Bombesin Antagonists for Targeting Gastrin-Releasing Peptide Receptor-Positive Tumors

Design, Synthesis, Preclinical Evaluation and Optimization of Imaging Agents

ZOHREH VARASTEH
This thesis is focused on the development, preclinical evaluation, and optimization of radiotracers for the detection of gastrin-releasing peptide receptor (GRPR)-expressing tumors. The work is divided into three distinct parts: (1) the development of bombesin (BN) antagonist (RM26)-based imaging radiotracers for the detection of GRPR-expressing tumors using different positron emission tomography (PET) and single photon emission computed tomography (SPECT) radionuclides ($^{68}$Ga, $^{18}$F and $^{111}$In), (2) the establishment of a method to monitor the ligand-G protein-coupled receptor (GPCR) interaction in real time without requiring purification and stabilization of the receptors, and (3) the evaluation of radiopeptide structure-related factors (length of mini-PEG linker and composition of chelator for metal labeling) affecting the in vitro and in vivo characteristics of RM26-based tracers.

We demonstrated the possibility of high-contrast in vivo imaging of GRPR-expressing xenografts despite the physiological expression of GRPR in abdominal organs. Fast radioactivity clearance from the blood and healthy organs, including receptor-positive organs, and long retention in the tumors resulted in high tumor-to-background ratios. A novel real-time assay for measuring the kinetics of the radiotracers targeting GPCR was evaluated. Living cells were used instead of purified receptors in this technology, bringing the developmental work one step closer to the true target environment (imaging in living systems). The comparative study of $^{68}$Ga-labeled NOTA-PEG$_n$-RM26 with di-, tri-, tetra- and hexaethylene glycol chains demonstrated that the addition of only a few units of ethylene glycol to the spacer is insufficient to appreciably affect the biodistribution of the radiopeptide. Finally, a comparative study of $^{68}$Ga-labeled PEG$_2$-RM26 analogs N-terminally conjugated to NOTA, NODAGA, DOTA or DOTAGA highlighted the influence of the chelator on the targeting properties of the radiopeptide.

The main conclusion that can be drawn from this thesis is that $^{68}$Ga-NOTA-PEG$_2$-RM26 has favorable biodistribution properties, such as rapid clearance from blood and tissues with physiological GRPR expression levels and long retention in GRPR-expressing tumors, and that this radiopeptide is potentially suitable for initial clinical investigation.

Keywords: Bombesin, Gastrin-releasing peptide receptor (GRPR), Antagonist, Radionuclide molecular imaging

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ISSN 1651-6192
urn:nbn:se:uu:diva-232123 (http://urn.kb.se/resolve?urn=nbn:se:uu:diva-232123)
To my beloved parents
and my soulmate
for supporting me all the way!
List of Papers

This thesis is based on the following papers, which are referred to in the text by their Roman numerals.


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List of papers not included in this thesis


VI. Rosik, D., Orlova, A., Malmberg, J., Altai, M., Varasteh, Z., Sandström, M., Karlström, AE., Tolmachev,


X. Andersson, G.K., Rosestedt, M., **Varasteh, Z.**, Malm1, M., Sandström, M., Tolmachev, V., Löfblom, J., Ståhl1, S., Orlova, A. Comparative Evaluation of 111In-Labeled NOTA-Conjugated Affibody Molecules for Visualization of HER3 Expression in Malignant Tumors. Conditionally accepted to *J Nucl Med*.


XII. **Varasteh, Z.**, Orlova, A. Comparing the measured affinity of 111In-labeled ligands for cellular receptors by monitoring gamma beta or x-ray radiation with three different LigandTracer® devices: A technical note on affinity measurement, submitted to *PLOS ONE*.

*Equal contribution to this work*
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<th>Description</th>
</tr>
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<tbody>
<tr>
<td>ADT</td>
<td>Androgen deprivation therapy</td>
</tr>
<tr>
<td>AR</td>
<td>Androgen receptor</td>
</tr>
<tr>
<td>BN</td>
<td>Bombesin</td>
</tr>
<tr>
<td>BPH</td>
<td>Benign prostatic hyperplasia</td>
</tr>
<tr>
<td>DOTA</td>
<td>1,4,7,10-tetraazacyclododecane-1,4,7,10-tetraacetic acid</td>
</tr>
<tr>
<td>DOTAGA</td>
<td>1,4,7,10-tetraazacyclododecane-1-glutaric acid-4,7,10-triacetic acid</td>
</tr>
<tr>
<td>EC</td>
<td>Extracellular</td>
</tr>
<tr>
<td>EGFR</td>
<td>Epidermal growth factor receptor</td>
</tr>
<tr>
<td>EPR</td>
<td>Enhanced permeability and retention</td>
</tr>
<tr>
<td>FDG</td>
<td>Fluorodeoxyglucose</td>
</tr>
<tr>
<td>FDHT</td>
<td>Fluorodihydrotestosterone</td>
</tr>
<tr>
<td>GRPR</td>
<td>Gastrin releasing peptide receptor</td>
</tr>
<tr>
<td>GPCR</td>
<td>G protein-coupled receptor</td>
</tr>
<tr>
<td>HRPC</td>
<td>Hormone-refractory prostate cancer</td>
</tr>
<tr>
<td>MET</td>
<td>Methionine</td>
</tr>
<tr>
<td>MDP</td>
<td>Methylene diphosphonate</td>
</tr>
<tr>
<td>NMBR</td>
<td>Neuromedins B receptor</td>
</tr>
<tr>
<td>NOTA</td>
<td>1,4,7-triazacyclononane-1,4,7-triacetic acid</td>
</tr>
<tr>
<td>NODAGA</td>
<td>1,4,7-triazacyclononane-1-glutaric acid-4,7-diacetic acid</td>
</tr>
<tr>
<td>PC</td>
<td>Prostate cancer</td>
</tr>
<tr>
<td>PSA</td>
<td>Prostate specific antigen</td>
</tr>
<tr>
<td>PET</td>
<td>Positron emission tomography</td>
</tr>
<tr>
<td>PEG</td>
<td>Polyethylene glycol</td>
</tr>
<tr>
<td>RAID</td>
<td>Radioimmunodetection</td>
</tr>
<tr>
<td>SPR</td>
<td>Surface plasmon resonance</td>
</tr>
<tr>
<td>SPECT</td>
<td>Single photon emission computed tomography</td>
</tr>
<tr>
<td>TRUS</td>
<td>Transrectal ultrasonography</td>
</tr>
</tbody>
</table>
Introduction

Prostate cancer

Prostate cancer (PC) is the most frequently diagnosed non-cutaneous cancer and the second leading cause of cancer-related mortality in men after lung and bronchus cancers [1]. Although PC can grow and spread rapidly and develop into fatal and painful disease, in most cases, it grows slowly, and symptoms do not affect men during their lifetime. PC has a heterogeneous nature, and the characteristics of the disease diverge during its progression. The progression of PC leads to a transition from tumors with androgen-responsive growth toward hormone-refractory prostate cancer (HRPC).

Screening of prostate cancer: Potential and limitations

PC can be indicated by the prostate-specific antigen (PSA) blood test, which measures the level of this well-characterized prostate-specific glycoprotein. Digital rectal examination (DRE), the physical examination of the patient’s prostate from the rectum to assess prostate size abnormalities, is the other standard screening test. In cases with an elevated PSA level and an enlarged prostate gland, the screening procedure will be followed by the histopathological examination of multiple transrectal ultrasonography (TRUS)-guided biopsy samples to confirm PC. However, none of the above-mentioned screening modalities are sufficiently reliable to rule out the presence of cancer. An enlarged prostate and an elevated PSA level can result from factors other than PC, e.g., prostatitis (prostatic inflammation) and benign prostatic hyperplasia (BPH) (enlargement of the prostate) [2, 3]. In addition, prostate growth is age-dependent [4], and the serum PSA level is related to prostate size [5]. Therefore, an increase in the PSA level due to prostate enlargement is common in advanced age and can lead to overdiagnosis. Due to the heterogeneous expression of genetic markers associated with PC even in the primary tumor, not all the biopsy samples are representative, which limits the utility of biopsy data [6].
Staging of prostate cancer: Potential and limitations

After confirming the presence of cancer, it is necessary to determine the extent of the disease. Staging, the detection of bone and soft tissue involvement, is essential for guiding optimal therapy for cancer patients. The stage of disease describes how far the cancer has spread. The staging of PC may also consider biopsy results (Gleason score), the PSA level, or certain imaging tests. Among imaging modalities, nuclear medicine procedures are widely used.

Bone is one of the strongly preferred sites for spreading of the PC. Bone scintigraphy with \([^{99m}Tc]\)methylene diphosphonate (\([^{99m}Tc]\)MDP) using a gamma camera is the initial, sensitive, and cost- and time-efficient imaging modality for the identification of altered skeletal metabolism and metastatic spread to the skeletal system [7]. Bone scanning with \([^{18}F]\)fluoride using PET is the other imaging test used for focal and generalized bone diseases [8].

Likewise, most of the tracers used in nuclear medicine for imaging soft tissue metastases visualize tumor cell metabolism. \([^{18}F]\)Fluorodeoxyglucose (FDG)-PET, a metabolic imaging technique, has shown great success for the detection of many types of hypermetabolic cancer. However, this method very poorly visualizes tumors with low metabolism or low FDG-avidity. Because PC in most cases is a slow-growing disease, it has a low glycolytic rate that results in low FDG uptake [9].

\([^{11}C]\)Acetate, another metabolic radiopharmaceutical, has been evaluated clinically for PC imaging. \([^{11}C]\)Acetate accumulation in tumor cells is the consequence of enhanced cell membrane and thus lipid (fatty acid) synthesis due to cell growth [10]. However, this tracer has shown low specificity for prostate tumors. Data showing high \([^{11}C]\)acetate uptake in normal prostate and hyperplastic tissues have been reported [11, 12].

\([^{11}C]/[^{18}F]\)Choline uptake in PC tumors has been extensively studied. The cellular uptake of \([^{11}C]/[^{18}F]\)choline is primarily due to increased phospholipid synthesis [13] and the upregulation of choline kinase induced by malignancy [9]. Depending on the lesion localization, the sensitivity of choline-based tracers in the detection of PC is as low as 45-65% [14].

\([^{11}C]\)Methionine (MET) accumulation in tumor cells is associated with increased amino acid active transport and metabolism, which corresponds with the number of tumor cells [15, 16]. \([^{11}C]\)MET-PET has shown higher sensitivity in PC imaging by identifying significantly more lesions than \([^{18}F]\)FDG-PET [17]. However, \([^{11}C]\)MET demonstrated low specificity
for the tumor cells. Methionine uptake increases by increasing the density of inflammatory cells [18].

To overcome these limitations regarding tumor metabolic imaging agents, a new approach based on receptor targeting was developed. This technique provides information concerning the extent and behavior of tumors by directing radiolabeled peptides and proteins to the pathological or modified antigens and over-expressed target receptors. Receptor-mediated tumor targeting concept showed higher specificity to the site of action in PC. Following this concept, $[^{18}\text{F}]$fluorodihydrotestosterone (FDHT)-based imaging was evaluated in prostatic carcinoma with increased androgen receptor (AR) expression. This imaging method was used to assess the receptor status and to quantify changes in the receptor density. However, AR mutations that occur during PC progression may affect the sensitivity of the ligand [19].

