, $^{177}$Lu-2 and $^{177}$Lu-3. In order to separate radiolabeled peptides from unlabeled peptides on HPLC, 4-5 mg CoCl$_2$ was then added and incubated for 5 min at 90 °C to increase the hydrophobicity of unlabeled conjugates. The resulting radioconjugates were peak purified using RP-HPLC (≥95%) and concentrated using C18 extraction disk. Elution of the extraction disk with ethanol/sterile saline solution (6:4, 200 μL) delivered the radioconjugates in high purity. A 200 μl solution of selemethionine (0.20mg/ml) and ascorbic acid (40mg/ml) in 90% PBS buffer was added to all radioconjugates to reduce radiolysis.
4.2.11 HPLC Purification and Quality Control

When necessary, bulk sample purification was performed using a semi-preparative Proteo column with a flow rate of 5.0 mL/min. Sample purification for in vitro studies was performed on an analytical Proteo column with a flow rate of 1.5 mL/min. For labeled $^{177/\text{nat}}$In-conjugates 0 and 1, an initial gradient of 80 % A : 20 % B linearly decreased to 70 % A : 30 % B over a 15 minute time period. For $^{177/\text{nat}}$Lu-conjugates 2 and 3, an initial gradient of 75 % A : 25 % B linearly decreased to 65 % A : 35 % B over a 15 minute time period. At the end of the run time for all HPLC experiments, the column was flushed with the gradient 5 % A : 95 % B and re-equilibrated to the starting gradient.

4.2.12 Radiochemical Stability Studies

Twenty five µg samples of $^{177}$Lu-0-3 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 a C18 extraction disk with ethanol/sterile PBS (6:4, 200 µL). The ethanol was evaporated using nitrogen gas and the resulting solutions were diluted with A: PBS, B: Ascorbic acid in 0.9% sodium chloride solution (final ascorbic acid concentration: 40mg/ml), C: Selenomethionine (Se-Met) in buffer B (final Se-Met concentration: 0.2mg/ml) yielding a final radio-concentration of 74 MBq/ml (2 mCi/ml). The purity of desired radioconjugates was determined by HPLC at 24, 48 and 72 h.

4.2.13 Partition Coefficient

Partition coefficient was determined for each $^{177}$Lu-labeled conjugates. 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 (500,000 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. P values < 0.05 were considered statistically significant.

4.2.14 In Vitro Competitive Cell-Binding Studies

The half maximum inhibitory concentration (IC$_{50}$) of the $^{\text{nat}}$Lu conjugates were determined by competitive displacement cell-binding assays using PC-3 cells. $^{\text{nat}}$Lu-conjugates were used as substitutes for the corresponding $^{\text{177}}$Lu-radioconjugates. Briefly, PC-3 cells (~3×10$^4$) 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 [${}^{125}$I-Tyr$_4$]-Bombesin and various concentrations of the $^{\text{nat}}$Lu-O-3. 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 IC$_{50}$ values determined by nonlinear regression using the one-binding site model of Graphpad PRISM 5 (U.S.).

4.2.15 Efflux Studies at Different Oxygen Concentrations

Efflux studies were carried out under atmospheres that contain 5% CO2 and 0.2, 1.0, 2.5, 5 and 21% O2 (21% O2 represents atmospheric oxygen concentrations). PC-3 cells were incubated in six-well plates (0.5 × 106 / well) in a hypoxic chamber 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 removed, and the cells were washed with medium and incubated for 2 h under different oxygen concentrations. Cells were incubated for an additional 2 h at 37°C in the presence of 100,000 cpm of each $^{177}$Lu-radioconjugate. Upon completion of the incubation at time points 0, 2, 4, and 8 h, the media was harvested to quantitate the amount of 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 0.5 % aqueous SDS solution and the lysates 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. Statistical analyses were performed by multiple t-test with Holm-Sidak correction using Graphpad PRISM 5 (U.S.). A P value of less than 0.05 was considered statistically significant.

4.2.16 Efflux Study under Various O₂ Concentrations

In this experiment, normoxic and hypoxic PC-3 cells were prepared as outlined in the efflux studies above. PC-3 cells were incubated for 2 h at 37°C in the presence of 100,000 cpm of each ¹⁷⁷Lu-radioconjugate under hypoxic condition (94.9% N₂, 0.1% O₂, 5% CO₂). Upon completion of the incubation, the 6 well plates were transferred to a normal CO₂ incubator. At 2, 4 and 8 h time points, effluxed, surface bound and internalized radioactivity was measured using the procedure stated above.

