Development of Neurotensin-Based Radiopharmaceuticals For Neurotensin-Receptor-1-Positive Tumors Targeting

Yinnong Jia
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DEVELOPMENT OF NEUROTENSIN-BASED RADIOPHARMACEUTICALS FOR NEUROTENSIN-RECEPTOR-1-POSITIVE TUMORS TARGETING

by

Yinnong Jia

A DISSERTATION

Presented to the Faculty of the University of Nebraska Graduate College in Partial Fulfillment of the Requirements for the Degree of Doctor of Philosophy

Pharmaceutical Sciences Graduate Program

Under the Supervision of Professor Jered Garrison

University of Nebraska Medical Center
Omaha, Nebraska

April, 2017

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Aaron Mohs, Ph.D.
To My Family Who Believed in Me
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The neurotensin receptor 1 (NTR1) is overexpressed in many cancers, due to its role as a growth pathway. These NTR1-positive cancers include pancreatic, colon, prostate and breast cancers. In the radiopharmaceutical field, the overexpression of NTR1 in cancer has prompted the development of NTR1-targeted diagnostics and therapeutics. The neurotensin (NT) peptide exhibits low nanomolar affinity for NTR1 and has been the paradigm for NTR1-targeted agents. Since the 1980’s, radiolabeled NT analogs have been developed and evaluated for targeting NTR1-positive cancers. Since native NT is rapidly degraded in vivo by a variety of peptidases, a tremendous amount of effort has been put forth to design stabilized NT analogs with increased in vivo efficacy. To further enhance NTR1-targeted agents for diagnosis and therapy, our work has focused on strategies to increase the binding affinity, stability and optimize the pharmacokinetic profile of NTR1-targeted radiopharmaceuticals. In this dissertation, our work includes: 1) the investigation of the structure-activity relationship of the spacer groups in the NTR1-targeted agent design; 2) the evaluation of the effect charge distribution has on NTR1 binding and the biodistribution profile and 3) the utilization of peptidase inhibitors to extend the activity of NTR1-targeted agents.
It is well known that 1,4,7,10-tetraazacyclododecane-1,4,7,10-tetraacetic acid (DOTA), and other radiometal chelators, can negatively impact the receptor binding of targeted peptides. Utilizing DOTA and a known metabolically stabilized NTR1-targeted peptide ([N-α-Me]Arg^8,Dmt^{11},Tle^{12}]NT(6-13)), we evaluated a series of spacer groups to examine what steric impact, if any, DOTA had on NTR1-targeted peptides. We observed that the binding affinity was negatively affected by the direct conjugation (i.e., absence of spacer) with DOTA. However, the optimal binding activity can be restored with the inclusion of β-Ala or longer spacer groups. Our following studies investigated the impact the Lys^6 charge had on NTR1 binding and the overall biodistribution profile. It was observed that translation of this amino acid further away from the peptide influenced the receptor binding, internalization and kidney retention profile of the NTR1-targeted peptide. Lastly, we examined if peptidase inhibitors, such as phosphoramidon (PA), would improve the targeting efficacy of NTR1-targeted agents. We observed that PA limited the degradation of the peptide and resulted in an increased NTR1-positive tumor uptake. Based on these findings, we increased our understanding of the structure-activity relationships and in vivo degradation of NTR1-targeted agents. Utilizing this knowledge, we plan on optimizing the design of future NTR1-targeted agents for diagnostic imaging and radiotherapy.
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LIST OF ABBREVIATION

% ID/g  percentage injected dose per gram
\(^{111}\text{In}\)  indium-111
\(^{177}\text{Lu}\)  lutetium-177
\(^{188}\text{Re}\)  rhenium-188
\(^{18}\text{F}\)  fluorine-18
\(^{201}\text{Ti}\)  thallium-201
\(5\)-Ava  5-aminoovaleric acid
\(^{64}\text{Cu}\)  copper-64
\(^{68}\text{Ga}\)  gallium-68
\(8\)-Aoc  8-aminooctanoic acid
\(^{99\text{m}}\text{Tc}\)  technetium-99m
ACE  angiotensin-converting enzyme
Arg  arginine
Asn  asparagine
BK  bradykinin
BSA  bovine serum albumin
COMU (1-cyano-2-ethoxy-2-oxoethylidenaminooxy) dimethylamino-morpholino-carbenium hexafluorophosphate
CT  computed tomography
DCC  \(N,N'\)-dicyclohexylcarbodiimide
DCM  dichloromethane
DIEA  \(N,N\)-diisopropylethylamine
DMF  \(N,N\)-dimethylformamide
Dmt  2,6-dimethyl tyrosine
DOTA  1,4,7,10-tetraazacyclododecane-1,4,7,10-tetraacetic acid
DTPA  diethylene triamine pentaacetic acid
ES-MS  electrospray ionization mass spectrometry
FDG  fluorodeoxyglucose
Fmoc  fluorenlymethyloxycarbonnyl
Glu  glutamic acid
<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Definition</th>
</tr>
</thead>
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<tr>
<td>HEPES</td>
<td>4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid</td>
</tr>
<tr>
<td>IC&lt;sub&gt;50&lt;/sub&gt;</td>
<td>half maximal inhibitory concentration</td>
</tr>
<tr>
<td>Ile</td>
<td>isoleucine</td>
</tr>
<tr>
<td>IPP</td>
<td>Ile-Pro-Pro</td>
</tr>
<tr>
<td>LC-MS</td>
<td>liquid chromatography-mass spectrometry</td>
</tr>
<tr>
<td>Leu</td>
<td>leucine</td>
</tr>
<tr>
<td>Lys</td>
<td>lysine</td>
</tr>
<tr>
<td>NEP</td>
<td>neprilysin</td>
</tr>
<tr>
<td>NMP</td>
<td>N-methylpyrrolidone</td>
</tr>
<tr>
<td>NT</td>
<td>neurotensin</td>
</tr>
<tr>
<td>NTR1</td>
<td>neurotensin receptor 1</td>
</tr>
<tr>
<td>PA</td>
<td>phosphoramidon</td>
</tr>
<tr>
<td>PBS</td>
<td>phosphate-buffered saline</td>
</tr>
<tr>
<td>PEG</td>
<td>polyethylene glycol</td>
</tr>
<tr>
<td>PET</td>
<td>positron emission tomography</td>
</tr>
<tr>
<td>PPRT</td>
<td>peptide receptor radionuclide therapy</td>
</tr>
<tr>
<td>Pro</td>
<td>proline</td>
</tr>
<tr>
<td>RAP</td>
<td>receptor-associated protein</td>
</tr>
<tr>
<td>RP-HPLC</td>
<td>reverse phase high performance liquid chromatography</td>
</tr>
<tr>
<td>SCID</td>
<td>severe combined immunodeficiency</td>
</tr>
<tr>
<td>SDS</td>
<td>sodium dodecyl sulfate</td>
</tr>
<tr>
<td>SP</td>
<td>short peptide</td>
</tr>
<tr>
<td>SPECT</td>
<td>single-photon emission computed tomography</td>
</tr>
<tr>
<td>sst2</td>
<td>somatostatin receptors</td>
</tr>
<tr>
<td>Tle</td>
<td>tert-leucine</td>
</tr>
<tr>
<td>TOP</td>
<td>thimet oligopeptidase</td>
</tr>
<tr>
<td>Tyr</td>
<td>tyrosine</td>
</tr>
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<td>β-Ala</td>
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Chapter 1:

INTRODUCTION
1.1 Peptide-based radiopharmaceuticals

Small molecules bearing radionuclides for targeting cancers have been of interest for diagnostic as well as therapeutic purposes [1–5]. One successful example of small molecule diagnostic agents is $^{18}$F-FDG ($^{18}$F-fluorodeoxyglucose), which exhibits high uptake in many cancers due to the reliance of cancers on glucose metabolism [6]. In order for a radiopharmaceutical to be considered successful, it must accumulate at a high level within the target tissues, as well as clear from the blood and other non-target organs in the time frame that is compatible with the half-life ($t_{1/2}$) of the radionuclide [7–9]. One class of small molecules being actively investigated are regulatory peptides, such as the somatostatin, bombesin, cholecystokinin and neurotensin (NT), due to the favorable pharmacokinetic properties of these molecules [9–14]. Most receptor-targeted peptides that have been investigated for diagnosis and therapy are based on endogenous bioactive molecules [8,9,12,15–17]. Because of this and their relatively small size, receptor-targeted peptides are generally not antigenic [18]. Advantages of peptides over monoclonal antibodies, another class of molecules employed for tumor scintigraphy and treatment, include ease of synthesis and modification, ability to withstand harsher chemical conditions (i.e., acids and heat), and the significantly faster plasma clearance [19].

In the early 1990s, the first clinically successful receptor-targeted peptide was obtained with $[^{111}$In-DTPA$^0$]octreotide (Octreoscan™), a somatostatin peptide binding with high affinity to somatostatin receptors found at high densities in several neuroendocrine tumors [20]. To date, $[^{111}$In-DTPA$^0$]octreotide has been clinically utilized for the detection of carcinoids and pancreatic neuroendocrine tumors [5,20–23].
Additionally, $^{90}\text{Y}$- and $^{177}\text{Lu}$-labeled somatostatin analogues have been developed for peptide receptor radionuclide therapy (PPRT) to treat gastroenteropancreatic neuroendocrine tumors [7,10,21,22]. To improve the targeting capabilities of radiolabeled pharmacophores to somatostatin receptors, the field has evolved to the examination of radiolabeled somatostatin receptor antagonists that have shown superior pharmacokinetics in pilot studies [24,25]. The successes related to somatostatin receptor targeting has propelled the field forward into the development of other applicable regulatory peptide derivatives for tumor diagnosis and therapy [16,20,26–30]. Such peptides or peptide analogues investigated include, but are not limited to, bombesin [28], substance P [29], cholecystokinin [26,27] and neurotensin [31,32]. A representative list of receptor-targeted peptides under development is given in Table 1.1.

In general, the components of receptor-targeted peptides include a peptide targeting vector, a chelator, a spacer group and a radiometal. The peptide targeting vector is responsible for obtaining target selectivity. The ideal peptide targeting vector is one with high in vivo affinity and stability to maximize target accumulation. Chelators, typically referred to as bifunctional chelating agents due to its metal binding and requisite conjugation chemistries, are utilized to stabilize radiometals and minimize the possibility of in vivo radiometal release [33–35]. For some receptor-targeted peptides, spacer groups are utilized to minimize steric inhibition of the pharmacophore by other structural components, such as the chelation system [36,37]. In addition, spacer groups can be a convenient handle to modify the hydrophilicity of the receptor-targeted agent without making structural changes to the targeting vector [36]. Lastly, the selection of the radiometal is based on the desired application, diagnostic imaging or radiotherapeutic treatment.
Table 1.1. Selected Peptides and Receptors in Tumors\(^a\) [19].

<table>
<thead>
<tr>
<th>Peptide</th>
<th>Receptor types</th>
<th>Tumor expression</th>
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<tbody>
<tr>
<td>Somatostatin</td>
<td>sst1, sst2, sst3, sst4, sst5</td>
<td>Neuroendocrine tumors (gastroenteropancreatic tumors), lymphoma, paraganglioma, carcinoids, breast, brain, renal, small cell lung cancer, medullary thyroid cancer</td>
</tr>
<tr>
<td>Bombesin/GRP</td>
<td>BB1, BB2, BB3, BB4</td>
<td>Prostate, breast, pancreas, gastric, colorectal, small cell lung cancer</td>
</tr>
<tr>
<td>VIP</td>
<td>VPAC1, VPAC2</td>
<td>Adenocarcinomas of breast, prostate, stomach and liver; neuroendocrine tumors</td>
</tr>
<tr>
<td>α-MSH</td>
<td>MC1-5R</td>
<td>Melanomas</td>
</tr>
<tr>
<td>CCK/gastrin</td>
<td>CCK1, CCK2</td>
<td>Medullary thyroid cancer, small cell lung cancer, gastrointestinal stromal tumor, stromal ovarian cancer, astrocytomas</td>
</tr>
<tr>
<td>Neurotensin</td>
<td>NTR1, NTR2, NTR3</td>
<td>Small cell lung cancer, colon, exocrine ductal pancreatic cancer, Ewing sarcoma, meningioma, astrocytoma, breast, prostate cancer</td>
</tr>
<tr>
<td>LHRH</td>
<td>LHRH-R</td>
<td>Prostate, breast cancer</td>
</tr>
<tr>
<td>Substance P</td>
<td>NK1, NK2, NK3</td>
<td>Glial tumors (glioblastoma, medullary thyroid cancer), pancreas, breast, small cell lung cancer</td>
</tr>
<tr>
<td>Exendin</td>
<td>GLP-1</td>
<td>Insulinomas, gastrinomas, pheochromocytomas, paragangliomas and medullary thyroid carcinomas</td>
</tr>
<tr>
<td>RGD</td>
<td>α(_v)(β_3)-integrin</td>
<td>Glioma, breast, prostate cancer</td>
</tr>
</tbody>
</table>

\(^a\) GRP, gastrin-releasing peptide; VIP, vasoactive intestinal peptide; α-MSH, α-melanocyte-stimulating hormone; CCK, cholecystokinin; LHRH, luteinizing hormone-releasing hormone; GLP, glucagon-like peptide; RGD, Arg-Gly-Asp
The types of radioisotope decays include $\gamma$, $\beta^+$, $\beta^-$, $\alpha$, and Auger emissions [7]. For diagnostic imaging, radiometals that emit $\gamma$ photons and $\beta^+$ particles are utilized for use with single photon emission computed tomography (SPECT) or positron emission tomography (PET), respectively. The commonly used radioisotopes and chelators of NT-based radiopharmaceuticals are listed in Table 1.2. To date, $^{68}$Ga, $^{64}$Cu and $^{18}$F have been used for the development of NT-based PET radiopharmaceuticals [38–40]; $^{99m}$Tc-, $^{111}$In and $^{177}$Lu have been investigated for SPECT imaging agent development [31,41–46]. In our studies, we utilized $^{177}$Lu ($t_{1/2}$= 6.67 days) which is a theranostic radioisotope due to its dual $\gamma$ photons and $\beta^-$ emission profile [47,48]. $^{177}$Lu emits $\gamma$ photons (113 and 210 keV) which have high enough energies to penetrate tissues and provide a signal that is compatible with SPECT detection. In addition, $^{177}$Lu emits $\beta^-$ particles, a form of ionizing radiation that damages cellular DNA thereby killing affected cells [48]. To stably chelate $^{177}$Lu, we selected the 1,4,7,10-tetraazacyclododecane-1,4,7,10-tetraacetic acid (DOTA) chelator, a common chelation system utilized in clinical medicine. DOTA has been shown to stably chelate $^{177}$Lu in vivo and has been a mainstay of radiopharmaceutical agent design [48].
### Table 1.2. Characteristics of Radioisotopes and Chelators used for NT-based peptides.

<table>
<thead>
<tr>
<th>Radioisotopes</th>
<th>Half-life</th>
<th>Chelators</th>
<th>Application</th>
</tr>
</thead>
<tbody>
<tr>
<td>Technetium-99m ((^{99m})Tc)</td>
<td>6.0 h</td>
<td>(NαHis)Ac, MAG, HYNIC</td>
<td>Diagnosis (SPECT)</td>
</tr>
<tr>
<td>Fluorine-18 ((^{18})F)</td>
<td>1.8 h</td>
<td>SFB</td>
<td>Diagnosis (PET)</td>
</tr>
<tr>
<td>Gallium-68 ((^{68})Ga)</td>
<td>1.1 h</td>
<td>NOTA, DOTA</td>
<td>Diagnosis (PET)</td>
</tr>
<tr>
<td>Copper-64 ((^{64})Cu)</td>
<td>12.7 h</td>
<td>DOTA</td>
<td>Diagnosis (PET)</td>
</tr>
<tr>
<td>Indium-111 ((^{111})In)</td>
<td>67.2 h</td>
<td>DTPA, DOTA</td>
<td>Diagnosis ((\gamma) imager)</td>
</tr>
<tr>
<td>Lutetium-177 ((^{177})Lu)</td>
<td>160.8 h</td>
<td>DOTA</td>
<td>Diagnosis (SPECT)</td>
</tr>
<tr>
<td>Rhenium-188 ((^{188})Re)</td>
<td>17.0 h</td>
<td>(NαHis)Ac</td>
<td>Therapy</td>
</tr>
<tr>
<td>Thallium-201 ((^{201})Ti)</td>
<td>73.1 h</td>
<td>DTPA</td>
<td>Diagnosis (SPECT)</td>
</tr>
</tbody>
</table>
1.2 Neurotensin receptors and cancers

Neurotensin (NT) receptors have been found to be overexpressed as a growth pathway for the proliferation, survival, migration, invasion and neoangiogenesis of numerous cancers [49]. Reubi and coworkers [50] have observed overexpression of NT receptors in clinically relevant human tumors, such as Ewing’s sarcoma (65%), meningioma (52%), astrocytoma (43%), medulloblastoma (38%), medullary thyroid carcinoma (29%) and small cell lung cancer (25%). In addition to the above cancers, the occurrence of NT receptors has been reported in high abundance in ductal pancreatic adenocarcinoma (75%) [51,52], invasive ductal breast cancers (91%) [53,54] and malignant mesothelioma (90%) [55]. To date, the increased expression of NT receptors has been shown to contribute to the tumor progression in non-small cell lung cancer [56], colon adenocarcinoma [57], head and neck squamous cell carcinoma [58] and prostate cancer [59]. The high occurrence of NT receptors in such prevalent cancers has led to an increased interest in the development of imaging moieties for targeting NT receptors in the last three decades. Despite the ongoing research concerning radiopharmaceuticals for targeting NT receptors, to date only two clinical studies have been performed [41,60], both lacking the desired pharmacokinetic performances required for a successful radiopharmaceutical agent.

Currently, there are four NT receptors that have been identified. Two of the identified receptors, NTR1 and NTR2, have been found in human and belong to the G-protein coupled receptor family, demarked by the seven putative transmembrane domains of this class [61,62]. NTR3, also found in human, belongs to sortilin family of proteins as an intracellular sorting protein and is composed of a single transmembrane
domain [62–65]. Recently, a fourth NT receptor (NTR4) belonging to the G-protein coupled receptor family was identified in bullfrogs [66].

Of most interest, with respect to cancer imaging and therapy, is the NTR1 (424-amino-acid protein in rat and 418-amino-acid protein in human) which mediates most of the NT biological effects and is found overexpressed in various cancers [61,67,68]. The high expression of NTR1 in cancerous tissues is linked to signaling pathways in which NTR1 facilitates several transforming functions such as cell proliferation, migration, tumor angiogenesis, and metastasis [49,69,70]. The high NTR1 expression in numerous cancers, the minimal or lack of expression in normal surrounding tissues, and the fact that NT exhibits high affinity (Kd = 0.2 nM) for the NTR1 [71,72] has prompted the development NTR1-targeted radiopharmaceuticals [26,50–52,73,74].

1.3  NT and NTR1

Initially isolated from the bovine hypothalamus [75] in 1973 and later obtained from the bovine intestines [76] in 1976 by Carraway and Leeman, NT is a 13-amino-acid, linear peptide with the sequence of Glu-Leu-Tyr-Glu-Asn-Lys-Pro-Arg-Arg-Pro-Tyr-Ile-Leu. It has been shown to bind to NTR1 receptors with high nanomolar affinity. Functioning as both a neuromodulator and local hormone in mammals, NT is found in the central nervous system and peripheral tissues (mainly the gastrointestinal tract) [77–79]. Structural information concerning the molecular binding of the ligand NT to NTR1 has recently been published and has increased our understanding of the ligand-receptor interactions [62,72,80,81]. The binding interactions of NT to NTR1 were first investigated by Bernassau, Kitabgi, and Labbé-Jullié [72] utilizing alanine scanning and receptor mutagenesis methods, but
some details concerning the receptor/ligand interaction remained unclear. It was not until White and coworkers were able to obtain the crystal structure of NTR1, from Rattus norvegicus, binding to NT(8-13) that a detailed analysis of the ligand-receptor interactions was possible [62]. In this study, conformation thermostabilization of NTR1 (NTR1-GW5-T4L) allowed the crystal structure of the active-like state to be obtained at 2.8 Å resolution (Figure 1.1). The structure of the NTR1 binding pocket was found to be composed of three extracellular loops (ECL1-ECL3) and six transmembrane helices (TM2-TM7). Of the findings, charge interactions were observed between the positive-charged arginine side chains of NT(8-13) and the electronegative rim of the binding site. The formation of a salt bridge between the C-terminus of NT(8-13) and R327 of the receptor is probable along with van der Waals interactions and three hydrogen bonds between the side chains of the central regions of NT(8-13) and the receptor [62]. As a consequence, these interactions result in directing the N-terminus end out of the binding pocket (Figure 1.2). These observations confirm previous studies that have shown that only the NT(8-13), C-terminus sequence (Arg-Arg-Pro-Tyr-Ile-Leu-OH) is needed for high binding affinity to the NTR1 [67]. Due to this, the majority of NTR1-targeted agents have utilized truncated NT sequence such as NT(8-13) and NT(6-13) [44,82–84].
Figure 1.1. Overview of the NTR1 structure bound to the peptide agonist NT8–13. (a) side view (b) extracellular view and (c) intracellular view of the NTR1. Adapted with permission from Nature Publishing Group [62]
Figure 1.2. The extracellular views of NTR1 binding pocket with NT(8–13). (a) the ligand-binding pocket (b) The interaction between NTR1 (green residues with grey labels) and NT(8-13) (grey residues with bold black labels). Dashed lines: hydrogen bonds and salt bridges. (c) The charge complementarity between NT(8-13) and its binding pocket (red, negative; blue, positive). Adapted with permission from Nature Publishing Group [62]
1.4 The optimization of NT-based radiopharmaceuticals

As with all targeted radiopharmaceuticals, the development of NT-based agents for cancer diagnosis and therapy has focused on increasing NTR1-positive tumor uptake, while minimizing non-target retention. In order to successfully translate the NT-based radiopharmaceuticals into the clinic, research have focused on increasing the stability and NTR1-affinity of agents under development.