Radiolabeled monoclonal antibodies were considered initially as target-specific imaging agents. The $^{111}\text{In}$-labeled monoclonal antibody capromab pendetide (ProstaScint) is an FDA-approved imaging radiopharmaceutical that has been introduced for staging PC. This antibody binds to prostate specific membrane antigen (PSMA), which is overexpressed in PC. Capromab pendetide is directed against the cytoplasmic domain of PSMA and is thought to bind primarily to the necrotic cells in prostate tumors. As is typical for other intact antibody-based tracers, the sensitivity and specificity of $^{111}\text{In}$-capromab pendetide is far from optimal [20, 21, 22]. The large size of the antibodies does not allow rapid kinetics, and the slow clearance of unbound molecules from blood and non-target compartments results in enhanced background signals and reduced target-to-background ratios. In addition, the enhanced permeability and retention (EPR) effect, which results in the nonspecific tumor uptake and retention of macromolecules (MW >40 kDa) due to defective vascular architecture in tumors, makes it difficult to evaluate the specific uptake of antibody-based radiotracers [23].

To overcome the limitations of radioimmunodetection (RAID) of tumors, a number of more efficient approaches have been investigated. The targeting of overexpressed receptors in tumors using radiolabeled peptide ligands for non-invasive cancer imaging has received an appreciable attention.
Treatment of prostate cancer

After PC diagnosis and staging, a treatment option, which differs for each individual, will be chosen. Selecting the treatment for PC patients depends heavily on the stage of the cancer. Radical prostatectomy (the surgical removal of the prostate gland and the seminal vesicles) and/or external radiation therapy are treatment modalities for early diagnosed locally confined prostate tumors, whereas androgen deprivation therapy (ADT) is the first-line treatment for the palliation of symptomatic late-stage PC that has already metastasized [24, 25]. Testosterone suppression can be achieved surgically (removal of testicles to suppress testosterone production) or chemically (treatment with female sex hormones).

Single photon emission computed tomography (SPECT) and positron emission tomography (PET)

Single photon emission computed tomography (SPECT) and positron emission tomography (PET) are two major modalities used in nuclear medicine for imaging. SPECT uses radioactive tracers labeled with gamma-emitting radionuclides and a gamma camera to obtain a projection image of the radioactivity distribution. The projections can be reconstructed into three-dimensional images by a computer (Figure 1A). Similar to SPECT, a PET scan creates computerized in vivo image of distribution of the tracers labeled with positron-emitting radionuclides. The detectors detect pairs of annihilation photons, which are produced when the emitted positrons interact with nearby electrons (Figure 1B). Due to the coincidence detection in the PET camera, it is the most sensitive method for the quantitative measurement of physiological processes in vivo. In other words, PET provides better spatial resolution, better sensitivity, and more accurate quantification of the radioactivity concentration in vivo. For this reason, PET imaging typically provides better sensitivity and improved diagnostic specificity.

The most widely used short-lived positron-emitting nuclides, $^{15}$O, $^{13}$N, $^{11}$C and $^{18}$F, are produced by a cyclotron. Thus, the PET scanner must be located near a cyclotron and a radiopharmaceutical laboratory that synthesizes tracers, which substantially increases the cost of PET diagnostics.

In contrast to cyclotron-produced positron emitters, $^{68}$Ga is a generator-produced radionuclide that can be eluted from a $^{68}$Ge/$^{68}$Ga generator on-site and does not require a cyclotron in the vicinity of the PET facility. In addition, the cyclotron-PET satellite concept enables a single cyclotron
production facility to regularly supply $^{18}\text{F}$ to several clinical PET centers and significantly increase the number of PET scans. The list of the most commonly used radionuclides for imaging is presented in Table 1.

**Figure 1.** A schematic overview of (A) gamma camera and (B) PET camera acquisition of images.

**Table 1.** A tabular overview of the most commonly used radionuclides for imaging [26, 27, 28].

<table>
<thead>
<tr>
<th>Radionuclide</th>
<th>Usage</th>
<th>Half-life</th>
<th>Gamma-ray energy (MeV)</th>
<th>maximum positron energy (MeV)</th>
<th>Mean range of particle in tissue (mm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>$^{11}\text{C}$</td>
<td>PET</td>
<td>20.3 min</td>
<td>0.97</td>
<td></td>
<td>1.1</td>
</tr>
<tr>
<td>$^{13}\text{N}$</td>
<td>PET</td>
<td>10 min</td>
<td>1.2</td>
<td></td>
<td>1.5</td>
</tr>
<tr>
<td>$^{15}\text{O}$</td>
<td>PET</td>
<td>2 min</td>
<td>1.7</td>
<td></td>
<td>2.5</td>
</tr>
<tr>
<td>$^{18}\text{F}$</td>
<td>PET</td>
<td>109.7 min</td>
<td>0.63</td>
<td></td>
<td>0.6</td>
</tr>
<tr>
<td>$^{68}\text{Ga}$</td>
<td>PET</td>
<td>68 min</td>
<td>1.9</td>
<td></td>
<td>2.9</td>
</tr>
<tr>
<td>$^{89}\text{Zr}$</td>
<td>PET</td>
<td>3.2 d</td>
<td>0.89</td>
<td></td>
<td>1</td>
</tr>
<tr>
<td>$^{124}\text{I}$</td>
<td>PET</td>
<td>4.2 d</td>
<td></td>
<td></td>
<td>3.4</td>
</tr>
<tr>
<td>$^{99m}\text{Tc}$</td>
<td>SPECT</td>
<td>6.0 h</td>
<td>0.141</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>$^{111}\text{In}$</td>
<td>SPECT</td>
<td>2.8 d</td>
<td>0.172 and 0.245</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>$^{123}\text{I}$</td>
<td>SPECT</td>
<td>13.2 h</td>
<td>0.159</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>$^{131}\text{I}$</td>
<td>SPECT</td>
<td>8 d</td>
<td>0.364</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>
Peptide ligand/receptor systems used for tumor imaging and treatment

Molecular imaging is a technique that enables the visualization of cellular and molecular disease mechanisms and normal biological processes. It is a rapidly growing discipline aiming toward developing new probes for imaging specific molecular pathways involved in disease processes and utilizing them for improved diagnostics.

The observation that some peptide receptors are overexpressed in certain tumors compared to endogenous levels in normal organs has encouraged the use of these receptors as targets and the design of peptide-based radiotracers for targeted molecular imaging. Peptides are molecules consisting of 3 to 100 amino acid residues with a molecular mass of less than 10 kDa. Peptides are designed by nature for inhibiting, stimulating, and regulating important fundamental biological processes through their corresponding receptors, which are located on the cell membrane. Due to their small size, peptides usually have rapid extravasation, tissue penetration, and clearance from blood and non-target tissues. These attributes result in high tumor-to-organ ratios—the primary requirement for diagnostic and therapeutic agents. Furthermore, the synthesis, modification, and stabilization of small peptides to obtain optimized pharmacokinetic parameters can be cheaper and faster than the modification of bulky antibodies. High binding affinity and low immunogenicity are other considerable advantages of the peptides.

There has been an exponential growth in the development of radiolabeled peptides providing in vivo histopathological information for diagnostic and therapeutic purposes [29]. Among these tailored radiopharmaceuticals, some G protein-coupled receptor (GPCR) radioligands have already made or are likely to make a contribution to the diagnosis and therapy of oncological disorders. Indium-111-labeled octreotide (\(^{111}\text{In-OctreoScan}\)) became the first FDA-approved peptide-based tracer. It is used primarily for the visualization of neuroendocrine tumors but also for many other tumors [30]. In addition, lutetium-177-labeled octreotate has been clinically used for many years to treat patients with somatostatin receptor-positive tumors [31]. This example of success in developing radiolabeled peptides for targeting receptor-expressing tumors has paved the way for the exploration of other receptor/peptide systems.

Bombesin (BN) analogs, other well-studied GPCR-targeting peptides, are the main focus of this thesis.
Bombesin, bombesin-like peptide ligands and their corresponding receptors

BN is a natural linear amidated tetradecapeptide initially isolated from skin extracts of the European discoglossid frog *Bombina bombina* [32, 33]. Subsequently, a search for BN counterparts in mammalian tissues was initiated. The neuromedins B (NMB) from the central nervous system and the gastrointestinal bombesin (so-called gastrin-releasing peptide, GRP) are the corresponding mammalian peptides. BN-like regulatory peptides exert a spectrum of physiological activities in the nervous system and the gut. These physiological activities are elicited after BN-like peptides bind to a group of three GPCRs in mammals and one GPCR in amphibians. The neuromedin B receptor (BB1R/NMBR) with high affinity for NMB, the gastrin-releasing peptide receptor (BB2R/GRPR) with high affinity for GRP, and the orphan BN receptor subtype 3 (BB3R/BRS3) are expressed in mammalian tissues. The BN receptor subtype 4 (BB4R/BRS4) is the amphibian equivalent [34, 35, 36, 37, 38, 39]. The receptor subtypes and the structures of their native peptide ligands are summarized in Table 2.

*Table 2*. The sequences for the native peptide ligands binding to different receptor subtypes (BB1R, BB2R and BB4R) are presented. Two distinct naturally occurring BN agonists have been identified for the BB4R differing in the second residue from C-terminal (either by Leu13 or Phe13). The potency of [Phe13]BN for BB4R is higher than [Leu13]BN [40]. The native peptide for BB3R has not been identified.

<table>
<thead>
<tr>
<th>Receptor subtype</th>
<th>Native peptide ligand</th>
<th>Structure of the corresponding ligand</th>
<th>Origin</th>
</tr>
</thead>
<tbody>
<tr>
<td>BB1R/NMBR</td>
<td>NMB</td>
<td>Gly-Asn-Leu-Trp-Ala-Thr-Gly-His-Phe-Met-NH₂</td>
<td>Mammalian</td>
</tr>
<tr>
<td>BB2R/GRPR</td>
<td>GRP</td>
<td>Val-Pro-Leu-Pro-Ala-Gly-Gly-Thr-Val-Leu-Thr-Lys-Met-Tyr-Pro-Arg-Gly-Asn-His-Trp-Ala-Val-Gly-His-Leu-Met-NH₂</td>
<td>Mammalian</td>
</tr>
<tr>
<td>BB3R/BRS3</td>
<td>Not identified</td>
<td></td>
<td>Mammalian</td>
</tr>
</tbody>
</table>
It is known that the normal human pancreas (most abundantly), stomach, adrenal cortex, and brain (weakly) express GRPR [41]. The abnormal overexpression of GRPR was reported in the vast majority of cancer cells from the breast, prostate, uterus, ovaries, colon, pancreas, stomach, lung (small and non-small cell), head and neck squamous cell cancer and in various CNS/neural tumors. These data suggest that GRPR could be a potential candidate as a clinically relevant molecular target for the visualization and therapy of several tumors [34].

GRP, the endogenous ligand for GRPR, and [Leu^{13}]BN share an amidated C-terminal seven amino acid sequence homology (highlighted in Table 2) that governs the agonist activity of the peptides. Previously investigated BN analogs can be categorized in two different groups based on their structures. Type A analogs are truncated with a portion of BN retained, and type B analogs are full-length tetradecapeptides in which one or more amino acid residues were replaced [42]. The C-terminal of BN is required for receptor recognition and binding affinity [43]. It has been shown that more than seven but no more than nine C-terminal amino acids from the BN sequence are required for high affinity receptor binding [44]. Thus, BN[7-14] is the sequence most widely used for tracer development. Previous structure-activity studies have demonstrated that modification in the C-terminal region affects the binding affinity, and changing L- to D-amino acids at the 8, 9, 13 or 14 positions reduced the receptor binding affinity dramatically [43]. Therefore, the N-terminal of these analogs was used for coupling to the chelators for loading with radiometals or modified for labeling with other radionuclides [45, 46].