4.3 RESULTS

4.3.1 Synthesis and Radiolabeling

In this study, we targeted the synthesis of four conjugates, each with increasing numbers of 2-nitroimidazoles. In our initial synthetic design, all conjugates were to be synthesized by SPPS techniques to yield the precursors (designated with an astericks i.e., 1*) which would be later conjugated to 2-nitroimidazole acetic acid (2-NIAA) post-assembly. Note that conjugate 0, which lacks 2-nitroimidazoles, is our experimental control and did not undergo 2-NIAA coupling. The desired peptides were easily synthesized by standard SPPS chemistry. Conjugate 0 and precursors 1*, 2* and 3* were purified by RP-HPLC and isolated with yields of 26.6, 25.7, 20.7
and 28.2 %, correspondingly. After purification, 2-NIAA was coupled to the ε-amino group of lysine residue(s) of the precursor conjugates using EDC/NHS in DMF. The coupling yields for conjugates 1 and 2 after purification by RP-HPLC were 37.8 and 16.9 %, respectively. However, conjugate 3 gave very poor yields. Despite attempts to improve the yield of 3 by optimizing concentrations of reactants and the coupling agent, as well as the exploration of other coupling agents (i.e., HBTU), isolated yields of 3 were never more that 1%.

As a result of this difficulty, an alternative strategy was pursued for the synthesis of conjugate 3. Instead of a post-conjugation strategy for introducing the 2-NIAA, we set out to synthesis a 2-NIAA derivative of the Fmoc-D-Lys-OH amino acid, compound III, which could be employed to directly synthesize conjugate 3 by SPPS. The synthetic scheme is given in Figure 4.2. Fmoc-D-lysine hydrochloride was reacted with trimethylsilyl chloride to yield compound I, a methyl ester derivative of Fmoc-D-lysine, in near quantitative yield as determined by LC/MS. Using EDC/NHS coupling chemistry, 2-NIAA was effectively conjugated to compound I with an overall yield of 87.4 %. Compound II was crystallographically characterized by x-ray diffraction studies and is depicted in Figure 4.3. Conversion of compound II to the acid derivative (compound III) was achieved by hydrolysis using hydrochloric acid. The progress of the reaction was monitored using LC-MS. The final product was extracted by methanol to give a yield of 37.4%. With compound III in hand, conjugate 3 was directly synthesized by SPPS with a yield of 23.9% after chromatographic purification. The RP-HPLC retention times and mass spectrometric identification of the conjugates and precursors are listed in Table 4.1.
Figure 4.2 Synthesis of Fmoc-D-lysine 2NIAA (compound III)

Reagents and conditions: (a) TMSCI, methanol, rt, overnight, 98%; (b) EDC, NHS, rt, overnight; 67%; (c) HCl, rt.
Figure 4.3 X-ray crystal structure of Compound II
<table>
<thead>
<tr>
<th>Analogue</th>
<th>LogD</th>
<th>Time / min</th>
<th>IC50 / nM</th>
<th>Molecular Formula</th>
<th>Calcd. M.W.</th>
<th>Found (M/z) +</th>
<th>RP-HPLC Retention</th>
<th>Analogue Mass Spectrometric and RP-HPLC Characterization of Conjugates</th>
</tr>
</thead>
</table>
| Lu-3     | 2.05 | 22.1 ± 1.2 | 1.09 ± 1.4 | \\text{Lu}^{177}\\text{H}^{9}\\text{N}^{14}\\text{BBN}\\text{PEG}\\text{Lu}^{177}\\text{H}^{9}\\text{N}^{14}\\text{BBN}\\text{PEG} | 3150.3 | 3153.4 | 1.4 ± 0.5 | | 3
| Lu-2     | 2.10 | 22.1 ± 1.2 | 1.09 ± 1.4 | \\text{Lu}^{177}\\text{H}^{9}\\text{N}^{14}\\text{BBN}\\text{PEG}\\text{Lu}^{177}\\text{H}^{9}\\text{N}^{14}\\text{BBN}\\text{PEG} | 3150.3 | 3153.4 | 1.4 ± 0.5 | | 3
| Lu-1     | 2.25 | 22.1 ± 1.2 | 1.09 ± 1.4 | \\text{Lu}^{177}\\text{H}^{9}\\text{N}^{14}\\text{BBN}\\text{PEG}\\text{Lu}^{177}\\text{H}^{9}\\text{N}^{14}\\text{BBN}\\text{PEG} | 3150.3 | 3153.4 | 1.4 ± 0.5 | | 3