1.4.1 Degradation of NT

The lack of plasma stability due to rapid degradation has been a major hurdle to overcome in the design of imaging and therapeutic agents based on NT. The rapid degradation of these peptides results in metabolites with no NTR1-affinity and, ultimately, leads to poor in vivo tumor uptake. For example, in a recent clinical study, the low tumor uptake of $^{99m}$Tc-Demotensin VI ($N_t$-$\beta$-Ala-Arg-Dab-Pro-Tyr-Tle-Leu-OH), a NTR1-targeted agent, was attributed to the rapid degradation of this NT-based radiopeptide in vivo [41]. Findings such as this have given the impetus for the development of “stabilized” NT analogs with replacements using natural and unnatural amino acids. However, even with these replacements, we and others have observed that the in vivo degradation of “stabilized” NTR1-targeted agents, while inhibited, still occurs [85].

To date, the major endogenous peptidases responsible for cleavages of NT have been revealed [86–88]. These four peptidases responsible for the NT degradation are zinc-dependent peptidase members of the metalloendopeptidase family [24,68,69]: (1) The neutral endopeptidase 24.11 (EC 3.4.24.11, neprilysin, NEP); (2) angiotensin-converting enzyme (EC 3.4.15.1, ACE); (3) metalloendopeptidase 24.15 (EC 3.4.24.15, thimet oligopeptidase, TOP); and (4) metalloendopeptidase 24.16 (EC 3.4.24.16, neurolysin). With respect to the NT(8-13) truncated sequence, TOP is
known to hydrolyze the Arg<sup>8</sup>-Arg<sup>9</sup> bond, neurolysin cleaves the Pro<sup>10</sup>-Tyr<sup>11</sup> site, ACE hydrolyses the Tyr<sup>11</sup>-Ile<sup>12</sup> amide bond and NEP can degrade both the Pro<sup>10</sup>-Tyr<sup>11</sup> and Tyr<sup>11</sup>-Ile<sup>12</sup> sites (Figure 1.3). To better understand the process and location of degradation, the distribution of these four peptidases have been reported [89]. NEP is a cell membrane-bound protein that is present on the vascular wall and is also distributed in tissues such as the kidney, intestine, lung and brain [89]. TOP is present in both soluble and membrane-bound forms that are widely distributed in tissues, such as the liver, brain, lung and kidneys [90,91]. Neurolysin and ACE are found in soluble forms located predominately in the cytoplasm of the cells. Neurolysin is distributed mostly in the kidney, brain and liver, while ACE is distributed in the lung, kidney, intestine and brain [89].

1.4.2 Modification of NT-based radiopharmaceuticals to increase stability

To radiolabel and stabilize NT from degradation, while retaining the high binding affinity of the targeting vector, many groups have focused on modifying the essential amino acid sequence of NT(8-13) [31,32,43,44,92]. The modifications that have been made to increase the metabolic stability of NT-based agents include introducing pseudopeptide bonds between amino acids, substituting selected amino acids with unnatural amino acids and/or N-methylation [31,32,43,44,92,93]. Below, we discussed how investigators have utilized these different modifications to stabilize the NT-based radiopharmaceuticals and the resulting biological effect caused by these alterations.
Figure 1.3. Peptide bond cleavage of NT.
1.4.2.1 Modifications at Arg\(^8\)-Arg\(^9\)

The most common modifications to stabilize NTR1-targeted agents were made between amino acids Arg\(^8\)-Arg\(^9\), due to the susceptibility of this peptide bond to proteolytic cleavage by TOP. Utilizing a DTPA-NT(8-13) analogue, Tourwé and coworkers modified the Arg\(^8\) by replacing it with a Lys. This modification was found to increase metabolically stability while still retaining high NTR1-affinity [94]. In addition, the amide bond (CO-NH) between Arg\(^8\)-Arg\(^9\) was replaced with a pseudopeptide isosteric bond Ψ(CH\(_2\)-NH) to make it even more resistant to enzymatic cleavage. With these modification, the plasma stability increased from 10 min for \(^{111}\)In-DTPA-NT(8-13) to 275 min for \(^{111}\)In-DTPA-Lys\(^8\)-Ψ(CH\(_2\)-NH)-Arg\(^9\)-NT(10-13), demonstrating that these modifications substantially increased in vivo stability [94]. In regards to NTR1-binding affinity, these modifications were found to be well tolerated and still resulted in low nanomolar binding affinity.

The investigation of Mindt’s group demonstrated the same conclusions that the modifications to Arg\(^8\)-Arg\(^9\) could be made to stabilize the peptide without significantly decreasing binding affinity. They replaced the amide bond with a triazole ring between Arg\(^8\)-Arg\(^9\) to evaluate the structure-activity relationship. The percentage of intact peptide increased from 0.9 ± 0.3 % after 4 h (\(t_{1/2}= 39.4\) min) to 6.5 ± 4.6% after 4 h (\(t_{1/2}= 64.9\) min) in blood serum [95].

Another approach to stabilize the Arg\(^8\)-Arg\(^9\) site has been to N-methylate the Arg\(^8\). Using DTPA-substituted NT analogues, Tourwé and coworkers found that N-methylation of the Arg\(^8\) did not influence the binding affinity significantly. The representative NT-based peptides, NT-20.1 (truncated structure), NT-20.2 (with one modification) and NT-20.3 (with two modifications) have been shown in Table 1.3. As
shown in the Table 1.4, the \textit{in vitro} and \textit{in vivo} stability has been improved more significantly in NT-20.3 with both N-methylation at Arg$^8$ and the Tle$^{12}$ modifications compared to NT-20.2 with only Tle$^{12}$ modification \cite{84}. The decreased binding affinity in NT-20.2 (41.2 ± 6.2 nM) has been restored with the dual-modifications in the NT-20.3 (15.9 ± 1.7 nM), as compared with the NT-20.1 (6.73 ± 0.31 nM). The internalization rate remained similar to an unmodified NT analog control, namely [Lys$^6$(DTPA(In))]$\text{N}$ \cite{84}. Most importantly, the DTPA-NT-20.3 exhibited the highest tumor uptake at 1 h (~3 fold higher than [Lys$^6$(DTPA(In))]$\text{N}$, ~ 7 fold higher than DTPA-NT-20.1 and ~ 3.5 fold higher than DTPA-NT-20.2). Additionally, due to the higher tumor uptake, DTPA-NT-20.3 demonstrated the highest tumor to normal tissues ratios\cite{84}. The improved stability and, as a consequence, the increased tumor uptake of DTPA-NT-20.3 demonstrates that this modification can substantially increase the diagnostic and therapeutic potential of NTR1-targeted agents.
Table 1.3. Selected NT Analogs with Arg⁸-Arg⁹ Modifications [44,84].

<table>
<thead>
<tr>
<th>Peptides</th>
<th>Sequences</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lys⁶(DTPA)-NT</td>
<td>pGlu-Leu-Tyr-Glu-Asn-Lys(DTPA)-Pro-Arg-Arg-Pro-Tyr-Ile-Leu-OH</td>
</tr>
<tr>
<td>DTPA-NT-20.1</td>
<td>Ac-Lys (DTPA)-Pro-Arg-Arg-Pro-Tyr-Ile-Leu-OH</td>
</tr>
<tr>
<td>DTPA-NT-20.2</td>
<td>Ac-Lys (DTPA)-Pro-Arg-Arg-Pro-Tyr-Ile-Leu-OH</td>
</tr>
<tr>
<td>DTPA-NT-20.3</td>
<td>Ac-Lys (DTPA)-Pro-(NMe)Arg-Arg-Pro-Tyr-Ile-Leu-OH</td>
</tr>
<tr>
<td>DOTA-NT-20.3</td>
<td>Ac-Lys (DOTA)-Pro-(NMe)Arg-Arg-Pro-Tyr-Ile-Leu-OH</td>
</tr>
<tr>
<td>DOTA-NT-20.4</td>
<td>Ac-Lys (DOTA)-Pro-Arg-(NMe)Arg-Arg-Pro-Tyr-Ile-Leu-OH</td>
</tr>
</tbody>
</table>
Table 1.4. The Binding Affinity, Stability and Tumor Uptake of the NT-Analogs with Modifications [44,84].

<table>
<thead>
<tr>
<th>Peptides</th>
<th>IC$_{50}$ (nM)</th>
<th>In vitro stability$^a$ (t$_{1/2}$)</th>
<th>In vivo stability$^b$ (15 min)</th>
<th>Tumor uptake$^c$ at 1 h</th>
<th>Tumor uptake$^c$ at 3 h</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lys$^6$(DTPA)-NT</td>
<td>17.0 ± 4.0</td>
<td>25.0 ± 2.0</td>
<td>4.0</td>
<td>1.0 ± 0.3</td>
<td>0.7 ± 0.2</td>
</tr>
<tr>
<td>DTPA-NT-20.1</td>
<td>6.7 ± 0.3</td>
<td>0.4 ± 0.02</td>
<td>0.8</td>
<td>0.5 ± 0.1</td>
<td>0.5 ± 0.1</td>
</tr>
<tr>
<td>DTPA-NT-20.2</td>
<td>41.2 ± 6.2</td>
<td>4.4 ± 0.6</td>
<td>10.0</td>
<td>0.9 ± 0.3</td>
<td>0.5 ± 0.1</td>
</tr>
<tr>
<td>DTPA-NT-20.3</td>
<td>15.9 ± 1.7</td>
<td>257.0 ± 71.0</td>
<td>26.5</td>
<td>3.3 ± 0.2</td>
<td>2.4 ± 0.2</td>
</tr>
<tr>
<td>DOTA-NT-20.3</td>
<td>15.0 ± 1.0</td>
<td>ND</td>
<td>22.0 ± 1.0</td>
<td>4.7 ± 0.8</td>
<td>2.5 ± 0.2</td>
</tr>
<tr>
<td>DOTA NT-20.4</td>
<td>190.0 ± 20.0</td>
<td>ND</td>
<td>16.0 ± 2.0</td>
<td>0.8 ± 0.1</td>
<td>0.5 ± 0.1</td>
</tr>
</tbody>
</table>

$^a$ In vitro stability is expressed as the half-life in human serum at 37 °C

$^b$ The percentage of intact peptide in plasma has been evaluated at 15 min after injection.

$^c$ The tumor uptake was depicted as the %ID/g.
In contrast to modification at Arg$^8$, the NT-20.4 utilizes the N-methylation of Arg$^9$. This modification was found to dramatically decrease the binding affinity of the resulting analog. The IC$_{50}$ value of In-DOTA-NT-20.4 increased dramatically to $190 \pm 20$ nM from $15 \pm 1$ nM for In-DOTA-NT-20.3 [44]. With the decreased binding affinity, the NTR1-positive, tumor uptake of In-DOTA-NT-20.4 was only $0.8 \pm 0.1 \%$ ID/g. In another study, the concurrent replacement of Arg$^8$ by Gly(PipAm) and Arg$^9$ by Gly(Pip) (as shown in Figure 1.4) resulted in a substantial loss of affinity relative to Arg$^8$ modification alone [83]. Thus, during the pursuit of higher stability NTR1-targeted agents, the effect these modifications have on binding affinity should be carefully considered.

Based on the discussion above, the results demonstrate that modifications to the Arg$^8$.Arg$^9$ site were feasible and effective in increasing in vivo stability. These modifications led to approximately 10-20 fold increase in in vivo plasma stability relative to the native sequence. However, modifications to the Arg$^8$ are generally more tolerated in terms of maintaining NTR1-binding affinity relative to Arg$^9$. 
Figure 1.4. Structures of the amino acids utilized to modify the Arg^8 and Arg^9.
1.4.2.2 Modifications at Tyr\textsuperscript{11}-Ile\textsuperscript{12}

Another site of the NT targeting vector that is sensitive to degradation is the Tyr\textsuperscript{11}-Ile\textsuperscript{12} position. However, unlike modifications to the Arg\textsuperscript{8}-Arg\textsuperscript{9} site, modifications to the Tyr\textsuperscript{11}-Ile\textsuperscript{12} position are less tolerated and as a result the numbers of successful modifications are fewer. The structures of the amino acids that replace the Tyr\textsuperscript{11} and Ile\textsuperscript{12} have been listed in Figure 1.5. These modifications are believed to inhibit the degradation of the NT based peptide by ACE, NEP and neurolysin.

For the discussion of the Tyr\textsuperscript{11}-Ile\textsuperscript{12} modifications, the structures of the NT-based examples are listed in Table 1.5. The replacement of Tyr\textsuperscript{11} by 2,6-dimethyltyrosine (Dmt) is a common modifications. Many analogs has shown that this modification improves stability while preserving NTR1-binding affinity. For example, NT-XIX, which includes the Dmt\textsuperscript{11} modification, has similar binding affinity, but substantially increased stability relative to NT-XII, which lacks this modification [43,96]. However, other attempts at modifying or substituting Tyr\textsuperscript{11} have included the insertion of triazole rings or the replacement with mTyr [83]. These modifications have resulted in negligible improvement to the stability of the peptide and complete or partial loss of binding affinity [95]. Interestingly, in one instance, the replacement L-Tyr\textsuperscript{11} with D-Tyr\textsuperscript{11} has been shown not to impact stability or NTR1-affinity [83]. This is somewhat unexpected since it has been shown that Tyr\textsuperscript{11} contributes (phenol group) to the binding affinity through hydrogen bonding to hydroxyl group (Thr) in the NTR1 binding pocket.
Figure 1.5. The structures of Tyr, Ile and their analogs.
Table 1.5. The NT Analogs with Modifications (the proposed alterations are in bold).

<table>
<thead>
<tr>
<th>Peptides</th>
<th>Sequences</th>
</tr>
</thead>
<tbody>
<tr>
<td>NT-XI</td>
<td>(NaHis)Ac-Lys-Ψ(CH₂NH)-Arg-Pro-Tyr-Tle-Leu</td>
</tr>
<tr>
<td>NT-XIX</td>
<td>(NaHis)Ac-Arg-(NMe)Arg-Pro-Dmt-Tle-Leu</td>
</tr>
<tr>
<td>NT-VIII</td>
<td>(NaHis)Ac-(NMe)Arg-Lys-Pro-Tyr-Tle-Leu</td>
</tr>
<tr>
<td>NT-LB119</td>
<td>Ac-Lys (Ahx-DOTA)-Pro-(NMe)Arg-Arg-Pro-Dmt-Tle-Leu-OH</td>
</tr>
</tbody>
</table>
The position of Ile\textsuperscript{12} has been shown to be one of the most, if not the most, effective sites to increase the metabolic stability of NTR1-targeted agents [95]. For example, the replacement of Ile\textsuperscript{12} by Tle (tert-Leu) resulted in a stability increase from 4.3% to 72% in human serum after 24 h incubation [83]. Although a significant reduction in the cell internalization rate and binding affinity was observed with this modification, the higher tumor uptake due to the increased stability made up for any loss in NTR1-affinity or internalization rate [95]. Interestingly, the insertion of a triazole at the position of Ile\textsuperscript{12}, resulted in a more stable peptide, but a complete loss in binding affinity [95].

1.4.2.3 Modifications at Leu\textsuperscript{13}

The modification at Leu\textsuperscript{13} is not commonly applied to increase the stability of NT-based radiopharmaceuticals considering that peptidase degradation at this site is limited and there is a risk of complete loss in NTR1 binding affinity. As mentioned previously, the crystal structure of NT(8–13) bound to NTR1 has demonstrated that the Leu\textsuperscript{13} participates in ionic and hydrogen bonding to the residues of the receptor [62]. To date, modifications of the Leu\textsuperscript{13} have not provide any substantial overall benefits in terms of metabolic stability and, in some cases, have adversely affected binding to the receptor. For example, Mindt’s group demonstrated that the insertion of triazole at the C-terminus resulted in a total loss of binding affinity [95]. In addition, the replacement of Leu\textsuperscript{13} by Cpa, Cha or tBuAla (Figure 1.6) decreased the binding affinity and did not improve the stability significantly [83].
Figure 1.6. The Cpa, Cha and tBuAla that used to modify Leu$^{13}$.
1.4.2.4 Enhancing stability using enzyme inhibitors

Rather than modifying the structure of the NT based targeting vector, which can sometimes negatively impact affinity, the utilization of enzyme inhibitors to temporarily protect radiopeptides from \textit{in vivo} peptidase degradation was first proposed by De Jong and colleagues [97]. Phosphoramindon (PA), the inhibitor of NEP has been shown to dramatically increase the \textit{in vivo} targeting efficacy of receptor-targeted peptides, such as bombesin, gastrin/CCK and somatostatin analogs [97,98]. The increased \textit{in vivo} targeting is associated with a decrease in NEP metabolism resulting in an increase in \textit{in vivo} metabolic stability [97–100]. Two other NEP inhibitors, thiorphan and its prodrug racacdotril, have also been shown to increase the \textit{in vivo} stability and tumor uptake of radiolabeled gastrin analogs (\textsuperscript{111}In-DOTA-MG11); however, the effects of these analogs are not as potent as PA [101,102]. The co-injection of the ACE inhibitor, lisinopril showed no effect on increasing the tumor uptake of \textsuperscript{111}In-DOTA-MG11, which indicates that ACE is not significantly involved in the degradation of \textsuperscript{111}In-DOTA-MG11 [102].

Because NEP and ACE are both responsible for the degradation of NT, we expect that co-formulating NEP and ACE inhibitors with NT-based peptides could be potentially beneficial. In our approach, we investigated PA (NEP inhibitor) and IPP (Ile-Pro-Pro, an ACE inhibitor) with NT analogs to evaluate the effect on the \textit{in vivo} tumor uptake and stability. In addition, we investigated the effect of bradykinin (a competitive substrate for NEP, ACE and TOP) as well as a short peptide containing the most common metabolic sites of the NT(8-13) sequence (Pro-Arg-Arg-Pro-Tyr). These studies will be explored in Chapter 4.
1.4.3 The utilization of spacer groups to decrease steric inhibition and modify hydrophilic properties

The insertion of spacer groups is considered an effective method to decrease the steric inhibition of receptor-targeted agents, thus restoring binding affinity. Common chelators, such as DOTA and DTPA, are ideal for stabilizing radiometals, such as $^{177}$Lu, $^{111}$In and $^{68}$Ga. However, the introduction of DOTA too close to the NT pharmacophore can negatively impact binding affinity, particularly for those utilizing the smallest NT(8-13) fragment as a targeting vector. This loss of affinity is attributable to the steric bulk of the chelator. The introduction of various spacer groups in some NT analogs have been shown to restore optimal binding affinity by increasing the distance between the macrocyclic chelator and the targeting vector. For example, our studies have shown that the binding affinity of a NT(6-13) derivative was restored by the insertion of spacers groups, such as β-Ala, 5-Ava or 8-Aoc. This work will be further discussed in Chapter 2.

In addition to reducing steric inhibition, spacer groups can also be utilized to easily adjust the hydrophilicity of receptor-targeted peptides. Hydrophilicity is an important factor to consider in order to achieve optimal target to non-target ratios. Analogs that are too hydrophilic may have reduced circulation times, while analogs that are too hydrophobic may have increased hepatobiliary clearance and poor tissue diffusion. To quantitate the hydrophilicity of a charged species in biological milieu, the distribution coefficient, LogD, is commonly used. The LogD values of various reported NTR1-targeted peptides are shown in Table 1.6. For example, DOTA-Ahx-NT(8-13) ($^{177}$Lu(2)) has a LogD of -2.30, while DOTA-PEG$_4$-NT(8-13) ($^{177}$Lu-NT2) has a LogD of -2.40, suggesting that the PEG$_4$ is able to increase the hydrophilicity of the analog relative the Ahx hydrocarbon.
Table 1.6. The Selected NT Analogs and LogD Values.

<table>
<thead>
<tr>
<th>Analogs</th>
<th>Sequences</th>
<th>LogD</th>
<th>Ref</th>
</tr>
</thead>
<tbody>
<tr>
<td>$^{177}$Lu(2)</td>
<td>DOTA-Ahx-NT(8-13)</td>
<td>-2.30</td>
<td>[103]</td>
</tr>
<tr>
<td>$^{177}$Lu-NT1</td>
<td>DOTA-NT(8-13)</td>
<td>-2.50 ± 0.30</td>
<td>[104]</td>
</tr>
<tr>
<td>$^{177}$Lu-NT2</td>
<td>DOTA-PEG$_4$-Arg-Arg-Pro-Tyr-Ile-Leu</td>
<td>-2.40 ± 0.20</td>
<td>[104]</td>
</tr>
<tr>
<td>$^{177}$Lu-NT3</td>
<td>DOTA-PEG$_4$-Arg-Arg-Pro-Tyr-Tle-Leu</td>
<td>-2.25 ± 0.03</td>
<td>[104]</td>
</tr>
<tr>
<td>$^{177}$Lu-NT4</td>
<td>DOTA-PEG$_4$-Lys-Pro-Tyr-Ile-Leu</td>
<td>-2.10 ± 0.10</td>
<td>[104]</td>
</tr>
<tr>
<td>$^{177}$Lu-NT5</td>
<td>DOTA-PEG$_4$-Lys-Pro-Tyr-Ile-Leu</td>
<td>-2.08 ± 0.01</td>
<td>[104]</td>
</tr>
<tr>
<td>$^{177}$Lu-NT6</td>
<td>DOTA-PEG$_4$-Lys-Pro-Tyr-Ile-Leu</td>
<td>-2.00 ± 0.10</td>
<td>[104]</td>
</tr>
<tr>
<td>[68]Ga4</td>
<td>NODA-PEG$_6$-Lys-Pro-Tyr-Tle-Leu</td>
<td>-4.20</td>
<td>[105]</td>
</tr>
<tr>
<td>[68]Ga6</td>
<td>DOTA-(ζ)Lys-Lys-Pro-Tyr-Tle-Leu</td>
<td>-4.00</td>
<td>[105]</td>
</tr>
<tr>
<td>[68]Ga8</td>
<td>NODA-PEG$_6$-(ζ)Lys-Lys-Pro-Tyr-Tle-Leu</td>
<td>-4.10</td>
<td>[105]</td>
</tr>
<tr>
<td>$^{177}$Lu-N0</td>
<td>DOTA-Lys-Pro-(N-Me)Arg-Arg-Pro-Dmt-Tle-Leu-OH</td>
<td>-3.45 ± 0.05</td>
<td>[45]</td>
</tr>
<tr>
<td>$^{177}$Lu-N1</td>
<td>DOTA-β-Ala-Lys-Pro-(N-Me)Arg-Arg-Pro-Dmt-Tle-Leu-OH</td>
<td>-3.15 ± 0.05</td>
<td>[45]</td>
</tr>
<tr>
<td>$^{177}$Lu-N2</td>
<td>DOTA-5-Ava-Lys-Pro-(N-Me)Arg-Arg-Pro-Dmt-Tle-Leu-OH</td>
<td>-3.11 ± 0.05</td>
<td>[45]</td>
</tr>
<tr>
<td>$^{177}$Lu-N3</td>
<td>DOTA-8-Aoc-Lys-Pro-(N-Me)Arg-Arg-Pro-Dmt-Tle-Leu-OH</td>
<td>-2.99 ± 0.03</td>
<td>[45]</td>
</tr>
<tr>
<td>$^{177}$Lu-K2</td>
<td>DOTA-Lys-DLeu-Dleu-Dleu-Dleu-Pro-(N-Me)Arg-Arg-Pro-Dmt-Tle-Leu-OH</td>
<td>-2.53 ± 0.02</td>
<td>[46]</td>
</tr>
<tr>
<td>$^{177}$Lu-K4</td>
<td>DOTA-DLeu-Dleu-Lys-Dleu-Dleu-Pro-(N-Me)Arg-Arg-Pro-Dmt-Tle-Leu-OH</td>
<td>-2.59 ± 0.02</td>
<td>[46]</td>
</tr>
<tr>
<td>$^{177}$Lu-K6</td>
<td>DOTA-DLeu-Dleu-Dleu-Dleu-Lys-Pro-(N-Me)Arg-Arg-Pro-Dmt-Tle-Leu-OH</td>
<td>-2.63 ± 0.01</td>
<td>[46]</td>
</tr>
</tbody>
</table>


1.5 The optimization of NT-based radiopharmaceuticals to decrease renal uptake

The high in vivo NTR1-targeting capabilities of NT-based radiopharmaceuticals has resulted in significant interest for the development of diagnostic and therapeutic agents based on this platform [44,45]. However, the high, non-specific kidney uptake, due to renal clearance, has been a concern with regard to the development of NTR1-targeted radiotherapeutic agents [44,45]. This situation is not new to the field of receptor-targeted peptide when considering that other peptides in clinical development, such as bombesin, somatosatin, minigastrin and CCK analogs, have been comprised by the high non-specific kidney uptake [106]. The significant amount of radioactivity remained in the kidney post-injection for peptide receptor radionuclide therapy (PPRT) could cause renal toxicity and possibly damage the kidneys after treatment [107].

