Chapter 5:

ALF-NOTA stabilized bombesin analogues for targeting GRP receptor-expressing Prostate Cancer

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Submitted
**ABSTRACT**

The gastrin-releasing peptide receptor (GRPR) is overexpressed in a variety of human tumors, including prostate cancer. Bombesin is a 14 amino acids peptide which selectively binds to GRPR. In this study we developed Al\(^{18}\)F-labeled lanthionine-stabilized bombesin analogues (C5 and C6 bombesins) for positron emission tomography (PET) imaging of GRPR expression in xenograft prostate cancer models. **Methods:** (Methyl)lanthionine-stabilized C5 and C6 bombesin analogues were conjugated with a NOTA-NHS chelator and radiolabeled with Al\(^{18}\)F by using aluminum fluoride one-pot method. Receptor-binding affinity of Al\(^{18}\)F-NOTA-BombesinC5 (Al\(^{18}\)F-NOTA-C5) and Al\(^{18}\)F-NOTA-BombesinC6 (Al\(^{18}\)F-NOTA-C6) was tested in PC-3 human prostate cancer cells. Internalization and efflux of both radiotracers were also evaluated. **Results:** Al\(^{18}\)F-NOTA-C5 and Al\(^{18}\)F-NOTA-C6 could be labeled with Al\(^{18}\)F in a single step strategy and with a radiochemical yield between 40-70% (EOB). After RP-HPLC purification, the observed specific activity was always >30 GBq/μmol for both radiotracers. The log P values calculated for Al\(^{18}\)F-NOTA-C5 and Al\(^{18}\)F-NOTA-C6 were -2.14± 0.14 and -2.34± 0.15, respectively. In athymic nude PC-3 xenografts, at 1h post injection (p.i.), the uptake of Al\(^{18}\)F-NOTA-C5 and Al\(^{18}\)F-NOTA-C6 in PC-3 tumors was 1.88±0.3 %ID/g and 2.34±0.54 %ID/g, respectively. GRPR binding specificity was evaluated after co-injection with unlabeled ε-aminocaproic acid-bombesin(7-14). In vivo metabolism studies at 1h p.i. showed high stability of both the analogues. No defluorination was observed. The accumulation of Al\(^{18}\)F-NOTA-C5 and Al\(^{18}\)F-NOTA-C6 in PC-3 tumors could be well visualized by PET imaging after 1h p.i. while in the blocking studies no significant uptake was observed. **Conclusion:** Lanthionine-stabilized full-length C5 and C6 bombesin peptides were rapidly and successfully labeled with \(^{18}\)F. The effect of stabilization was confirmed by in vitro and in vivo studies. Both tracers showed specific GRPR targeting potential.
INTRODUCTION

Prostate cancer (PCa) is the third-leading cause of cancer related deaths and the most frequently diagnosed cancer among men in the Western World (1). A promising diagnostic tool for cancer targeted imaging is the use of radiolabeled receptor-binding peptides. Bombesin-like receptors, such as the GRPR-subtype, were found to be overexpressed in several types of human cancer, such as lung, colon, gastric, pancreatic, breast and prostate cancer (3-4). The relatively low expression of GRPR in normal tissue and increased expression in cancer tissue makes GRPR an attractive target for cancer detection and treatment. Bombesin is a 14-amino acid peptide with high binding affinity to the gastrin-releasing peptide receptor (GRPR) (2). Thereby, several bombesin sequences have been investigated for GRPR-positive tumor-targeted imaging with positron emission tomography (PET) and single photon emission computed tomography (SPECT) (5-9).

One of the limitations of the full-length bombesin molecule is the poor in vivo stability and relatively poor pharmacokinetics properties due to the large molecular weight. However, full-length bombesin can be easily modified to obtain truncated or synthetic sequences where one or more amino acids can be substituted and/or eliminated. Most of the modifications occur on the N-terminus because the C-terminus is important for the binding affinity of the peptide.

The aim of this paper is to investigate two stabilized full-length bombesin analogues (C5 and C6) for their targeting ability to GRPR order to overcome the poor in vivo stability of full-length bombesin. These two analogues were selected from a library of synthesized bombesin peptides. These sequences were obtained by the insertion of internal thioether cross-links within either the N-terminal (C5, C6) or within the C-terminal of the amino acid sequence. C5 and C6 were chosen for this study based on the binding affinity to GRPR. Bombesin has been labelled with $^{99m}$Tc, $^{111}$In, $^{64}$Cu and $^{18}$F. $^{18}$F is the most widely used isotope for PET imaging due to its excellent characteristics. However, in general, most of the described procedures to label peptides with $^{18}$F do often require prosthetic groups (i.e. SFB or FBA) for coupling. These strategies are usually multistep time-consuming
protocols. Here we use a recently described one-pot process where $^{18}$F is complexed to a chelating agent via aluminium $^{18}$F-fluoride with a straight-forward efficient technique by McBride and coworkers (10-11). NOTA was used as chelating agent because of its properties to stabilize the $+2$ charge of the $\text{Al}^{18}\text{F}^{2+}$ complex. Here, we describe the targeting characteristics, \textit{in vitro and in vivo}, of the two different lanthionine stabilized bombesin peptides labeled by $^{18}$F.