For convenient mass spectra analysis, values represent mean ± SD (n=3). This table was replaced by manual Lu was replaced by manual.
Labeling and purification of the conjugates with natLu and ¹⁷⁷Lu were carried out under nearly identical conditions. ¹⁷⁷/natLuCl₃ was incubated with the conjugates, 0 - 3, in a 0.1 M ammonium acetate buffer (pH 5.5) at 90°C for 40 min. Purification of the ¹⁷⁷/natLu-conjugates was accomplished by RP-HPLC and solid phase extraction on a C18 column with typically ≥ 90% recovery. Prior to in vitro analysis, purity of each conjugate was determined by RP-HPLC and found to have ≥ 95% chemical purity. Radiolabeling yields for ¹⁷⁷Lu-0, ¹⁷⁷Lu-1, ¹⁷⁷Lu-2 and ¹⁷⁷Lu-3 were 90.5, 74.2, 79.6 and 77.3 %, correspondingly.

4.3.2 Partition Coefficient and Radiochemical Stability

Partition coefficient of the radiolabeled peptides was determined by the shake flask method and the log D values are given in Table 4.1. The partition ratio of ¹⁷⁷Lu-0 - 3 radioconjugates under physiological condition (pH = 7.4) were 0.006, 0.005, 0.007 and 0.009, respectively, indicating highly hydrophilic peptides. Slightly decreased hydrophilicity was observed with increasing number of incorporated 2-nitroimidazoles.

Previous studies demonstrated a significant increase in radiolytic degradation with increasing numbers of incorporated 2-NIAA into the structure of the peptide. To combat this degradation, we investigated the efficacy of known radiostabilizers, ascorbic acid and selenomethionine, to increase the stability of the modified BB2r-targeted agents. Two groups of potential stabilizers and control buffers were tested. Figure 4.4 shows the percentage of desired radioconjugates stored at 4°C for 24, 48 and 72 h in the presence of different stabilizing agents. Of the tested reducing agents, the free radical scavenger Se-Met was the most effective radiostabilizer for all ¹⁷⁷Lu-conjugates. Over 72 h at 4°C, 5.3, 7.5 15.8 and 30.1% were radiolyzed in the presence of Se-Met for ¹⁷⁷Lu-0 - 3 whereas only 60.1, 44.9, 50.3 and 15.9% of desired radioconjugates remained when using PBS. Increased radiolytic rate was observed to be
associated with increasing numbers of incorporated 2-NIAA. $^{177}$Lu-4 exhibited the highest radiolytic rate with 30% radiolysed at 72 h even with the presence of Se-Met (radio-HPLC chromatography shown in figure 4.5).
Radiochemical stability studies depicted as % of desired radioconjugates as a function of time.

Figure 4.4 Radiochemical stability studies
Figure 4.4 (continued) Radiochemical stability studies depicted as % of desired radioconjugates as a function of time.
Radio-stability studies of $^{177}$Lu-4 as determined by radio-HPLC at 24, 48, and 72 h post-incubation.
4.3.3 In Vitro Competitive Cell-Binding Studies

A competitive displacement binding assay (IC$_{50}$) of BBN(7-14)NH$_2$ was determined in GRPr-positive PC-3 cells using $^{125}$I-(Tyr4)-BBN radioligands. For both unlabeled and natLu-labeled conjugates, natLu-0 demonstrated the highest affinity binding (1nM) to the BB2r. The binding affinity slightly decreased with more 2-nitroimidazoles incorporated into the structure. The IC$_{50}$ values of natLu-0-3 were 1.0, 6.8, 10.9 and 22.1 nM, respectively. IC$_{50}$ values are summarized in Table 4.1.

4.3.4 Efflux Studies

Efflux studies were performed under various oxygen environments to determine the effect oxygen concentration has on the rate at which radioconjugates $^{177}$Lu-0-3 are effluxed from PC-3 cells. In these studies, $^{177}$Lu-0, is the control and was used to compare the relative effectiveness of the hypoxia trapping conjugates ($^{177}$Lu-1-3). The percentile efflux rates for $^{177}$Lu-1-3 are depicted in Figure 4.6.
Figure 4.6 Efflux assays for the $^{111}$In-radioconjugates in PC-3 cells