To further explain the reason of high kidney uptake in the next section, the general process of radiolabeled peptides after administration has been provided here [106,108]. After the injection and distribution, the majority of the radiolabeled peptides were excreted to the urine via kidney. The first step for kidney excretion is the glomerular filtration. The glomerulus is consisted of the loopy arteriole, where the ions, amino acids, glucose and water will be leaked under high pressure through the fenestrated endothelial cells on the vessel wall (with holes). During this process, the radiolabeled peptides are small enough to be efficiently filtered out from the blood in glomerulus to the Bowman’s Capsule and entered proximal tubule [106]. At the proximal tubule, majority of the filtered radiolabeled peptides will be reabsorbed back to blood, by multiple transport mechanisms (e.g., receptor-mediated endocytosis and
pinocytosis) [106,109]. The filtrate and water that are not reabsorbed at the proximal tubule will be excreted to the urine.

1.5.1 Mechanisms of renal uptake

The mechanism underlying the high kidney uptake of radiolabeled peptides has been of great interest in order to understand how to limit or decrease the kidney absorption of these agents. Based on our current understanding, two major uptake pathways have been identified: (1) receptor-mediated endocytosis by megalin and (2) fluid-phase endocytosis (pinocytosis) [106,107,109–111]. Other membrane transporters, such as organic anion and organic cation transporter, have been investigated, but none have yet to be found to be prominent players in the uptake of peptide-based radiopharmaceuticals [107]. Using \(^{111}\text{In-DTPA}\)octreotide, a somatostatin analogue, de Jong and co-workers determine that the primary site of uptake in the kidney was the cortex [111]. Their subsequent work unambiguously demonstrated that megalin, a multiligand receptor found on many absorptive cell types, was a major contributor to radiolabeled somatostatin retention in the kidney [112]. Courtoy and Jamar’s group first identified the equal role of pinocytosis to the renal uptake of radiolabeled somatostatin analogues by using the proximal tubule-derived opossum kidney (OK) cell line [113]. Though the initial work identifying these uptake mechanism centered on somatostatin analogs, the same mechanisms of uptake have been shown to be relevant for all peptide-based radiopharmaceuticals [22,106,107,110,114]. A more detailed discussion concerning the megalin-mediated endocytosis and pinocytosis mechanisms are presented below.
1.5.1.1 Receptor-mediated endocytosis via megalin receptor

The biodistribution of radiolabeled NT analogs in the kidneys has shown the same pattern of uptake as other radiopeptides (e.g., somatostatin, bombesin, neurotensin, minigastrin and CCK analogues), namely high retention in the cortex and low retention in the outer and inner medulla of the kidneys [22]. The high accumulation of radiopeptides in the cortex has supported the hypothesis that megalin, which is expressed predominately in the cortex, is one of the main uptake mechanisms. Megalin is a large, membrane-associated receptor (600 kDa) that is mostly expressed in the proximal tubule and involved in the reabsorption of numerous endogenous substances [106,110]. Unfortunately, numerous studies have demonstrated that megalin also has high affinity for many peptide-based radiopharmaceuticals [109]. In one study, the renal uptake of radiolabeled peptides was significantly reduced after pre-incubation with known megalin ligands, such as albumin or receptor-associated protein (RAP) [107]. In addition, the role of megalin-mediated kidney uptake of radiolabeled peptides was further demonstrated with the significant decrease of uptake of $[^{111}\text{In-DTPA}]\text{octreotide}$ in a megalin-deficient mouse model compared to wild-type mice [112]. Similar findings were observed when evaluating the renal uptake of $^{111}\text{In-neurotensin}$, exendin and minigastrin in megalin-deficient mice compared to wild-type mice [106]. While our understanding of megalin ligand-selectivity is imperfect, studies to date have suggested that the positive charge state of most receptor-targeted peptide could be a major contributing factor in the uptake by the negatively charged megalin receptor [107].
1.5.1.2 Pinocytosis (fluid-phase endocytosis)

Another transport mechanism that has been shown to contribute to the kidney uptake of radiolabeled peptides is fluid-phase endocytosis (pinocytosis) [107,113]. Using lucifer yellow, a pinocytosis marker, Courtoy and Jamar’s group demonstrated that a fluorescent somatostatin analog was taken up into proximal tubule endothelial cells by pinocytosis [113]. Following this study, Trejtnar and colleagues further proved the key role of pinocytosis in the renal uptake of several radiolabeled peptides by in vitro cellular studies [107]. They found that pre-incubation with an irreversible and selective pinocytosis inhibitor, rottlerin, significantly decreased pinocytotic uptake of several radiolabeled peptides, including $^{177}$Lu-labeled somatostatin, gastrin and bombesin analogs. Although the radiolabeled NT analogs were not tested in these studies, the generality of the uptake of numerous radiolabeled peptides supports the hypothesis that pinocytosis likely contributes to the accumulation of NT-based analogs in the renal proximal tubules [107].

1.5.2 Impact of charge and charge distribution on NT uptake in the kidney

To decrease the unfavorable kidney uptake of radiopeptides, the possible factors that influence the kidney uptake have been investigated. The impact of the charge state of radiopeptides on renal uptake has been reported by several different groups [17,22,27,107,115,116]. These studies revealed that the kidney uptake of these radiolabeled peptides were linearly increased with the number of charged amino acids [22,116]. For example, in one study which evaluates the addition of a negatively charged amino acid (i.e., Glu) to a radiolabeled bombesin construct, the decrease in net charge decreased the kidney uptake [117]. In contrast, increasing the positive charge of a somatostatin analog with the addition of basic amino acids increased the renal retention [106,115]. Considering the megalin discussion above, a large portion of the
renal uptake of radiolabeled peptides is carried out through a megalin-mediated process and this process is influenced by the charge of the peptide. Based on these and other studies, it seems highly likely that charge would be a factor in the uptake of NTR1-targeted agents. Recently, we have expanded on the investigation of the influence of charge by investigating how charge distribution in a radiolabeled NT analog could affect biological performance including its renal retention. Specifically, we designed NT analogs with the same overall charge state, but translated the charged amino acid (Lys) away from the targeting pharmacophore. A complete discussion of this study can be found in Chapter 3.

1.5.3 Strategies for reducing kidney uptake

Strategies to decrease the kidney uptake have been actively pursued. Some investigations have focused on modifying the peptide, particularly the charge state, to decrease renal retention. These modifications have general focused on substitution, addition or removal of charged amino acids [115,117]. However, these modifications can many times result in the loss of binding affinity and/or suboptimal biodistribution profiles. An alternative that has been actively explored is the utilization of blocking agents to reduce renal retention. Most of these agents are believed to reduce kidney uptake by blocking megalin-mediated uptake. Below are examples of the most commonly used approaches.

1.5.3.1 Arg and Lys administration

The administration of positively-charged amino acids, such as Arg, Lys, poly-L-lysine, D-Lys and their combination have been shown effective at decreasing the kidney uptake of some positively-charged radiopeptides [118–120]. The positively-charged Arg and Lys competitively bind to the megalin and saturate the receptor to decrease the non-specific binding of positively-charged radiopeptides (e.g.,
NT-based peptides). For example, a clinical study using a targeted radiotherapeutic somatostatin analog demonstrated that the co-administration of Lys and Arg significantly decreased the renal uptake in patients [120]. Currently, co-infusion of basic charged amino acids (e.g., Arg and Lys) is the standard and common strategy to protect the kidney during PRRT [106].

1.5.3.2 Other strategies

Besides the administration of Arg or Lys, pretreatment of albumin (a natural ligand for megalin) at low concentrations (0.5mg/ml) decreased kidney uptake for several investigated radiolabeled peptides (e.g., $^{111}$In-octreotide, $^{111}$In-exendin and $^{111}$In-minigastrin) [114]. The inhibitory effect of the administration of albumin was proportionally related to the concentration that was employed. Other megalin blocking agents such as RAP [107] and fragments of albumin have also demonstrated the capability of decreasing the kidney uptake of radiopeptides [114]. Other strategies that have been investigated to reduce renal uptake and retention of the proximal tubule endothelial cells include: disruption of microtubule formation by colchicine or nocodazole [121,122], the administration of NH$_4$Cl to neutralize the negatively charged binding site of megalin [123,124], and the inhibition of the citric acid cycle to disrupt the endocytic energy supply by maleate [123].

The studies on the mechanisms of renal uptake and the strategies that could efficiently decrease retention are still ongoing. More studies are needed to investigate if other transporters play a role in this endocytic process. Based on what is currently known of the proximal tubule cell absorption, there is no doubt that much work will be focused on the development of better methods and techniques for avoiding the retention of NTR1-targeted agents as well as other receptor-targeted peptides.
1.6 Current clinical studies

Although NT-based radiopharmaceuticals has been under development for more than 30 years, only two clinical studies have been carried out [41,60]. In 2003, the first clinical study investigating an NTR1-targeted agent was reported. $^{99m}$Tc-NT-XI (see Table 1.5) was evaluated in four patients with pancreatic cancer. NTR1-mediated uptake was found to correspond to the NTR1-expression of the pancreatic tumors. Unfortunately, the overall pancreatic tumor uptake was relatively low compared to the non-target uptake in the intestine and kidneys, as well as to a lesser extent, in the liver, spleen and bone marrow. Eight years later, an additional clinical study was carried out using $^{99m}$Tc-Demotensin VI (N$_4$-$\beta$-Ala-Arg-Dab-Pro-Tyr-Tle-Leu-OH) in patients with a variety of tumors types, including pancreatic adenocarcinoma ($n = 4$), small cell lung cancer (SCLC) ($n = 4$), non-small cell lung cancer (NSCLC) ($n = 4$), and colon carcinoma ($n = 2$). Tumor uptake was negligible for all patients. However, this was attributed to the rapid degradation of this analog in vivo and does not necessarily reflect on the potential of NTR1-targeted agents. These two clinical studies revealed that further development of NTR1-targeted agents is needed to reduce non-target retention, increase in vivo stability and determine the true potential of these agents for clinical translation.

1.7 Summary and current proposal

Due to its role as a growth pathway for cancer cell proliferation, invasion and migration, NTR1 has been shown to be overexpressed in many prominent cancers, including pancreatic, breast, and colon cancers. The high occurrence of NTR1 in these tumors provides a viable target for receptor-specific diagnosis and therapy. The NTR1-targeted ligands, typically based on the NT peptide, has been conjugated to
various radiometals and investigated for the diagnosis and therapy of NTR1-positive tumors. As discussed above, the development of NT-based radiopharmaceuticals has been comprised by the rapid degradation \textit{in vivo}, reduced binding affinity due to the chelator (e.g., DOTA) and the high uptake in non-target tissues (e.g., kidney). To investigate solutions for these drawbacks and improve the biological performance of NT-based radiopharmaceuticals, we focused on approaches to better elucidate the structure-activity relationships and improve the potential of NTR1-targeted agents.

In this dissertation, the work in the following chapters demonstrated the possible strategies that could be employed to increase the biological performance of NT-based radiopharmaceuticals. Specifically, we investigated: (1) the insertion of various spacers to optimize binding affinity, (2) the effect charge distribution of the NTR1-targeted agent had on pharmacokinetic performance and (3) the effect co-administration of enzyme inhibitors and competitive substrates had on the \textit{in vivo} stability of an NTR1-targeted agent. Through the strategies and methods that we have investigated, we hope to move the field toward a better understanding of NTR1-targeted agents and improve the potential of these agents for clinical applications.
Chapter 2:

Evaluation of DOTA-Chelated Neurotensin Analogs with Spacer-enhanced Biological Performance for Neurotensin-Receptor-1-Positive Tumors Targeting
2.1 Abstract

Neurotensin receptor 1 (NTR1) is overexpressed in many cancers types. Neurotensin (NT), a 13 amino acid peptide, is the native ligand for NTR1 and exhibits high (nM) affinity to the receptor. Many laboratories have been investigating the development of diagnostic and therapeutic radiopharmaceuticals for NTR1-positive cancers based on the NT peptide. To improve the biological performance for targeting NTR1, we proposed NT analogs with DOTA chelation system and different lengths of spacers.

We synthesized four NTR1-targeted conjugates with spacer lengths from 0 to 9 atoms (null (N0), β-Ala-OH (N1), 5-Ava-OH (N2), and 8-Aoc-OH (N3)) between the DOTA and the pharmacophore. In vitro competitive binding, internalization and efflux studies were performed on all four NT analogs. Based on these findings, metabolism studies were carried out on our best performing conjugate, $^{177}$Lu-N1. Lastly, in vivo biodistribution and SPECT/CT imaging studies were performed using $^{177}$Lu-N1 in an HT-29 xenograft mouse model.

As shown in competitive binding assay, the NT analogs with different spacers (N1, N2 and N3) exhibited lower IC$_{50}$ values than the NT analog without a spacer (N0). Furthermore, N1 revealed higher retention in HT-29 cells with more rapid internalization and slower efflux than the other NT analogs. In vivo biodistribution and SPECT/CT imaging studies of $^{177}$Lu-N1 demonstrated excellent accumulation (3.1 ± 0.4 %ID/g) in the NTR1-positive tumors at 4 h post-administration.

The DOTA chelation system demonstrated some modest steric inhibition of the pharmacophore. However, the insertion of a 4-atom hydrocarbon spacer group
restored optimal binding affinity of the analog. The in vivo assays indicated that \(^{177}\text{Lu-N1}\) could be used for imaging and radiotherapy of NTR1-positive tumors.

## 2.2 Introduction

The neurotensin receptor 1 (NTR1) is a G protein-coupled receptor of the neurotensin receptor family, of which there are three known members [77]. NTR1 has been shown to be up-regulated in numerous cancer types, including colon [57], breast [53], and pancreatic cancer [52]. The overexpression of NTR1 has been attributed to cancer cells as a crucial growth signaling pathway [59,125]. Studies have shown a strong link between NTR1 expression and the progression of many cancer types [54,57,59].

One area of NTR1 drug development being actively explored is NTR1-targeted diagnostic and therapeutic radiopharmaceuticals. The majority of these agents are based on neurotensin (NT), a 13-amino-acid peptide which exhibits nanomolar binding affinity to NTR1 [126]. The C-terminus of NT is responsible for binding to NTR1, thus research has focused largely on employing NT(8-13) and NT(6-13) fragments in drug development [68]. One major obstacle that has hampered the development of NTR1-targeted radiopharmaceuticals is the rapid proteolytic degradation of the NT in serum. The main degradation sites are the Arg\(^8\)-Arg\(^9\), Pro\(^{10}\)-Tyr\(^{11}\) and Try\(^{11}\)-Ile\(^{12}\) bonds in the NT structure [127]. To enhance the potential of NTR1-targeted radiopharmaceuticals, Schubiger, Garcia-Garayoa and others have reported promising strategies to metabolically stabilize the NTR1-targeted agents [42–44]. These modifications consist of replacing selected amino acids at the degradation sites with non-natural counterparts to inhibit proteolytic cleavage. Among these stabilizing modifications, replacement of a secondary amide with a tertiary
amide (N-CH₃) at the Arg⁸ position, replacement of Tyr¹¹ with 2,6-dimethyl tyrosine (Dmt), and replacement of Ile¹² with tert-leucine (Tle), have been shown to substantially inhibit degradation and improve in vivo stability [44,96].

A principal chelator used in the design of radiometal-based theranostic agents is DOTA [44]. The primary reason for the extensive utilization of this macrocycle is its inherent in vivo stability with a variety of clinically relevant radiometals. The incorporation of DOTA on the N-terminus of the pharmacophore has been accomplished with diverse receptor-avid peptides [128]. However, for some peptides, placement of the DOTA chelation system too close to the pharmacophore can sterically interfere with the binding of the agent to the receptor of interest [129]. In this circumstance, inclusion of a linking group to increase the distance between DOTA and the pharmacophore can restore optimal binding [130,131].

In this study, we investigated the extent to which distance between the DOTA chelation system and a NT(6-13) peptide fragment affects binding affinity. The NT(6-13) analog ([((N-α-Me)Arg⁸,Dmt¹¹,Tle¹²]NT(6-13)) chosen for this study includes all three of the stabilizing modifications listed above. Hydrocarbon linking groups of various lengths (0 – 9 atoms) were incorporated between the DOTA chelation system and the N-terminus of the NT pharmacophore. Once in hand, the in vitro binding and cellular uptake properties of the NTR1-targeted conjugates were investigated in the NTR1-positive HT-29 human colon cancer cell line. Based on these results, we report the preliminary biodistribution and SPECT/CT studies of our most promising radioconjugate.
2.3 Methods

2.3.1 Materials

Acetonitrile, formic acid, N,N-diisopropylethylamine (DIEA), N,N-dimethylformamide (DMF), dichloromethane (DCM), N,N’-dicyclohexyl-carbodiimide (DCC) , N-methylpyrrolidone (NMP), 4-(2-hydroxyethyl)-1-piperazineethane-sulfonic acid (HEPES), bovine serum albumin (BSA) and sodium dodecyl sulfate (SDS) were purchased from Fisher Scientific (Fair Lawn, NJ). Fluorenlymethyloxycarbonnyl (Fmoc)-protected natural amino acids, Fmoc-β-Ala-OH, Fmoc-Leu-Wang resin (100-200 mesh), and (1-cyano-2-ethoxy-2-oxoethylidenaminooxy) dimethylamino-morpholino-carbenium hexafluorophosphate (COMU), were purchased from NovaBiochem (Hoherbrunn, Germany). Fmoc-N-Me-Arg(Pbf)-OH was produced by ChemPep, Inc. (Wellington, FL). Fmoc-2,6-dimethyl-L-tyrosine (Dmt) was from Ontario Chemicals, Inc (Guelph, ON, Canada). Fmoc-Tle-OH, Fmoc-8-Aoc-OH, and Fmoc-5-Ava-OH were purchased from CreoSalus (Louisville, KY). Lutetium-177 chloride (177LuCl₃) was obtained from Perkin Elmer (Waltham, MA) with a specific activity of 32.3 Ci/mg. Naturally abundant lutetium chloride (natLuCl₃), triisopropylsilane and 3,6-dioxa-1,8-octanedithiol were from Sigma-Aldrich (St Louis, MO). Neurotensin (NT) was purchased from American Peptide, Inc. (Sunnyvale, CA). McCoy’s 5A medium (1X; Iwakata & Grace Mod.) with L-glutamine was obtained from Mediatech, Inc. (Manassas, VA). TrypLE™ Express was purchased from Invitrogen (Grand Island, NY).
2.3.2 Cell culture

The human colon cancer cell line HT-29 was obtained from American Type Culture Collection (Manassas, VA) and cultured under vendor-recommended conditions. Cells were passaged twice weekly in McCoy’s 5A medium supplemented with 10% FBS, 100 IU/ml penicillin and 100 μg/ml streptomycin at 37 °C in a humidified incubator containing 5% CO2.

2.3.3 Xenograft models

All animal experiments were conducted in accordance with the Principles of Animal Care outlined by the National Institutes of Health and approved by the Institutional Animal Care and Use Committee of the University of Nebraska Medical Center. Eight-week-old Institute of Cancer Research severely combined immunodeficient (ICR SCID) mice were obtained from Charles River Laboratories (Wilmington, MA). Animals were housed five per cage in a light- and temperature-controlled environment. Food and water were given ad libitum. Bilateral HT-29 tumors were induced by subcutaneous injection of $4.0 \times 10^6$ cells in Matrigel (BD Biosciences). The tumors were allowed to grow for 2-3 weeks reaching maximal 1 cm in diameter before the mice were utilized in pharmacokinetic studies.

2.3.4 Solid-phase peptide synthesis (SPPS)

Peptides were synthesized on an automated solid-phase Liberty microwave peptide synthesizer from CEM (Matthews, NC), employing traditional Fmoc chemistry. Briefly, the Fmoc-Leu-Wang resin (100 μmol of the resin-substituted peptide anchors) was deprotected by piperidine, resulting in the formation of a primary amine from which the C-terminus of the growing peptide was anchored. Fmoc-protected amino acids (300 μmol) were activated with COMU and sequentially
conjugated to the resin. The resulting peptide was orthogonally deprotected and cleaved from the resin by shaking in a cocktail consisting of triisopropylsilane (0.125 ml), water (0.125 ml), 3,6-dioxa-1,8-octanedithiol (0.125 ml), and trifluoroacetic acid (4.625 ml) for 3 h. The cleaved peptide was subsequently precipitated and washed thrice using cold (0 °C) methyl-tert-butyl ether (40 ml×3). The crude conjugate was dried by a CentriVap concentrator and weighed.