**MATERIALS AND METHODS**

**Chemicals**

NOTA-NHS was purchased from CheMatech (Dijon, France). All other solvents and chemicals were purchased from Sigma-Aldrich (St. Louis, Missouri, USA). C18 cartridges with 55-105 µm particle size was purchased from Waters Corporation (Milford, Massachusetts, USA).

**HPLC-systems**

Reversed phase high-performance liquid chromatography (RP-HPLC) was performed on a HITACHI L-2130 HPLC system (Hitachi High Technologies America Inc., Pleasanton, CA, USA) equipped with a Bicron Frisk-Tech area monitor. Purification of radiolabeled peptides was performed using a reversed phase AlltimaAlltech RP-C18 column (10 mm × 250 mm, 5 µm) (Delta Technical Products, Des Plaines, IL, USA). The flow was set at 2.5 mL/min using a gradient system starting from 90% solvent A (0.01 M phosphate buffer, pH = 6.0) and 10% solvent B (acetonitrile) (5 min), following by a linear gradient mobile phase going to 35% solvent A and 65% solvent B at 35 min and then back to 90% solvent A and 10% solvent B at 40 min. Quality control was performed using a reversed phase Grace Smart RP-C18 column (Grace, Lokeren, Belgium) (4.6 mm × 250 mm, 5 µm). The flow was set at 1.5 mL/min using a gradient system starting from 90% solvent A (0.01 M phosphate buffer, pH = 6.0) and 10% solvent B (acetonitrile) (2 min) to 35% solvent A and 65%
solvent B at 32 min. The column was then re-equilibrated afterwards and the gradient became 90 % solvent A and 10 % solvent B at 37 min.

**Synthesis of stabilized Bombesin analogues**

The peptides C5 bombesin (C5) and C6 bombesin (C6) were obtained from JPT Peptide Technologies (Berlin, Germany). The sequence of C5 is pEQkdCGNCWAVGHLM-NH₂ and C6 ispEdCKLGQWAVGHLM-NH₂ (pE stands for pyroglutamate and dC stands for D-cysteine). The two different sequences were obtained based on the difference of hydrophilicity values between the N- and the C-terminal part of bombesin. Nonspecific base-assisted sulfur extrusion was used for obtaining lanthionine-stabilized peptides with the lanthionines in the N-terminal half (12). Lanthionine-stabilized peptides with the lanthionine in the C-terminal half were produced stereospecifically by a three-steps method. Lanthionine was firstly introduced via a *Lactococcus lactis* production system (13-14) followed by pGlu formation and finally amidation (15). Briefly, both the bombesin modified peptides were dissolved in water [2 mg/mL]. Ammonia was added to a 0.3% final concentration and the reaction mixture incubated at 37 °C overnight. After that, ammonia was removed and the sample was then concentrated with speedvac concentrator (Thermo Fisher Scientific Inc., Logan, UT, USA), and, subsequently, purified by HPLC. The final isomeric mixtures of the thioether bridged bombesin analogues were analyzed by mass spectrometry. Introduction of a thioether cross-link by desulphurization, causes a loss in mass of 34 Dalton.

**Synthesis of NOTA-C5 and NOTA-C6**

NOTA-NHS (50 μmol) and C5 (2.6 μmol) or C6 (2.6 μmol) were dissolved in 1.5 ml of DMF. After addition of excess triethylamine (3 drops), the reaction mixture was stirred overnight at room temperature to make sure the reaction was complete. The product was purified by HPLC (HPLC method 1). The fraction at ~26 min was collected. Lyophilization of the collected fraction gave the final product (~24%) with the purity > 95% by HPLC. Final products were identified by Maldi-ToF Mass Spectrometry as NOTA-C5 and NOTA-C6.
**In vitro competitive binding assay**

The *in vitro* competitive binding assay was performed as reported previously (16). The 50% inhibitory concentration (IC$_{50}$) values were calculated for all of them by fitting the data with nonlinear regression using GraphPad Prism 5.0 (GraphPad Software, San Diego, CA, USA) and expressed as an average plus the standard deviation. Experiments were performed with triplicate samples.

