

Preclinical Evaluation of a Novel ^{111}In -Labeled Bombesin Homodimer for Improved Imaging of GRPR-Positive Prostate Cancer

G. Carlucci,^{*,†,‡} H. J. K. Ananias,[†] Z. Yu,^{†,‡} H. D. Hoving,[†] W. Helfrich,[§] R. A. J. O. Dierckx,^{‡,||} S. Liu,[⊥] I. J. de Jong,[†] and P. H. Elsinga^{‡,||}

[†]Department of Urology and [‡]Department of Nuclear Medicine and Molecular Imaging, University Medical Center Groningen, University of Groningen, Groningen, The Netherlands

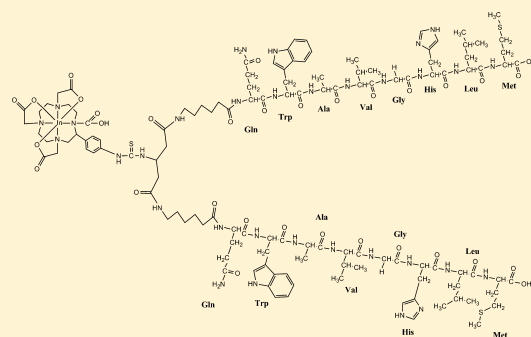
[§]Surgical Research Laboratory, University Medical Center Groningen, University of Groningen, Groningen, The Netherlands

^{||}Department of Nuclear Medicine, University Hospital Ghent, Ghent, Belgium

[⊥]School of Health Sciences, Purdue University, West Lafayette, Indiana, United States

ABSTRACT: Rational-designed multimerization of targeting ligands can be used to improve kinetic and thermodynamic properties. Multimeric targeting ligands may be produced by tethering multiple identical or two or more monomeric ligands of different binding specificities. Consequently, multimeric ligands may simultaneously bind to multiple receptor molecules. Previously, multimerization has been successfully applied on radiolabeled RGD peptides, which resulted in an improved tumor targeting activity in animal models. Multimerization of peptide-based ligands may improve the binding characteristics by increasing local ligand concentration and by improving dissociation kinetics. Here, we present a preclinical study on a novel radiolabeled bombesin (BN) homodimer, designated ^{111}In -DOTA-[(Aca-BN(7–14))₂], that was designed for enhanced targeting of gastrin-releasing peptide receptor (GRPR)-positive prostate cancer cells. A BN homodimer was conjugated with DOTA-NHS and labeled with ^{111}In . After HPLC purification, the GRPR targeting ability of ^{111}In -DOTA-[(Aca-BN(7–14))₂] was assessed by microSPECT imaging in SCID mice xenografted with the human prostate cancer cell line PC-3. ^{111}In labeling of DOTA-[(Aca-BN(7–14))₂] was achieved within 30 min at 85 °C with a labeling yield of >40%. High radiochemical purity (>95%) was achieved by HPLC purification. ^{111}In -DOTA-[(Aca-BN(7–14))₂] specifically bound to GRPR-positive PC-3 prostate cancer cells with favorable binding characteristics because uptake of ^{111}In -DOTA-[(Aca-BN(7–14))₂] in GRPR-positive PC-3 cells increased over time. A maximum peak with 30% radioactivity was observed after 2 h of incubation. The log *D* value was -1.8 ± 0.1 . ^{111}In -DOTA-[(Aca-BN(7–14))₂] was stable *in vitro* both in PBS and human serum for at least 4 days. *In vivo* biodistribution analysis and microSPECT/CT scans performed after 1, 4, and 24 h of injection showed favorable binding characteristics and tumor-to-normal tissue ratios. This study identifies ^{111}In -DOTA-[(Aca-BN(7–14))₂] as a promising radiotracer for nuclear imaging of GRPR in prostate cancer.

KEYWORDS: GRPR, bombesin homodimer, ^{111}In -DOTA, prostate cancer imaging, PC-3 tumor model



INTRODUCTION

Multimerization can be observed in antibody recognition, DNA–DNA duplex formation, and presentation of numerous viral peptides or the binding of one molecule or particle with several sites onto a target. The binding selectivity of a multimer is strongly correlated with different parameters such as its overall size, length, flexibility of the linker between ligand and target, and receptor density.^{1–4}

The aim of multimerization is to increase the binding affinity of the ligand to its targets. Several multimers have already been reported as good candidates for future targeting probes, and it has been shown that the tumor uptake of several dimeric and tetrameric labeled peptides was better than the monomer counterpart.^{1,5–7}

Bombesin (BN) is a 14-amino-acid peptide extracted from amphibian skin which binds to GRPR with high affinity.⁸ GRPR is an ideal candidate for imaging prostate cancer. This G protein-coupled receptor is overexpressed in prostate cancer and at a lower level expressed in healthy prostatic tissue and normal tissues. An overall increased expression in cancer tissues has validated GRPR as a target for cancer detection and treatment.^{8,9}

In the past years, a series of BN analogues were labeled with various isotopes such as $^{99\text{m}}\text{Tc}$, ^{111}In , ^{64}Cu , ^{177}Lu , ^{18}F , or ^{68}Ga .

Received: September 26, 2012

Revised: March 27, 2013

Accepted: April 2, 2013

Published: April 2, 2013

These radiotracers were investigated for GRPR-positive tumor targeted imaging and therapy in both animal models and human trials.¹⁰ Very few BN tracers have reached the clinical phase.

We applied the multimerization strategy to BN by synthesizing a dimeric BN with 2 identical Aca-BN(7–14) units (Figure 1). This dimer was chosen to determine if a dimeric bombesin

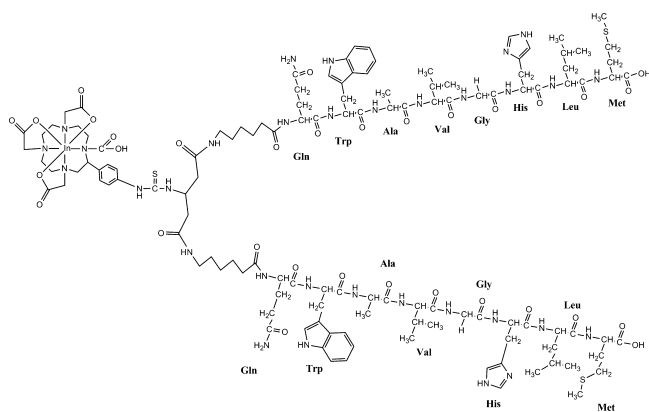


Figure 1. Chemical structure of ^{111}In -DOTA-[(Aca-BN(7–14))₂].

might improve the receptor binding, as the consequence of an increased local BN ligand concentration, leading to an increased tumor uptake and tumor-to-nontumor ratios.^{11–13}