2.3.5 HPLC purification and analysis methodology

HPLC/MS analyses were performed on a Waters (Milford, MA) e2695 system equipped with a Waters 2489 absorption detector and a Waters Q-Tof Micro electrospray ionization mass spectrometer. Sample purification for in vitro studies was performed on a Phenomenex (Torrance, CA) Jupiter 10 µm Proteo 250 × 4.6 mm C12 column with a flow rate of 1.5 mL/min. For bulk sample purification, a Phenomenex Jupiter 10 µm Proteo 250 × 10 mm C12 column was used with a flow rate of 5.0 mL/min. HPLC solvents consisted of H2O containing 0.1% formic acid (solvent A) and acetonitrile containing 0.1% formic acid (solvent B). For unlabeled and 177/177natLu-conjugates of N0 and N1, an initial gradient of 95% A: 5% B linearly decreased to 85% A: 15% B over a 15-minute time period. For unlabeled and 177/177natLu-conjugates of N2, an initial gradient of 92% A: 8% B linearly decreased to 87% A: 13% B over a 15-minute time period. For unlabeled and 177/177natLu-conjugates of N3, an initial gradient of 90% A: 10% B linearly decreased to 85% A: 15% 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.
2.3.6 Labeling with $^{\text{n}}$atLuCl$_3$

Naturally abundant $^{\text{n}}$atLu was used to substitute for $^{177}$Lu in the ES-MS and in vitro binding studies. A sample of conjugates (0.10 mg, 0.06 μmol) was dissolved in ammonium acetate buffer (0.5 M, 200 μL, pH 5.5) and mixed with a solution of $^{\text{n}}$atLuCl$_3$ (1.7 mg, 6 μmol). The solution was heated for 60 min at 90 °C. After cooling to room temperature, $^{\text{n}}$atLu-conjugates were then peak purified by RP-HPLC in the same conditions above. All $^{\text{n}}$atLu-conjugates were ≥ 95% pure before mass spectrometric characterization and in vitro binding studies were performed.

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

A sample of the conjugates (50 μg, 30 nmol) was dissolved in ammonium acetate buffer (0.5 M, 100 μL, pH 5.5). $^{177}$LuCl$_3$ (37 MBq, 1 mCi, 0.18 nmol) was added to the vial containing the conjugate, and the solution was heated for 60 min at 90 °C. To separate radiolabeled peptides from unlabeled peptides on HPLC, 4-5 mg CoCl$_2$ were then added and incubated for 5 min at 90 °C to increase the hydrophobicity of unlabeled conjugates[132]. After cooling to room temperature, evaluation and purification of radiolabeled conjugate were performed on a Waters 1525 binary pump equipped with a Waters 2489 absorption detector and a Bioscan (Poway, CA) Flow Count radiometric detector system. The collected radioconjugate was concentrated with an Empore (Eagan, MN) C18 high performance extraction disk followed by elution with ethanol/sterile saline solution (6:4, 400 μL) to provide the radiolabeled conjugates in high purity.

2.3.8 Distribution coefficient

The distribution coefficient was determined (n = 6, 2 technical (tech) and 3 biological (bio) repeats) for each $^{177}$Lu-labeled conjugate. In a 1.5 ml centrifuge tube, 0.5 mL 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 (8000 x g, 5 min) to yield two immiscible layers. Aliquots of 100 µL were taken from each layer and the radioactivity of each was quantified by an LTI (Elburn, IL) Multi-Wiper nuclear medicine gamma counter.

### 2.3.9 Receptor saturation

Receptor saturation studies were performed (n = 6, 2 tech and 3 bio repeats) using $^{177}$Lu-N1 on the human colon cell line HT-29. HT-29 cells ($1\times10^6$) were suspended in 1.5 ml low retention centrifuge tubes with 100 µl McCoy’s medium (pH 7.4, 4.8 mg/mL HEPES, and 2 mg/mL BSA). $^{177}$Lu-N1 (200,000 cpm, 100 µl) and a series of 100 µl N1 concentrations ranging from 0.469 nM to 120 nM were added for 45 min at 4 °C. Non-specific binding was determined using 3 µM N1 in the presence of the radioligand. The specific activity was determined as total binding activity minus non-specific binding. At the end of the incubation time, cells were aspirated and washed five times with cold medium and the remaining radioactivity was measured by gamma counter. Non-linear regression analysis was then performed using GraphPad Prism 5 (La Jolla, CA) to determine the $B_{\text{max}}$ and $K_d$ values for each experiment.

### 2.3.10 In vitro competitive binding studies

For in vitro binding studies, the half maximal inhibitory concentration ($IC_{50}$) for each conjugate was determined (n = 6, 2 tech and 3 bio repeats) for each $^{177}$Lu-labeled conjugate using the human colon cancer cell line, HT-29. In these studies, $^{177}$Lu-N1 served as the control and as the radioligand for comparing the relative effectiveness of the conjugates (N0-3 and NT). Briefly, HT-29 cells (~$1\times10^6$) were suspended in 100 µl McCoy’s medium (pH 7.4, 4.8 mg/mL HEPES, and
2 mg/mL BSA) and incubated at 37 °C for 45 min in the presence of radiolabeled 
$^{177}$Lu-N1 (100,000 cpm, 100 µl) and various concentrations of the unlabeled conjugates and nat-Lu-conjugates (100 µl). At the end of the incubation, the cells were centrifuged, aspirated and washed with media five times. The cell-associated radioactivity 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 (La Jolla, CA).

2.3.11  In vitro internalization and efflux studies

Internalization studies were performed (n = 6, 2 tech and 3 bio repeats) for each $^{177}$Lu-labeled conjugate using HT-29 cells in 1.5-ml centrifuge tubes. HT-29 cells ($\sim$1×10$^6$) were suspended in 100 µl McCoy’s media (pH 7.4, 4.8 mg/mL HEPES, and 2 mg/mL BSA). Cells were incubated for various times up to 2 h at 37°C in the presence of 100,000 cpm of each $^{177}$Lu-radioconjugates. During the incubation, at time points 15, 30, 60 and 120 min, cells were washed five times with media to remove the unbound peptide. Surface-bound radioactivity was removed by washing the cells twice with an acidic buffer (50 mM glycine-HCl/0.1 M NaCl buffer, pH 2.8). The amount of radioactivity in each cellular pellet was measured as the internalized fraction by gamma counter.

For efflux studies, HT-29 cells ($\sim$1×10$^6$) were incubated in six-well plates overnight. On the day of the experiment, HT-29 cells were first incubated for 2 h at 37°C in the presence of 100,000 cpm of each of the $^{177}$Lu-radioconjugates to reach the plateau of internalized radioactivity. Upon completion of the incubation, cells were washed five times with medium to remove the unbound peptide. Then, fresh medium was put in each well as the reservoir for efflux. At 0, 1, 2, 4 and 24 h, the medium for each time point was harvested for quantitative analysis of ligand efflux.
Surface-bound radioactivity was removed by washing the cells twice with an acidic buffer (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 to quantify the remaining internalized fractions. The radioactivity in the effluxed, surface-bound and internalized fractions for each radioconjugate was determined using a gamma counter. The effluxed fraction is expressed as a percentage of the total amount, which is the sum of the effluxed, surface-bound and fraction remaining in the cell.

2.3.12 Metabolic stability

The stability of $^{177}$Lu-N1 in human serum and cell medium was determined. Conditioned medium refers to medium in which cells were cultured at least for one day, as medium obtained under these conditions is believed to contain proteases excreted from HT-29 cells. The conditioned medium was prepared by centrifuging at 4000 x g for 2 min to remove cells or cell debris. Briefly, 50 µL of HPLC-purified peptide in PBS (1.85 MBq, 50 µCi, 15 ng) was added to 50 µL human serum (MP Biomedicals, LLC (Solon, OH)) or conditioned medium and incubated at 37 °C for up to 24 h. After the incubation, 100 µL of a mixture of ethanol and acetonitrile (v/v = 1:1) was added to precipitate the serum proteins. The resulting mixture was centrifuged at 12000 x g for 10 min. The supernatant was collected and purged with N$_2$ gas for 20 min to remove the ethanol and acetonitrile. The resulting sample was dissolved in 100 µL water and injected into RP-HPLC for analysis using the gradient described above.

2.3.13 In vivo biodistribution studies

Biodistribution studies were carried out using HT-29 tumor bearing SCID mice. Each mouse (average weight, 20 g) received an intravenous bolus, via the tail vein, of the radio-RP-HPLC purified $^{177}$Lu-N1 (280 kBq, 7.5 µCi, 2.25 ng) in 100 µL
of saline. Four-hour post injection, the mice were sacrificed and the amount of radioactivity in the tumors and other tissues (blood, heart, lung, stomach, pancreas, spleen, liver, kidneys, small intestine and colon) were counted by gamma counter. The excised tissues were weighed and results were expressed as percentage of injected dose per gram of tissue (%ID/g). Biodistribution radiation was measured with a NaI (Tl) well detector from AlphaSpectra, Inc. (Grand Junction, CO). Blocking studies were carried out by co-injection with excess unlabeled N1 (250 μg).

2.3.14 SPECT/CT imaging with $^{177}$Lu-N1

For SPECT/CT imaging studies, HT-29 tumor xenograft mice were injected i.v. with $^{177}$Lu-N1 (37 MBq, 1 mci, 300 ng) in 100 μL of saline and sacrificed for scanning 2 h post-injection. Images were acquired for 1 h using a FLEX Triumph single photon emission computed tomography system/X-ray computed tomography (SPECT/CT) and software (Gamma Medica, Inc., Northridge, CA).

2.3.15 Statistical analysis

Comparisons of each two groups for IC$_{50}$, internalization and efflux studies, were analyzed by the unpaired two-tailed Student’s t test, and $P$ values of less than 0.05 were considered statistically significant.

2.4 Results

2.4.1 Synthesis and radiolabeling

Four NT analogs were synthesized by SPPS using the DOTA-X-[(N-α-Me)Arg$^8$,Dmt$^{11}$,Tle$^{12}$]NT(6-13) paradigm depicted in Figure 2.1, where X represents a series of hydrocarbon linkers of increasing length. Initial attempts at the synthesis of the peptides by SPPS gave unacceptably poor yields. Evaluation of the crude material by LC-MS revealed that a significant portion was
composed of terminated peptide chains and deletion peptides that originated at or after, respectively, the N-methyl arginine. With the premise that the steric of the secondary amine were negatively affecting coupling yields, we made the following modifications to our SPPS protocol: 1) for all coupling reaction, the concentration of the coupling agent, COMU, was increased 4-fold, from 0.125 M to 0.5 M; 2) the time allowed for Fmoc-deprotection of all amino acids was increased 2-fold, from 3 min to 6 min; and 3) for the two amino acids directly following the N-methyl arginine, the coupling time was increased from 5 min to 10 min and the reaction temperature was increased from 75 °C to 95 °C. With these modifications, the isolated yields of conjugates N0-3, after RP-HPLC purification, were substantially improved from less than 1% to greater than 10%. The purified conjugates were subsequently labeled with $^{177/nat}\text{LuCl}_3$ ($t_{1/2} = 6.75$ days for $^{177}\text{LuCl}_3$) by co-incubation in an ammonium acetate buffer (pH = 5.5) for 60 min at 90 °C. The radiochemical yields for $^{177}\text{Lu-N0}$, $^{177}\text{Lu-N1}$, $^{177}\text{Lu-N2}$ and $^{177}\text{Lu-N3}$ were 91%, 94%, 98% and 95%, respectively. RP-HPLC retention time, mass spectrometric identification and yields of the unlabeled conjugates and natLu-conjugates are listed in Table 2.1.
**Figure 2.1.** Structures of the Lu-([(N-α-Me)Arg$^8$,Dmt$^{11}$,Tle$^{12}$]NT(6-13) analogs used in this study (Lu-N0, Lu-N1, Lu-N2 and Lu-N3).
### Table 2.1. Characterization, Yields, and IC$_{50}$ values of NT analogs.

<table>
<thead>
<tr>
<th>Analog</th>
<th>MS Cal.</th>
<th>MS Obs.</th>
<th>Retention (min)</th>
<th>Yields</th>
<th>IC$_{50}$ ± SD (nM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>N0</td>
<td>1469.9</td>
<td>1470.9</td>
<td>13.1</td>
<td>10.6%</td>
<td>52.3 ± 1.5</td>
</tr>
<tr>
<td>N1</td>
<td>1540.9</td>
<td>1541.9</td>
<td>13.9</td>
<td>11.1%</td>
<td>27.6 ± 1.3</td>
</tr>
<tr>
<td>N2</td>
<td>1568.9</td>
<td>1569.9</td>
<td>10.8</td>
<td>10.0%</td>
<td>20.8 ± 1.4</td>
</tr>
<tr>
<td>N3</td>
<td>1611.0</td>
<td>1611.9</td>
<td>11.3</td>
<td>10.2%</td>
<td>21.1 ± 1.7</td>
</tr>
<tr>
<td>natLu-N0</td>
<td>1641.9</td>
<td>1642.8</td>
<td>10.6</td>
<td>78.1%</td>
<td>47.2 ± 1.2</td>
</tr>
<tr>
<td>natLu-N1</td>
<td>1712.9</td>
<td>1713.8</td>
<td>11.7</td>
<td>71.2%</td>
<td>27.2 ± 1.1</td>
</tr>
<tr>
<td>natLu-N2</td>
<td>1740.9</td>
<td>1741.8</td>
<td>10.4</td>
<td>72.2%</td>
<td>24.3 ± 1.1</td>
</tr>
<tr>
<td>natLu-N3</td>
<td>1783.0</td>
<td>1783.8</td>
<td>9.6</td>
<td>86.7%</td>
<td>20.3 ± 1.2</td>
</tr>
<tr>
<td>NT</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td>22.3 ± 1.2</td>
</tr>
</tbody>
</table>
2.4.2 Distribution coefficient

To evaluate the impact of the hydrocarbon linkers on water solubility of the radioconjugates, the octanol-PBS distribution coefficients at pH 7.4 were measured. The log $D_{\text{oct/water}}$ values for $^{177}$Lu-N0, $^{177}$Lu-N1, $^{177}$Lu-N2 and $^{177}$Lu-N3 were $-3.45 \pm 0.05$, $-3.15 \pm 0.05$, $-3.11 \pm 0.05$ and $-2.99 \pm 0.03$, respectively.

2.4.3 Receptor saturation

To establish the surface expression of the NTR1 on the HT-29 cell line, receptor saturation studies were performed using $^{177/\text{nat}}$Lu-N1. $^{177/\text{nat}}$Lu-N1 bound well to the surface of HT-29 cells, and this binding could be blocked with the addition of unlabeled NT, thus implying the specific blockage of NTR1. From these studies, carried out at 4 °C to prevent receptor-mediated endocytosis, the NTR1 expression was found to be $85000 \pm 1000$ binding sites per HT-29 cell with a dissociation constant ($K_d$) of $42 \pm 8$ nM. The receptor saturation curve has been shown in Figure 2.2.
Figure 2.2. Receptor saturation study for N1.
2.4.4 *In vitro* competitive binding studies

The NTR1 binding affinity of the unlabeled conjugates and $^{177}$Lu-conjugates was investigated by competitive binding studies using HT-29 cells. Both unlabeled conjugates and $^{177}$Lu-conjugates demonstrated nanomolar binding affinities. The IC\textsubscript{50} values for unlabeled N1, N2 and N3 were in the same range as full NT peptide. In contrast, the IC\textsubscript{50} value for unlabeled N0 was higher than that of NT ($P < 0.01$), indicating lower binding affinity to the NTR1. A similar trend was observed for the $^{177}$Lu-labeled conjugates. $^{177}$Lu-N0 had the lowest affinity for the NTR1 relative to $^{177}$Lu-N1, $^{177}$Lu-N2 and $^{177}$Lu-N3 ($P < 0.01$) (Table 2.1).

2.4.5 *In vitro* internalization and efflux studies

The effect of the linker modifications on the rates of internalization (Figure 2.3) and efflux (Figure 2.4) of the radioconjugates was investigated in HT-29 cells. The cellular uptake of the radioconjugates, represented as a percentage of total activity added, was evaluated at 15, 30, 60, and 120 min time points. The cellular uptake consisted of internalized and surface-bound radioactivity. For $^{177}$Lu-N0, $^{177}$Lu-N2 and $^{177}$Lu-N3, the internalization of these radioconjugates was essentially identical over the initial 60 min time period. However, by 120 min post-incubation, a statistically significant increase was observed with overall uptake of $^{177}$Lu-N0 > $^{177}$Lu-N2 > $^{177}$Lu-N3 (3.69\% ± 0.17\%, 3.14\% ± 0.10\%, 2.74\% ± 0.02\%, respectively, $P < 0.0001$). After the 15 min time point, $^{177}$Lu-N1 demonstrated substantially higher levels of uptake (2.66\% ± 0.02\%) relative to the other radioconjugates. By the 120 min time point, $^{177}$Lu-N1 had the highest level of overall uptake with 4.38\% ± 0.10\% of total activity added.
Efflux studies were conducted to determine if a structure-activity relationship exists with regard to the externalization of the internalized radioconjugates. The efflux of these radioconjugates was investigated at the 1, 2, 4 and 24 h time points. At 1 h post-incubation, $^{177}$Lu-N2 demonstrated higher levels of effluxed radioactivity (26% ± 0.8%) relative to the other radioconjugates (18-21%). However, the percentage of externalized radioactivity was largely indistinguishable for all of the radioconjugates investigated by 2 h. At the 4 h time point, the effluxed fraction for $^{177}$Lu-N1 (29% ± 0.4%) was lower than that for $^{177}$Lu-N0, $^{177}$Lu-N2, $^{177}$Lu-N3 (33% ± 2.5%, 34% ± 0.9% and 36% ± 3.6%, respectively, $P < 0.01$). Overall, the percent of initial internalized radioactivity effluxed from the cells ranged from 25-32 % by the 24 h time point.

### 2.4.6 Metabolic stability

NT is known to be susceptible to serum proteases [127]. The targeting vector used in our radioconjugates contains modifications to inhibit metabolic cleavage [44]. To establish if the structural modifications, the DOTA, or the N-terminal linker altered metabolic stability, the stability of $^{177}$Lu-N1 was investigated over a 24 h period in PBS (not shown), the conditioned (used) medium, and human serum. Under the conditions of the study, > 95% of the radioactivity was recovered for analysis from each sample. Evaluation of these samples by radio-RP-HPLC revealed no degradation over a 24-h time period (Figure 2.5).
Figure 2.3. Time course following the uptake of $^{177}\text{Lu}$-radioconjugates, both internalization and surface bound, by HT-29 cells. Values are means ± SD (n = 6).
Figure 2.4. Time course following the percent efflux of the internalized $^{177}$Lu-radioconjugate by HT-29 cells. Values are means ± SD ($n = 6$).
Figure 2.5. Stability of $^{177}$Lu-N1 in conditioned medium and human serum. Panel A: chromatograph of $^{177}$Lu-N1 at start of the study, Panel B: chromatograph of $^{177}$Lu-N1 after 24 h in conditioned medium, and Panel C: chromatograph of $^{177}$Lu-N1 after 24 h in human serum. (x-axis: running time for sample in mins; y-axis: the intensity of radioactivity)
2.4.7 In vivo biodistribution and imaging studies

Based on the in vitro studies, $^{177}$Lu-N1 was deemed our best overall candidate as a balance of the affinity, internalization and efflux. Therefore, $^{177}$Lu-N1 was chosen for our preliminary in vivo investigation. Using the HT-29 xenograft mouse model, we evaluated the in vivo biodistribution of $^{177}$Lu-N1 at the 4 h time point, data given in Figure 2.6. The i.v. administered $^{177}$Lu-N1 effectively cleared from the blood ($0.02 \pm 0.01$ % ID/g) by 4 h. As expected given the hydrophilic nature of $^{177}$Lu-N1, the clearance of radioactivity preceded largely through the renal system with $86.3 \pm 6.0$ %ID being excreted in the urine. As a result of the high renal clearance, the kidneys exhibited significant retention ($17.0 \pm 2.2$ %ID/g) at the 4 h time point. In addition, both the small and large intestines, known to be NTR1-positive tissues, exhibited retention of $1.4 \pm 0.3$ and $0.6 \pm 0.1$ %ID/g. This retention was largely found to be NTR1-receptor mediated as it could be effectively blocked by co-injection of 250 µg of unlabeled N1. For all other non-target tissues, $^{177}$Lu-N1 demonstrated good overall clearance. At the 4 h time point, $^{177}$Lu-N1 displayed good in vivo tumor targeting and retention, with a total accumulation of $3.1 \pm 0.4$ %ID/g. Accumulation in the tumor was shown to be NTR1-receptor specific with $86.6 \pm 4.5$ % reduction in uptake upon blocking. The ratio of tumor to blood was $123 \pm 10$, tumor to kidney was $0.2 \pm 0.02$, tumor to liver was $9.3 \pm 1.6$, and tumor to muscle was $111 \pm 10$ (Table 2.2).
Figure 2.6. Graph of the biodistribution data at 4 h p.i. for $^{177}$Lu-N1 in SCID mice with HT-29 xenografts. The radioactivity accumulation is expressed as a percentage of injected dose per gram (%ID/g). Values are means ± SD ($n = 5$).
Table 2.2. The Ratios of Tumor to Organs in Biodistribution Studies.

<table>
<thead>
<tr>
<th>Tumor/organs</th>
<th>$^{177}$Lu-N1</th>
<th>blocking</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tumor/blood</td>
<td>123.07 ± 9.81</td>
<td>3.91 ± 1.14</td>
</tr>
<tr>
<td>Tumor/kidney</td>
<td>0.17 ± 0.02</td>
<td>0.02 ± 0.00</td>
</tr>
<tr>
<td>Tumor/liver</td>
<td>9.33 ± 1.57</td>
<td>0.82 ± 0.16</td>
</tr>
<tr>
<td>Tumor/muscle</td>
<td>110.97 ± 10.34</td>
<td>2.03 ± 0.70</td>
</tr>
</tbody>
</table>
Using microSPECT/CT, we evaluated the imaging potential of $^{177}\text{Lu}$-N1 at 2 h post-administration (Figure 2.7). Overall, the images correlated well with the biodistribution studies carried out at the 4 h time point. With the exception of the kidneys, little signal was observed in non-target tissues. The uptake of $^{177}\text{Lu}$-N1 in the HT-29 tumors was clearly visible in the flanks of the mice. As expected, the kidneys, the major pathway for biological clearance, were also readily observed.