**$^{18}$F Radiolabeling**

Aqueous $^{18}$F-fluoride was produced by irradiation of $^{18}$O-water with a Scanditronix MC-17 cyclotron via the $^{18}$O(p,n)$^{18}$F nuclear reaction. The $^{18}$F-fluoride solution was passed through a SepPak Light Accell plus QMA anion exchange cartridge (Waters, Milford, MA, USA), previously washed with 5mL of metal-free water, to recover the $^{18}$O-enriched water. Radiolabeled Al$^{18}$F-NOTA-C5 or Al$^{18}$F-NOTA-C6 were synthesized in a one-pot strategy based on the procedure described by McBride et Al. (18). $^{18}$F-fluoride was eluted from the QMA anion exchange cartridge with 200 μL of (NH$_4$)$_2$CO$_3$ [5 mg/mL] and collected in a metal-free eppendorf tube. To this eluate, 5-10 μL of metal-free glacial acetic acid were added to adjust the pH to ~4.1. Subsequently, 5-45 μL of 2mM AlCl$_3$ in 0.1M sodium acetate buffer (pH~4.1) were added. Finally, 75 μL of this solution were added to 400 μL of ACN and 25 μL of C5- or C6-bombesin dissolved in 0.5 M sodium acetate buffer (pH~4.1) [5mg/mL]. The reaction mixture was heated for 20 min at 95°C. After cooling the reaction mixture, the radiolabeled peptides were isolated and analyzed by using HPLC. To eliminate the acetonitrile, purified Al$^{18}$F-NOTA-C5 and Al$^{18}$F-NOTA-C6 were diluted with water and loaded on a Waters Sep-Pak Light C18 cartridge and eluted with 0.4 mL 60% ethanol. For cell experiments and animal experiments, Al$^{18}$F-NOTA-C5 and Al$^{18}$F-NOTA-C6 were diluted with saline solution.

**Octanol/Water Partition Coefficient**
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$^{18}$F-NOTA-C5or $^{18}$F-NOTA-C6 (37 kBq) was dissolved in a mixture of 0.5 mL $n$-octanol and 0.5 mL 25 mM PBS (pH 7.4) and well mixed for 5 min at room temperature. Afterwards, the mixture was centrifuged at 3000 rpm for 5 minutes. 100 μL samples were obtained from $n$-octanol and aqueous layers and counted in a $\gamma$-counter (Compugamma CS1282, LKB-Wallac, Turku, Finland). The $\text{log}D$ value is reported as an average of three different measurements (mean ± SD).

**In Vitro Stability**

$^{18}$F-NOTA-C5or $^{18}$F-NOTA-C6*in vitro* stability was studied in 0.9% NaCl and human serum as described previously (16). $^{18}$F-NOTA-C5or $^{18}$F-NOTA-C6 was incubated at room temperature in 0.9% NaCl solution or in human serum (at 37 °C). Samples were collected at different time points (0, 5, 15, 30, 60, 120, 180, 240 min) after incubation. Analysis of samples from 0.9% NaCl solution was done by HPLC system. 250 μL of human serum mixture sample was precipitated with 750 μL of ethanol/acetonitrile solution($V_{\text{ethanol}}: V_{\text{acetonitrile}}=1:1$). After centrifugation, the supernatants were collected, filtered by 0.22 μm filter and finally analysed by RP-HPLC analysis.

**Cell culture**

The GRPR positive human prostate cancer cell line PC-3 (ATCC, Manassas, VA, USA) was cultured in RPMI 1640 (Lonza, Verviers, France) supplemented with 10% fetal calf serum (Thermo Fisher Scientific Inc., Logan, UT, USA) at 37 °C in a humidified 5% CO₂ atmosphere.

**In Vitro Cell Uptake and Internalization Kinetics**

PC-3 cells were placed in 6-well plates (0.5 million cells/well) one day prior to the assay. The day after, the cells were washed twice with PBS and incubated with $^{18}$F-NOTA-C5or $^{18}$F-NOTA-C6 (0.04 MBq/well) to allow for cellular uptake at 37 °C up to 180 minutes (in triplicate). Unlabeled bombesin (20 μg) was co-incubated with $^{18}$F-NOTA-C5or $^{18}$F-
NOTA-C6 in the blocking group. The cells were washed twice with ice-cold PBS to remove unbound radioactivity at each of the selected time points. After washing twice, the cells were incubated for ~3 minutes with 1 mL glycine acid solution (50 mM glycine−HCl/100 mM NaCl, pH 2.8) and washed again with ice-cold PBS. Radioactivity of collected glycine acid and PBS solutions was measured in γ-counter as membrane receptor bound radioactivity. Then, the 6-well plates were washed with 1 M NaOH and put at 37 °C to allow cell lysis. The lysate was collected from each well and measured in gamma counter as internalized activity. Total cellular uptake of Al\textsuperscript{18F}-NOTA-C5 or Al\textsuperscript{18F}-NOTA-C6 was plotted as sum of internalized and membrane receptor bound radioactivity. Results were expressed as mean ± SD (n = 3).