Efflux assays depicted as percentage of initial internalized activity for the $^{111}$In-radioconjugates in PC-3 cells at different oxygen concentrations. Values are mean ± SD (n=5).
Figure 4.5 (continued) Efflux assays depicted as percentage of initial internalized activity for the $^{111}$In-radioconjugates in PC-3 cells at different oxygen concentrations. Values are mean ± SD (n=5).
The efflux rates for the control $^{177}\text{Lu-0}$ under various oxygen concentrations were similar over the timepoints investigated. After 2 h, approximately 40 % of the originally internalized radioactivity for $^{177}\text{Lu-0}$ was effluxed. By 8 h, the amount of effluxed radioactivity had reached approximately 60%. Overall for the control, $^{177}\text{Lu-0}$, the efflux of radioactivity from PC-3 cells over the various oxygen concentrations was found to be statistically identical between groups and thus was independent of oxygen concentration. For $^{177}\text{Lu-1-3}$, the radioconjugates demonstrated efflux rates that were highly dependent on oxygen concentration. At the 2 h time point, the efflux of radioactivity averaged 23 to 45% for $^{177}\text{Lu-1-3}$ under 0.2, 1, 2.5, 5 and 21% O$_2$, correspondingly. With exception of the studies carried out at 2.5% O$_2$, the efflux of radioactivity from the PC-3 cells largely plateaued by the 4 h time point. Efflux maxima for $^{177}\text{Lu-1-3}$ were observed at the 8 h time point for all O$_2$ concentrations investigated. From lower to higher pO$_2$, approximately 28, 39, 54, 60 and 69% of radioconjugates were effluxed. Overall 2-nitroimidazole containing radioconjugates $^{177}\text{Lu-1-3}$ showed a significantly higher retention at lower pO$_2$. Over the timepoints investigated, $^{177}\text{Lu-1-3}$ demonstrated a linear correlation with oxygen concentrations.

In order to gauge the impact of O$_2$ deprivation on the efflux rate, we performed a series of studies in which the $^{177}\text{Lu-0-3}$ conjugates were allowed to internalize in PC-3 cells under 0.2% O$_2$ conditions for 2 h. After which time, the efflux of the radioactivity from the cells was investigated under an oxygen-rich environment (i.e., 21%). The results of these studies for $^{177}\text{Lu-0-3}$ are shown in Figure 4.7.
Figure 4.7 Efflux study under “0.2 to 21%” O₂ concentration.
Figure 4.6 (continued) Efflux study under “0.2 to 21%” O₂ concentration
The control $^{177}\text{Lu-0}$ showed a similar efflux pattern as in efflux studies. The percentage of efflux radioactivity was 64% at 8 h which is close to 67% of efflux at 21% O$_2$. However, conjugates $^{177}\text{Lu-1-3}$ showed significantly lower efflux rates when pre-incubated under 0.2% O$_2$. The conjugate $^{177}\text{Lu-1-3}$ showed 41, 58 and 56% of effluxed radioactivity compared with 68, 68 and 71% of effluxed radioactivity under 21% O$_2$ at 8 h timepoint.

4.4 DISCUSSION

The success or failure of a receptor-targeted diagnostic and radiotherapeutic radiopharmaceutical for clinical application depends on many factors. These factors range from the basic chemical properties of the drug (e.g., chemical stability and radionuclide emission properties) all the way to commercial practicalities (e.g., cost and availability). While all of these various aspects are important, the achievable target-to-non-target ratios can be viewed as one of the most vital indicators of a drug’s potential success. Therefore, the development of approaches to increase the target-to-non-target ratios of receptor-targeted agents would be of significant interest to the radiopharmaceutical research community. Our laboratory is currently pursuing this avenue of research using the 2-nitroimidazoles as a hypoxia-selective trapping agent to selectively increase the residence time of the receptor-targeted radiotracer in hypoxic cancers thus yielding higher tumor-to-non-target ratios.

Recently, we have reported the development and evaluation of BB2r-targeted agents with 2-nitroimidazoles incorporated into the structure of the peptide [165, 166]. These studies have shown that these “hypoxia-enhanced” BB2r-targeted agents demonstrate significantly higher retention in hypoxic relative to normoxic prostate cancer cells. In addition, we found an increased association of the internalized radiotracer with intracellular proteins. Taken together,
these finding, while anecdotal, are consistent with the known trapping mechanisms of 2-nitroimidazoles. Furthermore, our laboratory has previously observed a substantial increase in radiolytic degradation with increasing number of incorporated 2-nitroimidazoles. In this report, we describe the synthesis of a new set of hypoxia-enhanced BB2r-targeted agents with 1, 3 and 5 2-nitroimidazole moieties incorporated into the structure of the peptide. With these agents in hand, we evaluated the radiolytic stability of these agents as well as examined the effect oxygen concentration has on cellular retention.