### 2.5 Discussion

To date, NTR1-targeted peptides have been labeled with a number of clinically relevant radiometals, such as $^{99m}\text{Tc}$, $^{111}\text{In}$ and $^{68}\text{Ga}$ [44,96,103,133,134]. In recent years, $^{177}\text{Lu}$, a theranostic radioisotope, has received increasing attention for potential clinical applications due to its dual emission characteristics [48,135–137]. Upon decay, $^{177}\text{Lu}$ emits therapeutic beta particles ($E_{\text{avg}} = 133$ keV) and gamma rays (113 and 210 keV, 6% and 10%) that are suitable for SPECT diagnostic imaging. Using $^{177}\text{Lu}$ allows the potential for one radiopharmaceutical to be used as both a diagnostic and radiotherapeutic, thus negating the need for a "matched-pair" system (e.g., $^{111}\text{In}$ and $^{90}\text{Y}$) for imaging and therapy. In general, the goal of any chelation system in a radiopharmaceutical design is to retain the radionuclide during its in vivo lifetime. The most common polyaminocarboxylate chelator utilized in NTR1-targeted agent design is diethylenetriamine pentaacetic acid (DTPA) [31,44,84,138]. Unfortunately, DTPA forms a complex with $^{177}\text{Lu}$ that is known to be unstable in vivo [139]. Conversely, DOTA, due to its cyclic nature and steric inhibition, is known to form in vivo stable $^{177}\text{Lu}$ complexes, as demonstrated by numerous preclinical and clinical studies [135,137,139]. At the same time, DOTA, when placed too closely to the pharmacophore, has been shown to sterically inhibit the efficacy of receptor-targeted peptides [37,129]. This has given us the impetus to investigate the impact of the
Figure 2.7. SPECT/CT image of $^{177}$Lu-N1 in a HT-29 xenograft mouse model at 2 h post-administration. Tu: tumor. Ki: kidney.
\[^{177}\text{Lu-DOTA \ chelation-complex on the performance of a NTR1-targeted pharmacophore (i.e., [(N-\alpha-Me)Arg}^8,\text{Dmt}^{11},\text{Tle}^{12}]\text{NT(6-13))}.\] To that end, we synthesized and evaluated a series of these NTR1-targeted conjugates, structurally depicted in Figure 2.1, with hydrocarbon linkers of 0, 4 (\(\beta\)-Ala), 6 (5-Ava) and 9 (8-Aoc) atoms in length.

Under our standard SPPS conditions, initial attempts to synthesize the conjugates, N0-3, in good yield was unsuccessful. Analysis of the crude material revealed that the majority of the peptides formed were termination or deletion products that stem from the incorporation of the N-methyl arginine at position 8. This is not too surprising given that it is recognized that the formation of N-methylated amides can be more difficult due to increased steric interference [140]. Adjustments to more vigorous conditions (i.e., increased temperatures and longer reaction times) gave substantially better yields of the desired peptides.

Receptor saturation studies of NTR1-positive HT-29 human colon cancer cells revealed a receptor density of approximately 85,000 ± 1000 per cell and a \(K_d\) value of 42 ± 8 nM. Relative to literature, our determined \(K_d\) is higher than values reported from other groups (1.5-15 nM), but this moderate difference in values is likely attributable to different experimental conditions (e.g., different ligands and temperature) [43,141,142]. Of particular note, our studies were performed at 4 °C to inhibit internalization; while, literature reports were carried out at 24 or 37 °C. At these elevated temperatures, the internalized activity would likely lead to an overestimate (lower value) in the disassociation constant.

Based on the competitive binding assays, \(^{177}\text{Lu-N0},\) as well as the unmetallated peptide (N0), exhibited binding affinities that were roughly two fold lower than the
corresponding analogs with hydrocarbon linkers. While this decrease in binding affinity is relatively modest, it does show that the steric bulk of the DOTA affects the NTR1 binding efficacy of the [(N-α-Me)Arg\textsuperscript{8},Dmt\textsuperscript{11},Tle\textsuperscript{12}]NT(6-13) pharmacophore. Inclusion of a 4 atom hydrocarbon linker (i.e., β-Ala) appears to alleviate the steric inhibition of the chelator. Extension of the hydrocarbon linker beyond 4 atoms, as is the case for N2, N3, nat Lu-N2 and nat Lu-N3, does not lead to substantial increases in binding affinities. Interestingly, linker length does seem to play a more important role in the internalization of the NTR1-targeted agents. For \textsuperscript{177}Lu-N1, the β-Ala linker gives a substantially higher percentage of internalization relative to the other analogs. This is followed by \textsuperscript{177}Lu-N0, \textsuperscript{177}Lu-N2 and \textsuperscript{177}Lu-N3. It is interesting that \textsuperscript{177}Lu-N0 out-performed \textsuperscript{177}Lu-N2 and \textsuperscript{177}Lu-N3 in spite of having a lower binding affinity. It appears that even with high binding affinities, radioconjugates with longer hydrocarbon linkers, \textsuperscript{177}Lu-N2 and \textsuperscript{177}Lu-N3, did not induce receptor-mediated internalization as well as those with short or no hydrocarbon linkers, \textsuperscript{177}Lu-N1 and \textsuperscript{177}Lu-N0, respectfully. Why the longer linker length would affect the ability of the pharmacophore to activate the NTR1 is not clear. Relative to internalization, linker length had little effect on efflux patterns of the radioconjugates. One noteworthy observation was the small decrease in percentage efflux from 4 to 24 h time points. This suggests that externalized radioactivity (decay normalized) is being recycled back into the cell. It seems unlikely that this is through a receptor-mediated process, but may be attributable to pinocytosis of effluxed radioactivity over time.

As previously mentioned, the [(N-α-Me)Arg\textsuperscript{8},Dmt\textsuperscript{11},Tle\textsuperscript{12}]NT(6-13) pharmacophore contains several modifications that significantly reduce enzymatic degradation of the pharmacophore \textit{in vitro} and \textit{in vivo} [44]. Utilizing \textsuperscript{177}Lu-N1, our best performing conjugate, the stability of this NTR1-targeted agent was evaluated in PBS,
conditioned medium and human serum. No discernible degradation was observed from these conditions over a 24 h period. This is expected based on the reported *in vitro* stability data of analogs with similar modifications [44,84]. Thus, as anticipated, the DOTA and the hydrocarbon linkers did not significantly alter *in vitro* stability of the pharmacophore at least over the monitored time frame. While no *in vitro* degradation was found for the [(N-α-Me)Arg^8,Dmt^{11},Tle^{12}]NT(6-13) peptide, Gruaz-Guyon and co-workers have demonstrated that this pharmacophore does exhibit significant *in vivo* metabolism in as little as 15 min post-injection [44]. This is an important consideration, as others have pointed out, when evaluating *in vivo* data [85]. Although the stability studies were not carried out with ^177^Lu-N0, ^177^Lu-N2 and ^177^Lu-N3, we assume similar stabilities given the relative similarity of the radioconjugates.

^177^Lu-N1 was chosen for preliminary *in vivo* investigations due to its superior *in vitro* properties. At the 4 h time point, very little of the administered radioactivity remained in the blood pool. The majority of the radioactivity (85% of the ID) was cleared by the renal system which contributed to the substantial level of accumulation in the kidneys (17.0 ± 2.2 %ID/g). Significant kidney accumulation was also observed in the SPECT/CT imaging studies. Curiously, an analog (LB119) reported with the same [(N-α-Me)Arg^8,Dmt^{11},Tle^{12}]NT(6-13) pharmacophore demonstrated substantially less renal accumulation (2.4 ± 0.2 %ID/g at 3 h p.i.) [44]. The distinct differences between the two analogs may be explained by the charge of each radioconjugate. On the N-terminal portion of ^177^Lu-N1, the peptide contains three positively charged amino acid residues (i.e. lysine and two arginines) yielding a total +2 charge for the radioconjugate. Comparatively, the N-terminus of LB119 has the same three amino acids, but the charge of the lysine amino acid has been neutralized, by conjugation, to yield a total +1 charge for the agent. For radiolabeled peptides, the
effect of charge and charge distribution on kidney accumulation has been well established [106,110,117]. In general, radiolabeled peptides with increasing positive charges tend to exhibit higher levels of kidney accumulation. This has been attributed, at least in part, to non-specific uptake by the surface proteins megalin and cubilin [111]. The non-specific nature of $^{177}$Lu-N1 uptake in the kidney was confirmed by observation that blocking studies did not reduce accumulation.

$^{177}$Lu-N1 demonstrated excellent tumor uptake and retention with $3.1 \pm 0.4 \% \text{ID/g}$ at the 4 h time point. In comparison to LB119, $^{177}$Lu-N1 demonstrated a 2 fold higher HT-29 tumor retention at 4 h than LB119 ($1.41 \pm 0.05 \% \text{ID/g}$) at 3 h post-administration. Given the structural similarities of the pharmacophores, the significant ($P < 0.0001$) increase in tumor uptake and retention of $^{177}$Lu-N1 may be attributable to the charge of the radioconjugate as observed with the kidneys. Alternatively, this dissimilarity may be due to the purity of the prepared radioconjugates. $^{177}$Lu-N1 was obtained as a peak-purified product from HPLC whereas LB119 was prepared without HPLC purification. The lack of purification of LB119 likely resulted in a small amount of unlabeled conjugate being co-injected with the radioconjugate, which may negatively impact NTR1 uptake. To what extent these factors play into the higher retention of the $^{177}$Lu-N1 is not clear at this time. Small animal SPECT/CT imaging studies demonstrated significant tumor accumulation at 2 h post-administration and overall agrees well with the biodistribution data obtained at the 4 h time point.
2.6 Conclusion

We investigated the steric impact of the DOTA chelator and the length of hydrocarbon spacers on the biological performance of the \([(N-\alpha-\text{Me})\text{Arg}^8,\text{Dmt}^{11}, \text{Tle}^{12}]\text{NT(6-13)}\) pharmacophore. In conclusion, we found that the DOTA does negatively impact the binding affinity of the studied NTR1-targeted peptide. Optimal binding affinity could be restored by incorporation of a 4-atom hydrocarbon linker. Our best candidate \(^{177}\text{Lu-N1}\) demonstrated substantial tumor accumulation, but the increase in renal retention relative to other literature analogs is likely due to the higher overall positive charge of the agent.
Chapter 3:

Investigation of the Biological Impact of Charge Distribution on a NTR1-Targeted Peptide
3.1 Abstract

The neurotensin receptor 1 (NTR1) has been shown to be a promising target, due to its increased level of expression relative to normal tissue, for pancreatic and colon cancers. This has prompted the development of a variety of NTR1-targeted radiopharmaceuticals, based on the neurotensin (NT) peptide, for diagnostic and radiotherapeutic applications. A major obstacle for the clinical translation of NTR1-targeted radiotherapeutics would likely be nephrotoxicity due to the high levels of kidney retention. It is well-known that for many peptide-based agents, renal uptake is influenced by the overall molecular charge. Herein, we investigated the effect of charge distribution on receptor binding and kidney retention. Using the [(N-α-Me)Arg\(^8\),Dmt\(^{11}\),Tle\(^{12}\)]NT(6–13) targeting vector, three peptides (\(^{177}\)Lu–K2, \(^{177}\)Lu–K4, and \(^{177}\)Lu–K6), with the Lys moved closer (K6) or further away (K2) from the pharmacophore, were synthesized. In vitro competitive binding, internalization and efflux, and confocal microscopy studies were conducted using the NTR1-positive HT-29, human colon cancer cell line. The \(^{177/\text{nat}}\)Lu–K6 demonstrated the highest binding affinity (21.8 ± 1.2 nM) and the highest level of internalization (4.06% ± 0.20% of the total added amount). In vivo biodistribution, autoradiography, and metabolic studies of \(^{177}\)Lu-radiolabeled K2, K4, and K6 were examined using CF-1 mice. \(^{177}\)Lu–K4 and \(^{177}\)Lu–K6 gave the highest levels of in vivo uptake in NTR1-positive tissues, whereas \(^{177}\)Lu–K2 yielded nearly 2-fold higher renal uptake relative to the other radioconjugates. In conclusion, the position of the Lys (positively charged amino acid) influences the receptor binding, internalization, in vivo NTR1-targeting efficacy, and kidney retention profile of the radioconjugates. In
addition, we have found that hydrophobicity likely play a role in the unique biodistribution profiles of these agents.

3.2 Introduction

The neurotensin receptor 1 (NTR1) is a G-protein coupled receptor that has been linked to proliferative action in a number of cancers, including colon and pancreatic cancer [49,125]. In addition, or as a consequence of this proliferative pathway, NTR1 has also been shown to be overexpressed on the cellular surface of these cancers. These findings have resulted in significant interest in developing NTR1-targeted radiopharmaceuticals for diagnostic and therapeutic purposes [41,43–45,92,96,103,133,143]. To date, NTR1-targeted agents have been largely based on neurotensin (NT), a thirteen amino acid peptide that has demonstrated nanomolar binding affinity to the receptor. Researchers have principally focused on targeting vectors based on the last six (NT(8-13)) or eight (NT(6-13)) amino acids of the NT sequence. Of the two targeting vectors, the NT(6-13) peptide demonstrates a marginally higher binding affinity to the NTR1 [144]. One major obstacle that hampered the development of NTR1-targeted radiopharmaceuticals early on was the rapid proteolytic degradation of the NT in the serum [32,145]. This situation is aggravated by the inability of in vitro serum studies to adequately predict in vivo stability which led to significant underperformance of the NTR1-targeted agents in preclinical and clinical studies [41,85]. The in vivo instability of these agents has led to the development of more stable, synthetic derivatives of the NT pharmacophore, which demonstrate significantly better in vivo performance [43,84,92,143].

The development of receptor-targeted, radiotherapeutic agents based on small peptide targeting vectors has been an active area of research over the last three decade
However, the translation of these radiotherapeutic peptides, including NTR1-targeted agents, into the clinic has been, in many cases, hampered by high renal uptake which can result in dose-limiting toxicities [106,146]. A variety of reports have investigated both the mechanism and structure-activity relationships corresponding to the renal uptake and retention of radiolabeled peptides [106,110,116]. From these studies, it has been recognized that the molecular charge is a significant factor in the renal uptake of radiolabeled peptides, including NTR1-targeted agents which typically contain two to three positively-charged amino acids (Arg and Lys) [110,115]. This high renal uptake has been a major hurdle for the potential development and translation of radiotherapeutic, NTR1-targeted agents.

Our laboratory has recently begun exploring the development of NTR1-targeted agents using a stabilized [(N-α-Me)Arg⁸,Dmt¹¹,Tle¹²]NT(6-13) targeting vector (Lys-Pro-(N-α-Me)Arg-Arg-Pro-Dmt-Tle-Leu) [45]. Although this pharmacophore exhibits excellent NTR1-positive tumor uptake, it has a total charge of +2 and exhibits significant renal uptake, reducing its potential for radiotherapeutic applications. While the molecular charge/renal uptake relationship for NTR1-agents has been established, we are interested in determining if charge distribution impacts the receptor avidity and renal uptake and retention of these agents. Herein, we investigate the biological impact of the translation of the Lys⁶ relative to the rest of the pharmacophore. Using a series of NTR1-targeted agents, Figure 3.1, we explore the structure-activity relationship of these agents using in vitro and in vivo models of the HT-29 human colon adenocarcinoma cell line.
3.3 Methods

3.3.1 Materials

Acetonitrile, formic acid, \( N,N\)-diisopropylethylamine (DIEA), \( N,N\)-dimethylformamide (DMF), dichloromethane (DCM), \( N,N'\)-dicyclohexylcarbodiimide (DCC), methyl-t-butyl ether, \( N\)-methylpyrrolidone (NMP), bovine serum albumin (BSA), sodium dodecyl sulfate (SDS), LysoTracker\textsuperscript{TM} Blue DND-22, CellLight\textsuperscript{®} early endosome-green fluorescent protein (GFP) and CellLight\textsuperscript{®} late endosome-red fluorescent protein (RFP) were purchased from Fisher Scientific (Fair Lawn, NJ). Fluorenylmethyloxycarbonyl (Fmoc)-protected natural amino acids, Fmoc-(D)leu-OH, Fmoc-Leu-Wang resin (100-200 mesh), and (1-cyano-2-ethoxy-2-oxoethylidenaminooxoxy) dimethylamino-morpholino-carbenium hexafluorophosphate (COMU) were purchased from NovaBiochem (Hoherbrunn, Germany). Fmoc-N-Me-Arg(Pbf)-OH was produced by ChemPep, Inc. (Wellington, FL). Fmoc-2,6-dimethyl-L-tyrosine (Dmt) was from Ontario Chemicals, Inc (Guelph, ON, Canada). Fmoc-Tle-OH was purchased from CreoSalus (Louisville, KY). Lutetium-177 chloride (\(^{177}\text{LuCl}_3\)) was obtained from Perkin Elmer (Waltham, MA) with a specific activity of 32.3 Ci/mg. Naturally abundant lutetium chloride (\(^{nat}\text{LuCl}_3\)), triisopropylsilane and 3,6-dioxa-1,8-octanedithiol were from Sigma-Aldrich (St Louis, MO). McCoy’s 5A medium (1X; Iwakata & Grace Mod.) with L-glutamine was obtained from Mediatech, Inc. (Manassas, VA). TrypLE\textsuperscript{TM} Express was purchased from Invitrogen (Grand Island, NY). Penicillin-Streptomycin solution and 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) were from HyClone Laboratories, Inc (Logan, UT). Heparin was purchased from Elkins-Sinn, Inc (Cherry Hill, NJ). Fetal Bovine Serum (FBS) was purchased from Gibco\textsuperscript{TM} by Life Technologies Corporation (Grand Island, NY). BD Cytofix\textsuperscript{TM} Fixation buffer was
purchased from BD Biosciences (San Jose, CA). The O.C.T compound for tissue embedding was from Sakura Finetek USA, Inc. (Torrance, CA).

3.3.2 Cell culture

The human colon cancer cell line HT-29 was obtained from American Type Culture Collection (Manassas, VA) and cultured under vendor-recommended conditions. Cells were passaged twice weekly in McCoy’s 5A medium supplemented with 10% FBS, 100 IU/ml penicillin and 100 μg/ml streptomycin at 37 °C in a humidified incubator containing 5% CO₂.

3.3.3 Mouse model

All animal experiments were conducted in accordance with the Principles of Animal Care outlined by the National Institutes of Health and approved by the Institutional Animal Care and Use Committee of the University of Nebraska Medical Center. Eight-week-old CF-1 mice were obtained from Charles River Laboratories (Wilmington, MA). Animals were housed five per cage in a light- and temperature-controlled environment. Food and water were given ad libitum.

3.3.4 Solid-phase peptide synthesis (SPPS)

Peptides were synthesized on an automated solid-phase Liberty microwave peptide synthesizer from CEM (Matthews, NC), employing traditional Fmoc chemistry. Briefly, the Fmoc-Leu-Wang resin (100 μmol of the resin-substituted peptide anchors) was deprotected by piperidine, resulting in the formation of a primary amine from which the C-terminus of the growing peptide was anchored. Fmoc-protected amino acids (300 μmol) were activated with COMU and sequentially conjugated to the resin. The resulting peptide was orthogonally deprotected and cleaved from the resin by shaking in a cocktail consisting of triisopropylsilane (0.125 ml), water (0.125 ml),
3,6-dioxa-1,8-octanediol (0.125 ml), and trifluoroacetic acid (4.625 ml) for 3 h. The cleaved peptide was subsequently precipitated and washed thrice using cold (0 °C) methyl-tert-butyl ether (40 ml×3). The crude peptides were dried under vacuum.

### 3.3.5 Synthesis of Cyanine5-K6 as fluorescent dye

Cyanine5-K6 was synthesized by conjugation of the Cyanine5 (Cy5) carboxylic acid to the NH₂-K6-Wang resin. Briefly, a mixture of Cy5-COOH (6 µmol), NH₂-K6-Wang-resin (6 µmol), COMU (30 µmol) and DIEA (30 µmol) was dissolved in DMF and shaken overnight at room temperature. After the reaction, the Cy5-K6 was cleaved from the Wang resin and purified by HPLC.

### 3.3.6 HPLC purification and analysis methodology

HPLC/MS analyses were performed on a Waters (Milford, MA) e2695 system equipped with a Waters 2489 absorption detector and a Waters Q-Tof Micro electrospray ionization mass spectrometer. Sample purification for *in vitro* studies was performed on a Phenomenex (Torrance, CA) Jupiter 10 µm Proteo 250 × 4.6 mm C12 column with a flow rate of 1.5 mL/min. For bulk sample purification, a Phenomenex Jupiter 10 µm Proteo 250 × 10 mm C12 column was used with a flow rate of 5.0 mL/min. HPLC solvents consisted of H₂O containing 0.1% formic acid (solvent A) and acetonitrile containing 0.1% formic acid (solvent B). For unlabeled and \(^{177/171}\text{Lu}\)-conjugates of K2, K4 and K6, an initial gradient of 85% A: 15% 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.
3.3.7 Labeling with $^{\text{n}}\text{LuCl}_3$

Naturally abundant Lutetium ($^{\text{n}}\text{Lu}$) was substituted for $^{177}\text{Lu}$ in the ES-MS and $\text{in vitro}$ binding studies. The conjugate (0.10 mg, 0.06 μmol) was dissolved in ammonium acetate buffer (0.5 M, 200 μL, pH 5.5) and mixed with a solution of $^{\text{n}}\text{LuCl}_3$ (1.7 mg, 6 μmol). The solution was heated for 45 min at 90 °C. After cooling to room temperature, $^{\text{n}}\text{Lu}$-conjugates were then peak purified by RP-HPLC. All $^{\text{n}}\text{Lu}$-conjugates were ≥ 95% pure before mass spectrometric characterization and $\text{in vitro}$ binding studies were performed.

3.3.8 Radiolabeling with $^{177}\text{LuCl}_3$

The conjugate (50 μg, 30 nmol) was dissolved in ammonium acetate buffer (0.5 M, 100 μL, pH 5.5). $^{177}\text{LuCl}_3$ (37 MBq, 1 mCi, 0.18 nmol) was added to the vial containing the conjugate, and the solution was heated for 45 min at 90 °C. To separate radiolabeled peptides from unlabeled peptides on the HPLC, 4-5 mg of CoCl$_2$ was added and incubated for 5 min at 90 °C to increase the hydrophobicities of the unlabeled conjugates [132]. After cooling to room temperature, evaluation and purification of the radiolabeled conjugates were performed on a Waters 1525 binary pump equipped with a Waters 2489 absorption detector and a Bioscan (Poway, CA) Flow Count radiometric detector system. The collected radioconjugate was concentrated with an Empore (Eagan, MN) C18 high performance extraction disk followed by elution with ethanol/sterile saline solution (6:4, 400 μL) to provide the radiolabeled conjugates in high purity. When required, a BSA-saline solution was added to the radioconjugate to give a solution containing 0.5% BSA.