**Efflux Kinetics**

PC-3 cells at confluence were placed in 6-well plates (0.5 million cells/well) 24 hours before the assay. The cells were washed with PBS and incubated with Al\textsuperscript{18F}-NOTA-C5 or Al\textsuperscript{18F}-NOTA-C6 (0.04 MBq/well) at 37 °C for 60 minutes for maximal internalization. After incubation, the cells were washed twice with ice-cold PBS to eliminate unbound radioactivity and then incubated in the pre-warmed culture medium at 37 °C for 0, 15, 30, 45, 60, 90, 120, and 240 minutes for externalization. Then, cells were incubated in the prewarmed culture medium at 37 °C for 0, 15, 30, 45, 60, 90, 120, and 180 minutes in triplicate to allow for externalization. At each time point, the cells were washed twice with ice-cold PBS and then washed twice for 3 minutes with acid (50 mM glycine−HCl/100 mMNaCl, pH 2.8) to remove cell-surface bound radiotracer. Finally, the cells were incubated with 1 M NaOH at 37 °C for to allow cell lysis. Afterwards, the lysate was aspirated and measured by γ-counter (Compugamma CS1282, LKB-Wallac, Turku, Finland). Results are calculated as percentage of maximum intracellular radioactivity (remaining activity at specific time-point / activity at time-point 0) (mean±SD).

**Animal Model**
6 weeks old Athymic nude mice (Harlan, Zeist, The Netherlands) were injected subcutaneously with $1 \times 10^6$ PC-3 cells (in 0.1 mL of sterile saline) in the left shoulder. Animals were anesthetized with gas (3.5 % isoflurane in an air/oxygen mixture) during inoculation. 4 weeks after inoculation, mice were subjected to μPET scan and biodistribution studies. All animal experiments were performed in accordance with the regulations of Dutch law on animal welfare and the institutional ethics committee for animal procedures approved the protocol.

**MicroPET/CT imaging and Biodistribution**

The PC-3 xenografted athymic mice were used for animal experiments when the tumor volume reached 250-300 mm$^3$ (4-5 weeks after inoculation). Mice with subcutaneous PC-3 were injected intravenously with ~ 5-8 MBq of $^{18}$F-NOTA-C5 or $^{18}$F-NOTA-C6. A constant 300 µg amount of unlabeled ε-aminocaproic acid-bombesin(7-14) was pre-injected (30 minutes before the tracer injection) for the receptor-blocking imaging studies. Control mice and blocking group were scanned by using a small-animal PET scanner (Inveon PET, Siemens Preclinical Solution, Knoxville, TN, USA) for 60 min after tracer injection. PET image confusio was accomplished with Inveon Research Workplace Software (Siemens Inveon Software, Erlangen, Germany). Mice were terminated 1h p.i. and biodistribution studies were also performed. Blood, tumor, major organs and tissue samples were collected, weighed and measured by γ-counter. Percentage of injected dose per gram (%ID/g) was determined for each sample. For each mouse, radioactivity of the tissue samples was calibrated against a known aliquot of radiotracer. The specificity of $^{18}$F-NOTA-C5 or $^{18}$F-NOTA-C6 was assessed by a receptor-blocking study. Mice were co-injected with excess of ε-aminocaproic acid-bombesin(7-14) as described above and terminated 1h p.i.. Biodistribution data are reported as mean ± SD (n=4).

**Metabolic Stability**
Male nude athymic mice bearing PC-3 tumors were injected intravenously with approximately 25 MBq of the $^{18}$F-bombesin derivative. Animals were sacrificed and dissected at 60 min after injection. Blood, tumor, pancreas, liver, kidney and urine were collected. Blood was immediately mixed with acetonitrile for protein precipitation. The samples were centrifuged for 10 min at 13,200 rpm. The supernatant was analyzed by HPLC with a Grace C18 analytical column after Millex 0.22 μm filtration. Organs were homogenized using an IKA Ultra-Turrax T8 (IKA-Werke GmbH and Co. KG, Staufen, Germany), suspended in 1 mL of PBS, and centrifuged for 5 min at 13,200 rpm. After removal of the supernatants, the pellets were washed with 500 μL of PBS. For each sample, supernatants of both centrifugation steps were combined and passed through Sep-Pak C$_{18}$ cartridges. The urine sample was directly diluted with 1 mL of PBS and passed through Sep-Pak C$_{18}$ cartridge. The cartridges were washed with 5 mL of H$_2$O and eluted with 500 μL of pure acetonitrile. The combined solutions were passed through a 0.22-μm Millipore filter, and injected onto an analytic HPLC column at a flow rate of 1 mL/min using the gradient described earlier. Radioactivity was monitored using a solid-state radiation detector. Eluent was collected every minute, and γ-counter was used to measure the activity of each fraction. HPLC chromatograms were obtained by plotting the data obtained from the γ-counter fraction measurements. The HPLC analysis and extraction efficiency was determined in triplicate. Control experiments were also performed to test the extraction protocol and elution efficiency by using Al$^{18}$F-NOTA-C5 and Al$^{18}$F-NOTA-C6 as standards and by adding them to the same tissue samples. Mass spectrometry analysis were afterwards performed to assess the identity of the recovered HPLC fractions.