From our previous study on $^{99\text{m}}\text{Tc}$ -HYNIC(Tricine/TPPTS)-Glu[Aca-BN(7–14)]₂,¹⁴ it was postulated that the increased molecular size of BN dimer (compared to that of the BN monomer) would result in an increased circulation time, a slower clearance, and a slower accumulation rate. Other authors demonstrated that multimeric peptides, such as somatostatin analogues, can visualize tumors better than monomeric probes used for neuroendocrine tumors.⁵

This phenomenon may be explained by the fact that a multimeric peptide probe binds to a larger number of binding sites on its respective target, which is associated with lower dissociation rates. Indeed, this has been observed for multimeric RGD peptides. Previously, the 50% inhibitory concentration of cyclo(-RGDfK)- and cyclo(-RGDfE)-monomers, dimers, tetramers, and octamers was compared, and the results showed a significant increase in binding affinity in the order monomer < dimer < tetramer < octamers.¹⁵ In the current report, we conjugated a dimeric bombesin probe to DOTA-NHS, labeled with ^{111}In .

^{111}In was selected because of its high compatibility and stability with the DOTA chelator used. The DOTA-ester is a bifunctional chelator with a reactive functionality, high thermodynamic stability, and kinetic inertness.¹⁶

MATERIALS AND METHODS

Chemicals. DOTA-NHS was purchased from Macrocyclics (Dallas, Texas, USA). All other solvents and chemicals were purchased from Sigma-Aldrich (St. Louis, Missouri, USA). C18 cartridges with 55–105 μm particle size were purchased from Waters Corporation (Milford, Massachusetts, USA). The peptide Glu[Aca-BN(7–14)]₂ (denoted as Aca-BN₂) was purchased from Peptides International (Louisville, Kentucky, USA).

Synthesis of Glu[Aca-BN(7–14)]₂. Boc-Glu(OSu)-OSu was prepared according to a previously reported procedure.¹⁷

Briefly, Aca-BN(7–14) (Aca-Gln-Trp-Ala-Val-Gly-His-Leu-Met-NH₂) (31.6 mg, 30 μmol) was added to a solution of Boc-Glu(OSu)-OSu (8.8 mg, 20 μmol) in anhydrous DMF (2 mL). The pH of the resulting mixture was adjusted to 8.5–9.0 by the addition of diisopropylethyl amine (DIPEA). The reaction was stirred overnight at room temperature, and the compounded product, Boc-Glu(Aca-Gln-Trp-Ala-Val-Gly-His-Leu-Met-NH₂)₂, was isolated by semipreparative HPLC, and the collected fractions were lyophilized to give a white powder. Deprotection from the Boc-group was made by adding anhydrous TFA for 5 min. The crude product was purified by HPLC. The collected fractions were combined and lyophilized to afford H-Glu(Aca-Gln-Trp-Ala-Val-Gly-His-Leu-Met-NH₂)₂ as a white powder. The yield was about 50% based on Boc-Glu(OSu)-OSu starting material. LC–MS characterization was performed with a Waters Qtof Premier (Waters, Milford, Massachusetts, USA) coupled with a Waters Acquity UPLC system. LC–MS analysis employed an Acquity BEH Shield RP18 column (150 $\times$ 2.1 mm, 1.7 μm) interfaced to the Waters Q-TOF MS. ESI-MS (positive mode): m/z = 2217.63 for $[\text{M} + \text{H}]^+$ (C₁₀₃H₁₅₇N₂₉O₂₂S₂, calculated MW = 2217.65).

Synthesis of DOTA-[Aca-BN(7–14)]₂. DOTA-NHS (24.5 mg, 50 μmol) and E(BBN)₂ (5.8 mg, 2.6 μmol) were dissolved in 1.5 mL of DMF. After the addition of excess triethylamine (3 drops), the reaction mixture was stirred for 7 days at room temperature to make sure the reaction was complete. The product was purified by HPLC. Fraction at ~28 min was collected. Lyophilization of the collected fractions gave the final product 1.5 mg (13%) with the purity >95% by HPLC. ESI-MS: C₁₁₃H₁₇₁N₃₃O₂₉S₂, calculated 2519.9, observed 2521 ($[\text{M} + \text{H}]^+$).

The HPLC method used a LabAlliance semiprep HPLC system equipped with a UV–vis detector (λ = 254 nm) and Zorbax C₁₈ semiprep column (9.4 mm $\times$ 250 mm, 100 Å pore size). The flow rate was 2.5 mL/min. The gradient mobile phase goes from 90% solvent A (0.1% acetic acid in water) and 10% solvent B (0.1% acetic acid in acetonitrile) at 0 min to 75% solvent A at 5 min, followed by a gradient mobile phase going from 75% solvent A to 65% solvent A at 40 min.

In Vitro Competitive Cell-Binding. The *in vitro* GRPR binding affinity of DOTA-[Aca-BN(7–14)]₂ was assessed via a competitive binding assay with ^{125}I -Tyr4-BN(1–14) as the GRPR specific radioligand. Experiments were performed with PC-3 human prostate cancer cells according to a method previously described.¹⁸ The 50% inhibitory concentration (IC₅₀) values were calculated by fitting the data with nonlinear regression using GraphPad Prism 5.0 (GraphPad Software, San Diego, California, USA) (Figure 3). Experiments were performed in triplicate. IC₅₀ values are reported as an average of these samples plus the standard deviation (SD).

^{111}In Radiolabeling. Twenty-five micrograms of conjugated DOTA-BN dimer [1 mg/mL] was dissolved in ammonium acetate buffer (0.1 M, 275 μL , pH 5.5). $^{111}\text{InCl}_3$ (37 MBq) was added to the vial containing the DOTA-BN conjugate. Then, the solution was incubated at 85 $^\circ\text{C}$ for 30 min and allowed to cool down to room temperature. The reaction mixture was purified by using HPLC (system 1) and concentrated using the sep-pak C18 light cartridge. Elution of the radiolabeled bombesin dimer was made by ethanol (60%)/sterile saline solution (40%) in a total volume of 400 μL . The purified ^{111}In -DOTA-[(Aca-BN(7–14))₂] was obtained in high purity ($\geq 95\%$) and therefore diluted by sodium chloride 0.9%

to diminish the presence of ethanol at the lowest concentration (<5%).