3.3.9 Distribution coefficient

The distribution coefficient was determined (n = 6, 2 technical (tech) and 3 biological (bio) repeats) for each $^{177}\text{Lu}$-labeled radioconjugate. In a 1.5 ml centrifuge
tube, 0.5 mL of 1-octanol was added to 0.5 mL of phosphate-buffered saline (pH 7.4) containing the radiolabeled peptide (500,000 cpm). The solution was vigorously stirred for 2 min at room temperature and subsequently centrifuged (8000 x g, 5 min) to yield two immiscible layers. Aliquots of 100 µL were taken from each layer and the radioactivity of each was quantified by an LTI (Elburn, IL) Multi-Wiper nuclear medicine gamma counter.

3.3.10 In vitro competitive binding studies

As described previously [45,132], the half maximal inhibitory concentration (IC_{50}) for each conjugate was determined (n = 6, 2 tech and 3 bio repeats) for each $^{177}$Lu-labeled radioconjugate using the HT-29 human colon cancer cell line. In these studies, $^{177}$Lu-N1 ($^{177}$Lu-DOTA-β-Ala-[(N-α-Me)Arg^8,Dmt^11,Tle^12]NT(6-13)) served as the competitive radioligand for comparing the relative effectiveness of the unlabeled and labeled conjugates. HT-29 cells (~1×10^6) were suspended in 100 µL of McCoy’s 5A medium (pH 7.4, 4.8 mg/mL HEPES, and 2 mg/mL BSA) and incubated at 37 °C for 45 min in the presence of $^{177}$Lu-N1 (100,000 cpm, 100 µL) and various concentrations of the unlabeled conjugates and $^{nat}$Lu-conjugates (100 µL). At the end of the incubation, the cells were centrifuged, aspirated and washed with media five times. The cell-associated radioactivity 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 (La Jolla, CA).

3.3.11 In vitro internalization and efflux studies

The in vitro internalization and efflux studies were performed as stated previously [45,132]. HT-29 cells (~1×10^6) were suspended in 100 µL of McCoy’s 5A medium (pH 7.4, 4.8 mg/mL HEPES, and 2 mg/mL BSA). Cells were incubated at 37°C with each $^{177}$Lu-radioconjugate (100,000 cpm) for up to 2 h. At 15, 30, 60 and
120 min time points, cells were washed five times with media to remove the unbound peptide. Surface-bound radioactivity was removed by washing the cells twice with an acidic buffer (50 mM glycine-HCl/0.1 M NaCl buffer, pH 2.8). The amount of radioactivity remaining in each cellular pellet was assigned as the internalized fraction. The cellular uptake of the radioconjugates was presented as a percentage of total activity added with groups representing internalized and surface-bound radioactivity.

For efflux studies, HT-29 cells (~1×10^6) were incubated in six-well plates overnight. On the day of the experiment, HT-29 cells were incubated for 2 h at 37°C in the presence of 100,000 cpm of each ¹⁷⁷Lu-radioconjugate. Cells were washed five times with medium (1 ml) to remove the unbound peptide followed by the addition of fresh medium. At 0, 0.5, 1, 2, 4 and 24 h, the medium for each time point was harvested for quantitative analysis of ligand efflux. Surface-bound radioactivity was removed by washing the cells twice with an acidic buffer (50 mM glycine-HCl/0.1 M NaCl buffer, pH 2.8). The cells were then lysed using a 10 % aqueous SDS solution to quantify the remaining internalized fractions. The radioactivity in the effluxed, surface-bound and internalized fractions for each radioconjugate was determined using a gamma counter. The effluxed fraction is expressed as a percentage of the total radioactivity in the well, which is the sum of the effluxed, surface-bound and fraction remaining in the cell.

### 3.3.12 Confocal microscopy images of the Cy5-K6

HT-29 cells were cultured on a Lab-Tek chambered #1.0 borosilicate coverglass disk (4 well) overnight at a concentration of 0.05 million cells per well. The CellLight™ early endosome-GFP and CellLight™ late endosome-RFP reagents were added to each well and incubated overnight in a humidified atmosphere with 5% CO₂ at 37 °C. After incubation, HT-29 cells were washed with fresh medium and incubated
with LysoTracker Blue DND-22 for 2 h at a concentration of 1 nM. After again washing the cells, the Cy5-K6 was diluted in McCoy’s 5A medium and incubated with HT-29 cells. At 0.5, 2 and 24 h, the unbound Cy5-K6 was washed off. The formaldehyde fixation buffer was added to the cells and incubated for 10 min. The cells were washed with PBS and fluorescent images were obtained using an excitation wavelength of 373 nm (blue), 488 nm (green), 555 nm (red) and 650 nm (Cy5). Confocal microscopy images were taken on a Leica LSM 510 META Microscope equipped with an argon laser.

### 3.3.13 In vivo biodistribution studies

Biodistribution studies were carried out using healthy CF-1 mice. Each mouse (average weight, 25 g) received an intravenous bolus, via the tail vein, of $^{177}$Lu-radiolabeled K2, K4 or K6 (370 kBq, 10 µCi) in 100 µL of saline. At 1, 4 and 24 h post injection, the mice were sacrificed and the amounts of radioactivity in the tissues were counted with a NaI (Tl) well detector from AlphaSpectra, Inc. (Grand Junction, CO). The excised tissues were weighed and results were expressed as percentage of injected dose per gram of tissue (%ID/g). Blocking studies were carried out by co-injection with excess unlabeled K6 (250 µg).

### 3.3.14 Metabolic studies in mice

For metabolic evaluation assays, $^{177}$Lu-K2, $^{177}$Lu-K4 or $^{177}$Lu-K6 (37 MBq, 1mCi) was injected in the tail vein of CF-1 mice. After 10 min, blood was drawn from the heart of the animals under anesthesia and collected with prechilled polypropylene tubes, containing heparin, and placed on ice. Blood samples were centrifuged at 2000 g/ 4°C for 10 mins, plasma was collected, mixed with chilled acetonitrile (1:1 v/v ratio), and centrifuged at 15,000 g/4°C for 10 min. Supernatants were concentrated to a smaller volume under a gentle N$_2$ gas, diluted with saline, and filtered through a Millex
GV filter (0.22 μm). After 1 h, urine was collected, filtered with a Millex GV filter (0.22 μm). All samples were subsequently analyzed by RP-HPLC using the conditions previously stated.

### 3.3.15 Autoradiography of the kidney

The kidneys were collected from the metabolic studies, washed with deionized water, dried and immediately embedded in O.C.T compound on dry ice. Cryostat sections (10 μm) of tumor sample were exposed to the phosphor plate for 2 days. The phosphor plate was subsequently scanned by a Typhoon FLA 9500 variable mode imager (GE Lifesciences) at a 10 μm resolution.

### 3.3.16 Statistical analysis

Comparisons of groups for *in vitro* and *in vivo* studies were analyzed by the unpaired two-tailed Student’s t test. *P* values of less than 0.05 were considered statistically significant.

### 3.4 Results

#### 3.4.1 Synthesis and radiolabeling

Three NT analogs (Figure 3.1) with linking groups consisting of one lysine (Lys) and four D-leucine ((D)Leu) in the paradigm DOTA-X-[(N-α-Me)Arg⁸,Dmt¹¹,Tle¹²] NT(7-13), where X= Lys-(D)Leu-(D)Leu-(D)Leu-(D)Leu, K2; (D)Leu-(D)Leu-Lys-(D)Leu-(D)Leu, K4; and (D)Leu-(D)Leu-(D)Leu-(D)Leu-Lys, K6, were synthesized by SPPS. The three analogs, K2, K4 and K6, were named based on the position of Lys in the peptide sequence. RP-HPLC retention time, mass spectrometric identification and yields of the unlabeled conjugates and natLu-conjugates are listed in Table 3.1. The isolated yields of the conjugates were relatively poor, likely due to the sterics resulting from conjugation to the secondary amines of the prolines and (N-α-Me)Arg [3]. The
purified conjugates were subsequently radiolabeled with $^{177/\text{n}}\text{LuCl}_3$ ($t^{1/2} = 6.75$ d) by co-incubation in an ammonium acetate buffer (pH = 5.5) for 45 min at 90 °C. The radiochemical yields for $^{177}\text{Lu-K2}$, $^{177}\text{Lu-K4}$, and $^{177}\text{Lu-K6}$ were 77.5%, 79.14% and 75.88%, respectively. In addition to the radioconjugates, a Cy5 fluorescent analog of K6 (Cy5-K6) was synthesized in order to perform cell trafficking studies. Cy5-K6 was prepared directly from SPPS, Figure 3.2, with an overall isolated yield of 2.5%.

### 3.4.2 Distribution coefficient

To evaluate the impact of the Lys position on the water solubility of the radioconjugates, octanol-PBS distribution coefficients at pH 7.4 were measured. The log $D_{\text{oct/water}}$ values for $^{177}\text{Lu-K2}$, $^{177}\text{Lu-K4}$, and $^{177}\text{Lu-K6}$ were $-2.53 \pm 0.02$, $-2.59 \pm 0.02$ and $-2.63 \pm 0.01$, ($P < 0.05$), respectively. While, as expected, the distribution coefficients were similar, the relative hydrophilicities were $^{177}\text{Lu-K6} > ^{177}\text{Lu-K4} > ^{177}\text{Lu-K2}$, indicating that movement of the Lys closer to pharmacophore increases hydrophilicity, albeit slightly.

### 3.4.3 In vitro competitive binding studies

The NTR1 binding affinity of the unlabeled conjugates and $^{\text{n}}\text{Lu}$-labeled conjugates was investigated by competitive binding studies using HT-29 cells. The IC$_{50}$ values are given in Table 3.1. For the unlabeled conjugates, the IC$_{50}$ value for K2 was the highest ($P < 0.001$) signifying the poorest binding analog investigated. In contrast, K6 had the lowest IC$_{50}$ value ($P < 0.001$) indicating the highest affinity to the NTR1. For the $^{\text{n}}\text{Lu}$-labeled K2, K4 and K6, the same trend was found (i.e., binding affinity of $^{\text{n}}\text{Lu-K6} > ^{\text{n}}\text{Lu-K4} > ^{\text{n}}\text{Lu-K2}$). This reveals that the proximity of the Lys to the pharmacophore plays a role in increasing the binding strength of the conjugate to the NTR1.
Figure 3.1. Structures of the $^{177}$Lu-DOTA-X-([(N-$\alpha$-Me)Arg$^{8}$,Dmt$^{11}$,Tle$^{12}$]NT(7-13) analogs (Lu-K2, Lu-K4 and Lu-K6) used in this study.
Figure 3.2. Synthesis of the Cy5-K6 that was utilized in cell trafficking studies.
Table 3.1. Characterization of K2, K4 and K6.

<table>
<thead>
<tr>
<th>Analog</th>
<th>Mass Cal. ([M+2H]^+)</th>
<th>Mass Obs. ([M+2H]^+)</th>
<th>Retention Time (min)</th>
<th>Yields</th>
<th>IC_{50} ± SD (nM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>K2</td>
<td>962.2</td>
<td>962.5</td>
<td>8.9</td>
<td>5%</td>
<td>48.6 ± 1.2</td>
</tr>
<tr>
<td>K4</td>
<td>962.2</td>
<td>962.5</td>
<td>8.8</td>
<td>3%</td>
<td>31.0 ± 1.1</td>
</tr>
<tr>
<td>K6</td>
<td>962.2</td>
<td>962.0</td>
<td>8.0</td>
<td>4%</td>
<td>21.8 ± 1.2</td>
</tr>
<tr>
<td>natLu-K2</td>
<td>1048.2</td>
<td>1048.5</td>
<td>8.8</td>
<td>69%</td>
<td>35.6 ± 1.1</td>
</tr>
<tr>
<td>natLu-K4</td>
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<td>1048.6</td>
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<td>23.5 ± 1.1</td>
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<tr>
<td>natLu-K6</td>
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<td>1048.6</td>
<td>7.5</td>
<td>71%</td>
<td>14.8 ± 1.1</td>
</tr>
</tbody>
</table>
3.4.4 In vitro internalization and efflux studies

The effect of the position of the positive charged amino acid (Lys) on the rates of internalization (Figure 3.3) and efflux (Figure 3.4) of the radioconjugates was investigated in HT-29 cells. At time points 15, 30, 60 and 120 min, the cellular uptake (internalized and surface-bound radioactivity) of the radioconjugates was represented as a percentage of total activity added. $^{177}$Lu-K6 exhibited a statistically higher level of uptake ($P < 0.0001$) relative to $^{177}$Lu-K2 and $^{177}$Lu-K4 at each time point from 15 min to 120 min. The internalization of $^{177}$Lu-K2 and $^{177}$Lu-K4 was not significantly different over the initial 60 min time period. However, by 120 min post-incubation, a statistically significant increase was observed for $^{177}$Lu-K4. At the 120 min post-incubation time point, the overall uptake for the radioconjugates were $^{177}$Lu-K6 ($4.06 \pm 0.20\%$) > $^{177}$Lu-K4 ($2.64 \pm 0.04\%$) > $^{177}$Lu-K2 ($1.91 \pm 0.09\%$).

Efflux studies were conducted by incubating the radioconjugates with HT-29 cells for 2 h, washing the cells with media to remove extracellular radioactivity and monitoring the release of radioactivity from the cells into the extracellular environment. The effluxed fractions of $^{177}$Lu-K2, $^{177}$Lu-K4 and $^{177}$Lu-K6 were calculated at the 0.5, 1, 2, 4 and 24 h time points. By the 1 h time point, the effluxed fraction for $^{177}$Lu-K6 ($15.74 \pm 1.13\%$) was substantially lower than $^{177}$Lu-K2 and $^{177}$Lu-K4 ($24.78 \pm 0.43\%$ and $25.15 \pm 0.93\%$ respectively, $P < 0.0001$). Statistically lower levels of efflux were observed for $^{177}$Lu-K6 relative to the other radioconjugates for the remaining time points. By the 24 h time point, $^{177}$Lu-K6 had effluxed only 20% of the internalized radioactivity relative to 28% and 31% for $^{177}$Lu-K4 and $^{177}$Lu-K2, correspondingly.
Figure 3.3. The uptake of $^{177}$Lu-K2, $^{177}$Lu-K4 and $^{177}$Lu-K6 (internalization and surface bound) by HT-29 cells. Values are means ± SD ($n = 6$).
Figure 3.4. The efflux of the internalized $^{177}$Lu-K2, $^{177}$Lu-K4 and $^{177}$Lu-K6 by HT-29 cells. Values are means ± SD ($n = 6$).
3.4.5 Confocal studies of the Cy5-K6

Using HT-29 cells, confocal microscopy images of the Cy5-K6 (pink) were taken at 0.5, 2 and 24 h, Figure 3.5. Additionally, early endosome (Rab5a, green), late endosome/lysosome (Rab7a, red) and acidic compartments (LysoTracker™, blue) were stained and their colocalization with Cy5-K6 quantified, Table 3.2. At 30 min post-incubation, the Cy5-K6 was shown primarily at the surface of the HT-29 cells with a small fraction of the Cy5-K6 entering the cell and co-localizing with the early endosome marker (green). By 2 h post-incubation, Cy5-K6 had distributed into vesicles that contained all three markers with low to moderate correlation. The Cy5-K6 demonstrated strong correlation/retention in vesicles that were both Rab7a- and LysoTracker™-positive by the 24 h post-incubation time point.

3.4.6 In vivo biodistribution studies

We investigated the in vivo biodistribution of $^{177}$Lu-K2, $^{177}$Lu-K4 and $^{177}$Lu-K6 at the 1, 4 and 24 h time points (Figure 3.6, 3.7 & 3.8). All three compounds, $^{177}$Lu-K2, $^{177}$Lu-K4 and $^{177}$Lu-K6, were effectively cleared from the blood after 4 h post-injection (0.04 ± 0.02 %ID/g, 0.06 ± 0.08 %ID/g and 0.03 ± 0.03 %ID/g, respectively). At all time points investigated, $^{177}$Lu-K2 and $^{177}$Lu-K6 exhibited substantially higher levels of liver uptake and retention, relative to $^{177}$Lu-K4. At 1 h, the liver uptake values for $^{177}$Lu-K2 and $^{177}$Lu-K6 were 5.64 ± 0.47 %ID/g and 6.65 ± 0.71 %ID/g, correspondingly. By the 24 h time point, the liver uptake for $^{177}$Lu-K2 and $^{177}$Lu-K6 had dropped modestly to 4.73 ± 0.75 %ID/g and 5.67 ± 0.57 %ID/g, respectively.
**Figure 3.5.** Confocal microscopy images of HT-29 cells with GFP-Rab5a, RFP-Rab7a, LysoTracker and Cy5-K6 at 30 min, 2 h and 24 h.
### Table 3.2. Colocalization of Cy5-K6

<table>
<thead>
<tr>
<th>Overlap coefficient</th>
<th>30 min (n=4)</th>
<th>2 h (n=4)</th>
<th>24 h (n=6)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Rab7a+ and LysoTracker+</td>
<td>0.80 ± 0.12</td>
<td>0.75 ± 0.07</td>
<td>0.73 ± 0.09</td>
</tr>
<tr>
<td>Rab7a+ and Cy5-K6+</td>
<td>0</td>
<td>0.56 ± 0.18</td>
<td>0.75 ± 0.13</td>
</tr>
<tr>
<td>LysoTracker+and Cy5-K6+</td>
<td>0</td>
<td>0.49 ± 0.14</td>
<td>0.76 ± 0.15</td>
</tr>
<tr>
<td>Rab5a+ and Cy5-K6+</td>
<td>0.51 ± 0.03</td>
<td>0.36 ± 0.11</td>
<td>0.62 ± 0.07</td>
</tr>
</tbody>
</table>
Additionally, $^{177}$Lu-K6 demonstrated significantly higher lung uptake ($5.08 \pm 1.31 \text{ %ID/g}$) at 1 h post-injection relative to the other conjugates ($<1.11 \pm 0.15 \text{ %ID/g}$). The substantial level of uptake in the liver and lung prompted us to investigate if the biodistribution of the radioconjugates was hindered by aggregate formation. BSA has been shown to inhibit the aggregation of peptides/proteins in solution [147,148]. The three radioconjugates were mixed with saline containing 0.5% BSA, to impede any potential aggregation of the radioconjugates. With this in hand, the 1 h biodistribution experiments were repeated (data presented in the supporting information) and yielded no substantial changes in organ uptake and overall distribution profiles.

The kidney uptake of $^{177}$Lu-K4 and $^{177}$Lu-K6 were statistically lower than that of $^{177}$Lu-K2 at all time points. At 1 h, the kidney uptake of $^{177}$Lu-K2 was $127.51 \pm 13.14 \text{ %ID/g}$, two fold higher than that of $^{177}$Lu-K4 and $^{177}$Lu-K6 ($64.8 \pm 10.81 \text{ %ID/g}$ and $68.98 \pm 6.22 \text{ %ID/g}$, respectively). By 24 h post-injection, the kidney retention of $^{177}$Lu-K2 had decreased substantially to $77.75 \pm 10.15 \text{ %ID/g}$, but still significantly higher than $^{177}$Lu-K4 and $^{177}$Lu-K6 ($48.75 \pm 9.59 \text{ %ID/g}$ and $43.96 \pm 6.92 \text{ %ID/g}$, correspondingly).

To evaluate the in vivo targeting capabilities of the NTR1-targeted agents, the uptake of the radioconjugates in the small and large intestines (NTR1-positive tissues) were evaluated. At the initial 1 h time point, $^{177}$Lu-K4 and $^{177}$Lu-K6 demonstrated a statistically higher uptake ($P < 0.001$) in the small intestines (e.g., $4.25 \pm 0.75 \text{ %ID/g}$ and $4.46 \pm 0.58 \text{ %ID/g}$, correspondingly) relative to $^{177}$Lu-K2 ($2.72 \pm 0.48 \text{ %ID/g}$). By 24 h post-injection, $^{177}$Lu-K4 and $^{177}$Lu-K6 retained 67.1 and 57.0 % of the 1 h uptake, while $^{177}$Lu-K2 preserved 66.2 % of the initial uptake. Similar trends of uptake and
retention for the radioconjugates were observed in the large intestines data. A blocking study was performed to demonstrate the NTR1-mediated nature of the intestinal uptake. Using the $^{177}$Lu-K6 as our model radioconjugate, the blocking study at 1 h demonstrated a substantial reduction of 82.1% and 62.6% in small and large intestinal uptake, respectively. Important to note, substantial reduction in uptake due to NTR1-blocking was not observed in the kidneys or liver, suggesting this uptake is not NTR1-mediated. However, interestingly, the lung uptake of $^{177}$Lu-K6 was shown to be substantially lower upon NTR1-blocking implying it was NTR1-mediated.