**BN receptor agonists vs. antagonists**

In the past few decades, peptide receptor agonists have been the ligands of choice for tracer development and utilization in nuclear medicine. The paradigm regarding the use of agonist-based constructs was primarily due to receptor-radioligand complex internalization and the high accumulation of radioactivity inside the target cells. In the case of radiometal-labelled peptides, the efficient receptor-mediated endocytosis in response to agonist stimulation provides high *in vivo* radioactivity uptake in the targeted tissue, which is a prerequisite for the optimal imaging and treatment of malignancies. In contrast, antagonists, which do not trigger internalization, have not been considered for tumor targeting. Subscribing to this paradigm, a majority of radiolabeled BN derivatives for the molecular imaging of GRPR overexpression in human tumors were agonists.
However, a paradigm shift occurred when somatostatin receptor-selective peptide antagonists showed preferable biodistribution, including considerably higher *in vivo* tumor uptake, compared with highly potent agonists [47]. Similar results were observed for the BN antagonist Demobesin1 [48]. These results suggest that the same preference change from agonists to antagonists could be justified for other GPCRs such as BN-based radiopharmaceuticals. The tumor-to-kidney ratio for Demobesin 1 was more than 7-fold higher than the ratio for the corresponding BN agonist Demobesin 4 [48]. Furthermore, it has been hypothesized that side effects observed during a clinical phase I dose escalation study of BN[7-14] agonist, including abdominal cramps, nausea and diarrhea, might be avoided by using antagonists [49]. In addition, antagonistic GRPR-based peptides may not induce the endocrinological side effects, such as the stimulation of tumor-growth, that are known to occur with agonists [50]. The BN-induced activation of MAP kinase pathway by transactivating epidermal growth factor receptor (EGFR) was reported by Xiao et al [50]. This side effect could be even more crucial when a therapeutic dose is administrated. In contrast, BN receptor antagonists may be able to suppress tumor growth [51]. A considerable decrease in hormone-dependent human prostate tumor cell (PC-82) proliferation was reported upon treatment with a BN receptor antagonist [D-Tpi6, Leu13 ψ(CH2NH) Leu14]BN[6-14] (RC-3095) alone or in combination with an agonist of luteinizing hormone-releasing hormone [D-Trp6]-LH-RH and the somatostatin analog D-Phe-Cys-Tyr-D-Trp-Lys-Val-Cys-Trp-NH2 (RC-160) [52]. Therefore, there has been considerable interest in the design and development of BN analogs with antagonistic activity based on the tetradecapeptide BN agonist.

There are additional strategies for BN receptor antagonist design beyond the classical multiple side chain modification approach. Peptide backbone modification through carbonyl reduction was found to be the design approach of choice for BN antagonist discovery. It has been reported that the C-terminal region of [Leu14]BN (peptide bond between residues 13 and 14) is directly responsible for triggering the receptor response, and the replacement of this bond (CO-NH group) by its reduced form (CH2-NH group) using rapid solid-phase methods resulted in the first sufficiently potent (IC50 = 35±7 nM) BN antagonist [Leu13-ψ(CH2NH)Leu14]BN [53]. In this work, the readily oxidized Met14 residue was also replaced by a Leu14 residue.

BN receptor antagonists can also be obtained by manipulating C-terminal amino acid residues. Broccardo et al. demonstrated that the Trp8 and His12 residues play essential roles in the biological activity of the
peptide [54], and substitution with D-Phe in these positions (especially His12) resulted in a BN receptor antagonist [D-Phe12, Leu14]BN (IC50=2±0.2 µM) [55]. Additional amino acid replacements improved the binding affinity of the antagonistic analog [D-Phe6, D-Phe12, Leu14]BN (IC50 = 0.3±0.05 µM) [55]. The lipophilicity of position 12 was also reported to be important because substitution with a more hydrophilic residue in this position resulted in more than 40-fold reduced binding affinity [55].

It was observed that the peptide bond between the last two amino acids can be hydrolyzed by an activating enzyme system, and H-Leu14-NH2 was released leading to the hypothesis that these two residues are important as an active moiety responsible for the biological activity of BN. Therefore, replacing this bond with a non-hydrolysable bond would result in BN receptor antagonists. Several partial BN antagonist derivatives based on [D-Phe6-Leu14]BN[6-14] have been reported in which Leu14 was replaced by non-natural amino acid residues or the peptide bond between Leu13 and Leu14 was modified [56]. However, the replacement of Leu13 with a statyl residue in [D-Phe6-Leu14]BN[6-14] ([D-Phe6, Sta13, Leu14]BN[6-14], IC50=2.9±0.6 nM) or introducing a hydroxyl group in place of the amide bond between Leu13-Leu14 residues resulted in potent BN antagonistic analogs [57]. The spatial orientation of the hydroxyl group is not important for biological activity [57].

It has also been shown that the C-terminal amide group is important for the agonist activity of BN, and by deleting this amide function, several potent BN receptor antagonists with preserved high affinity for the BN receptor have been obtained [57].

This thesis is focused on the development of high-affinity BN receptor antagonist-based imaging agents using D-Phe-Gln-Trp-Ala-Val-Gly-His-Sta-Leu-NH2 ([D-Phe6, Sta13, Leu14]BN[6-14]NH2), which is also known as JMV594 or RM26 [46, 58, 59].

Molecular basis for selectivity of RM26 for GRPR

The BN receptor antagonist RM26 has more than 5000 times higher selectivity for GRPR compared to NMBR, despite the fact that these receptors share 56% overall amino acid sequence identity [60, 61]. It has been shown that the fourth extracellular (EC) domain of GRPR is principally involved in selectively discriminating GRPR from NMBR and responsi-
able for high affinity binding. Substituting the fourth EC domain of GRPR into NMBR (e4-NMBR)GRPR significantly decreased the binding affinity for RM26 [61]. In the reverse study, substituting the fourth EC domain of NMBR into GRPR (e4-GRPR[NMBR] resulted in a 300-fold improvement in binding affinity for the same peptide analog [61]. In the same study, site-directed mutagenesis investigations showed that among 11 divergent amino acids between GRPR and NMBR in the fourth EC domain, Thr297, Phe302 and Ser305 are critical residues for determining the GRPR selectivity of RM26. It is also important to mention that both human and mouse GRPRs are identical in the fourth EC domain [62, 63].

The influence of the structure of radiolabeled peptides on targeting properties and biodistribution

Radiolabeling receptor-specific peptides and proteins provides a method for the selective delivery of radionuclides to the tissues (over-) expressing the target receptors. This method may also be used for imaging tumors overexpressing certain receptors. It may also be used for the delivery of nuclides with cytotoxic particulate emission for therapeutic purposes. Sensitivity and specificity to the target are essential features for a successful diagnostic and therapeutic application of radiopeptides. In other words, the success of the detection and treatment of malignancies using radiolabeled peptides and proteins depends upon the delivery of the radionuclide specifically to the cancer cells while sparing healthy tissue. The degree of discrimination between target and normal tissue highly depends on the structure of the radioconjugate.

![Radiotracer structure](image)

*Figure 2. Radiotracer structure.*

A radiopeptide is composed most importantly of the target recognition moiety, which is conjugated with a radionuclide using different labeling
strategies. The labeling technique used depends primarily on the radionuclide used. Radiohalogens, e.g., iodine, can be directly integrated into a peptide by the electrophilic substitution of a tyrosine or indirectly via a prosthetic group. Radioactive metals, in contrast, are incorporated into a protein via complexation by a chelating agent. In addition, a spacer could be introduced to the structure to separate the chelating moiety from the target-binding fragment (Figure 2).

The pharmacokinetics of radiopeptides can be tuned by altering the tracer structure using different spacer moieties and/or different chelators with variable chemical natures [64, 65].

The influence of the spacer between the receptor binding sequence and the chelating moiety on the biological characteristics of BN analogs

There are many structure-related parameters that control the behavior of peptide-based radiopharmaceuticals in the biological system. One of these parameters is the structure and physicochemical properties of a spacer moiety. A chelator or a prosthetic group might be attached to a peptide using a covalent bond (e.g., amide), but a spacer could also be introduced to modify the pharmacological properties of the radiotracer.

It is known that the direct coupling of the radiometal-chelator complex to the N-terminus of the BN analogs decreases the receptor binding affinity [66], whereas the introduction of a linking group prevents the interference of the chelator with the binding site of the targeting vector to the GRPR. To maintain the radiolabeling site distant from the receptor recognition and binding site, different spacers have been used. The analog containing no spacer (X=0) for $^{111}$In-DOTA-X-BN[7-14]NH$_2$ has 100-fold reduced binding affinity to GRPR compared to its counterpart containing an 8-carbon aliphatic spacer [67]. A Lys(sha)-βAla-βAla linker for coupling retro[N$^\alpha$-carboxymethyl-histidine] to a BN analog for $^{99m}$Tc(CO)$_3$ labeling improved the affinity of the conjugate by a factor of 23 compared to the analog without the linker, [Leu$^{13}$, Nle$^{14}$]BN[7-14] [68].

Subsequently, it was demonstrated that an excessive increase of the lipophilicity of the linker group impairs the binding affinity [67]. Significantly high liver uptake for hydrophobic linker analogs is an additional
problem beyond the suboptimal pharmacokinetics of these tracers [67]. To improve the pharmacokinetic profiles for BN analogs, more hydrophilic spacer moieties were introduced. Schweinsbery et al. have shown that a hydrophilic carbohydrate introduced into the linker sequence of $^{99m}$Tc-labeled BN analogs decreased liver uptake significantly [69]. Increasing the polarity by inserting a negatively charged $\beta^3$hGlu to the linker provided favorable biodistribution compared to the reference analog (N$^{\alpha}$His)Ac-$\beta$Ala-$\beta$Ala-[Leu$^{13}$, Nle$^{14}$]BN[7-14] labeled using $^{99m}$Tc-tricarbonyl [70]. Consequently, modifying the spacer length and composition may influence the binding affinity of BN to GRPRs and the in vivo kinetics.

This thesis is focused on the development of RM26-based imaging agents using linear polyethylene glycol (PEG$_n$)-spacers of different molecular weights. The hypothesis was that the incorporation of the PEG-spacer would increase tracer hydrophilicity and decrease unspecific off-target uptake in healthy organs, particularly in the liver.

The influence of the chelator on the biological characteristics of peptide-based targeting agents

The pharmacokinetics of radiotracers can also be tuned by choosing the proper chelation system. The chemical structure of chelators is different, and this can influence the targeting properties of tracers substantially. Different chelators are reported to influence the receptor binding affinity, internalization rate, and even the functional (agonistic/antagonistic) profile of targeting peptides. The effect of chelator modification on the biodistribution of peptide-based imaging agents has been widely investigated for somatostatin analogs. For example, it has been reported that increasing the number of carboxylates on a triazacyclononane-based chelator seems to decrease the overall receptor binding affinity and internalization of $^{68}$Ga-labeled [Tyr$^3$]octreotide conjugates, which results in unfavorable biodistribution profiles [71]. Tumor-to-normal tissue (blood, liver, kidney, and muscle) ratios were significantly higher for the $^{64}$Cu-NODAGA-conjugated than for the $^{64}$Cu-CB-TE2A-conjugated somatostatin-based antagonist LM3 [72].

The antagonistic somatostatin analog Cpa-DCys-Asn-Phe-Phe-DTrp-Lys-Thr-Phe-Thr-Cys-2Nal-NH$_2$ (406-040-15) alternated to a full agonist when coupled to DOTA (406-051-20), as determined in the sst(3) recep-
tor internalization assay [73]. This finding demonstrates that it is not possible to predict whether the modification of a targeting peptide by the addition of a chelator will activate or antagonize the internalization process.