Initially, the synthesis of conjugates 1-3 was pursued by first assembling the BB2r-targeted peptide by SPPS and subsequently coupling the 2-nitroimidazoles to the construct. Using this approach, we found that yields of the conjugates decreased with increasing numbers of 2-nitroimidazoles per peptide. While this approach was successful for obtaining adequate quantities of 1 and 2, conjugate 3 gave relatively very poor yields (≤ 1 %) by this route. Evaluation of the reaction mixture of 3 by LC-MS revealed numerous structural isomers with incomplete 2-nitroimidazole conjugation. Attempts to increase the yield of 3 by increasing the ratio of 2-NIAA to peptide, performing the coupling reaction with higher concentrations of EDC or increasing the reaction time were unsuccessful. Moreover, the exploration of other coupling agents (e.g., HBTU) did not lead to improvements in the synthetic yield. Overall, these results suggest to us that steric inhibition may be a significant factor limiting conversion to conjugate 3.

As a consequence of our inability to synthesize 3 by post-modification, we alternatively chose to synthesize the conjugate by a pre-modification approach. Using the scheme in Figure 4.1, the Fmoc-D-lysine was conjugated to the 2-NIAA prior to SPPS assembly. With the altered amino acid in hand, we assembled conjugate 3 by SPPS achieving an isolated yield of 23.9 %. This yield is consistent with those attained for conjugate 0 (26.6 %) and the precursor conjugates 1*-3* (20.7 - 28.2 %). This result suggests that the increased steric bulk of compound
III, relative to the unaltered Fmoc-Lysine, did not significantly influence the coupling efficiency of the amino acid during SPPS. Given the overall poor yields by the post-modification approach, particularly when conjugating more than one 2-NIAA, the pre-modification approach appears to be the desirable method for incorporating this agent into the structure of targeted peptides.

The conjugates were radiolabeled with $^{177}$LuCl$_3$ to obtain $^{177}$Lu-0-3 with good radiochemical yields (74.2 - 90.5 %). All of the radioconjugates synthesized were found to have hydrophilic distribution coefficients ($\log D = -2.25 \pm 0.07$ - $-2.02 \pm 0.05$) at physiological pH. As more 2-nitroimidazoles were added to the peptide the radioconjugates exhibited increased hydrophobicity. This observation is likely due to the fact that 2-nitroimidazole (pKa ~ 9) remains largely uncharged under physiological conditions. The incremental incorporation of the uncharged 2-nitroimidazole ring systems is increasing the hydrophobicity of $^{177}$Lu-1-3. However, even with some small increases in hydrophobicity being observed, all of the radioconjugates synthesized have distribution coefficient that are low enough to discount passive diffusion as a significant mechanism of entry across cellular membranes. Therefore, cellular uptake of $^{177}$Lu-0-3, as with most reported BB2r-targeted agents, is expected to be primarily attributed to receptor-mediated endocytosis. This is in contrast with most hypoxia imaging agents based on 2-nitroimidazole which are relatively hydrophobic and rely on passive diffusion as the primary route of distribution. This mechanism of cellular uptake for the hypoxia-enhanced BB2r-targeted agents is important for tissue specificity. The dependence on the radioconjugates on receptor mediated uptake, instead of passive diffusion, dictates that the agents will be primarily taken up into BB2r-positive tissues.

It is well established that ionizing radiation ($\alpha^{2+}$ and $\beta^-$ particles) can directly or indirectly, through the generation of solvated electrons and free radicals species, degrade peptides and proteins [167]. Previous studies showed that $^{111}$In-labeled bombesin conjugates were very
radiosensitive and the radiolytic rate was associated with the amount of incorporated 2-nitroimidazoles. Without radiostabilizers, degradation occurred both during radiolabeling and after HPLC purification. Se-Met has been widely used to protect against radiation- and chemical-induced carcinogenesis in both preclinical and clinical studies. It has been previously demonstrated that Se-Met is very effective in protecting $^{177}$Lu-labeled bombesin conjugates from radiolytic degradation when combined with ascorbic acid. In this study, the Se-Met containing AA buffer showed the best stabilizing capability.

Investigations into the BB2r-avidity of the synthesized conjugates and radioconjugates revealed that a small incremental decrease in binding affinity was observed as more 2-NIAAs were added to the peptide, particularly in the case of the radioconjugates. However, the inclusion of the 2-NIAA moieties did not seem to substantially impact the overall affinity of the conjugates or radioconjugates studied. Moreover, internalization studies of $^{177}$Lu-0-3 revealed that all of the radioconjugates had statistically identical percentages of cellular accumulation after 2 h of incubation, demonstrating that the inclusion of 2-NIAA did not inhibit the ability of the radioconjugates to activate the BB2r-mediated endocytotic pathway.

The efflux profiles for conjugates $^{177}$Lu-0-3 in PC-3 cells under 0.2, 1, 2.5, 5 and 21% $O_2$ concentration over 8 h are displayed in Figure 4.5. At all examined time points, the control $^{177}$Lu-0 showed similar efflux pattern at different $O_2$ concentrations. At end of the studies, $61.19\pm 6.59$, $63.07\pm 3.23$, $63.42\pm 1.57$, $62.32\pm 1.64$ and $67.32\pm 2.33$ % radioactivities were effluxed under 0.2, 1, 2.5, 5 and 21% $O_2$ concentration respectively. Overall efflux rate of $^{177}$Lu-0 was independent of $pO_2$ in this study.