HPLC Analysis. Semipreparative reversed-phase high-performance liquid chromatography (RP-HPLC) was performed on a HITACHI L-2130 HPLC system (Hitachi High Technologies America Inc., Pleasanton, California, USA) equipped with a Bicorn Frisk-Tech area monitor. Isolation and quality control of ^{111}In -DOTA-[Aca-BN(7-14)]₂ was performed using an Alltech reversed-phase Alltima C18 column (10 mm × 250 mm, 5 μm) (Delta Technical Products, Des Plaines, Illinois, 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) and ramped to 45% solvent A and 55% solvent B at 35 min (HPLC Method 1). Quality control was performed on a reversed phase Grace Smart RP-C18 column (Lokeren, Belgium) (4.6 mm × 250 mm, 5 μm). The flow was set at 1 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) and ramped to 45% solvent A and 55% solvent B at 35 min (HPLC Method 1). HPLC Method 2 used a LabAlliance semipreparative HPLC system equipped with a UV-vis detector (λ = 218 nm) and Zorbax C18 semiprep column (9.4 mm × 250 mm, 100 Å pore size). The flow rate was 2.5 mL/min. The mobile phase was isocratic with 60% solvent A (0.1% acetic acid in water) and 40% solvent B (0.1% acetic acid in acetonitrile) at 0–5 min, followed by a gradient mobile phase going from 60% solvent A and 40% solvent B at 5 min to 20% solvent A and 80% solvent B at 30 min.

Octanol–Water Partition Coefficient (log $D_{\text{octanol/water}}$). The lipophilicity of the ^{111}In -DOTA-[Aca-BN(7-14)]₂ was determined using the method described previously.¹⁸ Briefly, 0.002 MBq of ^{111}In -DOTA-[Aca-BN(7-14)]₂ was diluted in a mixture of 0.5 mL of 1-octanol and 0.5 mL of 25 mM phosphate buffered saline (pH 7.4) and well mixed for 5 min. Then, the mixture was centrifuged at 3000 rpm for 5 min. One hundred microliter samples were obtained from organic and aqueous layers, and the radioactivity of the samples were measured in a γ -counter (Compugamma CS1282, LKB-Wallac, Turku, Finland). The experiment was performed in triplicate, and the resulting log $D_{\text{octanol/water}}$ was calculated as the mean $\pm$ SD.

In Vitro Stability. ^{111}In -DOTA-[Aca-BN(7-14)]₂ (0.002 MBq) was dissolved in 1 mL of saline or PBS, incubated at room temperature, and analyzed by RP-HPLC (HPLC Method 1) at 1, 6, 24, 48, and 72 h postincubation.

Human serum from healthy donors was incubated at 37 °C with ^{111}In -DOTA-[Aca-BN(7-14)]₂ for different time periods (1, 2, 4, 6, 24, 48, and 72 h). After incubation, a sample of 250 μL was precipitated with 750 μL of acetonitrile, stirred in a vortex mixer, and then centrifuged at 3000 rpm for 3 min to pellet the precipitated serum proteins. The supernatants were passed through a filter and analyzed by RP-HPLC (HPLC Method 1).

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

Cellular Uptake and Efflux Studies. One day prior to the assay, PC-3 cells at confluence were placed in 6-well plates (0.5 million cells/well). To evaluate the cellular uptake, cells were washed twice with PBS and then incubated with ^{111}In -DOTA-

[Aca-BN(7-14)]₂ (0.002 MBq/well) at 37 °C for 0, 15, 30, 45, 60, 90, 120, and 240 min in RPMI1640 buffer + 10% fetal calf serum to allow for internalization. Twenty micrograms of unlabeled Glu[Aca-BN(7-14)]₂ was coincubated with ^{111}In -DOTA-[Aca-BN(7-14)]₂ as blocking agent for GRPR in the blocking group. To remove unbound radioactivity, the cells were washed twice afterward with ice-cold PBS and lysed by incubation with 1 M NaOH at 37 °C, and the resulting lysate in each well was aspirated to determine the radioactivity in a γ -counter.

For the internalization study, 1 million PC-3 cells were placed in 6-well plates 1 day before the experiments and kept at 37 °C. The cells were washed with PBS and incubated with 0.002 MBq/well of ^{111}In -DOTA-[Aca-BN(7-14)]₂. Cells were kept for 2 h at 4 °C. After this time, the cells were washed twice with ice-cold PBS to remove unbound radioactivity and were then incubated in the prewarmed culture medium at 37 °C for 0, 15, 30, 45, 60, 90, 120, and 240 min for internalization. To remove cell-surface bound radiotracer, the cells were washed twice for 3 min with acid (50 mM glycine-HCl/100 mM NaCl, pH 2.8), and the acid solution was collected and measured with a γ -counter (Compugamma CS1282, LKB-Wallac, Turku, Finland). The removed cell-surface bound radiotracer was measured as the surface-bound activity. In the final step, the cells were incubated and lysed by adding 1 M NaOH at 37 °C. The resulting lysate obtained from each well was measured as a value of the internalized radioactivity by a γ -counter. Results are analyzed and expressed as the percentage of total radioactivity (internalized activity/(surface-bound activity + internalized activity), (mean $\pm$ SD).

For the efflux study, 1 million PC-3 cells were placed in 6-well plates 1 day before the experiments and kept at 37 °C. The cells were washed with PBS and then incubated with ^{111}In -DOTA-[Aca-BN(7-14)]₂ (0.002 MBq/well) for 1 h at 37 °C for maximum internalization. To remove unbound radioactivity, the cells were washed twice afterward with ice-cold PBS and were then incubated in the prewarmed culture medium at 37 °C for 0, 15, 30, 45, 60, 90, 120, and 240 min for externalization. To remove the cell-surface bound radiotracer, the cells were washed twice for 3 min with acid (50 mM glycine-HCl/100 mM NaCl, pH 2.8). Then, the cells were lysed by incubation with 1 M NaOH at 37 °C, and the resulting lysate in each well was recovered and measured by a γ -counter (Compugamma CS1282, LKB-Wallac, Turku, Finland). The results shown are expressed as the percentage of maximum intracellular radioactivity (remaining activity at specific time-point/activity at time-point 0) (mean $\pm$ SD).

Xenograft Model. The PC-3 tumor model was generated by subcutaneous injection of 2×10^6 PC-3 cells (suspended in 0.1 mL sterile saline) into the left front flank of male athymic mice (Harlan, Zeist, The Netherlands). During injection, animals were anesthetized with a gas composed of ~3.5% isoflurane in an air/oxygen mixture. Approximately 4 weeks after inoculation, when tumors were 8–10 mm in diameter, mice were used for biodistribution experiments and microSPECT/CT imaging.

The local animal welfare committee has evaluated and approved the animal experiments according to Dutch regulations on animal welfare.

MicroSPECT/CT and Biodistribution. Subcutaneous tumor bearing mice were used for biodistribution when the tumor volume reached 8–10 mm diameter (250–300 mm³ as volume after 4–5 weeks from inoculation).