The chelator has been shown to have little impact on the integrin $\alpha_\nu\beta_3$-targeting capacity of multimeric cyclic RGD peptides. However, DOTA-conjugated 3P-RGD$_2$ showed favorable tumor-targeting properties compared to DTPA- and DTPA-Bn-coupled counterparts [74]. Both $^{111}$In-DTPA-3P-RGD$_2$ and $^{111}$In-DTPA-Bn-3P-RGD$_2$ showed a much faster tumor washout and lower tumor-to-background ratios than $^{111}$In-DOTA-3P-RGD$_2$. However, a rapid increase of tumor-to-organ ratios over time for $^{64}$Cu-NODAGA-c(RGDfK) was not observed for $^{64}$Cu-DOTA-linked RGD peptide [75]. Long retention in the blood is most likely a consequence of the limited in vivo stability of the $^{64}$Cu-DOTA complex and the transchelation of $^{64}$Cu to blood proteins.

The effect of varying the chelator on the stability and radiochemical properties of the $^{68}$Ga-labeled BN agonist AMBA has been reported [76]. Although NODAGA showed remarkable advantages over DOTA- and NOTA-coupled AMBA (from a radiochemical point of view), further in vivo comparison is required to confirm the superiority of NODAGA over other chelators.

![Figure 3. Schematic overview illustrating the structural differences of the four chelators used in this study, NOTA, NODAGA, DOTA and DOTAGA. The chelators are shown as conjugated to a peptide via one of the carboxylic acid groups.](image)

The profound influence of the labeling chemistry on the tumor-targeting properties observed for small targeting agents was less expected for larger molecules. However, this effect was also reported for larger targeting agents such as affibody molecules [77]. The $^{111}$In-labeled anti-HER2 affibody molecule NODAGA-Z$_{\text{HER2:2395}}$ demonstrated higher tumor-to-organ ratios compared to $^{111}$In-DOTA-Z$_{\text{HER2:2395}}$ despite the lower uptake in HER2-expressing tumors. Due to rapid blood clearance and
lower uptake in normal organs, the NODAGA-conjugated affibody molecule improved biodistribution compared to the DOTA-coupled counter-part [78]. Similar results were obtained when the radionuclide was changed to $^{68}$Ga. The $Z_{HER2:2395}$ affibody molecule, which was labeled with $^{68}$Ga using a maleimido derivative of NODAGA, showed a higher tumor-to-liver ratio compared to the $^{68}$Ga-DOTA-conjugated analog [79].

In this thesis, we investigated the influence of chelators on the in vitro and in vivo performance of PEG2-RM26. As chelators, we have chosen four homologous macrocyclic chelators: NOTA, NODAGA, DOTA and DOTAGA (Figure 3).

Detecting ligand interactions with G protein-coupled receptors on living cells in real time to measure the binding affinity

The development of radiotracers for the visualization of cancer-associated molecular abnormalities or the treatment of target-expressing cancers requires careful characterization of the interaction of a potential targeting probe with its target. In the development of novel radiolabeled ligands, the measurement of their affinity to the target is critical.

There are some end-point assays in which either direct ligand-receptor binding is measured, as in saturation assays, or binding is measured indirectly by signaling assays that measure the activity of the downstream signaling pathway. Both saturation and activity assays for affinity measurement typically rely on equilibrium being reached at the time of treatment. This requirement has been identified as a potential source of error for binders with subnanomolar $K_D$ values. In saturation binding studies, the time to equilibrium ($T$) depends on the association rate ($k_a$), the dissociation rate ($k_d$), and the radioligand concentration ($C$). According to the approximate expression, $T \approx \frac{3.5}{k_a \times C + k_d}$. For the strong interactions ($K_D << 1$ nM), the time to equilibrium can sometimes be many hours or even days, leading to practical difficulties using equilibrium-based end-point assays [80, 81].

Monitoring the ligand-receptor binding in real time is an alternative method to end-point assays that does not require the interaction to reach equilibrium to estimate the affinity. In addition, the time-resolved assays
provide information about not only the affinity but also the binding kinetics.

The heterogeneous nature of living cells is another important issue that should be considered. The information on the kinetics provided by surface plasmon resonance (SPR) instruments is based on purified proteins, which may be a poor representation of the real conditions in living cells. Therefore, it is also more accurate to determine the kinetics from ligand-receptor binding when receptors are located on intact living cells [82].

Furthermore, it is known that radiolabeling can change receptor-ligand binding affinity, and these changes can be more pronounced for small peptides than for larger proteins [83, 84]. Hence it is important to measure the affinity of the ligands after being labeled with radionuclides.

**Figure 4.** Schematic principle of real-time kinetic assay for radioligands. Target-bound signals are collected with detectors mounted above the highest position of a sloping and rotating cell dish in the LigandTracer devices. The detectors are collimated to read a certain liquid-depleted area of the cell dish. Cells are seeded in a local well-defined part of 10-cm cell dishes and placed inside the instruments on a tilted support. The opposite side of the cell dish is used as reference (background radioactivity). Finally amount of bound protein to the cells are registered after repeated differential measurements over time.
Thus far, most of the real-time interaction measurements have been made in cell-free systems using label-free technologies. These measurements have proved challenging for GPCRs. The difficulty of functionally expressing, purifying, and stabilizing GPCRs in sufficient quantities creates an obstacle to research using SPR technology for the detailed analysis of ligand-receptor interactions [85, 86, 87].

In this thesis, a novel simple and accurate technology (LigandTracer) was used to demonstrate how radioligand binding to GPCRs can be followed in real-time on living cells (Figure 4).

Bombesin analogs in the clinic

A limited number of radiolabeled BN analogs have been studied in humans. Most of these, including [Leu$^{13}$]BN [88], [Lys$^3$]BN [89], RP527 [90], and BZH3 [91] are BN agonists. It was reported that agonists were capable of eliciting physiological responses. In addition, high accumulation in the liver and/or hepatobiliary clearance have been reported for most of the studied tracers [92]. These biodistribution characteristics make imaging in the suprabadominal and/or abdominal region problematic.

BAY 86-7548, also known as RM2, was the first BN antagonist that was first studied in healthy men [93] and later in prostate cancer patients [94]. The pilot clinical study confirmed the favorable biodistribution of the $^{64}$Cu-labeled BN antagonist RM26 ($^{64}$Cu-CB-TE2A-AR06) with rapid clearance from organs with physiological GRPR expression and significantly longer retention in human PC, which resulted in steadily increasing tumor-to-normal tissue ratios over time [95]. Tumors were visualized with high contrast (tumor-to-prostate ratio > 4) at 4 h p.i. While these studies showed interesting results, more systematic studies involving more patients are needed to determine whether a BN agonist or antagonist is better for GRPR targeting.
Aims of this thesis

The aims of this thesis project were as follows:

- To develop BN antagonist-based imaging radiotracers for the detection of GRPR-expressing tumors using different PET and SPECT radionuclides. (Papers I and II)
- To establish a method to monitor the ligand-GPCR interaction in real time on cell-associated receptors without requiring the purification and stabilization of the receptors. (Paper III)
- To evaluate radiopeptide structure-related factors affecting the in vitro and in vivo characteristics of RM26; to study the influence of the length of the spacer moiety and the effect of different chelators on the targeting properties and biodistribution of RM26. (Papers IV and V)

Figure 5. Schematic representation of the projects regarding papers 1, 2, 3, 4 and 5.
The present investigation

Development of imaging radiotracers based on the BN antagonist RM26 for the detection of GRPR-expressing tumors (Papers I and II)


Rationale and aims
As was mentioned in the Introduction, there was a general consensus until recently that agonistic peptide ligands are preferable targeting moieties for the development of imaging agents. However, BN agonist-based radiotracers cause undesirable side effects when administered intravenously to patients, even in trace amounts. It was hypothesized that these issues could be avoided with radiopeptides based on BN antagonists.

The aim of this first study was to develop BN antagonist-based PET and SPECT tracers labeled with gallium-68 and indium-111 by the site-specific coupling of the macrocyclic chelator NOTA via a PEG₂ linker. We also aimed to establish a preclinical model for the in vitro and in vivo evaluation of GRPR-imaging agents.

Our reasoning in the selection of this tracer design was based on the following. NOTA is a versatile chelator, which permits labeling with ⁶⁸Ga and ¹¹¹In, and it is also capable of forming stable chelates with Al¹⁸F and ⁶⁴Cu. NOTA was coupled to the N-terminal of the peptide via PEG₂. This linker is long enough to provide distance between the chelator and the binding site of the RM26 antagonist. It was also used with the aim to decrease the lipophilicity of the conjugate and to modulate its pharmacokinetics.
Methods
The RM26 conjugated with NOTA via PEG₂ (NOTA-PEG₂-RM26, Figure 6) was synthesized and labeled with ⁶⁸Ga and ¹¹¹In. The labeling stability, binding specificity to GRPR-expressing cells, inhibition efficiency (IC₅₀), and retention and internalization by PC-3 cells of both compounds were investigated. The dissociation constant (Kₐ) of ¹¹¹In-NOTA-PEG₂-RM26 was measured using LigandTracer technology. The biodistribution of the dual isotope ¹¹¹In/⁶⁸Ga-NOTA-PEG₂-RM26 in both normal NMRI mice and PC-3-xenografted BALB/c nu/nu mice was studied.

Figure 6. Structural formula of NOTA-PEG₂-[D-Phe⁶, Sta¹³, Leu¹⁴]bombesin [6-14] (NOTA-PEG₂-RM26).

Results and discussion
This study was the first step toward developing an imaging radiotracer based on the BN-antagonist RM26.

NOTA-PEG₂-RM26 was labeled with ¹¹¹In and ⁶⁸Ga in greater than 98% yield. The labels were stable under challenge with a 500-fold excess of EDTA. Both ¹¹¹In- and ⁶⁸Ga-labeled analogs demonstrated saturable uptake in GRPR-expressing PC-3 cells (Figure 1 in paper I). The antagonistic activity of the radiotracers was confirmed by an in vitro internalization assay (Figure 2 in paper I). More than 6-fold lower radioactivity internalization was detected for ⁶⁸Ga- and ¹¹¹In-labeled NOTA-PEG₂-RM26 compared to the agonistic analog ¹²⁵I-Tyr⁴-BBN after 30 min incubation with a 1 nM concentration. More than twice as many binding sites were found for the BN-based antagonist compared to the universal agonist ¹²⁵I-Tyr⁴-BBN. These data are in agreement with earlier findings for a somatostatin-based antagonist [47] and a BN-based antagonist [58].

Almost 4-fold lower IC₅₀ values were measured for metal-loaded analogs compared to their non-labeled counterpart after 5 h incubation at 4°C (Figure 7). The positive charges at the N-terminal of BN analogs was reported to be beneficial for GRPR targeting by increasing the affinity, whereas negative charges decrease this affinity [46]. The overall charge of the gallium- or indium-NOTA compounds is +1, which could explain
the higher affinity for the metal-loaded analogs compared to non-labeled NOTA-PEG2-RM26. A K_D of less than 36 pM was determined for the 111In-labeled compound.

![Figure 7. Inhibition of 125I-Tyr^4-BBN binding to PC-3 cells with natGa-NOTA-PEG2-RM26, natIn-NOTA-PEG2-RM26, and non-labeled NOTA-PEG2-RM26.](image)

The biodistribution studies in NMRI mice (Table 3, Figure 4 in paper I) demonstrated fast blood and whole body clearance for both tracers via renal excretion. Kidney reabsorption was low for both counterparts. Moreover, the uptake of both 68Ga- and 111In-labeled peptides was specific in the abdominal area organs (pancreas, stomach and small intestine), which express GRPR.

The influence of the administered peptide dose on the biodistribution at 2 h p.i. of 111In-NOTA-PEG2-RM26 was minor. The tumor uptake after the injection of 15 pmol was significantly higher than uptake after the injection of lower or higher peptide doses. However, differences in tumor-to-organ ratios were not significant for doses between 3.6 and 45 pmol per mouse (Table 4, Figure 5 in paper I).