$^{177}$Lu-1-3 shows lower efflux rates at 0.2% $O_2$. At 8 h radioactivity effluxed were $28.41\pm 2.32$, $27.98\pm 1.55$, and $29.58 \pm$ % respectively. Under 1, 2.5 and 5 % $pO_2$ $^{177}$Lu-1 exhibits steadily
increased oxygen dependent efflux rates, but it was substantially lower than the trend observed in normoxic cells (21% O₂). The conjugate ^177Lu-2 demonstrates 42.91± 3.88 % cellular efflux under 21% O₂ at 2 h which increased to 68.51 ± 2.94 % at 8 h. However, at 0.2% O₂ only 27.98 ±1.55 % was effluxed at 8 h. Furthermore at 1, 2.5 and 5 % O₂ concentrations, ^177Lu-2 radioactivity efflux rates were increased to 36.90±0.97, 53.20 ±0.75 and 59.85 ±1.62 % correspondingly at 8h.

At the beginning of experiment at 2 h ^177Lu-3 exhibited significantly higher cellular efflux which is 55.36 ± 2.18 % at 21% O₂ compared with ^177Lu-1 and ^177Lu-2. Although by 8 h, ^177Lu-3 exhibited an efflux rate of 71.09 ±2.81% matching with the radioconjugates ^177Lu-1 and ^177Lu-2. Furthermore at 1, 2.5 and 5 % O₂, the percentage of effluxed ^177Lu-3 were 42.07±1.48, 58.10±3.18 and 63.80±1.75 respectively. Interestingly, when the partial oxygen pressure is above 5%, the percentage of effluxed radioactivities were found to be significantly higher compared with ^177Lu-1-2 (p < 0.05 to 0.001).

Overall in this experiment, lower efflux rates for the radioconjugates ^177Lu-1-3 was observed when the cells are exposed at 5 % O₂ or less. Furthermore, the retention of the ^177Lu 1-3 in the cells under 0.2% oxygen was twice that of the cells under 2.5 % oxygen indicating the hypoxia trapping efficiency of these designed radio-conjugates is dependent on the O₂ concentrations.

In another set of experiment where PC-3 cells were pre-incubated with ^177Lu-0-3 for 2 h under hypoxic (0.2 % O₂) conditions, followed by measuring efflux rate over normoxic (21 % O₂) at 2, 4 and 8 h. At 2 h, the cellular efflux of ^177Lu-0 was 35. 48 ±0.83 at 0.2 - 21 % O₂ which is consistent with what has previously been observed at 0.2 and 21 % O₂ concentrations. With the introduction of increasing numbers of 2-nitroimidazole moieties to the conjugates, that is, ^177Lu1-3, the reflux rate was decreased remarkably from 35.17±2.84, 43.01±1.76, 46.21±4.33
respectively which is significantly lower than the efflux profile observed at 21 % O\textsubscript{2} concentrations. The same trend was noted for timepoints at 4 and 8 h. The efflux profile of \textsuperscript{177}Lu\textsuperscript{3} at varying oxygen concentrations 0.2 -21 % O\textsubscript{2} were identical, with all three conjugates displaying their 1.3 -1.5 fold decrease in efflux rate between 21 and 0.2-21 % O\textsubscript{2} .This study indicates that the 2-nitroimidazole derivatives showed lower efflux rates under low O\textsubscript{2} levels compared with 21% O\textsubscript{2}. Rationale behind this is 2-nitroimidazoles bind irreversibly to intracellular proteins, which is consistent with the known trapping mechanism of 2-nitroimidazole. These results indicate that retention of these compounds in hypoxic cells were oxygen concentration dependent.