MicroSPECT scans were performed on a three-head γ -camera (MILabs, U-SPECT-II, Utrecht, The Netherlands) equipped with a multipinhole high-resolution collimator. The microSPECT scans were performed after penis vein injection of $\sim 20\text{MBq}$ ^{111}In -DOTA-[Aca-BN(7-14)]₂ under isoflurane anesthesia. Sixty minutes of dynamic microSPECT data was acquired immediately after injection. Sixty minute-static images were acquired at 4 and 24 h after injection of the peptide. All the mice were sacrificed just before acquisition of the static images. Mice that were used for the dynamic scans were sacrificed immediately afterward by an overdose of isoflurane anesthesia and were then scanned by microCT. Biodistribution was performed after the CT scan. Blood, tumor, major organs (heart, liver, spleen, lung, kidney, small and large intestines, stomach, bone, muscle, and pancreas), and tissue samples were collected, weighed, and counted by using a γ -counter. The 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.

Images were reconstructed by using U-SPECT-Rec v 1.34i3 (MILabs, Utrecht, The Netherlands) with a pixel-based ordered-subsets expectation maximum (POSEM) algorithm. Final images were 1 mm slices, made with Inveon software.

For the receptor-blocking experiment, 300 μg of unlabeled Aca-BN(7-14) was preinjected 30 min before the tracer injection. Animals of the blocking group were sacrificed just before each scanning time-point. Sixty minute dynamic scans microSPECT were acquired every 10 min, and 60 min static microSPECT & CT images were acquired at 4 and 24 h after tracer injection. Biodistribution was performed after the CT scan.

$^{111}\text{In}^{3+}$ Dissociation and *in Vivo* Plasma Stability.

Plasma was freshly collected from sacrificed mice injected with ^{111}In -DOTA-[Aca-BN(7-14)]₂ at 1 h postinjection (p.i.). Two hundred fifty microliter plasma sample was precipitated with 750 μL of acetonitrile, stirred in a vortex mixer, and then centrifuged (3 min at 3000 rpm) to pellet the precipitated serum proteins. The supernatants were collected and loaded on a preactivated Sep-Pak Classic C18 cartridge (Waters Corporation, Milford, Massachusetts, USA). The cartridge was passed by 5 mL of 100% water for collecting the dissociated ^{111}In . Subsequently, the cartridge was eluted by 60% ethanol to wash out and collect the ^{111}In labeled peptide bound to the cartridge. The radioactivity of eluted recovered fractions was finally measured by using a γ -counter. The ^{111}In labeled peptide was confirmed to be ^{111}In -DOTA-[Aca-BN(7-14)]₂ by RP-HPLC analysis (HPLC Method 1).

Statistical Analysis. Quantitative data are expressed as mean $\pm$ SD. Statistical analysis was performed by Student's *t* test or one-way ANOVA using GraphPad InStat software (version 5.0; Graph-Pad software). *P* < 0.05 was considered significant.

RESULTS

Synthesis, Radiolabeling, Partition Coefficient, and *in Vitro* Stability. ^{111}In -DOTA-(Aca-BN)₂ was prepared within 30 min at 85 $^{\circ}\text{C}$ with good radiochemical yield ($\sim 40\%$) and high radiochemical purity after purification ($>95\%$). ^{111}In labeling was a straightforward strategy and gave a good specific activity of $\geq 150\text{ GBq } \mu\text{mol}^{-1}$. The overall synthesis time was around 90 min including HPLC purification and formulation. The partition coefficient was determined in a mixture of *n*-octanol and phosphate buffer (pH 7.4). The log *D* value of

^{111}In -DOTA-[Aca-BN(7-14)]₂ was -1.8 ± 0.1 . The *in vitro* stability of ^{111}In -DOTA-[Aca-BN(7-14)]₂ was evaluated in saline and human serum. ^{111}In -DOTA-[Aca-BN(7-14)]₂ was stable in PBS buffer and human serum for at least 4 h (RCP > 95%). The radiochemical purity of ^{111}In -DOTA-[Aca-BN(7-14)]₂ in serum slowly decreased to $\sim 85\%$ after 48 h (Figure 2).

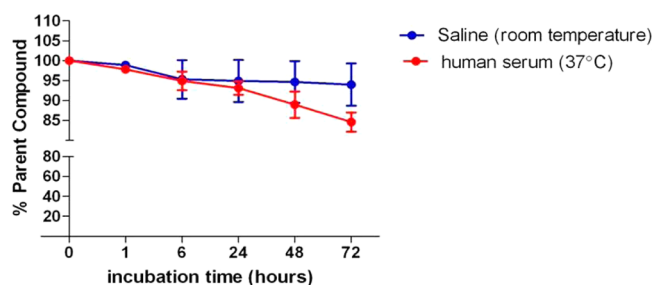


Figure 2. *In vitro* stability of ^{111}In -DOTA-[Aca-BN(7-14)]₂ in saline (at room temperature) and human serum (at 37 $^{\circ}\text{C}$). Results are plotted as the percentage of parent compound or radiochemical purity at different time points.

***In Vitro* Competitive Receptor Binding Assay.** Using ^{125}I -Tyr⁴-BN(1-14) as the GRPR specific probe, the binding affinity of DOTA-Glu[Aca-BN(7-14)]₂ for GRPR was evaluated via an *in vitro* competitive binding assay. Results are plotted as sigmoid curves for the displacement of ^{125}I -Tyr⁴-BN(1-14) as a function of increasing concentrations of DOTA-[Aca-BN(7-14)]₂. The IC₅₀ value observed was 20 nM for DOTA-[Aca-BN(7-14)]₂, while the IC₅₀ value obtained with ^{125}I -Tyr⁴-BN(1-14) was 14 nM (Figure 3).

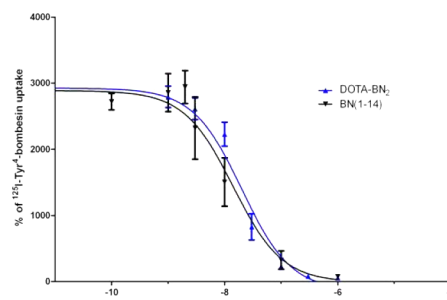


Figure 3. Inhibition of ^{125}I -[Tyr⁴]-BN(1-14) binding to GRPR on PC-3 cells by DOTA-Glu[Aca-BN(7-14)]₂ and BN(1-14). log[M] is expressed as the mean $\pm$ SD.

Cellular Uptake, Internalization, and Efflux Kinetics.