The biodistribution of both 111In-NOTA-PEG2-RM26 and 68Ga-NOTA-PEG2-RM26 in tumor-bearing mice was similar to the biodistribution in NMRI mice (Table 5, Figure 6 in paper I). The rapid blood and whole body clearance along with specific uptake in receptor-expressing organs and tumors was shown for both counterparts. However, 111In-NOTA-PEG2-RM26 showed more rapid blood clearance, and higher tumor uptake was measured for the 68Ga-labeled analog, which resulted in higher tumor-to-organ ratios. We speculate that the difference in blood clearance resulting in different tumor uptake could be due to the difference in the coordination geometries of 68Ga- and 111In-NOTA complexes. These co-
ordination differences might result in different strengths of interaction with blood proteins.

The high potential of $^{68}$Ga/$^{111}$In-NOTA-PEG$_2$-RM26 for the in vivo imaging of GRPR was confirmed by microPET/CT and gamma camera images (Figure 8). Based on biodistribution data, 2-3 h p.i. was a suitable time point for image acquisition, when higher tumor-to-organ ratios were obtained. These data demonstrate that $^{68}$Ga is a suitable label for NOTA-PEG$_2$-RM26, despite its short half-life.

**Figure 8.** Imaging of GRPR expression in PC-3-xenografted BALB/c nu/nu mice. The gamma camera image (A) was acquired at 3 h p.i. from an animal injected with 45 pmol of $^{111}$In-NOTA-PEG$_2$-RM26 (260 kBq). MicroPET/CT images were acquired at 1 h (B) and 2 h (C) p.i. from animal injected with $^{68}$Ga-NOTA-PEG$_2$-RM26 (45 pmol, 300 kBq).

**Conclusions**

This study demonstrated the potential of using NOTA-PEG$_2$-RM26, a BN antagonist-based imaging probe, for the visualization of GRPR-expressing tumors with SPECT and PET. It was shown that the biodistribution and kinetics of a peptide can be altered by small modifications, e.g., exchanging the radionuclide.
Table 3. Biodistribution of $^{68}$Ga-NOTA-PEG$_2$-RM26 and $^{111}$In-NOTA-PEG$_2$-RM26 (total injected mass 23 pmol) in male NMRI mice. One group of animals was pre-injected (i.v.) with 20 nmol of non-labeled NOTA-PEG$_2$-RM26 (designated as blocked). Data are presented as mean percentage of injected dose per gram of tissue (%ID/g ± SD, n=4).

<table>
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<th>Organ and tissue</th>
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<th></th>
<th></th>
<th>$^{111}$In-NOTA-PEG$_2$-RM26</th>
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<td>1 h (blocked)</td>
<td></td>
<td></td>
<td>1 h (blocked)</td>
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$^a$Significant difference (p<0.05) between 1 h and 1 h blocked for $^{68}$Ga-NOTA-PEG$_2$-RM26. $^b$Significant difference between 1 h and 2 h for $^{68}$Ga-NOTA-PEG$_2$-RM26. $^c$Significant difference between 1 h and 1 h blocked for $^{111}$In-NOTA-PEG$_2$-RM26. $^d$Significant difference between 1 h and 2 h for $^{111}$In-NOTA-PEG$_2$-RM26.
Table 4. Dose escalation study in BALB/c nu/nu male mice bearing PC-3 xenografts at 2 h p.i. of $^{111}$In-NOTA-PEG$_2$-RM26. Uptake values are expressed as an average percentage of injected dose per gram of tissue and standard deviation based on data from four mice (% ID/g ± SD).

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</tr>
<tr>
<td>Kidney</td>
<td>3.8±0.6</td>
<td>5±1</td>
<td>3.9±0.3</td>
<td>5±1</td>
</tr>
<tr>
<td>Tumor</td>
<td>7±2</td>
<td>11±1$^{ab}$</td>
<td>9±1</td>
<td>9±1</td>
</tr>
<tr>
<td>Muscle</td>
<td>0.12±0.03</td>
<td>0.15±0.05</td>
<td>0.14±0.04</td>
<td>0.13±0.04</td>
</tr>
<tr>
<td>Bone</td>
<td>0.26±0.10</td>
<td>0.30±0.08</td>
<td>0.4±0.2</td>
<td>0.27±0.06</td>
</tr>
</tbody>
</table>

$^{a}$Significant difference (p<0.05) between 3.6 and 15 pmol. $^{b}$Significant difference between 15 and 25 pmol. $^{c}$Significant difference between 15 and 45 pmol.
Table 7. Biodistribution of $^{68}$Ga-NOTA-PEG$_2$-RM26 and $^{111}$In-NOTA-PEG$_2$-RM26 (total injected mass 45 pmol) after injection in male BALB/c nu/nu male mice bearing PC-3 xenografts. One group of animals was co-injected (i.v.) with 20 nmol of non-labeled NOTA-PEG$_2$-RM26 (designated as blocked). Data are presented as mean percentage of injected dose per gram of tissue (%ID/g ± SD, n=4).

<table>
<thead>
<tr>
<th>Organ &amp; tissue</th>
<th>$^{68}$Ga-NOTA-PEG$_2$-RM26</th>
<th>$^{111}$In-NOTA-PEG$_2$-RM26</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>1 h (blocked)</td>
<td>3 h</td>
</tr>
<tr>
<td>Blood</td>
<td>0.68±0.04b</td>
<td>0.32±0.05c</td>
</tr>
<tr>
<td>Lung</td>
<td>0.78±0.07</td>
<td>0.7±0.1</td>
</tr>
<tr>
<td>Liver</td>
<td>1.83±0.07</td>
<td>1.7±0.1</td>
</tr>
<tr>
<td>Spleen</td>
<td>1.04±0.06b</td>
<td>0.8±0.1c</td>
</tr>
<tr>
<td>Pancreas</td>
<td>5.7±0.8</td>
<td>0.25±0.02d</td>
</tr>
<tr>
<td>Stomach</td>
<td>1.89±1.04</td>
<td>0.5±0.1</td>
</tr>
<tr>
<td>Small intestine</td>
<td>1.4±0.5</td>
<td>0.27±0.05</td>
</tr>
<tr>
<td>Kidney</td>
<td>2.7±0.4</td>
<td>1.4±0.1</td>
</tr>
<tr>
<td>Tumor</td>
<td>8.1±0.4b</td>
<td>0.3±0.1a</td>
</tr>
<tr>
<td>Muscle</td>
<td>0.09±0.01</td>
<td>0.03±0.01</td>
</tr>
<tr>
<td>Bone</td>
<td>0.28±0.05</td>
<td>0.25±0.07</td>
</tr>
</tbody>
</table>

*Significant difference (p<0.05) between 1 h and 1 h blocked for each of the compounds. *Significantly (p<0.05) higher uptake in comparison with counterpart for 1 h p.i. *Significantly (p<0.05) higher uptake in comparison with counterpart for 3 h p.i.
Paper II. In vitro and in vivo evaluation of a \(^{18}\text{F}\)-labeled high affinity NOTA-conjugated bombesin antagonist as a PET ligand for GRPR-targeted tumor imaging.

**Rationale and aims**

The potential of NOTA-PEG\(_2\)-RM26 as an imaging agent when labeled with gallium-68 and indium-111 was demonstrated in Paper I. The second study was designed to test the hypothesis that using the more long-lived positron-emitting radionuclide fluorine-18 as a label would further improve the sensitivity of imaging using NOTA-PEG\(_2\)-RM26.

The sensitivity of imaging depends directly on the contrast. The image contrast is determined by the ratio of accumulated radioactivity in the tumor to that in non-target normal tissue. Fast clearance of the \(^{111}\text{In}/^{68}\text{Ga}\)-NOTA-PEG\(_2\)-RM26 from blood and the receptor-expressing organs together with high uptake and long retention of the radioactivity in tumors resulted in increasing tumor-to-normal tissue ratios over time. The use of \(^{18}\text{F}\), with its longer half-life of 109.7 min compared to 67.6 min for \(^{68}\text{Ga}\), may provide an opportunity for imaging at later time points when the radiotracer would clear better from normal organs and higher image contrast could be obtained. In addition, the low positron energy of \(^{18}\text{F}\), which limits tissue penetration (2.3 mm in water) compared to \(^{68}\text{Ga}\) (8.9 mm in water) is well suitable for high-resolution PET images.

The availability of \(^{18}\text{F}\) is another important attribute. \(^{18}\text{F}\) is the most widely used radioisotope for PET scans, which is produced in large quantities for the synthesis of \(^{18}\text{F}-\text{FDG}\) (the main radiopharmaceutical for PET scans). In addition, radiolabeling peptides with \(^{18}\text{F}\) using chelate-based AlF-chemistry provides a relatively high yield of fluorinated peptides. This one-step method utilizes the strength of the Al-F bond and the ability of NOTA to chelate aluminum [96].

The aim of this study was to test the hypothesis that using \(^{18}\text{F}\) would enable imaging at later time points compared to \(^{68}\text{Ga}\), which would improve imaging contrast due to better clearance of non-bound tracer.
Methods
NOTA-PEG2-RM26 was labeled with $^{18}$F using aluminum-fluoride chelation. In vitro binding specificity and cellular processing tests were performed. The inhibition efficiency (IC$_{50}$) of the Al$^{nat}$F-NOTA-PEG2-RM26 was compared to previously tested natGa-loaded peptide using $^{125}$I-Tyr$^4$-BBN as the radioligand displacer. The biodistribution in NMRI mice and the targeting of GRPR-overexpressing PC-3 xenografts in nude mice were studied.

Results and discussion
The peptide was labeled with $^{18}$F within 1 h with a decay-corrected yield of 60-65%. The radiochemical purity was greater than 98%. Receptor binding capacity of NOTA-PEG2-RM26 was preserved after radiolabeling (Figure 3A in Paper II).

The low internalization of radioactivity into the cells was in good agreement with $^{68}$Ga- and $^{111}$In-labeled analogs (3.2 ± 1.4% of cell-bound radioactivity at 30 min of incubation). The IC$_{50}$ value measured for Al$^{nat}$F-NOTA-PEG2-RM26 was in the same low nanomolar range as natGa-NOTA-PEG2-RM26 after 3 h incubation at 4°C (Figure 9).

![Figure 9. Inhibition of $^{125}$I-Tyr$^4$-BBN binding to PC-3 cells with natGa-NOTA-PEG2-RM26 or Al$^{nat}$F-NOTA-P2-RM26. Data are mean values ± SD of 3 culture dishes.](image)

<table>
<thead>
<tr>
<th>Variant</th>
<th>IC$_{50}$ (nM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>natGa-NOTA-PEG2-RM26</td>
<td>3.5±0.5</td>
</tr>
<tr>
<td>Al$^{nat}$F-NOTA-PEG2-RM26</td>
<td>4.4±0.8</td>
</tr>
</tbody>
</table>

Al$^{18}$F-NOTA-PEG2-RM26 demonstrated fast blood clearance via renal excretion, low kidney retention and liver uptake, and high and specific uptake in the tumors and receptor-positive organs similar to $^{111}$In/$^{68}$Ga-NOTA-PEG2-RM26 (Table 6, Figure 4 in paper II). Low uptake in bones was an indication of the high in vivo stability of the Al$^{18}$F-NOTA chelation in Al$^{18}$F-NOTA-PEG2-RM26. However, the washout of Al$^{18}$F-
NOTA-PEG₂-RM26 from PC-3 xenografts was faster than $^{111}$In-NOTA-PEG₂-RM26 (the biodistribution data obtained in previous project) (Table 7, Figure 6A in paper II). This result contradicts the results obtained for affibody molecules [97]; Al$^{18}$F-NOTA-ZHER2:2395 showed tumor retention comparable with $^{111}$In-NOTA-ZHER2:2395 in SKOV-3 xenografts.