**Statistical Analysis**

Quantitative data are expressed as mean ± Standard Deviation (SD). Means were compared using the Student t test. P values, <0.05 were considered as significant.

**RESULTS**
Radiolabeling, partition coefficient, *in vitro* stability

The lanthionine-stabilized peptides C5, C6 were synthesized and their identity was confirmed by HPLC and mass spectrometry, as previously described (12).

The most critical steps affecting the yield of the reaction synthesis of $\text{Al}^{18}\text{F-NOTA}$ conjugates were the metal-free conditions and the pH value. $\text{Al}^{18}\text{F-NOTA-C5}$ and $\text{Al}^{18}\text{F-NOTA-C6}$ were synthesized with similar good radiochemical yield (between 40-70% yield depending on the amount of starting NOTA-BombesinC5/C6 used. Higher amounts of precursor produced exponentially higher radiochemical yields). The overall synthesis time was around 100 min including formulation. The collected peak of $\text{Al}^{18}\text{F-NOTA-C5}$ and $\text{Al}^{18}\text{F-NOTA-C6}$ was separated from cold precursor and was collected at a retention time of ~26 min. The cold precursor was typically eluting with a shift of 0,5min at ~25min for $\text{Al}^{18}\text{F-NOTA-C5}$ and of 1 min for $\text{Al}^{18}\text{F-NOTA-C6}$. The radiochemical purity was >95%, as determined by HPLC. The calculated specific activity was ≥32 GBq/μmol for $\text{Al}^{18}\text{F-NOTA-C5}$ and ≥88 GBq/μmol for $\text{Al}^{18}\text{F-NOTA-C6}$ based on the HPLC analysis of purified product.

The partition coefficient was determined in 1:1 mixture solution of *n*-octanol and PBS solution. Log-$\text{P}_{\text{octanol/water}}$ value of $\text{Al}^{18}\text{F-NOTA-C5}$ and $\text{Al}^{18}\text{F-NOTA-C6}$ was -2.14± 0.14 and -2.34± 0.15, respectively.

The *in vitro* stability of $\text{Al}^{18}\text{F-NOTA-C5}$ and $\text{Al}^{18}\text{F-NOTA-C6}$ was evaluated in saline and human serum as shown in Figure 1. The results are plotted as the percentage of parent $\text{Al}^{18}\text{F}$ labeled NOTA-C5 and NOTA-C6 compounds at different time points. For both tracers, more than 90 % of the radioactivity kept its initial form after 4 hours incubation in saline. In human plasma, at 37°C, more than 75% of radioactivity was still parent compound after 4 hours.
Figure 1: Panel A shows the in vitro stability of Al$^{18}$F-NOTA-C5 in saline and human serum solution and panel B shows the in vitro stability of Al$^{18}$F-NOTA-C6 in saline and human serum solution.

**In Vitro Competitive Receptor Binding Assay**

In this study, $^{125}$I-Tyr$^4$-Bombesin was used as golden standard in a competitive binding assay to GRPR. The IC$_{50}$ values of NOTA-C5 and NOTA-C6 were significantly decreased and in the nanomolar range. The IC$_{50}$ for C5 was 251±8 nM and for NOTA-C5 was 114±3 nM. The IC$_{50}$ observed for C6 was 23±4 nM and for NOTA-C6 15±2 nM indicating good binding affinity to GRPR.
Cell Uptake, Internalization and Efflux Kinetics

Uptake of $^{18}$F-NOTA-C5 and $^{18}$F-NOTA-C6 in PC-3 cells gradually increased within 120 min of incubation and reached a plateau after this time point. The measured total cellular activity was $\sim$30% for $^{18}$F-NOTA-C5 and $\sim$41% for $^{18}$F-NOTA-C6. For $^{18}$F-NOTA-C5 the internalized activity was $\sim$23% and for $^{18}$F-NOTA-C6 was $\sim$32%.

The internalization was already observed within 15 min after incubation with both the analogues. The maximum cellular uptake observed with $^{18}$F-NOTA-C5 was lower than that of $^{18}$F-NOTA-C6. The cellular uptake was negligible with co-injection of both tracers with blocking agent.