^{111}In -DOTA-[Aca-BN(7-14)]₂ uptake in PC-3 cells gradually increased within 120 min of incubation and reached a plateau afterward. The measured total cellular activity was $\sim 30\%$. Within this 30% of total cell uptake, $\sim 5\%$ was calculated as receptor bound activity, and the remaining 25% was internalized activity. Nonspecific binding was negligible (Figure 4A). The efflux kinetics are shown in Figure 4B. For this tracer, $\sim 65\%$ of the intercellular radioactivity remained internalized in PC-3 cells during the 240 min experimental period.

Biodistribution Experiments. Biodistribution of ^{111}In -DOTA-[Aca-BN(7-14)]₂ was evaluated in PC-3 tumor bearing athymic nude mice at different time points and after performing the microSPECT and CT scans. The results are shown in Figure 5. Figure 5A shows the biodistribution of

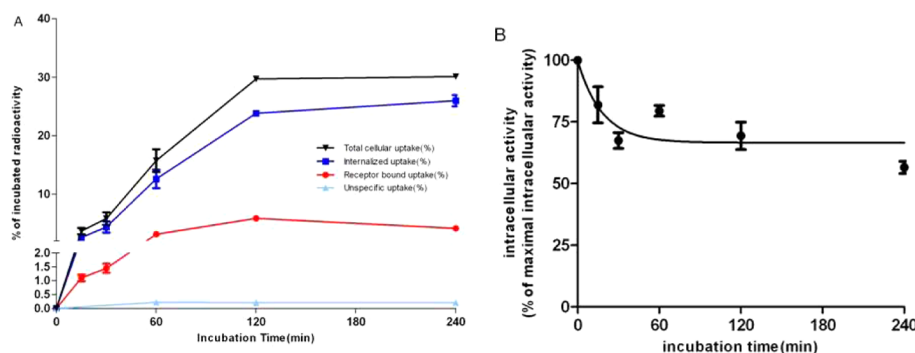


Figure 4. (A) Cell uptake assay of ^{111}In -DOTA-[Aca-BN(7-14)]₂ in PC-3 tumor cells. The cellular uptake results are expressed as the percentage of total radioactivity (internalized activity/(surface bound activity + internalized activity)) (mean $\pm$ SD). (B) Efflux kinetics of ^{111}In -DOTA-[Aca-BN(7-14)]₂ in PC-3 tumor cells. Results are expressed as the percentage of maximum intracellular radioactivity (remaining activity at specific time-point divided by activity at time point 0) (mean $\pm$ SD).

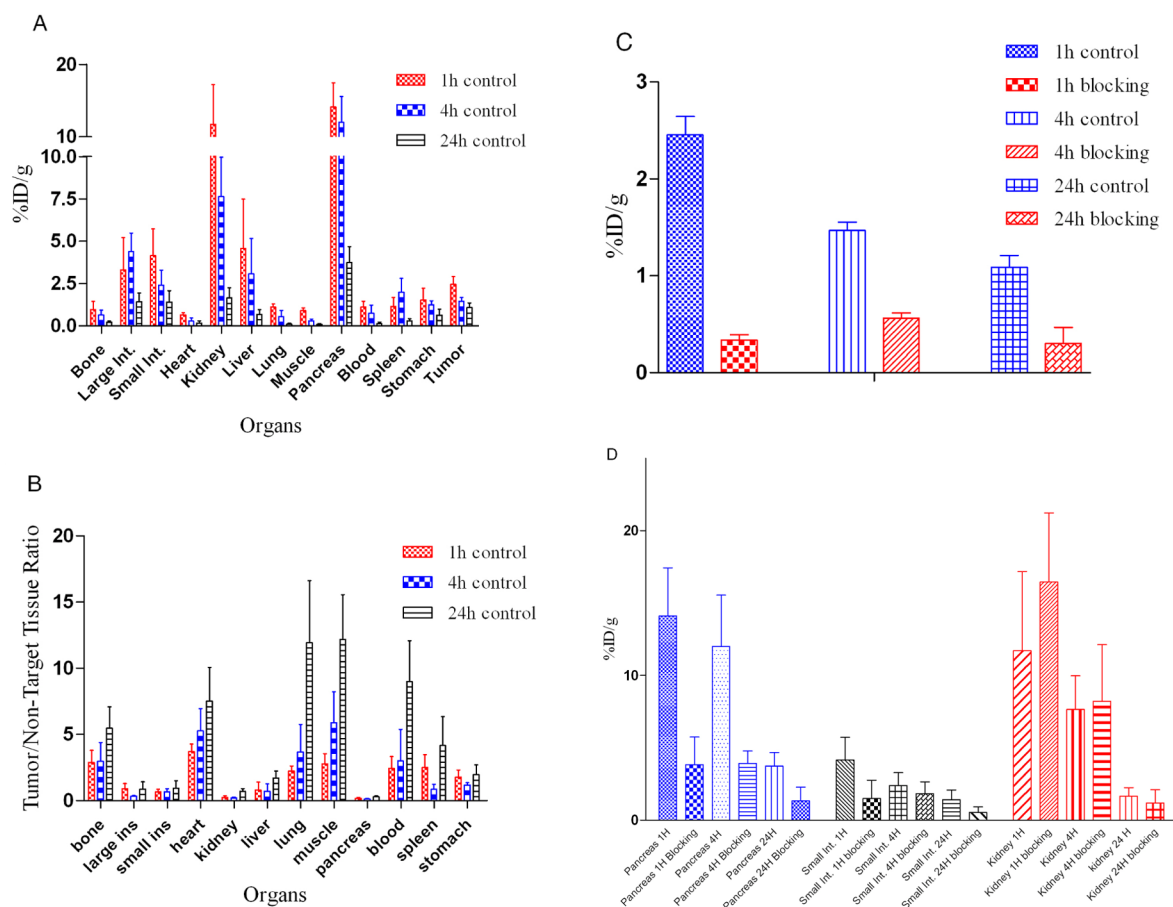


Figure 5. (A) Biodistribution of ^{111}In -DOTA-[Aca-BN(7-14)]₂ at 1, 4, and 24 h p.i. in a subcutaneous PC-3 xenograft model (mean $\pm$ SD %ID/g). (B) Tumor-to-nontumor ratios of ^{111}In -DOTA-[Aca-BN(7-14)]₂ at 1, 4, and 24 h p.i. (mean $\pm$ SD). (C) Tumor uptake at 1, 4, and 24 h after ^{111}In -DOTA-[Aca-BN(7-14)]₂ injection and after coinjection of tracer and blocking agent (mean $\pm$ SD %ID/g). (D) Uptake of ^{111}In -DOTA-[Aca-BN(7-14)]₂ in the pancreas, small intestine, and kidneys at 1, 4, and 24 h p.i. without and with blocking agent (mean $\pm$ SD).