4.5 CONCLUSION

In conclusion, we have synthesized and evaluated three BB2r-targeted radioconjugates that have 2-nitroimidazole hypoxia trapping moieties conjugated to the linking group of the peptide, for the purpose of enhancing retention in hypoxic cancers. Three hypoxia trapping enhanced BB2r-targeted radioconjugates with mini-PEG linkers were synthesized. All conjugates demonstrated hydrophilic nature under physiological conditions. The synthesized \textsuperscript{177}Lu-0-3 bombesin conjugates exhibited nanomolar binding affinities towards the BB2r in the PC-3 prostate cancer cell line. This study signifies the use of selenomethionine and ascorbic acid in combination can efficiently reduce the radiolytic rate associated with 2-nitroimidazole. In addition, in vitro efflux studies under different oxygen concentrations demonstrated that these conjugates were preferentially retained in hypoxic cancer cells which is an oxygen dependent process. The work carried out suggests a potential new avenue to significantly increase efficacy of diagnostic and therapeutic BB2r-targeted drugs in a variety of cancers.
Chapter 5 DISCUSSION

Among many techniques for oncological imaging, nuclear medicine techniques are of growing importance. Compared to MRI and optical imaging, nuclear imaging has good sensitivity for deep tissue due to continuously generated signals by the decay process of radionuclides. Tremendous effort has been made for development of radiolabeled antibodies and their fragments during the past 40 years [168]. Although promising results have been published, the radiolabeled antibodies have disadvantages, mainly associated with their high molecular weight, such as sequestration by reticuloendothelial cells and long elimination half-life. Low-molecular-weight based radiotracers, such as receptor-targeted regulatory peptides, are rapidly taken up in the target tissues and usually have fast plasma clearance through renal excretion, which leads to high tumor-to-non-target ratios quickly after administration. Unfortunately, one of the major challenges, especially for therapeutic application, of many low-molecular-weight, receptor-targeted peptides is the short tumor residence times due to intrinsically high diffusion and efflux rates. After receptor mediated uptake of radiotracers, a rapid tumor washout is often observed which substantially reduces the theranostic efficacy and clinical potential.

We seek to address this problem by incorporating hypoxiatrapping agents into the peptidic radiotracer, which is expected to selectively increase the residence time in hypoxic tumor tissues. The main idea behind this approach is depicted in Figure 5.1. After receptor mediated uptake of radiotracers, the 2-nitroimidazole trapping moieties will become activated and irreversibly bind to intracellular nucleophiles, such as cytoplasmic proteins. The expectation of this approach was that the binding to the intracellular macromolecules will substantially increase the retention time of radiolabeled peptides in hypoxic tumor tissues leading to an increase in diagnostic/therapeutic efficacy and clinical potential.
Figure 5.1 Trapping mechanism of hypoxia enhanced $^{111}$In-BB2r-targeted conjugates
As the first attempt of development of the hypoxia trapping enhanced system, we chose the widely investigated bombesin(7-14)NH$_2$ as the targeting peptide and 2-nitroimidazole as the hypoxia trapping agent. Bombesin(7-14)NH$_2$ peptides are able to agonistically bind to the BB2 receptor that is overexpressed on a variety of cancers with high (typically nanomolar) affinity. Derivatives of 2-nitroimidazole have been actively studied as hypoxia targeted agents that are able to quantify the hypoxic burden present in tumors. A total of four hypoxia trapping-enhanced bombesin conjugates were synthesized in chapter 2. Receptor saturation studies reveal that the BB2r expression is independent of oxygen level. In vitro internalization assays revealed significantly improved residence time of 2-nitroimidazole containing conjugates in PC-3 hypoxic cells. However, our studies indicate that incorporation of the 2-nitroimidazole moieties close to the pharmacophore impairs the BB2r binding affinity of these radioconjugates. Additionally, we found significantly higher protein association of 2-nitroimidazole incorporated conjugates under hypoxic conditions which suggests the trapping mechanism is at least partially associated with the 2-nitroimidazole moiety.

To eliminate any steric interference which the 2-nitroimidazoles might impose on the BB2r-targeting vector, a new series of conjugates with an extended linker (8-Aoc) incorporated between the 2-nitroimidazole-amino acid residue and the pharmacophore were synthesized in chapter 3. Based on the results of competitive binding studies, the incorporation of an extended linker has eliminated the detrimental effect of the 2-NIAA side chain on pharmacophore binding. In vitro studies demonstrated substantially lower efflux rates and higher protein association for 2-nitroimidazole containing conjugates under hypoxic conditions. As a result of the promising in vitro performance, we carried out the first in vivo investigation of these radioconjugates in a PC-3 xenograft mouse model. Significant tumor retention enhancement was observed at 72 h post injection for radioconjugates $^{111}$In-2 and $^{111}$In-4. Up to 20% higher tumor retention for $^{111}$In-4 was
observed relative to the control $^{111}$In-1. All radioconjugates exhibited substantial accumulation of radioactivity in the tumor site based on the MicroSPET/CT images. It is interesting to note that the unusual high radiolytic rate and kidney retention seem to be associated with the incorporated 2-nitroimidazoles. Further studies are needed to elucidate the underlying molecular mechanisms.