The biodistribution data suggest that 3 h p.i. would be the optimal time point for in vivo imaging when the tumor-to-organ ratio was the highest (Table 7, Figure 6B in paper II). The biodistribution data were confirmed by in vivo imaging using a clinical PET scanner (Figure 10).

Among compounds tested in this part of the project ($^{111}$In, $^{68}$Ga and Al$^{18}$F-labeled NOTA-PEG₂-RM26), $^{68}$Ga-NOTA-PEG₂-RM26 showed slightly higher decay-corrected tumor uptake (Table 1 in paper II). However, a non-decay-corrected tumor uptake of Al$^{18}$F-NOTA-PEG₂-RM26 at 3 h p.i. would not be lower due to the longer half-life of $^{18}$F. Al$^{18}$F-NOTA-PEG₂-RM26 provided higher tumor-to-blood, tumor-to-lung, tumor-to-liver, and tumor-to-spleen ratios compared with $^{68}$Ga-NOTA-PEG₂-RM26 at 3 h p.i (Figure 11). There was no difference in tumor-to-muscle and tumor-to-bone ratios for these two PET imaging agents. In addition, the short positron range of $^{18}$F compared to $^{68}$Ga can improve image quality significantly.

**Figure 10.** Imaging of GRPR-expression in PC-3-xenografted BALB/c nu/nu male mice 3 h p.i. The animal was injected with 45 pmol of Al$^{18}$F-NOTA-PEG₂-RM26 (~2 MBq).
Table 6. Biodistribution of Al\textsuperscript{18}F-NOTA-PEG\textsubscript{2}-RM26 in male NMRI mice 1 h p.i.

<table>
<thead>
<tr>
<th>Organ &amp; tissue</th>
<th>1 h</th>
<th>1 h blocked</th>
<th>Total injected mass of radiolabeled conjugate was 45 pmol and animals in blocked group were co-injected with 20 nmol of non-labeled peptide. Data are presented as the mean percentage of the injected dose per gram of tissue (%ID/g ± SD, n=4). The asterisks denote significant differences between the blocked and non-blocked animals 1 h p.i (p&lt;0.05).</th>
</tr>
</thead>
<tbody>
<tr>
<td>Blood</td>
<td>0.3±0.1</td>
<td>0.3±0.1</td>
<td></td>
</tr>
<tr>
<td>Lung</td>
<td>0.3±0.2</td>
<td>0.34±0.13</td>
<td></td>
</tr>
<tr>
<td>Liver</td>
<td>1.2±0.3</td>
<td>1.4±0.4</td>
<td></td>
</tr>
<tr>
<td>Spleen</td>
<td>0.6±0.3</td>
<td>0.16±0.08*</td>
<td></td>
</tr>
<tr>
<td>Pancreas</td>
<td>14±6</td>
<td>0.4±0.1*</td>
<td></td>
</tr>
<tr>
<td>Stomach</td>
<td>2±1</td>
<td>0.3±0.1*</td>
<td></td>
</tr>
<tr>
<td>Small intestine</td>
<td>4.05±1.53</td>
<td>1.7±0.7*</td>
<td></td>
</tr>
<tr>
<td>Kidney</td>
<td>2.8±0.5</td>
<td>3.4±0.8</td>
<td></td>
</tr>
<tr>
<td>Muscle</td>
<td>0.09±0.03</td>
<td>0.08±0.03</td>
<td></td>
</tr>
<tr>
<td>Bone</td>
<td>0.3±0.2</td>
<td>0.20±0.07</td>
<td></td>
</tr>
</tbody>
</table>

Figure 11. Tumor-to-organ ratios for \textsuperscript{68}Ga-NOTA-PEG\textsubscript{2}-RM26 (data from paper I) and Al\textsuperscript{18}F-NOTA-PEG\textsubscript{2}-RM26 (data from paper II) in male BALB/c nu/nu mice with PC-3 xenografts, 3 h p.i.

Conclusions

Because of the wide availability and superior imaging characteristics of \textsuperscript{18}F together with the simplicity of the facile Al\textsuperscript{18}F-NOTA chelation labeling chemistry compared to classical multi-step labeling methods, Al\textsuperscript{18}F-NOTA-PEG\textsubscript{2}-RM26 may be a good alternative or supplement to \textsuperscript{68}Ga-NOTA-PEG\textsubscript{2}-RM26 for clinical PET imaging.
**Table 7. Biodistribution of Al\(^{18}\)F-NOTA-PEG\(_2\)-RM26 in male BALB/c nu/nu mice bearing PC-3 xenografts.**

<table>
<thead>
<tr>
<th>Organ &amp; tissue</th>
<th>1 h</th>
<th>1 h (blocked)</th>
<th>3 h</th>
<th>6 h</th>
</tr>
</thead>
<tbody>
<tr>
<td>Blood</td>
<td>0.48±0.08</td>
<td>0.40±0.15</td>
<td>0.08±0.03</td>
<td>0.02±0.00</td>
</tr>
<tr>
<td>Lung</td>
<td>0.36±0.06</td>
<td>0.44±0.25</td>
<td>0.21±0.17</td>
<td>0.11±0.08</td>
</tr>
<tr>
<td>Liver</td>
<td>1.9±0.4</td>
<td>1.6±0.3</td>
<td>0.4±0.2</td>
<td>0.10±0.02</td>
</tr>
<tr>
<td>Spleen</td>
<td>1.0±0.2</td>
<td>0.67±0.23</td>
<td>0.10±0.03</td>
<td>0.15±0.08</td>
</tr>
<tr>
<td>Pancreas</td>
<td>13.1±0.2</td>
<td>0.29±0.15*</td>
<td>2.7±0.7</td>
<td>0.15±0.05</td>
</tr>
<tr>
<td>Stomach</td>
<td>2.4±0.4</td>
<td>0.38±0.14*</td>
<td>1.4±0.3</td>
<td>0.33±0.08</td>
</tr>
<tr>
<td>Small intestine</td>
<td>4.06±1.76</td>
<td>2.6±0.9</td>
<td>0.9±0.4</td>
<td>0.13±0.06</td>
</tr>
<tr>
<td>Kidney</td>
<td>3.61±0.26</td>
<td>3.9±0.8</td>
<td>1.7±0.2</td>
<td>0.27±0.06</td>
</tr>
<tr>
<td>Tumor</td>
<td>6.32±0.86</td>
<td>0.75±0.15*</td>
<td>5.53±0.75</td>
<td>2.25±0.65</td>
</tr>
<tr>
<td>Muscle</td>
<td>0.10±0.03</td>
<td>0.09±0.04</td>
<td>0.03±0.01</td>
<td>0.02±0.01</td>
</tr>
<tr>
<td>Bone</td>
<td>0.30±0.08</td>
<td>0.2±0.2</td>
<td>0.17±0.08</td>
<td>0.22±0.08</td>
</tr>
</tbody>
</table>

| Tumor to organ   |         |               |           |           |
| Blood            | 13.27±1.24|             | 87±42     | 89±25     |
| Lung             | 17.4±0.8 | 36±20        | 32.5±28.8 |           |
| Liver            | 3.4±0.2  | 14±8         | 23±3      |           |
| Spleen           | 6.5±1.2  | 61±19        | 15.5±8.3  |           |
| Pancreas         | 0.49±0.04| 2.2±0.6      | 14.7±2.3  |           |
| Stomach          | 2.7±0.1  | 4.25±1.16    | 6.4±0.6   |           |
| Small intestine  | 1.7±0.6  | 7.4±3.7      | 15.5±6.5  |           |
| Kidney           | 1.74±0.16 | 3.2±0.6    | 8.4±0.8   |           |
| Muscle           | 66.9±12.8 | 159±47      | 138±108   |           |
| Bone             | 21.7±2.8 | 38±16        | 10.8±1.7  |           |

Total injected mass of radiolabeled conjugate was 45 pmol and animals in blocked group were co-injected with 20 nmol of non-labeled peptide. Data are presented as the mean percentage of the injected dose per gram of tissue (%ID/g ± SD, n=4). The asterisks denote significant differences between the blocked and non-blocked animals 1 h p.i (p<0.05).
Paper III. Detecting ligand interactions with G protein-coupled receptors on living cells in real time.

Rationale and aims

The careful characterization of the interaction of a potential targeting probe with its target, such as measuring its affinity to the corresponding target, is essential for the development of novel radiotracers. The methods typically used to measure ligand-receptor affinity are end-point assays, which rely on equilibrium being reached during incubation. This requirement is a potential source of error for high-affinity binders, leading to underestimation of the affinity. In contrast, time-resolved interaction measurements using intact cells would provide information about binding kinetics and overcome the difficulties of the expression, purification and stabilization of GPCRs in sufficient quantities.

The aim of the present study was to establish a method for real-time measurements of radioligand-GPCR (RM26-GRPR) interactions in living cells.

Methods

$^{111}$In-labeled BN-antagonist ($^{111}$In-NOTA-PEG$_6$-RM26) binding to the naturally expressed GRPR system was used to evaluate the time-resolved ligand-GPCR binding assays in their true environment. The affinity and kinetic properties, such as association and dissociation rates were measured by following the interactions over time using LigandTracer technology.

Results and discussion

The interaction of $^{111}$In-NOTA-PEG$_6$-RM26 with GRPR was monitored on PC-3 cells (Figure 12A). The data fit to the one-to-one kinetic model was poor, suggesting that the interaction is not homogeneous and more than one type of interaction exists simultaneously. Later, when the real-time interaction data were analyzed with the Interaction Map method, two peaks were visible, indicating two parallel interaction types (Figure 12B).
Figure 12. (A) Binding of $^{111}$In-NOTA-PEG$_6$-RM26 to GRPR on PC-3 cells. Data is presented as duplicates, measured in separate dishes (grey and black). (B) Interaction Map, calculated from the black curve of (A). The map shows the presence of two parallel interactions, i and ii, with different kinetic constants ($k_a$ and $k_d$). The darker peak (peak i), represents the stronger contribution which has higher recognition ($k_a$) and stability ($k_d$), thus higher affinity.

Kinetic parameters are presented in Table 8. The two different interactions for the BN analog on PC-3 cells can be explained either by different conformations of the receptors or post-translational modification.

Table 8. The summary of kinetic parameters for $^{111}$In-NOTA-PEG$_6$-RM26–GRPR interaction.