^{111}In -DOTA-[Aca-BN(7-14)]₂ performed at 1, 4, and 24 h p.i. The tumor-to-nontarget tissues ratio was above 10 at 24 h p.i. in muscle as well as in other tissues such as blood and lung (Figure 5B). The specificity of ^{111}In -DOTA-[Aca-BN(7-14)]₂ was evaluated by coinjection with an excess of unlabeled Aca-BN(7-14) (300 $\mu\text{g}/\text{mouse}$). During the 24 h experiment, the tumor uptake dropped from $2.45 \pm 0.18\%$ ID/g at 1 h to $1.1 \pm 0.1\%$ ID/g at 24 h postinjection. The tumor uptake in PC-3 xenografted animals was $0.3 \pm 0.1\%$ ID/g after the coinjection

of the blocking agent. At 24 h postinjection, tumor uptake was more evident compared to the other time points (Figure 5C). Similar findings were observed in other GRPR expressing tissues such as the pancreas, small intestine for both control and blocked animals, and in kidneys, which are important in terms of clearance (Figure 5D).

In Vivo Plasma Stability. The *in vivo* stability of ^{111}In -DOTA-[Aca-BN(7-14)]₂ was evaluated in PC-3 tumor bearing athymic nude mice at 1 h p.i. The measured percentage

of dissociated ^{111}In at 1 h p.i. was $14.1 \pm 5.1\%$. The percentage of intact ^{111}In -DOTA-[Aca-BN(7-14)]₂ recovered from mouse serum 1 h p.i. (indicated in Figure 6 as ^{111}In -DABN₂) was $59 \pm 14\%$.

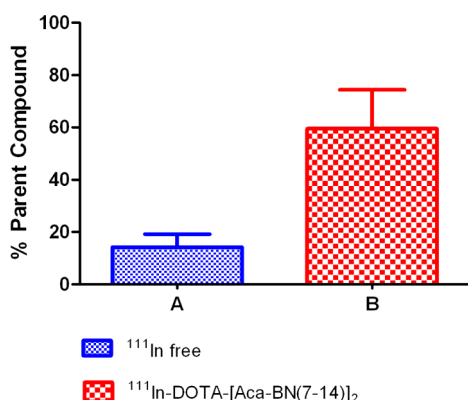


Figure 6. $^{111}\text{In}^{3+}$ dissociation at 1 h p.i. from ^{111}In -DOTA-[Aca-BN(7-14)]₂. $^{111}\text{In}^{3+}$ (A) and ^{111}In -DOTA-[Aca-BN(7-14)]₂ (B) are expressed as the percentage (mean ± SD) of *in vivo* plasma stability.

MicroSPECT Imaging. Coronal microSPECT/CT images of PC-3 tumor bearing mice at different time points after tracer injection are shown in Figure 7. The tumors were clearly visible

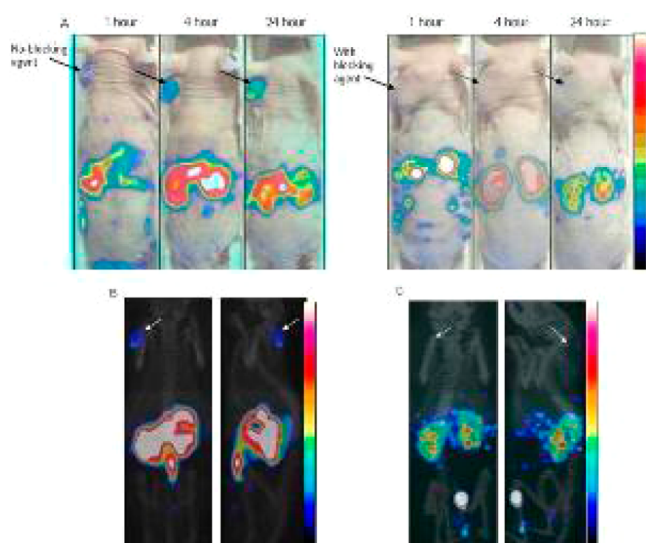


Figure 7. (A) Static coronal microSPECT images of ^{111}In -DOTA-[Aca-BN(7-14)]₂ on PC-3 tumor bearing athymic mice acquired at 1, 4, and 24 h p.i. (A) with and without blocking agent combined to optical photo. Arrows show the tumor site. (B and C) Static coronal microSPECT/CT images acquired at 24 h p.i. without (B) and with blocking agent (C). Arrows indicate the tumor site.

from static microSPECT images acquired at 4 and 24 h p.i. (A and B; left image). Prominent uptake of ^{111}In -DOTA-[Aca-BN(7-14)]₂ was also observed in the kidneys and pancreas at all time-points. With excess blocking agent, a reduction of tumor uptake was observed (A and B; right image).

DISCUSSION

We developed a DOTA-BN conjugate that might be utilized in single photon emission computed tomography (SPECT) ($^{111}\text{In}^{3+}$, $^{90}\text{Y}^{3+}$, $^{149}\text{Pm}^{3+}$, and $^{67}\text{Ga}^{3+}$), positron emission

tomography (PET) ($^{68}\text{Ga}^{3+}$ and $^{64}\text{Cu}^{3+}$), and targeted radionuclide therapy ($^{177}\text{Lu}^{3+}$) to diagnose, treat, and measure the treatment response of BN receptor-positive tumors such as prostate, breast, or gastrointestinal stromal tumors.^{18–20} 1,4,7,10-Tetraazacyclododecane-1,4,7,10-tetraacetic acid, also known as DOTA, forms M^{3+} radiometal labeled complexes with high *in vitro* and *in vivo* stability.^{16,22,23} Therefore, DOTA was the chelating agent selected to perform the radiolabeling with ^{111}In . Our results show that ^{111}In -DOTA-BN₂ is a hydrophilic compound ($\log D = -1.8 \pm 0.1$), which may be due to the conjugation of the peptide using the DOTA chelator. Our results show that the introduction of DOTA-NHS decreased the lipophilicity of our BN-dimer. This difference was apparent after analytic HPLC as a shift of ~2 min in the elution profile of the labeled peptide compared to the native BN unconjugated sequence. The retention time shifted from 30 to 27.9 min.

Similar to other ^{111}In -indium labeled BN analogues,^{23,24} ^{111}In -DOTA-[Aca-BN(7-14)]₂ is obtained within ~60 min (including RP-HPLC purification), with a good radiochemical yield (35%–40%), high radiochemical purity (>99%), and with high specific activity (150 GBq/μmol). The radiochemical yield of 40% was relatively low due to the presence of two stereoisomers, which almost coeluted from the column. The collection of both these peaks would increase the yield, but we chose to collect only the isomer with best *in vitro* binding properties.