To address the high kidney retention issue, a miniPEG linker was adopted due to the fact that the hydrophilic linker can increase the clearance rate from non-target organs. Stabilizing buffer containing selenomethionine was introduced to protect against the radiolysis of the targeting peptides. Considering the exhibited long term tumor retention in chapter 2, Lutetium-177 was utilized to examine the therapeutic potential of these radioconjugates. A total of four hypoxia enhanced bombesin conjugates were synthesized including all the modifications mentioned above in **Chapter 4**. Storage buffer containing selenomethionine was demonstrated to be very effective in protecting these $^{177}$Lu-labeled bombesin conjugates from radiolytic degradation when combined with ascorbic acid. Efflux studies were performed under various pO$_2$ (0.2, 1, 2.5, 5 and O$_2$) to evaluate the efficacy of the hypoxia trapping moieties to enhance retention in hypoxic PC-3 cells. Overall in this experiment, lower efflux rates for the radioconjugates $^{177}$Lu-1-3 were observed following a pO$_2$-dependent manner, while the efflux rate of the control was unchanged at various pO$_2$.

In summary, a hypoxia trapping enhanced BB2r-targeted radiopeptide system was established. These radioconjugates exhibited significantly higher retention in hypoxic PC-3 tumor cells compared to the control. Strong cellular protein association was observed for all hypoxia trapping enhanced conjugates which suggest the important role of 2-nitroimidazole for this enhancement. In vivo biodistribution studies revealed great long term tumor retention improvement, which suggests the therapeutic potential of the developed system.
Future Studies

1. Cell trafficking studies using confocal microscopy

As discussed earlier, the hypoxia trapping mechanism of 2-nitroimidazole is that it can bind to macromolecules such as protein in the cytoplasm compartment. Previous studies demonstrated that GRP are rapidly internalized into early endosomes and then trafficked and degraded in the lysosomes [169]. Subcellular localization and quantification of hypoxia trapping enhanced peptides is therefore needed to investigate a potential peptide recycling route which may affect the hypoxia trapping efficacy. We have already established the staining protocol for early endosomes, late endosomes and lysosomes using CellLight® Early Endosomes-GFP, CellLight® Late Endosomes-RFP, and LysoTracker® Blue DND-22. We have also synthesized peptide analogs that are labeled with Cy®5 dye. Calculation of colocalization coefficients of peptides with each organelle is ongoing using the Zen® colocalization analysis module.

2. Colocalization of radioactive signal with tumor hypoxic region

The extent of hypoxia in tumors appears to be strongly associated with the aggressiveness of the tumor phenotype, therapeutic resistance and patient prognosis [105]. In the clinic, the development of tumor hypoxia is independent of clinical size, stage, histology, grade and various patient demographics [170]. The hypoxic burden in tumor xenograft mouse models has been shown to correlate with tumor size, but it is highly dependent on the cell-line used [151]. It is therefore very important to quantify the tumor hypoxia burden for the xenograft model to better interpret the trapping efficiency of our system. We have already developed an immuno-histochemistry staining protocol for quantification of tumor hypoxia using pimonidazole. We have also established an autoradiography method to colocalize the radioactive signal with pimonidazole staining.
3. Synthesis of nitrogen mustard based hypoxia trapping agents

It has been previously demonstrated that only a fraction (~10%) of activated nitroimidazoles can form adducts with protein, partially because of the fast reaction of hydroxylamine intermediates with advantageous water molecules [131]. Activated nitrogen mustards have substantially longer lifetimes relative to activated nitroimidazoles, which may offer increased selectivity and lead to increased retention in hypoxic cells [171, 172]. Several nitrogen mustard agents such as TH302 and PR-104H are currently being evaluated in several phase II and phase III clinical trials [173-177]. We have begun to modify TH302 with addition of carbocyclic acid functionality to facilitate the conjugation to the peptide backbone.

4. Evaluation of the therapeutic efficacy using a patient-derived orthotopic xenograft model

The advantages of using subcutaneous xenograft models are (1) easy to establish; (2) easy to monitor; (3) convenient to quantify the tumor burden. However, the drug response on a subcutaneous xenograft model using human cell lines does not often correlate with clinical activity in human patients [178]. One reason is that the tumor is not orthotopically localized and specific molecules, such as hormones, which are crucial for development of breast and prostate cancer, are not expressed. Tremendous efforts have been made to develop techniques of surgical implantation of patient-derived tumors to the corresponding organ of rodents [179-181]. Strong predictive response values of clinical activity in patients were observed when using orthotopic xenograft tumor model [182-184]. Due to the fact that BB2r expression is androgen-dependent, the hypoxia enhanced BB2r-targeted agents will be investigated in a patient-derived orthotopic xenograft model in the future.
REFERENCES