The efflux kinetics of $^{18}$F-NOTA-C5 and $^{18}$F-NOTA-C6 were also compared in the PC-3 prostate cancer cell line (Figure 3). Both tracers showed similar efflux characters in the PC-3 cell line. For $^{18}$F-NOTA-C5 and $^{18}$F-NOTA-C6, 40±5 % and 48±9 % of internalized radioactivity remained in PC-3 cells after 3 h incubation.
Figure 2: A) Cellular uptake of $^{18}$F-NOTA-C5 and $^{18}$F-NOTA-C6 in the PC-3 prostate cancer cell line. B) Efflux kinetics $^{18}$F-NOTA-C5 and $^{18}$F-NOTA-C6 in the PC-3 prostate cancer cell line.

**Biodistribution experiments**

Biodistribution of $^{18}$F-NOTA-C5 and $^{18}$F-NOTA-C6 at 1 hour p.i. was measured in PC-3 xenografted athymic nude mice. The specificity of both tracers was estimated by coinjection with an excess (300 µg) of unlabeled ε-aminocaproic acid-bombesin(7-14). Biodistribution values of $^{18}$F-NOTA-C5 and $^{18}$F-NOTA-C6 are presented in Figure 3. The tumor uptake of $^{18}$F-NOTA-C5 at 1 hour p.i. was 1.8± 0.3 %ID/g. For $^{18}$F-NOTA-C6, at 1 hour p.i., the tumor %ID/g was 2.1± 0.4. These findings were also confirmed by SUV calculation. In a blocking study, uptake of $^{18}$F-NOTA-C5 and $^{18}$F-NOTA-C6 was significantly reduced in tumor (>70%) and in pancreas (>75%) (Table 1 and Figure 3). No significant lowered uptake was observed in non-GRPR expressing organs, such as heart, kidney, liver, muscle after co-injection with blocking agent (Fig. 3).
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![Graph showing the distribution of %ID/g in different organs for Control and Blocking Al\(^{18}\)F-NOTA-C5.](image1)

![Graph showing the distribution of %ID/g in different organs for Control and Blocking Al\(^{18}\)F-NOTA-C6.](image2)
Figure 3: Biodistribution of $^{18}$F-NOTA-C5 and $^{18}$F-NOTA-C6 in PC-3 prostate tumor bearing athymic nude mice at 1 hour after injection (n=6).

MicroPET/CT imaging

MicroPET images of $^{18}$F-NOTA-C5 and $^{18}$F-NOTA-C6 in prostate tumor-bearing athymic nude mice are shown in Figure 4. High abdomen background was observed for images of both tracers in a 60min experimental period. PC-3 tumor xenografts were visible from PET images of both tracers. With excess blocking agent, the radioactivity signal in PC-3 tumors was specifically lower compared to the control group (Table 1). For $^{18}$F-NOTA-C5 the observed tumor uptake value of the control group was 1.9 ± 0.3 %ID/g at 1h p.i., while, for the blocking group, the calculated tumor uptake value was 0.3 ± 0.2 %ID/g. For $^{18}$F-NOTA-C6, the tumor uptake value that was observed was 2.3 ± 0.3 %ID/g in the control group and 0.5 ± 0.3 %ID/g in the blocking group.

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<th>$^{18}$F-NOTA-C5</th>
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<td>In vitro stability</td>
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<td>Maximal cellular</td>
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<td>41±8 %</td>
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<td>Tumor uptake</td>
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<td>2.3 ± 0.3 %ID/g (1h post injection)</td>
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<td>Specific tumor uptake</td>
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<td>78 % blocked</td>
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<td>(with blocking agent)</td>
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<td>Pancreas uptake</td>
<td>2.0 ± 1.7 %ID/g (1h post injection)</td>
<td>7.2± 3.9 %ID/g (1h post injection)</td>
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<td>uptake (with blocking</td>
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<td>0.6 ± 0.1 %ID/g(1h post injection)</td>
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<td>0.7± 0.4 %ID/g(1h post injection)</td>
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Table 1: Summary highlighting the most important *in vitro*/*in vivo* characteristics of Al\(^{18}\)F-NOTA-C5 and Al\(^{18}\)F-NOTA-C6
Figure 4: Top panel shows microPET dynamic scans of Al\textsuperscript{18}F-NOTA-C5 acquired every 10 minutes (10 minutes frame). Middle panel represents microPET dynamic scan of Al\textsuperscript{18}F-NOTA-C6 (10 minutes frame). Bottom panels show on the left the microPET scan of Al\textsuperscript{18}F-NOTA-C5 and on the right the microPET scan of Al\textsuperscript{18}F-NOTA-C6. Both the in PC-3 prostate tumor- bearing athymic nude mice images were reconstructed at 60 minutes after injection as static scan. Arrows point at PC-3 tumors.