<table>
<thead>
<tr>
<th>Radiotracer</th>
<th>$^{111}$In-NOTA-PEG$_6$-RM26</th>
</tr>
</thead>
<tbody>
<tr>
<td>Interaction</td>
<td>i</td>
</tr>
<tr>
<td>$k_a$ (M$^{-1}$s$^{-1}$)</td>
<td>$(5.9 \pm 0.9) \times 10^5$</td>
</tr>
<tr>
<td>$k_d$ (s$^{-1}$)</td>
<td>$(1.4 \pm 0.2) \times 10^{-5}$</td>
</tr>
<tr>
<td>$K_D$ (M)</td>
<td>$(24.2 \pm 0.2) \times 10^{-12}$</td>
</tr>
<tr>
<td>Weight (%)</td>
<td>$73.4 \pm 4.0$</td>
</tr>
<tr>
<td>Interaction</td>
<td>ii</td>
</tr>
<tr>
<td>$k_a$ (M$^{-1}$s$^{-1}$)</td>
<td>$(2.4 \pm 1.8) \times 10^5$</td>
</tr>
<tr>
<td>$k_d$ (s$^{-1}$)</td>
<td>$(2.1 \pm 0.7) \times 10^{-3}$</td>
</tr>
<tr>
<td>$K_D$ (M)</td>
<td>$(15.6 \pm 13.4) \times 10^{-9}$</td>
</tr>
<tr>
<td>Weight (%)</td>
<td>$25.2 \pm 3.7$</td>
</tr>
</tbody>
</table>

The IC$_{50}$ value is another parameter that reflects the affinity of the conjugate for its receptor. However, the accuracy of IC$_{50}$ measurements relies on equilibrium being reached at the time of the treatment (end-point). This requirement may lead to the underestimation of binding affinity because the time needed to reach equilibrium could be several hours for high-affinity binders. In contrast, $K_D$ values (obtained with the Ligand-Tracer instrument) were measured by monitoring the ligand-receptor binding in real time and did not depend on equilibrium being achieved.
For instance, in our first study, the measured association ($k_a$) and dissociation ($k_d$) rates for $^{111}$In-NOTA-PEG$_2$-RM26 were approximately $3.3 \times 10^5$ s$^{-1}$M$^{-1}$ and $1.2 \times 10^{-5}$ s$^{-1}$, respectively, which would indicate that more than 8 h of incubation is necessary to achieve equilibrium ($T \approx 3.5\sqrt{\frac{k_a \times C + k_d}{k_d}}$) for a 0.3 nM concentration of the ligand. This duration necessary to reach equilibrium could explain the more than 25-fold lower IC$_{50}$ value (where cells were incubated for 5 h) for $^{nat}$In-NOTA-PEG$_2$-RM26 compared with the K$_D$ value measured in a real-time interaction assay for $^{111}$In-labeled NOTA-PEG$_2$-RM26. However, IC$_{50}$ measurement is a relatively appropriate method to compare the affinity of different conjugates and exclude binders with lousy affinities.

**Conclusions**

This project presents a method for providing information about binding kinetics by measuring real-time interactions with GPCRs in a living system without needing to purify and stabilize the receptors. With the use of advanced tools, e.g., the Interaction Map, the interaction heterogeneity related to the complexity of living cells can be further evaluated. The application of this method might improve the understanding of ligand-GPCR interactions.
Evaluation of tracer structure-related factors affecting binding specificity and sensitivity of RM26 (Paper IV and V)

Paper IV. The effect of mini-PEG-based spacer length on binding and pharmacokinetic properties of a $^{68}$Ga-labeled NOTA-conjugated antagonistic analog of bombesin.

**Rationale and aims**

As was indicated in the Introduction, the spacer between the receptor binding site and the chelator can be used not only to prevent the interference of the radiometal-chelator complex with the binding site of the peptide but also to improve the pharmacokinetic properties of the radiopeptides. The introduction of hydrophilic spacer moieties may improve the suboptimal biodistribution of BN analogs.

In this project, we aimed to investigate whether increasing the mini-PEG-spacer length (reducing the lipophilicity of the tracer) would further suppress the uptake of radioactivity in the liver, which would improve the visualization of liver metastases of GRPR-expressing breast cancer (BC). Because the liver is one of the major metastatic sites for BC, less radioactivity accumulation in the liver is desirable for tracer sensitivity. We have therefore studied the effect of varying the length of the mini-PEG spacer moiety on *in vitro* binding affinity as well as biodistribution, particularly the targeting properties and excretion pathway of the peptide.

Due to the flexibility of the PEG chain, which can mask the biologically active moiety of the peptide and impair the binding affinity, no more than 6 units of ethylene glycol were used.

**Methods**

Four NOTA-conjugated RM26 analogs with different mini-PEG-lengths (di-, tri-, tetra- and hexaethylene glycol) were synthesized, radiolabeled with $^{68}$Ga, and evaluated in *in vitro* and *in vivo* comparative studies. $^{111}$In was used in some *in vitro* studies, in which the short half-life of $^{68}$Ga was insufficient.
Results and discussion

All variants were labeled with high yield with $^{68}$Ga and $^{111}$In, and the labels were stable under EDTA challenge (Table 2 in paper IV). The log D values were -2.27±0.07, -2.47±0.06, -2.49±0.10 and -2.50±0.09 for PEG2-, PEG3-, PEG4- and PEG6-containing $^{68}$Ga-labeled variants, respectively. Small but significant increases in the overall hydrophilicity of the conjugates were observed by increasing the spacer length from two to three ethylene glycol units. A further decrease of lipophilicity was not significant.

Because the short half-life of $^{68}$Ga is not enough to measure the slow dissociation rate of the high-affinity conjugates, the $K_D$ values were measured for $^{111}$In-labeled analogs using LigandTracer technology. The calculated equilibrium dissociation constant values were in the low picomolar range due to rapid on-rates and slow off-rates ($K_D$ values were 23±13, 5±3, 8±5 and 18±8 pmol for PEG2, PEG3, PEG4 and PEG6 variants, respectively). The IC$_{50}$ values of natGa-loaded conjugates slightly increased as the number of PEG units increased (Figure 13).

Figure 13. Inhibition of $^{125}$I-Tyr$^4$-BBN binding to PC-3 cells with natGa-NOTA-PEG$_n$-RM26 ($n = 2$, 3, 4 and 6). Cell monolayers were incubated 3 h at 4°C.

The biodistribution profiles of the $^{68}$Ga-labeled analogs in NMRI mice were similar. No significantly different uptake was observed in the majority of the organs, neither in non-target organs nor in receptor-positive organs (Figure 14, Table 5 in paper IV). However, the accumulation of radioactivity for the PEG3 analog in the liver was slightly but significantly lower (p<0.05) than the uptake of PEG4 and PEG6 analogs.

On the basis of the measured affinity and biodistribution data in NMRI mice, $^{68}$Ga-NOTA-PEG3-RM26 was selected as the most promising conjugate for biodistribution, in vivo binding specificity, and imaging studies.
in tumor-bearing mice. Specific uptake was found in receptor-positive organs and the tumor xenografts formed by PC (PC-3) and BC (BT-474) cells (Figure 15, Table 6 in paper IV). Both tumor xenografts were clearly visualized with a microPET/CT camera at 2 h p.i (Figure 16). We speculate that implanted estradiol pellets may affect experimental outcomes because the radioactivity accumulation in the liver and pancreas was significantly lower in mice implanted with BT-474 cells both in *ex vivo* measurements and in imaging studies.

Figure 14. Biodistribution of $^{68}$Ga-NOTA-PEG$_n$-RM26 ($n = 2, 3, 4$ and $6$) in female NMRI mice at (A) 1 h and (B) 2 h p.i. Data are presented as the mean percentage of the injected dose per gram of tissue (%ID/g ± SD, $n=3$). Uptakes in GI tract and carcass are presented as %ID per whole sample.

**Conclusions**

This study demonstrates that the addition of only a few PEGs to the spacer sequence is not sufficient to change appreciably the biodistribution of NOTA-PEG$_n$-RM26. The hepatic uptake was low for all the conjugates, and the difference in the radioactivity accumulated in the liver was marginal when di-, tri-, tetra- and hexaethylene glycol were compared. However, the modification of the spacer moiety may be suitable for fine-tuning the targeting properties of tracers.
Figure 15. In vivo binding specificity test and biodistribution of $^{68}$Ga-NOTA-PEG$_3$-RM26 in female BALB/c nu/nu mice bearing (A) PC-3 or (B) BT-474 xenografts.

Figure 16. Imaging of GRPR-expression 2 h p.i. in (A) PC-3 and (B) BT-474 xenografts in BALB/c nu/nu female mice. The mice were injected with 45 pmol of $^{68}$Ga-NOTA-PEG$_3$-RM26.
Paper V. The effect of macrocyclic chelators on targeting properties of $^{68}$Ga-labeled gastrin-releasing peptide receptor antagonist RM26

**Rationale and aim**

The chelators are used to coordinate metal radionuclides and attach them to the targeting peptides. However, they may also modify the targeting properties of a radiopeptide. Different chelator-metal complexes have different charge and structures, which might affect their interaction with adjacent amino acids in the targeting peptides and determine their preferred conformation in solution, thus influencing their interaction with a target receptor. Furthermore, the variation of charge, conformation, and distribution of lipophilic patches along a radiopeptide may influence its off-target interactions, e.g., binding to the blood proteins and the scavenger receptors in excretory organs.

The aim of this study was to select an optimal chelator for labeling PEG$_2$-RM26 with $^{68}$Ga. The commercially available macrocyclic chelators NOTA, NODAGA, DOTA and DOTAGA, which provide thermodynamically stable and kinetically inert chelates with gallium, were selected for this study. The highest tumor-to-organ ratios were selected as an optimization criterion.

**Methods**

In this investigation, four homologous macrocyclic chelators, NOTA, DOTA, NODAGA and DOTAGA, were coupled to PEG$_2$-RM26. The properties of the $^{68}$Ga-labeled analogs, including labeling efficiency, stability, *in vitro* binding specificity, and affinity, were compared among the four radioligands. Their biodistributions were compared in normal and tumor-bearing mice.

**Results and discussion**

All four conjugates were labeled with $^{68}$Ga with high yield and demonstrated high stability in EDTA challenge (Table 1 in paper V). The compounds demonstrated specific uptake and slow internalization after binding to the receptors (Figure 1 and 3 in paper V). However, the DOTA-conjugated analog showed two-fold greater internalization compared with NOTA-, NODAGA- and DOTAGA-conjugated peptides. This effect is
similar to that of the same chelator on the full antagonist somatostatin analog 406-040-15, which converted to a full agonist when coupled with DOTA (406-051-20) [73].

This study demonstrates that the tracer affinity depends on the chelator. Although all natGa-loaded variants showed subnanomolar IC50 values, natGa-NOTA-PEG2-RM26 demonstrated the highest affinity (Figure 17). It is already known that the positive charge at the N-terminus of BN analogs increases the affinity whereas negative charge decreases their affinity [46, 98]. The overall charges of the NOTA, NODAGA, DOTA and DOTAGA complexes with gallium are +1, 0, 0, and −1, respectively (Chart 2 in paper V), which can explain almost 4-fold lower IC50 value of natGa-NOTA-PEG2-RM26 compared to natGa-DOTAGA-PEG2-RM26.

<table>
<thead>
<tr>
<th>Variant</th>
<th>IC50 (nM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>natGa-NOTA-PEG2-RM26</td>
<td>2.3±0.2</td>
</tr>
<tr>
<td>natGa-NODAGA-PEG2-RM26</td>
<td>3.0±0.3</td>
</tr>
<tr>
<td>natGa-DOTA-PEG2-RM26</td>
<td>2.9±0.3</td>
</tr>
<tr>
<td>natGa-DOTAGA-PEG2-RM26</td>
<td>10.0±0.6</td>
</tr>
</tbody>
</table>

Biodistribution studies in NMRI mice demonstrated rapid blood clearance via kidney ultrafiltration for all 68Ga-labeled conjugates (Figure 18, Table 2 in paper V). However, NOTA- and NODAGA-conjugated analogs showed lower normal organ (lung, muscle and bone) uptake compared to DOTA- and DOTAGA-coupled counterparts. The radioactivity in blood was twice as high for 68Ga-DOTAGA-PEG2-RM26 compared to the NOTA-conjugated counterpart 2 h p.i.