The *in vitro* cellular uptake, internalization, and efflux kinetics were evaluated in a PC-3 human prostate cancer cell line expressing GRPR. Specific cellular accumulation of ^{111}In -DOTA-[Aca-BN(7-14)]₂ is slow and reaches a plateau within 120 min. The efflux kinetics of ^{111}In -DOTA-[Aca-BN(7-14)]₂ was also slow, and only 35% of internalized tracer was excreted by the cells. The increased trapping of this radiopharmaceutical into the cells in comparison to the Aca-BN(7-14) monomer^{12,18,24,25} may be a direct consequence of the characteristics of the tracer and the slow washout from the targeted tissues. The increased trapping of a bombesin dimer compared to that of a bombesin monomer was already evident in our previous comparison with $^{99\text{m}}\text{Tc}$ -HABN₂. Compared to $^{99\text{m}}\text{Tc}$ -HABN, $^{99\text{m}}\text{Tc}$ -HABN₂ has slower cellular uptake and continued to internalize in PC-3 cells over the 4 h experiment period. While the monomer was quickly internalized within the first 15 min, ^{111}In -DOTA-[Aca-BN(7-14)]₂ showed slower internalization and longer efflux. When compared to $^{99\text{m}}\text{Tc}$ -HABN₂, the excretion of ^{111}In -DOTA-[Aca-BN(7-14)]₂ by PC-3 cells was similar (~30%) to that of PC-3 cells. This may be the result of the higher bombesin peptide “local concentration” in the proximity of available receptors. Also, in the case of $^{99\text{m}}\text{Tc}$ -HABN₂, we observed an increase in specific accumulation within the first 4 h of the experiment period (uptake plateau for $^{99\text{m}}\text{Tc}$ -HABN was reached already after 30 min). Previous studies with ^{111}In -cycloheximide-B-diethylenetriaminepentaacetic acid-8-Aoc-BN(7-14)NH₂ and $^{99\text{m}}\text{Tc}$ -N3S-X-BN(7-14)NH₂ analogues also showed GRPR-mediated trapping of radioactivity.^{26–28} In 1993, Duncan and Welch demonstrated that radiometals, such as ^{111}In , were retained intracellularly even after reaching the lysosomes.²⁹ After trafficking to lysosomes, the ^{111}In -BN homodimer molecule can be hydrolyzed to products with small molecular weight by the lysosomal enzymes. These metabolites are retained by the lysosome and slowly released from the cell. Most likely ^{111}In -DOTA-aca-amino acid(s) are an unlikely substrate for carrier mediated

transport, so they accumulated into lysosomes. These *in vitro* results suggest that ^{111}In -DOTA-[Aca-BN(7–14)]₂ is selectively taken up and trapped efficiently in GRPR positive cells. The identity of ^{111}In -DOTA-[Aca-BN(7–14)]₂ fragments retained by the cell is not known, and further studies are necessary to clarify the structure of these metabolites. The identification of these fragments might help also to describe better the ^{111}In trapping mechanisms in general.

The performed preclinical evaluation of this tracer in a cancer xenograft animal model overexpressing GRPR showed that the best imaging quality was obtained at relatively late time-points. This may be a consequence of the ^{111}In trapping and of the slow efflux. At a later time-point, optimal imaging quality may be achieved as a result of optimal tumor-to-nontarget tissue ratios. This may be the direct result of the prolonged intracellular retention of ^{111}In -DOTA-[Aca-BN(7–14)]₂ in tumors. However, the increased stability and the relative improved blood circulation may also be involved.

High and specific uptake of ^{111}In -DOTA-[Aca-BN(7–14)]₂ was also observed in other GRPR expressing tissues such as the pancreas, where it showed relatively slow excretion within the first 4 h p.i. The molecular size of the peptide, which results in an increased circulation time compared to its monomeric counterpart, and the relatively slow accumulation could explain this prolonged retention in several organs (Figure 4A), although the tracer slowly washes out from the nontarget organs overtime.

The uptake in the tumor, pancreas, and intestine was specific and receptor mediated, as shown by the coinjection of blocking Aca-BN(7–14) peptide, indicating that these organs are also GRPR positive. Similar high uptake in the GRPR-rich pancreas has been observed in other studies with different BN analogues.^{11,18,25,30}

Similar to our findings, studies carried out with ^{111}In -DOTA-labeled BN(7–14) tracers by Hoffman and co-workers showed a particularly high uptake in the normal pancreas; they achieved the best *in vivo* characteristics (PC-3 tumor uptake was $3.63 \pm 1.1\%$ ID/g) at 1 h, but instability of the probe was a main issue.²⁴ A γ -aminobutyric acid as spacer was tested by Zhang et al.²⁷ In their case, a BN(6–14) truncated sequence was labeled by Indium-111. The ^{111}In -DOTA-labeled BN dimer, compared to the tracer described by Zhang et al., showed relatively higher *in vivo* stability and slower tumor washout.

The agonist properties of ^{111}In -DOTA-labeled BN(7–14) were confirmed by its internalization properties. Although there is still a controversy as to whether agonists or antagonists are more suitable for the imaging of GRPR-expressing cancer, a few radiolabeled bombesin antagonists, such as demobesin-1,³⁰ have already been described as possible candidates. Our bombesin probe showed similar tumor accumulation when compared with Demobesin-1 but less favorable pharmacokinetics as a consequence of slower washout and different hydrophilicities. Ideally, tumor-to-normal ratios of BN analogues should increase over time with high retention in tumor. ^{111}In -DOTA-[Aca-BN(7–14)]₂ showed proportionally increased ratios over time from 1 h to 24 h. This is a known limitation of antagonists, like for example Demobesin-1, which showed a smaller change in tumor-to-normal tissue ratios compared to those of ^{111}In -AMBA and ^{111}In -PESIN.³¹

The uptake in the kidney could be explained by the excretion of peptides and therefore also for ^{111}In -DOTA-[Aca-BN(7–14)]₂. Small proteins (<60 kDa) and peptides are rapidly excreted into the urine by glomerular filtration. Reabsorption,

internalization, and lysosomal degradation of small peptide are a well-known process that takes place in the proximal tubule preventing the excretion of peptides. On the basis of these assumptions, we predict that the main radiolabeled catabolite of ^{111}In -DOTA-[Aca-BN(7–14)]₂ is positively charged and would be retained in the lysosomes. The reason is that positively charged peptides are more efficiently reabsorbed in the proximal renal tubular cells, which are negatively charged.³²

Currently, many efforts are carried out to modify the BN(7–14) sequence in order to improve receptor-targeting ability. For example, Schweinsberg et al. replaced Leu¹³ and Met¹⁴ by Cha and Nle in their [^{99m}Tc (CO)₃-Ala(NTG)- β Ala-BN(7–14)NH₂]. By doing this, they achieved an increased metabolic stability in human plasma and PC-3 cells. Together with an overall increased PC-3 tumor uptake (3.6% ID/g, 1.5 h), they observed a reduced liver accumulation and improved tumor-to-background ratios *in vivo*.³³

Our homodimer showed reasonable metabolic plasma stability and similar % ID/g to AMBA and PESIN with no changing in the primary amino acid sequence. The advantage of the unchanged BN(7–14) sequence is that the binding affinity between receptor and ligand remains unaltered. The stability might result from the increased ligand concentration, which allows a higher steric obstruction for the proteolytic enzymes to the peptide cleavage sites.