**Metabolism of Al\textsuperscript{18}F-NOTA-C5 and Al\textsuperscript{18}F-NOTA-C6**

The *in vivo* stability of Al\textsuperscript{18}F-NOTA-C5 and Al\textsuperscript{18}F-NOTA-C6 was evaluated in PC-3 tumor bearing athymic nude mice at 1h after injection. The metabolic stability was determined in mouse blood, urine, liver, kidney, tumor and pancreas homogenates (Fig.5 and Fig.6). The extraction efficiency was also monitored and was 63±13% for all samples tested. The eluted fractions were plotted in a chromatogram and the *in vivo* stability was measured in each organ of interest as percentage of parent compound. Every minute fraction was reported and the chromatogram was depicted as described by Zhang and coworkers (9).
**ALF- NOTA stabilized bombesin analogues for targeting GRPR expressing prostate cancer**

Figure 5: HPLC profiles of soluble fractions of tumor, blood and urine homogenates. Samples were collected at 1h p.i. of $^{18}$F-NOTA-C5 to a male athymic PC-3 tumor-bearing nude mouse. As a comparison, the quality control HPLC profile of intact tracer is shown as $^{18}$F-NOTA-C5.
**Figure 6:** HPLC profiles of soluble fractions of tumor, blood and urine homogenates. Samples were collected at 1h p.i. of Al\(^{18}\)F-NOTA-C6 to a male athymic PC-3 tumor-bearing nude mouse. As a comparison, the quality control HPLC profile of intact tracer is shown as Al\(^{18}\)F-NOTA-C6.

**DISCUSSION**

In the last years, an enormous growth has been observed in production and design of peptide radiopharmaceuticals which may be useful for diagnostic and therapeutic applications. Peptide radiopharmaceuticals have some advantages as low antigenicity, fast clearance, rapid tissue penetration. Their production is relatively quick and cheap. Unfortunately, there are also some disadvantages with peptides, such as degradation, chelator and physiologic effects. The discovery of GRPR as overexpressed receptor in prostate cancer disease, has led to the rapid development of radiolabeled bombesin analogues derived either from the full tetradecapeptide sequence or from truncated C-term sequence of the peptide as peptidic radiopharmaceutical for both diagnosis and therapy (6), (9), (17-20). As result of the disadvantages listed above, of those analogues, only a few have entered the clinical phase. The clinical translation of bombesin radiopharmaceuticals is still limited due to unfavorable *in vivo* kinetics of bombesin tracers. The lack of stability of radiolabeled bombesins has been extensively investigated resulting in a variety of chelators, linkers and, more in general, prosthetic groups to improve pharmacokinetics and *in vivo* stability (21-25). Our approach consisted in using two different stabilized bombesin analogues named C5 and C6, which were synthesized by the desulphurization method described by Galande et al (12). \(^{18}\)F was selected as PET radionuclide in this study because of the physical half-life of 110 min and the optimal characteristics which fit the *in vivo* biological half-life of peptides.

Therefore, these two bombesin analogues were conjugated to NOTA and radiolabeled with \(^{18}\)F using the one-pot Al\(^{18}\)F method described by McBride et al (10,11). This method is a fast radiolabeling strategy which allowed us to obtain \(^{18}\)F-labeled analogues with high radiochemical purity and high yield. The probes were both highly hydrophilic, as indicated
by their Log-P values (-2.14±0.14 for Al\(^{18}\text{F}\)-NOTA-C5 and -2.34±0.15 Al\(^{18}\text{F}\)-NOTA-C6). The total synthesis, including RP-HPLC purification and formulation, took approximately 60 min which can be considered reasonable compared to other procedures based on prosthetic groups. Fluorinations with \([^{18}\text{F}]\)-SFB, \([^{18}\text{F}]\)-FBA or \([^{18}\text{F}]\)-FDG include all multi-step reactions and longer synthesis time. In McBride’s approach, the longest time consuming part was the HPLC purification, which was also the most crucial step to get the analogues with high specific activity. However, McBride and coworkers evaluated also other chelators for this procedure and stated that the overall synthesis still has high margins of improvement. They demonstrated that different NOTA ligands form different stable ligand complexes with yields ranging between 5.8% to 87%, depending on the ligand used. Chelators such as C-NETA or pentadentate bifunctional chelator NODA-MPAA showed enhanced binding kinetics for some metals and form highly stable Al\(^{18}\text{F}\) chelates (26,27).

In our study, C5 and C6 were obtained by introducing a thioether linkage via the desulphurization method described by Galande et al (12) and were chosen based on their binding affinity to GRPR. They were radiolabelled and compared in vitro and in vivo. Al\(^{18}\text{F}\)-NOTA-C5 and Al\(^{18}\text{F}\)-NOTA-C6 acted as agonists as demonstrated by internalization studies and were quickly internalized into PC-3 cells through GRPR. The cellular uptake of Al\(^{18}\text{F}\)-NOTA-C6 was slightly higher than Al\(^{18}\text{F}\)-NOTA-C5. This can be due to the different IC\(_{50}\) values observed in the competitive binding assay. It has been shown that the IC\(_{50}\) for NOTA-C6 is significantly better than the one observed for NOTA-C5 (15±2 nM and 114±3 nM respectively). Another reason may be the difference in specific activity between the two evaluated analogues. Improvements in product purification of Al\(^{18}\text{F}\)-NOTA-C5 may hamper also its specific activity value. Due to the hydrophilic behavior and to the high metabolic stability, both Al\(^{18}\text{F}\)-NOTA-C5 and Al\(^{18}\text{F}\)-NOTA-C6 resulted in high radioactivity uptake in targeting organs already at 1h p.i., even if the contrast-to-non-target tissues cannot be considered as optimal at 1h post injection.