3.4.7 Autoradiography of the kidney

The distribution of radioconjugates/radiometabolites in the kidneys at 10 and 60 min post-injection was investigated using autoradiography, depicted in Figure 3.9. The autoradiographs of the $^{177}$Lu-K2, $^{177}$Lu-K4 and $^{177}$Lu-K6 show that even in the early time points, the radioactivity is largely localized in the cortex of the kidney. While the position of the Lys group does impact the extent of kidney retention, based on the biodistribution studies, these autoradiographic images demonstrate that it does not influence residual localization in the kidney.
Figure 3.6. Biodistribution studies at 1 h p.i. for $^{177}$Lu-K2, $^{177}$Lu-K4 and $^{177}$Lu-K6 in CF-1 mice. The blocking study was performed with co-administration of 250 µg of K6. The radioactivity uptake of tissues is expressed as a percentage of injected dose per gram of tissue (%ID/g). Values are means ± SD ($n = 5$).
Figure 3.7. Biodistribution studies at 4 h p.i. for $^{177}$Lu-K2, $^{177}$Lu-K4 and $^{177}$Lu-K6 in CF-1 mice. The radioactivity uptake of tissues is expressed as %ID/g. Values are means ± SD (n = 5).
Figure 3.8. Biodistribution studies at 24 h p.i. for $^{177}\text{Lu-K2}$, $^{177}\text{Lu-K4}$ and $^{177}\text{Lu-K6}$ in CF-1 mice. The radioactivity uptake of tissues is expressed as %ID/g. Values are means ± SD (n = 5).
Figure 3.9. Autoradiographic images of kidneys sections from CF-1 mice obtained at 10 and 60 min post-administration of $^{177}$Lu-K2, $^{177}$Lu-K4 and $^{177}$Lu-K6.
3.4.8 Metabolic studies in mice

To evaluate the metabolism of the radioconjugates in vivo, metabolic analyses were performed on the blood and urine at 10 and 60 min, respectively, Figure 3.10. For the blood metabolism studies, the recovery efficiencies of the radioactive components from the samples were ~70%. At 10 min post-injection, there were substantially higher levels of radioactivity observed in the blood for $^{177}$Lu-K2 and $^{177}$Lu-K4 (AUC: 22.56 and 14.99, respectively) compared to $^{177}$Lu-K6 (AUC: 2.61). However, the relative percent of intact radioconjugate was similar, ranging from 70.6 – 74.4%. Three primary metabolites were observed in the blood for $^{177}$Lu-K2 and $^{177}$Lu-K6, while five were found for $^{177}$Lu-K4. The radiometabolites isolated from the blood had shorter retention time relative to the intact radioconjugate suggesting an increased hydrophilicity. Urine analysis at the 1 h time point demonstrated that 43.2 %, 49.8 % and 34.0 % of the signal corresponded to the intact $^{177}$Lu-K2, $^{177}$Lu-K4 and $^{177}$Lu-K6, respectively. Three to four major radiometabolites were observed for each radioconjugate. Correlative identification based on retention times of the radiometabolites in the blood and urine was somewhat obfuscated due the different experimental conditions (e.g., sample matrices, analyte concentrations, etc.) leading to shifts in retention times. However, the major radiometabolite peaks for both the blood and urine samples had similar retention times and, for the most part, shift patterns suggesting that many of the radiometabolites were likely chemically identical.
Figure 3.10. Metabolism of the $^{177}$Lu-K2, $^{177}$Lu-K4 and $^{177}$Lu-K6. (A) Metabolites of $^{177}$Lu-K2 in the blood at 10 min (upper) and in the urine at 60 min (lower); (B) Metabolites of $^{177}$Lu-K4 in the blood at 10 min (upper) and in the urine at 60 min (lower); (C) Metabolites of $^{177}$Lu-K6 in the blood at 10 min (upper) and in the urine at 60 min (lower).
3.5 Discussion

To date, NTR1-targeted radiopharmaceuticals have been largely based on two targeting vector constructs: NT(6-13) and NT(8-13) [43–45,84,103,133,143]. While the NT(8-13) contains all of the necessary interactions to ensure low nanomolar binding affinity to the NTR1, the NT(6-13) fragment includes a terminal Lys\(^6\) which has been used by a variety of investigators as a convenient functionalization site for the incorporation of chelation systems (e.g., DTPA and DOTA) [31,44,84]. Recently, our laboratory reported a DOTA incorporated \([\text{N-( }\alpha\text{-Me)}\text{Arg}^8,\text{Dmt}^{11},\text{Tle}^{12}]\)NT(6-13) analog, based on work from Gruaz-Guyon and co-workers [44], in which the Lys\(^6\) remained unfunctionalized (charged) [45]. This analog exhibited significantly higher uptake in NTR1-positive tissues and kidneys compared to reported analogs that contain a functionalized (non-charged) Lys\(^6\) [44,84]. In order to better understand the role Lys\(^6\) plays on tissue uptake, we investigated the impact of positional translation of the Lys, relative to the pharmacophore, on the biological performance of a series of NTR1-targeted peptides, \(^{177}\text{Lu-K2, }^{177}\text{Lu-K4 and }^{177}\text{Lu-K6, Figure 3.1}

Evaluation of the receptor binding affinity of the NTR1-targeted analogs revealed that the position of the lysine has a modest impact on receptor binding. Binding affinities were found to decrease correlatively as the lysine is translated away from the main pharmacophore suggesting that Lys\(^6\) has a role in stabilizing the receptor-ligand complex. Based on what is known concerning NTR1-ligand interactions [62], we posit that the lysine is able to form weak charge-charge interactions with the electronegative rim of the binding pocket.
Evaluation of the receptor binding affinity of the NTR1-targeted analogs revealed that the position of the lysine has a modest impact on receptor binding. Binding affinities were found to decrease correlativelly as the lysine is translated away from the main pharmacophore suggesting that Lys$^6$ has a role in stabilizing the receptor-ligand complex. Based on what is known concerning NTR1-ligand interactions [62], we posit that the lysine is able to form weak charge-charge interactions with the electronegative rim of the binding pocket. The internalization efficacies of the NTR1-targeted agents were also found to correlativelly decrease as the lysine moved toward the N-terminus of the peptide. This signifies that the $^{177}$Lu-K6, with Lys$^6$, is more efficient at inducing the internalization of the receptor, possibly due to enhanced stabilization/residence time of the NTR1-ligand complex. Efflux studies revealed that both $^{177}$Lu-K2 and $^{177}$Lu-K4 exhibited significantly higher levels of externalization relative to $^{177}$Lu-K6. However, it is unclear to us how the structure of the NTR1-targeted agents resulted in this observation.

The cellular trafficking of fluorescently labeled NTR1-targeted agents has been previously reported by Vandenbulcke, Falciani and co-workers [149,150]. From our multicolor confocal microscopy studies, the initial uptake (i.e., 30 minute time point) of the Cy5-K6 demonstrated that internalized fluorescence was taken up, as expected, into the early endosomal compartments. Interestingly, the rate of Cy5-K6 uptake was much slower in HT-29 cells than what was observed by Vandenbulcke and co-workers using NTR1-transfected COS-7 cells [150]. This may be due to a number of factors including inherent differences in the two cell line phenotypes and levels of NTR1 expression. At 2h post-incubation, the Cy5-K6 demonstrated moderate levels of signal overlap ($0.36 \pm 0.11 \text{ -- } 0.56 \pm 0.18$) with the three compartmental markers. However, by 24 h of
continuous exposure to Cy5-K6, strong levels of overlap, 0.75 ± 0.13 and 0.76 ± 0.15, was observed for the Rab7a-positive (red) and Lysotracker™-positive (blue) vesicles, correspondingly. Both Rab7a and Lysotracker™ are markers for late endosomes/lysosomes. Given the 24 h time point, it seems likely that most of the Cy5-K6 has matriculated to its terminal location in the lysosomes.

Using biodistribution studies in CF-1 mice, the in vivo targeting efficacy of the NTR1-targeted agents were evaluated. For our NTR1-positive tissue, we utilized the small and large intestines, which are known to be endogenously expressed in the mouse [151]. The small intestinal uptake for 177Lu-K4 and 177Lu-K6 were statistically higher than 177Lu-K2 at all time points with \( P < 0.01 \). Similarly, uptake in the large intestines followed the same trend, but did not meet statistical significance. Blocking studies for 177Lu-K6 confirmed the NTR1-mediated nature of the uptake in the intestinal tissues. Given the observed binding affinity trend of 177Lu-K6 > 177Lu-K4 > 177Lu-K2, the uptake in the intestines suggests other factors than just binding affinity play a role in determining the in vivo NTR1-targeting efficacy of these agents.

The comparison of 177Lu-K6 with our previously published analog 177Lu-N1, which used the same [(N-α-Me)Arg\(^8\).Dmt\(^{11}\).Tle\(^{12}\)]NT(6-13) pharmacophore, yielded interesting disparities [45]. The in vitro binding and internalization characteristics of 177natLu-N1 were similar to 177Lu-K6. However, the uptake of 177Lu-K6, as well as 177Lu-K2 and 177Lu-K4, in the small and large intestines were roughly two-fold or more higher than observed for 177Lu-N1. This increased uptake is likely associated with the decreased hydrophilicity of this series of agents relative to 177Lu-N1 (\( \log D_{oct/water} = -3.15 ± 0.05 \)) leading to enhanced circulation time. Though, it is important to note, this speculation is based on NTR1-positive intestinal uptake from two different strains of mice (CF-1 and SCID) which may differ in the level of NTR1-expression.
All of the NTR1-targeted agents demonstrated significantly ($P < 0.0001$) higher kidney uptake (3-6 fold) than observed with the previously reported analog, $^{177}$Lu-N1. This uptake in the kidneys is not NTR1-mediated as demonstrated by our blocking study. It is well-known that radiolabeled peptides are taken up by the kidney through active (e.g., megalin and cubulin) and passive (e.g., pinocytosis) means [107,110]. Additionally, the overall charge of the radioconjugate has been shown to influence the extent of renal uptake [110]. Since the overall charge (+2) of $^{177}$Lu-N1 and this series of NTR1-targeted agents are identical, we attribute this substantial increase in uptake to increased hydrophobicity of $^{177}$Lu-K2, $^{177}$Lu-K4 and $^{177}$Lu-K6. Currently, it is unknown which renal uptake mechanism(s) were enhanced as a result of the increased hydrophobicities of the NTR1-targeted agents.

As demonstrated by autoradiography studies, the retention of the radioactivity in the kidney occurred in the cortex, which is consistent with literature reports for neurotensin and other small receptor-targeted peptides [22]. $^{177}$Lu-K2 demonstrated approximately two-fold higher kidney uptake/retention relative to $^{177}$Lu-K4 and $^{177}$Lu-K6. Based on the distribution coefficients, the differences in the hydrophobicity of the radioconjugates were negligible. Additionally, no substantial differences in the metabolism of $^{177}$Lu-K2 relative to the other radioconjugates were noted. Currently, it is unclear why the structure of $^{177}$Lu-K2 yields such a significant increase in kidney uptake. We hypothesize that this dissimilarity is due to unexpected higher renal uptake by one of the uptake mechanisms for $^{177}$Lu-K2 and/or one of its resulting radiometabolites.

For both $^{177}$Lu-K2 and $^{177}$Lu-K6, unexpected retention was observed in the liver and/or lung. For the DTPA and DOTA containing NTR1-targeted agents reported to date, significant uptake in these tissues has not been observed [44,45,84,85,103]. Since
these peptides are significantly more hydrophobic than previously reported analogs, initial thoughts were that aggregation of the peptides might be contributing to the uptake in these non-target tissues. Yet, co-formulation with BSA did not substantially alter the biodistribution profiles of the three radioconjugates. Interestingly, some reported $^{99m}$Tc-NTR1-targeted agents have been shown to have high liver and lung uptake [42,92]. Distribution coefficients were not reported for these analogs. However, based on their structures, we conjecture that hydrophobicity may be a contributing factor.

### 3.6 Conclusion

In summary, we investigated the impact of Lys charge distribution on the biological performance of a series of NTR1-targeted peptides. It was found that localization of the Lys closer to the pharmacophore lead to modest, but significant, increases in NTR1 affinity and rates of NTR1-mediated internalization. *In vivo* NTR1-targeting was best for radioconjugates ($^{177}$Lu-K4 and $^{177}$Lu-K6) with the Lys functionality closest to the targeting vector. The charge distribution of the Lys had an unexpected and substantial impact on uptake in the liver, lungs and kidneys. Additionally, the comparison of these findings with the literature suggests that the hydrophobicity plays a significant role in uptake in both NTR1-positive and NTR1-negative tissues.
Chapter 4:

Design and Development of Competitive inhibitors to protect NTR1-Targeted Peptide from Degradation \textit{in vivo}.
4.1 Abstract

The application of radiolabeled NT analogs for detecting and treating NTR1-positive tumors has been comprised by rapid \textit{in vivo} degradation. Some of the enzymes responsible for the degradation of NTR1-targeted analogs are known, including thimet oligopeptidase (TOP), neutral endopeptidase (NEP) and the angiotensin 1-converting enzyme (ACE). To investigate if inhibiting these enzymes can improve the efficacy of NT based analogs, we evaluated the co-administration of several known inhibitors and competitive substitutes of these enzymes. \textit{In vivo} biodistribution studies using HT-29 tumor xenograft mice demonstrated that the co-administration with enzyme inhibitor, phosphoramidon (PA) significantly increased the \textit{in vivo} tumor uptake from $5.55 \pm 1.54 \% \text{ID/g}$ (control) to $8.39 \pm 0.63 \% \text{ID/g}$ (with PA). The other agents tested, including the IPP (ACE inhibitor), bradykinin (competitive substrates for the peptidases that degraded NT) and peptide fragment of NT (Pro-Arg-Arg-Pro-Tyr), were not effective at inhibiting \textit{in vivo} degradation and increasing NTR1-mediated uptake.

4.2 Introduction

The development of targeted diagnostic and radiotherapeutic agents for cancers has long been an objective for biomedical sciences [9,16,20,26,30,152,153]. Neurotensin (NT) receptors have been an active area of interest for targeted agent development due to the overexpression of these receptors on a variety of cancers [50,51,53,55,57–59,154]. There are currently three known neurotensin receptors (NTR1, NTR2 and NTR3) [64,67,155]. However, to date, NTR1 has received the most interest for radiopharmaceutical development due to: 1) the higher NTR1 expression on
prominent cancers, such as prostate and pancreatic tumors, and 2) the higher affinity of NTR1 for NT and NT-based analogs [67,156].

NT was discovered over 40 years ago and has since been shown to have extensive neuromodulatory effects on both the central and peripheral nervous systems [67,75,79]. This neural hormone is a 13 amino acid peptide (Glu-Leu-Tyr-Glu-Asn-Lys-Pro-Arg-Arg-Pro-Tyr-Ile-Leu) which exhibits nanomolar affinity for the NTR1 [75,76]. Because of this, NT was the logical starting point for the development of targeted agents for NTR1-positive cancers. Early work revealed that only the NT(8-13) fragment was necessary to achieve nanomolar binding affinity [82]. However, it was also found that the native NT(8-13) sequence was rapidly degraded *in vivo*, thus limiting the *in vivo* targeting efficacy of the agent [87,156]. Investigation into the enzymes responsible for this degradation implicated several peptidases, including thimet oligopeptidase (TOP) 3.4.24.15, neutral endopeptidase (NEP) 3.4.24.11 and the angiotensin-1-converting enzyme (ACE) 2.4.15.1 [87,88,127,157]. Specifically, TOP has been shown to degrade at the Arg$^8$-Arg$^9$ site; NEP cleaves at the Pro$^{10}$-Tyr$^{11}$ position and ACE is capable of degrading the Tyr$^{11}$-Ile$^{12}$ amide bond. These findings have given the impetus for the development of stabilized NTR1-targeted analogs that employ single or multiple modification with alternative natural and/or non-natural amino acids to reduce *in vivo* degradation by these peptidases [31,32,43,44,93]. For example, our laboratory, have utilized the triply modified [(N-$\alpha$-Me)Arg$^8$,Dmt$^{11}$,Tle$^{12}$]NT(6-13) targeting vector [45,46]. These modifications have imparted substantially higher *in vivo* stability and targeting efficacy relative to the unmodified NT peptide, but are still relatively quickly degraded *in vivo*. Another recent approach, put forth by Nock, de Jong and co-workers, is the administration of enzymatic inhibitors to increase the *in vivo* stability of receptor-targeted peptides [97–100]. Using
analogs of targeted peptides that are in the clinic or under clinical development, these researchers have demonstrated that phosphoramidon (PA), a known inhibitor of NEP, could drastically increase the \textit{in vivo} stability and targeting efficacy of the investigated agents.

In this study, we explored the use of the enzymatic inhibitor approach in the context of NTR1-targeted peptides. Specifically, we examined whether the utilization of inhibitors can decrease the enzymatic degradation and increase the \textit{in vivo} targeting efficacy of current “stabilized” NTR1-targeted radioconjugates, such as the $^{177}\text{Lu}$-DOTA-$\beta$-Ala-[(N-$\alpha$-Me)Arg$^8$,Dmt$^{11}$,Tle$^{12}$]NT(6-13) radioconjugate. As part of this study, we investigated the enzymatic inhibitors PA (NEP inhibitor) and IPP, a tripeptide known to inhibit ACE. In addition, we investigated two peptide substrates: 1) bradykinin (BK, Arg-Pro-Pro-Gly-Phe-Ser-Pro-Phe-Arg) a known substrate for both NEP and ACE [158–160]; and 2) a shortened peptide (SP) fragment (Pro-Arg-Arg-Pro-Tyr) of NT(6-13) that contains two of the three known cleavage sites. It was our hypothesis that these sacrificial substrates, which are co-administered at amounts magnitudes higher than the radioconjugate, would be able to temporarily saturate the enzyme(s) responsible for the degradation of the NTR1-targeted agents leading to an increase in \textit{in vivo} stability. Herein, we report our findings of the \textit{in vivo} investigation of the $^{177}\text{Lu}$-DOTA-$\beta$-Ala-[(N-$\alpha$-Me)Arg$^8$,Dmt$^{11}$,Tle$^{12}$]NT(6-13) radioconjugate, designated as $^{177}\text{Lu}$-N1, in conjunction with the above mentioned enzymatic inhibitors.
4.3 Methods

4.3.1 Materials

Acetonitrile, formic acid, N,N-diisopropylethylamine (DIEA), N,N-dimethylformamide (DMF), dichloromethane (DCM), N,N'-dicyclohexyl-carbodiimide (DCC), methyl-t-butyl ether, N-methylpyrrolidone (NMP). Fluorenylmethyloxycarbonnyl (Fmoc)-protected natural amino acids, Fmoc-β-Ala-OH, Fmoc-Leu-Wang resin (100-200 mesh), and (1-cyano-2-ethoxy-2-oxo-ethylidenaminoxy) dimethylamino-morpholino-carbenium hexafluorophosphate (COMU) were purchased from NovaBiochem (Hoherbrunn, Germany). Fmoc-N-Me-Arg(Pbf)-OH was obtained from ChemPep, Inc. (Wellington, FL). Fmoc-2,6-dimethyl-L-tyrosine (Dmt) was purchased from Ontario Chemicals, Inc (Guelph, ON, Canada). Fmoc-Tle-OH was procured from CreoSalus (Louisville, KY). Lutetium-177 trichloride ($^{177}\text{LuCl}_3$) was obtained from Perkin Elmer (Waltham, MA) with a specific activity of 32.3 Ci/mg. Naturally abundant lutetium trichloride ($^{\text{n}\text{at}}\text{LuCl}_3$), triisopropylsilane and 3,6-dioxa-1,8-octanedithiol were purchased from Sigma-Aldrich (St Louis, MO). McCoy’s 5A medium (1X; Iwakata & Grace Mod.) with L-glutamine was obtained from Mediatech, Inc. (Manassas, VA). TrypLE™ Express was procured from Invitrogen (Grand Island, NY). Penicillin-Streptomycin solution and 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) were obtained from HyClone Laboratories, Inc (Logan, UT). Fetal Bovine Serum (FBS) was purchased from Gibco™ by Life Technologies Corporation (Grand Island, NY). Phosphoramidon sodium salt and bradykinin were procured from MP Biomedicals, LLC (Solon, OH).
4.3.2 Cell culture

The human colon cancer cell line HT-29 was obtained from American Type Culture Collection (Manassas, VA) and cultured under vendor-recommended conditions. Cells were passaged twice weekly in McCoy’s 5A medium supplemented with 10% FBS, 100 IU/mL penicillin and 100 μg/mL streptomycin at 37 °C in a humidified incubator containing 5% CO₂.

4.3.3 Mouse model

All animal experiments were conducted in accordance with the Principles of Animal Care outlined by the National Institutes of Health and approved by the Institutional Animal Care and Use Committee of the University of Nebraska Medical Center. Eight-week-old CF-1 mice and five-week-old Institute of Cancer Research severely combined immunodeficient (ICR SCID) mice were obtained from Charles River Laboratories (Wilmington, MA). Animals were housed five per cage in a light- and temperature-controlled environment. Food and water were given ad libitum. For xenograft model, bilateral HT-29 tumors were induced by subcutaneous injection of 5.0×10⁶ cells in Matrigel (BD Biosciences). The tumors were allowed to grow for 2-3 weeks reaching a maximal of 1 cm in diameter before the mice were utilized in pharmacokinetic studies.

4.3.4 Solid-phase peptide synthesis (SPPS)

As published previously, peptides were synthesized on an automated solid-phase Liberty microwave peptide synthesizer from CEM (Matthews, NC), employing traditional Fmoc chemistry. Briefly, the Fmoc-Leu-Wang resin (100 μmol of the resin-substituted peptide anchors) was deprotected by piperidine, resulting in the formation of a primary amine on the anchored amino acid. Fmoc-protected amino acids (300 μmol) were activated with COMU and sequentially conjugated to the resin. The
resulting peptide underwent cleavage from the resin and orthogonal group deprotection by shaking in a cocktail consisting of triisopropylsilane (0.125 mL), water (0.125 mL), 3,6-dioxo-1,8-octanedithiol (0.125 mL), and trifluoroacetic acid (4.625 mL) for 3 h. The cleaved peptide was subsequently precipitated, filtered and washed thrice using cold (0 °C) methyl-tert-butyl ether (40 mL×3). The crude peptides were dried under vacuum.

4.3.5 HPLC purification and analysis methodology

HPLC/MS analyses were performed on a Waters (Milford, MA) e2695 system equipped with a Waters 2489 absorption detector and a Waters Q-Tof Micro electrospray ionization mass spectrometer. Sample purification for in vitro studies was performed on a Phenomenex (Torrance, CA) Jupiter 10 µm Proteo 250 × 4.6 mm C12 column with a flow rate of 1.5 mL/min. For bulk sample purification, a Phenomenex Jupiter 10 µm Proteo 250 × 10 mm C12 column was used with a flow rate of 5.0 mL/min. HPLC solvents consisted of H2O containing 0.1% formic acid (solvent A) and acetonitrile containing 0.1% formic acid (solvent B). For unlabeled and ¹⁷⁷Lu-conjugates of N1, an initial gradient of 95% A: 5% B linearly decreased to 85% A: 15% B over a 15-minute time period. For unlabeled SP, an initial gradient of 95% A: 5% B linearly decreased to 90% A: 10% B over a 15-minute time period. For unlabeled IPP, an initial gradient of 98% A: 2% B linearly decreased to 92% A: 8% 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.
4.3.6 Radiolabeling with $^{177}$LuCl$_3$

The conjugate (50 µg, 30 nmol) was dissolved in ammonium acetate buffer (0.5 M, 100 µL, pH 5.5). $^{177}$LuCl$_3$ (37 MBq, 1 mCi, 0.18 nmol) was added to the vial containing the conjugate, and the solution was heated for 45 min at 90 °C. To separate radiolabeled peptides from unlabeled peptides on the HPLC, 4-5 mg of CoCl$_2$ was added and incubated for 5 min at 90 °C to increase the hydrophobicity of the unlabeled conjugate. After cooling to room temperature, evaluation and purification of the radiolabeled conjugates were performed on a Waters 1525 binary pump equipped with a Waters 2489 absorption detector and a Bioscan Flow Count (Poway, CA) radiometric detector system. The collected radioconjugate was concentrated with an Empore (Eagan, MN) C18 high performance extraction disk followed by elution with ethanol/sterile saline solution (6:4, 400 µL) to provide the radiolabeled conjugates in high purity.