In good agreement with the biodistribution in NMRI mice, NOTA- and NODAGA-conjugated analogs demonstrated lower uptake in normal organs in tumor-bearing mice biodistribution studies (Figure 19, Table 3 in paper V). Better blood clearance was also observed for these analogs compared to DOTA- and DOTAGA-coupled compartments. The higher affinity of NOTA-PEG2-RM26 was reflected in a higher tumor uptake and resulted in higher tumor-to-non-tumor ratios (Figure 20, Table 4 in paper V).
Figure 18. The biodistribution results of $^{68}$Ga-X-PEG$_2$-RM26 (X = NOTA, NODAGA, DOTA and DOTAGA) in NMRI mice at (A) 1 h and (B) 2 h p.i.
**Figure 19.** The biodistribution and in vivo binding specificity test results of $^{68}$Ga-X-PEG$_2$-RM26 (X = NOTA, NODAGA, DOTA and DOTAGA) in PC-3 xenografted BALB/c nu/nu mice at 2 h p.i.
Conclusions

This study suggests that the modification of the chelating moiety can appreciably alter the targeting properties of a peptide. In this study, the triaza chelators NOTA and NODAGA provided better imaging properties (higher tumor-to-organ ratios) compared to the tetraaza chelators DOTA and DOTAGA for $^{68}$Ga-labeled PEG$_2$-RM26. $^{68}$Ga-NOTA-PEG$_2$-RM26 was the best conjugate, providing significantly higher tumor-to-lung, tumor-to-kidney and tumor-to-muscle ratios compared with $^{68}$Ga-NODAGA-PEG$_2$-RM26.
Conclusions and perspectives

Accurately defining the location and extent of cancer is an important factor in treatment selection. Thus, the ability to visualize disease throughout the body is increasingly important for treatment planning, and many radiotracers have been developed for this purpose.

This thesis describes an in vitro and in vivo evaluation of radiolabeled antagonistic BN-analogs for PET and SPECT imaging of GRPR. The main aims were to develop radiotracers for the visualization of GRPR-expressing tumors and to optimize their targeting properties using different lengths of mini-PEG spacers and chelator moieties. It has been shown that BN receptor-based antagonists can perform as well as or even better in terms of high in vivo tumor uptake and long tumor retention than the corresponding agonists [47, 48]. Despite poor internalization into tumor cells, optimal pharmacokinetics of the antagonists suggests that the consensus of tracer development may be changed for GPCR targeting.

In this thesis, a high affinity, hydrophilic, antagonistic, BN-based imaging agent, RM26, was used. NOTA, allowing stable labeling with several radio- nuclides, e.g., $^{68}$Ga, $^{111}$In and $^{18}$F(AIF), was coupled to RM26 via a PEG$_2$ spacer. It was hypothesized that the incorporation of the mini-PEG spacer would decrease the lipophilicity and thus decrease the non-specific off-target uptake in healthy organs, particularly in the liver. NOTA-PEG$_2$-RM26 was labeled with $^{68}$Ga for PET and $^{111}$In for SPECT imaging, and the targeting properties of these peptides were directly compared in pre-clinical models.

The significant conclusion from this part of the study was that the radiometal-labeled NOTA-PEG$_2$-RM26 was rapidly cleared from the blood and whole body (even from receptor positive organs), but tumor uptake was high and stable, thus resulting in high tumor-to-organ ratios shortly after administration. The biodistribution profile of radiometal-labeled NOTA-PEG$_2$-RM26 demonstrated that short-lived positron-emitting isotopes (such as $^{68}$Ga and $^{18}$F) could be used for imaging of GRPR-expressing tumors using NOTA-PEG$_2$-RM26. However, tumor-to-organ ratios increased over time, which indicates that more long-lived positron-emitting radionuclides than $^{68}$Ga might be preferable radioisotopes for PET.
To test the hypothesis that a more long-lived label would provide better contrast and therefore better sensitivity, short-lived $^{68}$Ga ($T_{1/2} \sim 68$ min) was exchanged with the longer half-life radionuclide $^{18}$F ($T_{1/2} \sim 110$ min) for PET studies. The Al$^{18}$F-NOTA concept developed by McBride et al [93] was used for radiolabeling NOTA-PEG$_2$-RM26.

The main observation with regard to the biodistribution of Al$^{18}$F-NOTA-PEG$_2$-RM26 in PC-3 xenografted mice was that the radioactivity retention in the tumors was not as high as it was for the $^{111}$In-labeled counterpart. Therefore, an optimal imaging time for Al$^{18}$F-NOTA-PEG$_2$-RM26 should be approximately 3 h p.i. Al$^{18}$F-NOTA-PEG$_2$-RM26 provided higher tumor-to-blood, tumor-to-lung, tumor-to-liver, and tumor-to-spleen ratios compared with $^{68}$Ga-NOTA-PEG$_2$-RM26 at 3 h p.i. There was no difference in tumor-to-muscle and tumor-to-bone ratios for these two PET imaging agents. Wide availability and a short positron range in tissue make $^{18}$F an attractive label for NOTA-PEG$_2$-RM26. Al$^{18}$F-NOTA-PEG$_2$-RM26 might be alternative for $^{68}$Ga-NOTA-PEG$_2$-RM26 if a $^{68}$Ga generator is not available.

The literature suggests that the in vivo targeting properties of a tracer are not determined solely by the receptor targeting moiety of the tracer. These properties can be radically modified by a spacer or chelator. The influence of a mini-PEG spacer length and a macrocyclic chelator structure on the targeting properties of $^{68}$Ga-labeled RM26 were studied to further optimize its imaging properties.

The study comparing the biodistribution of $^{68}$Ga-labeled RM26 conjugated with NOTA via a mini-PEG polymer of different lengths showed that the length of the PEG-spacer had minor influence on the biological outcome when two, three, four and six ethylene glycol units were compared.

Comparing NOTA, NODAGA, DOTA and DOTAGA as chelators for labeling PEG$_2$-RM26 with $^{68}$Ga demonstrated the strong influence of the chelator on the targeting properties of this BN analog. In this study, triaza chelators provided appreciably better targeting properties than tetraaza counterparts. For imaging the local metastases of PC, high ratios of radioactivity in tumors to radioactivity in blood, muscle and bone are critical. The best variant, $^{68}$Ga-NOTA-PEG$_2$-RM26, provided almost 4-fold higher tumor-to-bone and tumor-to-blood and more than 7-fold higher tumor-to-muscle ratios compared to the poorer variant, $^{68}$Ga-DOTAGA-PEG$_2$-RM26.

The favorable biodistribution of $^{68}$Ga-NOTA-PEG$_2$-RM26, including rapid clearance from blood and tissues with physiological GRPR expression and long retention in GRPR-expressing prostate tumors, observed in the preclini-
clinical studies, make it the best candidate for imaging PC among tested variants. Clinical studies using $^{68}$Ga-NOTA-PEG$_2$-RM26 to image PC are currently planned in Uppsala University Hospital.

SPECT remains a more available clinical imaging modality than PET. $^{111}$In is a commonly used radionuclide for SPECT. We need to take into consideration that the choice of radionuclide is equally important as the choice of chelator. The substitution of $^{68}$Ga with $^{111}$In in complex with structurally different chelates might influence the biodistribution of targeting agents by interfering in different ways with both on-target and off-target interactions. Future studies should evaluate the possible effect of different radionuclides on the pharmacokinetics of the analogs with different chelators.

NOTA is also a suitable chelator for $^{61}$Cu and $^{64}$Cu, as long-lived PET radionuclides. Imaging with $^{61}$Cu- or $^{64}$Cu-labeled RM26 can be performed over several hours or next day because of the relatively long half-life of $^{61}$Cu (3.33 h) and $^{64}$Cu (12.7 h). This long half-life would allow for better radioactivity clearance from receptor-expressing organs and provide images with better contrast. $^{99m}$Tc (a cheap and widely used radionuclide in nuclear medicine) and $^{55}$Co (relatively long-lived PET radioisotope that forms stable complex with DOTA) should also be considered.

Another important study that needs to be considered is using RM26 in combination with PSMA inhibitors as dual targeting radiopharmaceuticals for the diagnosis, staging, and treatment of PC. PSMA inhibitors based on glutamate-urea-lysine analogs are promising targeting agents that were recently developed [99, 100]. The phase 1 study using two derivatives, $^{123}$I-MIP-1072 and $^{123}$I-MIP-1095, demonstrated the capability of PSMA inhibitors to detect lesions in soft tissue, bone and the prostate gland as early as 1-4 h after injection [101]. PSMA is expressed by nearly all prostate cancers, and its expression is further increased in late-stage, poorly differentiated, metastatic, and hormone-refractory carcinomas [102, 103]. In contrast, GRPR, with almost 100% expression in primary prostate tumors and over 50% in metastases, is highly expressed in early-stage PC, and its expression decreases during disease progression [103]. Therefore, a bi-specific heterodimeric molecule addressing both targets (PSMA and GRPR) simultaneously may significantly improve PC imaging and therapy. Targeting vectors covering two receptor entities, which could be used for the whole period of PC progression from onset to recurrent PC, might lead to an improved diagnostic sensitivity and therapeutic efficiency.
Acknowledgements

Over these past years I have learnt so much from so many and I would like to thank all those who supported me in my personal journey as well as a scientific or educational one.

I would like to express my deepest sense of gratitude to my supervisor Anna Orlova, for her support and her guidance during my work in the cell lab and animal experiments. Thanks a bunch for the everyday patience and inspiration.

My very special appreciation goes to my co-supervisor Vladimir Tolmachev for his bottomless scientific knowledge, his support and always helpful advice. I would also like to thank you and Anna for your hospitality and interesting discussions in and out of research at your home.

Special thanks go to my co-supervisors Bo Stenerlöw, for all the interesting radiobiology lectures and Irina Velikyan, for sharing her precious knowledge of $^{68}$Ga-labeling, purification and analysis methods.

Many thanks to Ola Åberg for the long hours you spent teaching me Al$^{18}$F-NOTA labeling.

I would like to thank Gunnar Lindeberg and Ulrika Rosenström for our fruitful collaboration and for providing us with the peptides we investigated in this thesis.

Thanks to Karl Andersson, Hanna Björkelund and Jonas Stenberg for their precious help and knowledge in experiments regarding LigandTracer technology.

Many thanks to all my group-mates at PPP and BMS, especially Bogdan Mitran who made all (that I don’t remember how many) inhibition experiments joyful, Mohamed Altai who always has simple and understandable explanations for complicated chemistry problems, Ram Kumar Selvaraju for all your helps in the imaging studies and of course disturbing me and my computer every now and then, Jennie Andersson, Maria Rosestedt, Hadis
Honarvar, Joanna Strand, Javad Garousi for all your helps in animal experiments.
Special thanks to my co-workers at PPP for the help and support they offered.

I want to express my love to each and every member of the women’s exclusive group, Tonge, Fernanda, Vicky, Maryam and Ruta. I am feeling home among you.

Nazila! Sanırım artık seninle Türkçe irtibat kura bilirim. Hastalıktan ve sağlaktan, İsveç’in karanlık ve soğuk günlerinde bir vefali koca gibi 😊 yanımda olduğun için memnunum. Seni her zaman özleyeceğim!

Gamzecik! Senin yerin benim için apayrı, Hep ‘’Mesut(la)’’ 😊 ol sen!

Many thanks to Maria, who made me meet the sweetest γιαγιά in the world. We should have asked her to make τραχανάς for us!

Mari and Ankur! Thanks for the cozy evenings with Italian food we had together. I still remember the sky trip 😊! Hope to have a chance to repeat it.

Erika, Arash and Anahita, I am really glad to know you. Arash! I promise one day I will start taking proteins with carbs, but I cannot promise about Creatine 😊!

Mom. and Dad. What can I say other than thank you! You have given me the greatest gifts of all, the chance at education and the freedom of choice. I would not be who and where I am today without your support.

Eshgham! My truly better other half, thanks for your unwavering support, patience and humor. I can boldly say that ‘’Hamsahoo loves you!’’
References


A doctoral dissertation from the Faculty of Pharmacy, Uppsala University, is usually a summary of a number of papers. A few copies of the complete dissertation are kept at major Swedish research libraries, while the summary alone is distributed internationally through the series Digital Comprehensive Summaries of Uppsala Dissertations from the Faculty of Pharmacy. (Prior to January, 2005, the series was published under the title “Comprehensive Summaries of Uppsala Dissertations from the Faculty of Pharmacy”.)

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