The high tumor-to-muscle ratio of both Al\(^{18}\text{F}\)-NOTA-C5 and Al\(^{18}\text{F}\)-NOTA-C6 at 1 hour after injection allowed to detecting clearly the tumor in the PET images of PC3 tumor-bearing mice. The reason may be the fast washout from non-targeting organs compared to tumor which is a highly GRPR expressing target. Both tracers showed specific tumor uptake in
PC-3 tumors: the coinjection of an excess of unlabeled bombesin peptide resulted in a significantly lower tumor uptake of \( \text{Al}^{18}\text{F-NOTA-C5} \) and \( \text{Al}^{18}\text{F-NOTA-C6} \) as shown in Table 1. The tumor uptake of \( \text{Al}^{18}\text{F-NOTA-C6} \) \( (2.3 \pm 0.3 \ %\text{ID/g}) \) at 1h p.i. was similar to the tumor uptake observed by Zhang et al. with \( \text{18}^\text{F-FB-[Lys3]BBN} \) \( (\pm 2.61 \ %\text{ID/g}) \) (9). However, \( \text{Al}^{18}\text{F-NOTA-C6} \) as well as \( \text{Al}^{18}\text{F-NOTA-C5} \) showed increased metabolic stability and less hepatobiliary uptake as result of less lipophilic behavior. The use of truncated sequences such as Aca-BN(7-14) is a tool to increase the metabolic stability of bombesin peptide; however, \( \text{Al}^{18}\text{F-NOTA-C6} \) and \( \text{Al}^{18}\text{F-NOTA-C5} \) show higher tumor uptake and improved stability than \( \text{18}^\text{F-FB-Aca-BN(7-14)} \) which had 0.78 %ID/g at 1h p.i. (9). It would be of interest to evaluate if the application of the method described by McBride et al. (26-27) might confer different uptake properties to the mentioned Aca-BN(7-14) peptide. A higher tumor uptake with \( \text{18}^\text{F} \) labeled BN(7-14) peptide was achieved in the study of Dijkgraaf et al.(6). In the subcutaneous PC-3 xenograft model, the tumor uptake of \( \text{18}^\text{F}-\text{labeled NOTA-8-Aoc-BN(7-14)NH}_2 \) was \( 2.15 \pm 0.55\%\text{ID/g} \) at 1h p.i.. Overall, their results are similar to those that we achieved and differences might be due to amounts of peptides used, different characteristics of mouse strains, tumor cells used and tumor size. Further studies should be performed in order to evaluate which peptide dose is optimal to have the best tumor uptake without the interference of the unspecific background signal from surrounding tissues. Other factors as vascularization may play a role in the in vivo studies effecting tumor uptake and tumor–to–non-tumor ratios. It would be of interest to directly compare the metabolic stability of these different analogues in order to define which radiotracer could most successfully be applied in the clinical phase.

Both \( \text{Al}^{18}\text{F-NOTA-C5} \) and \( \text{Al}^{18}\text{F-NOTA-C6} \) are metabolically stable after intravenous administration. Some metabolites were found but not characterized and identified. The characterization of the degradation products may be important to identify the cleavage sites to design more stable peptides.

In the PET images of tumor-bearing the presence of both probes in kidney and bladder was clearly demonstrated. This indicated that the renal clearance was the main route for excretion. These observations were supported from the data obtained from biodistribution studies.
CONCLUSION

To overcome the stability issues of radiolabeled peptide for receptor-mediated cancer imaging, we synthesized series of lanthionine-stabilized full-length bombesin like peptides and two promising candidates were successfully labeled with $^{18}$F. The effect of stabilization was evaluated in vitro and in vivo. In vitro studies and dynamic PET scans demonstrated the ability of both the tracers to visualize PC-3 tumor in xenograft models showing GRPR targeting potential.

Acknowledgement

This work was made possible by a financial contribution from CTMM, project PCMM, project number 03O-203. We thank V. Wiersma and L.D. Kluskens for initial studies on synthesis of lanthionine-stabilized bombesin analogues, D.F. Samplonius for technical assistance on cell culturing, D. De Paula Faria and W. A. Sijbesma for assisting animal experiments.

References:


Chapter 5


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