4.3.7 In vitro competitive binding studies

As described previously, the half maximal inhibitory concentration (IC$_{50}$) for SP was determined (n = 6, 2 tech and 3 bio repeats) using the HT-29 human colon cancer cell line. In these studies, $^{177}$Lu-N1 ($^{177}$Lu-DOTA-β-Ala-[N-α-Me]Arg$^8$,Dmt$^{11}$, Tle$^{12}$]NT(6-13)) served as the competitive radioligand for comparing the relative effectiveness of the unlabeled conjugates. HT-29 cells (~1×10$^6$) were suspended in 100 µL of McCoy’s 5A medium (pH 7.4, 4.8 mg/mL HEPES, and 2 mg/mL BSA) and incubated at 37 °C for 45 min in the presence of $^{177}$Lu-N1 (100,000 cpm, 100 µL) and various concentrations (from 10$^{-4}$ M to 10$^{-12}$ M) of the SP (100 µL). At the end of the incubation, the cells were centrifuged, aspirated and washed with media five times. The cell-associated radioactivity 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 (La Jolla, CA).

**4.3.8 In vivo biodistribution studies on mice**

Biodistribution studies were carried out both using healthy CF-1 mice and xenograft SCID mice. For biodistribution studies on CF-1 mice, each mouse (average weight, 30 g) received an intravenous bolus, via the tail vein, of $^{177}$Lu-radiolabeled N1 (370 kBq, 10 µCi) in 100 µL of saline alone as a control, or with PA (300 µg), IPP (300 µg), SP (500 µg) or BK (500 µg). For biodistribution studies on xenograft mice, each mouse (average weight, 25 g) received an intravenous bolus, via the tail vein, of $^{177}$Lu-radiolabeled N1 (370 kBq, 10 µCi) in 100 µL of saline alone as a control, or with PA (300 µg), or BK (500 µg). At 1 h post injection, the mice were sacrificed and the amounts of radioactivity in the tissues were counted with a NaI (Tl) well detector from AlphaSpectra, Inc. (Grand Junction, CO). The excised tissues were weighed and results were expressed as percentage of injected dose per gram of tissue (%ID/g).

**4.3.9 Statistical analysis**

Comparisons of groups for *in vitro* and *in vivo* studies were analyzed by the unpaired two-tailed Student’s t test. *P* values of less than 0.05 were considered statistically significant.

**4.4 Results**

**4.4.1 Synthesis of the peptides and radiolabeling of N1**

The structures of the N1, PA, IPP, BK and SP peptides are depicted in Figure 4.1. N1, IPP and SP were synthesized by SPPS and purified and characterized by LC-MS. PA and BK were purchased from commercial sources. Mass spectrometry, HPLC retention times and overall synthetic yields for the synthesized peptides are
given in Table 4.1. Yields for N1 were significantly lower than IPP or SP, which can be attributed to the relative length and increased steric restraints of the N1 peptide [45].

Radiolabeling of N1 with $^{177}$LuCl$_3$ was carried out in an ammonium acetate buffer (pH 5.5) at 90°C for 45 min. The radioconjugation reaction was examined by HPLC and found to give > 85% radiochemical yield. The radioconjugate was purified and characterized by HPLC. The retention time of the radioconjugate (9.8 min) was consistent with what was previously reported [45].

4.4.2 *In vitro* competitive binding studies

To confirm NTR1 binding affinity of N1, competitive binding studies were performed for both N1 and $^{177}$Lu-N1 using HT-29 human colorectal cancer cells. The resulting IC$_{50}$ value for both compounds were consistent with previous accounts [45]. Since the SP is a fragment of the NT(8-13) targeting vector, the IC$_{50}$ of this fragment was evaluated to confirm negligible binding to the NTR1. SP demonstrated no binding over the concentrations investigated and was found to have an IC$_{50}$ of > 0.1 mM.

4.4.3 *In vivo* biodistribution studies using CF-1 mice

The 1 h p.i. biodistribution profiles of $^{177}$Lu-N1 in a CF-1 mouse model alone and co-administered with PA, BK, SP or IPP are shown in Figure 4.2. $^{177}$Lu-N1 cleared from the blood rapidly with only 0.08 ± 0.01 %ID/g remaining at 1 h. The co-administration of IPP and SP had no effect on the blood retention of $^{177}$Lu-N1. However, co-injection with PA and BK did increase the blood retention of the radioconjugate to 0.17 ± 0.06 %ID/g and 0.29 ± 0.11 %ID/g, respectively. The tissues that demonstrated the highest degree of uptake of the NTR1-targeted agents is the small and large intestines and the kidney. Intestinal uptake has been shown to be due to the expression of NTR1 in the tissue of the mouse [151].
Figure 4.1. The structures of $^{177}\text{Lu-N1}$, enzyme inhibitors and competitive substrates.
Table 4.1. Characteristics of N1, SP and IPP.

<table>
<thead>
<tr>
<th>Analog</th>
<th>Sequence</th>
<th>Mass Cal.</th>
<th>Mass Obs.</th>
<th>Retention Time (min)</th>
<th>Yield</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>N1</strong></td>
<td>DOTA-β-Ala-Lys-Pro-(N-Me)Arg-Arg-Pro-Dmt-Tle-Leu-OH</td>
<td>1540.9</td>
<td>1541.9</td>
<td>13.9</td>
<td>11.1%</td>
</tr>
<tr>
<td><strong>SP</strong></td>
<td>Pro-Arg-Arg-Pro-Tyr</td>
<td>686.4</td>
<td>687.4</td>
<td>7.0</td>
<td>75.5%</td>
</tr>
<tr>
<td><strong>IPP</strong></td>
<td>Ile-Pro-Pro</td>
<td>324.2</td>
<td>325.2</td>
<td>6.7</td>
<td>88.9%</td>
</tr>
</tbody>
</table>
Thus, intestinal uptake, particularly the small intestines, can serve as a positive control from which to judge the \textit{in vivo} targeting efficacy of the various formulations. $^{177}$Lu-N1 demonstrated a small intestine uptake of $3.21 \pm 0.20 \text{ %ID/g}$. Co-administration of the SP and IPP had either no effect or a slightly negative effect, in the case of IPP, on the NTR1-mediated uptake of $^{177}$Lu-N1. Conversely, co-formulation with PA and BK increased small intestine uptake to $4.20 \pm 0.34 \text{ %ID/g}$ and $5.06 \pm 0.54 \text{ %ID/g}$, correspondingly. While similar, the trend in large intestinal uptake of $^{177}$Lu-N1 largely reflected that of the small-intestines, with exception to the BK co-formulation. The co-administration of BK yielded a substantial increase in uptake of the radioconjugate ($4.90 \pm 0.85 \text{ %ID/g}$).

The observed kidney uptake ($13.21 \pm 0.36 \text{ %ID/g}$) of $^{177}$Lu-N1 alone reflects the fact that $^{177}$Lu-N1, as with most small peptides, are excreted primarily through the renal system. It has been demonstrated that this kidney uptake is not NTR1-mediated, but rather is based on non-specific mechanisms of uptake [45]. Co-injection of SP and IPP with $^{177}$Lu-N1 had no substantial impact on kidney uptake. However, the co-administration with PA and BK yielded significantly ($P < 0.005$) higher levels of kidney uptake, $20.60 \pm 1.93 \text{ %ID/g}$ and $16.87 \pm 1.94 \text{ %ID/g}$, respectively, compared to $^{177}$Lu-N1.
Figure 4.2. Biodistribution studies on CF-1 mice at 4 h post-injection. (n=7 for $^{177}$Lu-N1, n=5 for the other group).
4.4.4 In vivo biodistribution studies on HT-29 human colon cancer xenograft SCID mice

The $^{177}$Lu-N1 formulations with the enzymatic inhibitors were next evaluated in an HT-29 human colon cancer xenograft mouse model (Figure 4.3). Taking the results from the CF-1 biodistribution studies into account, only $^{177}$Lu-N1 alone and formulations with PA and BK were investigated in the xenograft model. Biodistribution profiles of the SCID model were largely congruent with the data obtained from the CF-1 mouse model. $^{177}$Lu-N1 uptake in the NTR1-positive, small intestine was $5.26 \pm 1.08 \%$ID/g. Co-administration of $^{177}$Lu-N1 with PA ($7.04 \pm 1.13 \%$ID/g, $P < 0.05$) and BK ($8.04 \pm 1.11 \%$ID/g, $P < 0.01$) demonstrated a corresponding 1.3-fold and 1.5-fold higher radioactivity accumulation compared to $^{177}$Lu-N1 alone. In the large intestine, the $^{177}$Lu-N1 co-injection with BK exhibited the highest uptake ($9.34 \pm 1.13 \%$ID/g, $P < 0.0001$). In contrast, no significant increase in large intestine uptake was observed for the $^{177}$Lu-N1 co-injection with PA ($2.22 \pm 0.29 \%$ID/g), as compared to $^{177}$Lu-N1 alone ($2.03 \pm 0.55 \%$ID/g).

Uptake of the $^{177}$Lu-N1 in the NTR1-positive, HT-29 tumors was $5.55 \pm 1.54 \%$ID/g. The tumor uptake increased approximately 1.5-fold for the co-administered formulation with PA ($8.39 \pm 0.63 \%$ID/g). However, the tumor uptake of $^{177}$Lu-N1 co-injected with BK ($3.95 \pm 0.57 \%$ID/g) was not significantly different with the $^{177}$Lu-N1 alone. Interestingly, compared to the CF-1 data, the kidney uptake of the three groups was not significantly different with uptake values ranging from $23.19 \pm 6.97$ to $25.65 \pm 5.48 \%$ID/g.
Figure 4.3. Biodistribution studies on colon cancer xenograft SCID mice at 1 h post-injection (n=5).
4.5 Discussion:

During the development of NTR1-targeted peptides, it was quickly realized that analogs based on the native NT targeting vector were rapidly degraded. This prompted the development of NT targeting vectors that are resistant to proteolytic degradation to increase in vivo stability and maintain targeting viability. While this approach has resulted in more stable and effective imaging and therapeutic agents, degradation of the structurally modified peptides still occurs and is a determinant to optimal NTR1-targeting. For example, our laboratory, along with others, have utilized [(N-α-Me)Arg\textsuperscript{8},Dmt\textsuperscript{11},Tle\textsuperscript{12}]NT(6-13) targeting vector and have demonstrated that this agent is still quickly degraded (e.g., 30% in 10 min) in the blood [46]. The goal of this study was to investigate if inhibition of the enzymes responsible for the degradation of NTR1-targeted agents resulted in a significant increase in in vivo targeting. This premise is based on prior work that demonstrated that receptor-targeted peptides susceptible to metabolism by NEP demonstrated significantly higher in vivo targeting when PA, a known NEP inhibitor, was co-administered [97,98,101,161].

The metabolism of NT based analogs has been attributed to several metallopeptidases, including TOP, neurolysin, NEP and ACE. TOP is a metalloendopeptidase that is responsible for the degradation of NT at the Arg\textsuperscript{8}-Arg\textsuperscript{9} position [127,157,162]. Degradation at the Pro\textsuperscript{10}-Tyr\textsuperscript{11} of NT is attributed to the metalloendopeptidases neurolysin and NEP [127,157]. Lastly, ACE has been shown to degrade NT at the Tyr\textsuperscript{11}-Ile\textsuperscript{12} positions. For the aims of this study, we utilized several inhibitors of these peptides to evaluate the biological impact on the in vivo efficacy of NTR1-targeted agents. PA is a potent and relatively selective inhibitor of NEP (IC\textsubscript{50} = 34 nM), but has also shown inhibitor activity for other metalloproteases.
including ACE (IC\textsubscript{50} = 78 µM) [163]. IPP is a tripeptide that had demonstrated inhibitory effects (IC\textsubscript{50} = 5 µM) against ACE [164]. BK is a potent vasodilator and a substrate of ACE and NEP [165,166]. It is envisioned that BK would act as a “sacrificial” inhibitor, temporarily saturating ACE and NEP, thus temporally reducing the rate of degradation of NTR1-targeted peptides. Lastly, in the same vein, SP, a fragment of the NT(8-13) targeting vector, contains two of the three known cleavage sites. However, due to these proteases being endopeptidases, it is expected that SP would be a good inhibitor of TOP, but not necessarily neurolysin or NEP.

Our initial investigation into the ability of enzymatic inhibitors to increase the \textit{in vivo} targeting efficacy of NTR1-targeted agent was carried out using a CF-1 mouse model. Utilization of SP and IPP demonstrated no discernable effects with regard to the biodistribution profile of \textsuperscript{177}Lu-N1. Uptake of the radioconjugate into the NTR1-positive, small and large intestines remained unchanged. This establishes that the inhibitors, given at relatively high doses, were ineffective at increasing the \textit{in vivo} efficacy of the NTR1-targeted agent utilized in this study. The reason for this failure may be due to \textsuperscript{177}Lu-N1, which uses a modified (i.e., stabilized) targeting vector, no longer being a good substrate for ACE and TOP. If so, this implies that ACE and TOP are not the primary enzyme(s) responsible for the \textit{in vivo} degradation of stabilized, NTR1-targeted agents. Conversely to this finding, the utilization of PA and BK had a marked impact on the uptake in the small intestines. Co-administration of \textsuperscript{177}Lu-N1 with PA led to a 30.8% increase in radiotracer uptake, while co-formulation with BK led to a 57.6% increase. BK also had a substantial impact on the uptake in the large intestines with an increase of 301.6%. Given the selectivity of PA for NEP, it seems reasonable to conclude that NEP, through proteolytic cleavage, does effect the \textit{in vivo} efficacy of stabilized NTR1-targeted agents. This is consistent with what has been
observed for other neuropeptide-based analogs [97,98,101,102]. Utilization of BK resulted in the largest increase in NTR1-mediated uptake. However, the interpretation of the BK impact was obscured, due to its known vasodilation effects. Whether the increased NTR1-mediated uptake of $^{177}$Lu-N1 was due to inhibition of ACE and NEP and/or its potent vasodilation effect increasing tissue perfusion was unclear.

Taking the results from the CF-1 biodistribution studies into account, we decided to investigate $^{177}$Lu-N1 alone and the co-formulations including PA and BK in the HT-29 human colon cancer xenograft mouse model. The co-administration of PA and BK largely gave similar $^{177}$Lu-N1 uptake profiles compared to the CF-1 studies. One notable difference was the renal uptake. Co-administration of PA or BK did increased $^{177}$Lu-N1 kidney retention in CF-1 mice, but, interestingly, did not lead to a significant impact on renal uptake in SCID mice. The reason for the divergence in the renal uptake is unknown, but is possibly due to differences in the mouse strains. For the formulations investigated, the small and large intestines uptake profiles of the radiotracer were essentially identical for both mouse models. Using PA, HT-29 tumor uptake of $^{177}$Lu-N1 increased by 51.2% confirming that PA plays a positive role in increasing NTR1-mediated uptake through inhibition of NEP. However, the use of BK demonstrated no increase in HT-29 tumor uptake. BK is able to produce its vasodilation effect in normal vasculature by indirectly causing the vascular smooth muscle to relax [167,168]. In contrast, the vasculature of most solid tumors are known to generally lack smooth muscle [169]. Based on this, our finding strongly suggests that the positive effect of NTR1-mediated uptake in the intestines is a result of the vasodilation effect of BK and not on any inhibitory effect.
4.6 Conclusion

Utilizing inhibitors and competitive substrates for proteases known to degrade NTR1-targeted agents, we investigated the impact of these agents on the in vivo receptor-mediated uptake and biodistribution profile of $^{177}$Lu-DOTA-[(N-$\alpha$-Me)Arg$^8$.Dmt$^{11}$.Tle$^{12}$]NT(6-13). At the doses utilized, IPP (ACE inhibitor) and SP (NT fragment) did not demonstrate a significant effect on NTR1-mediated uptake or the biodistribution profile of the radioconjugate. Co-injection of the radioconjugate with BK (ACE and NEP substrate) did demonstrate an increase in uptake in NTR1-positive tissues, but this increase was determined to be largely due to the increased perfusion due to the vasodilation effect of BK. However, in vivo studies in CF-1 and HT-29 tumor xenograft mice did demonstrate a substantial increase NTR1-mediated uptake in NTR1-positive non-target tissues and tumors. Overall, these studies demonstrate that PA (NEP and ACE inhibitor) is effective at enzymatically stabilizing and improving the in vivo efficacy of NTR1-targeted agents.
Chapter 5:

Conclusions and Future Directions
NTR1 is overexpressed in several cancers due to its role as a growth pathway for cancer cells proliferation, invasion and migration [54,56,57,69,138,170,171]. The native ligand, NT, has been utilized as a base platform for the development of NTR1-targeted agents for the detection and treatment of receptor positive cancers [31,43,60,84,92,96]. However, the application of NT-based radiopharmaceuticals has been comprised by the decreased binding affinity due to the steric hindrance of various chelators [44,103]. In addition, the unstable nature of NT-based radiopharmaceuticals has led to a diminished amount of intact peptides that can accumulate at the target sites (e.g., tumors) [42–44,92,95]. Lastly, NT-based radiopharmaceuticals, as with most radiopeptides of small size, are primarily excreted via the renal system. As a consequence, the high kidney uptake of NTR1-targeted agents occurs which is a concern when considering its application for targeted radiotherapy [15,22]. To shed further light onto these problems as well as explore approaches that may improve the clinical potential of NT-based radiopharmaceuticals, we investigated the structure-activity relationships of various NTR1-targeted agents. Specifically, we explored possible strategies to restore binding affinity, determine the effect of charge distribution on the biological profile of NTR1-targeted agents and evaluate if peptidase inhibitors were able to increase the in vivo stability of a NTR1-targeted analog.

As demonstrated in the bombesin analogs, the negative effect of macrocyclic chelator on the binding affinity of receptor-targeted agents could be diminished by the insertion of spacer groups between the macrocyclic chelator and the binding sequence [37,172]. To evaluate the effect of inserted spacers on the binding affinity of NTR1-targeted agents, we synthesized and investigated a stabilized NT-based analog [(N-α-Me)Arg^8,Dmt^{11},Tle^{12}]NT(6-13)) with various spacers of different length (e.g.,
null, β-Ala, 5-Ava and 8-Aoc) in Chapter 2. *In vitro* competitive binding, internalization and efflux studies were performed on all four NT analogs using HT-29 human colon cancer cells. The *in vitro* competitive binding assays demonstrated that the binding affinity was significantly increased compared with the analog without a spacer. Specifically, the analog with a β-Ala spacer ($^{177}$Lu-N1) could restore a binding affinity similar to that of the intact NT. In addition, the $^{177}$Lu-N1 internalized more rapidly and effluxed more slowly than the other analogs tested, indicating the high retention of this agent in the HT-29 cells. $^{177}$Lu-N1 also exhibited *in vitro* stability for 24 h in PBS, conditioned medium and human serum. *In vivo* biodistribution and SPECT/CT imaging studies of $^{177}$Lu-N1 demonstrated excellent tumor accumulation (3.1 ± 0.4 %ID/g) in a NTR1-positive colon cancer xenograft model. We concluded that a spacer of β-Ala or longer could restore the binding affinity of NTR1-targeted agents containing DOTA. In the future, other spacers with different properties (e.g., hydrophobicity and charge) could be investigated to determine the effect these changes have on biological performance.

From the *in vivo* biodistribution study of $^{177}$Lu-N1, we observed the high kidney uptake, which could comprise the clinical therapeutic application of this agent. The high, non-specific kidney uptake is a common hurdle for the development of peptide-based radiopharmaceuticals. Based on numerous investigations concerning the mechanism and cause of peptide uptake in the kidneys, the charge of the NT-based radiopharmaceuticals has been shown to play an important role in the renal uptake. However, the effect of charge distribution of the NT-based radiopharmaceuticals on the biological profile, including the binding affinity and retention in non-target tissues (e.g., kidneys), has not been investigated. To investigate the influence of charge distribution on NTR1-targeted agents, we synthesized three analogs with the spacers
composed of Lys and D-Leu in Chapter 3. These analogs had the same net charge, but with different charge distributions through the sequence (K2, K4 and K6). The effect of the charge distribution was evaluated on the NTR1 binding, receptor-mediated uptake and \textit{in vivo} biodistribution. With the movement of Lys towards the binding sequence, the binding affinity increased. The \textit{in vivo} biodistribution of the non-target organs and receptor-positive organs showed different uptake patterns, indicating the influence of the charge distribution of the peptide. The noticeable uptake in the lung and liver of \textsuperscript{177}Lu-K2, K4 and K6 was most likely due to the hydrophobicity of these agents. The \textit{in vivo} radiometabolites generated by the \textsuperscript{177}Lu-K2, K4 and K6 were also investigated. These studies demonstrated that even these “stabilized” NTR1-targeted agents undergo degradation \textit{in vivo}. However, further studies to determine the chemical identity of the radiometabolites generated, including the molecular weight and sequence, have yet to be performed. With such information, the design of improved NT-based radiopharmaceuticals with higher \textit{in vivo} stability will be possible.

In the last study, we investigated the effect of selective inhibitors and competitive substrates for the enzymes responsible for the degradation of the NT-based radiopharmaceutical in Chapter 4. Using \textsuperscript{177}Lu-N1, the co-administration of PA (a NEP inhibitor) was found to increase NTR1-positive, tumor uptake 1.6 fold in colon cancer xenograft mice. However, with the co-administration of the other inhibitor, IPP (an ACE inhibitor), and competitive substrates (SP and BK), no increase in the receptor-mediated uptake was observed. The promising result from the co-administration of PA will encourage future studies to optimize the use of PA as well as the investigation of other inhibitors to improve the \textit{in vivo} stability and potential of NTR1-targeted agents.
With the studies performed herein, we evaluated the structure-activity relationships of NTR1-targeted agents as well as explored approaches that could ultimately increase translational potential. It is expected that the strategies and insights found in this work will inspire the design of the next generation of NTR1-targeted agents. Ultimately, it is hoped that, with these new and improved analogs, the successful translation of NTR1-targeted agents into the clinic will be achieved.
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