The intracellular trapping combined with high stability of the labeled dimer inside the target cells leads to a higher exposure of the cells to ionization and subsequently to a higher DNA damage and consequently apoptosis. One of the most common isotopes used in radiotherapy is ¹⁷⁷Lu because of its high energy and longer range.²¹

Therefore, GRPR-targeted radiotherapy might be feasible using dimer BN in combination with ¹⁷⁷Lu. However, the long retention of the tracer could also be a disadvantage because of the high radiation burden to nontarget tissues, for example, kidneys leading to nephropathy at therapeutic dose.

This study showed enhanced retention of ^{111}In -DOTA-[Aca-BN(7–14)]₂ in tumor compared to monomeric BN probes. Our probe revealed enhanced targeting of the dimer to the GRPR-expressing PC-3 prostate cancer tumors. Although ^{111}In -DOTA-[Aca-BN(7–14)]₂ shows enhanced retention compared to that of the monomeric counterpart, there are similarities with other BN monomers studied in our group (which have faster penetration and efflux kinetics). However, the current bombesin dimer may lead to the design of even better analogues, which may increase the introduction of dimers as tools for the detection of GRPR-expressing prostate cancer tumors.

Clinical evaluation is being planned to study the potential of ^{111}In -DOTA-[Aca-BN(7–14)]₂ for targeting GRPR-expressing tumors on the basis of an easy synthesis procedure, a high radiochemical yield, and consistent tumor uptake.

CONCLUSIONS

We have developed and evaluated an ^{111}In radiolabeled homodimeric BN tracer ^{111}In -DOTA-[Aca-BN(7–14)]₂, which showed excellent *in vitro* binding characteristics to GRPR-positive PC-3 prostate cancer cells. We have demonstrated ^{111}In -DOTA-[Aca-BN(7–14)]₂ as a stable probe for PC tumor imaging. The *in vivo* efficiency of this tracer as a tumor imaging tool was proven in a PC-3 xenograft animal model. ^{111}In -DOTA-[Aca-BN(7–14)]₂ has potential as a clinical radiotracer for targeting GRPR-expressing tumors.

■ AUTHOR INFORMATION

Corresponding Author

*University Medical Center Groningen, Department of Urology, Hanzeplein 1 9713EZ, Groningen, The Netherlands. Tel: +31 50 3612380. Fax: +31 50 3619607. E-mail: g.carlucci@umcg.nl.

Notes

The authors declare no competing financial interest.

■ ACKNOWLEDGMENTS

This work was made possible by a financial contribution from CTMM, project PCMM, project number 03O-203-02. We thank C. Wu for technical assistance on micro-SPECT image reconstruction, D.F. Samplonius for technical assistance on cell culturing, and J.W.A. Sijbesma for assisting in animal experiments. All animal experiments were approved by the local animal welfare committee in accordance with the Dutch legislation and carried out in accordance with their guidelines.

■ REFERENCES

- (1) Carrithers, M. D.; Lerner, M. R. Synthesis and characterization of bivalent peptide ligands targeted to G-protein-coupled receptors. *Chem. Biol.* **1996**, *3*, 537–542.
- (2) Mammen, M.; Choi, S.; Whitesides, G. M. Polyvalent interactions in biological systems: implications for design and use of multivalent ligands and inhibitors. *Angew. Chem., Int. Ed.* **1998**, *37*, 2754–2794.
- (3) Vance, D.; Shah, M.; Joshi, A.; Kane, R. S. Polyvalency: A promising strategy for drug design. *Biotechnol. Bioeng.* **2008**, *101*, 429–434.
- (4) Carlucci, G.; Ananias, H. J.; Yu, Z.; Van de Wiele, C.; Dierckx, R. A.; de Jong, I. J.; Elsinga, P. H. Multimerization improves targeting of peptide radio-pharmaceuticals. *Curr. Pharm. Des.* **2012**, *18*, 2501–2516.
- (5) Chatenet, D.; Cescato, R.; Waser, B.; Ercegyi, J.; Rivier, J. E.; Reubi, J. C. Novel dimeric DOTA-coupled peptidic Y1-receptor antagonists for targeting of neuropeptide Y receptor-expressing cancers. *EJNMMI Res.* **2011**, *1*, 21.
- (6) Cheng, C.; Pan, L.; Dimitrakopoulou-Strauss, A.; Schafer, M.; Wangler, C.; Wangler, B.; Haberkorn, U.; Strauss, L. G. Comparison between ⁶⁸Ga-bombesin (⁶⁸Ga-BZH3) and the cRGD tetramer ⁶⁸Ga-RGD4 studies in an experimental nude rat model with a neuroendocrine pancreatic tumor cell line. *EJNMMI Res.* **2011**, *1*, 34.
- (7) Dijkraaf, I.; Wester, H. J. Peptides, multimers and polymers. *Handb. Exp. Pharmacol.* **2008**, *185/2*, 61–92.
- (8) Majumdar, I. D.; Weber, H. C. Biology of mammalian bombesin-like peptides and their receptors. *Curr. Opin. Endocrinol. Diabetes Obes.* **2011**, *18*, 68–74.
- (9) Ananias, H. J.; van den Heuvel, M. C.; Helfrich, W.; de Jong, I. J. Expression of the gastrin-releasing peptide receptor, the prostate stem cell antigen and the prostate-specific membrane antigen in lymph node and bone metastases of prostate cancer. *Prostate* **2009**, *69*, 1101–1108.
- (10) Ananias, H. J.; de Jong, I. J.; Dierckx, R. A.; van de Wiele, C.; Helfrich, W.; Elsinga, P. H. Nuclear imaging of prostate cancer with gastrin-releasing-peptide-receptor targeted radiopharmaceuticals. *Curr. Pharm. Des.* **2008**, *14*, 3033–3047.
- (11) Nanda, P. K.; Pandey, U.; Bottenus, B. N.; Rold, T. L.; Sieckman, G. L.; Szczodroski, A. F.; Hoffman, T. J.; Smith, C. J. Bombesin analogues for gastrin-releasing peptide receptor imaging. *Nucl. Med. Biol.* **2012**, *39*, 461–471.
- (12) Liu, Z.; Yan, Y.; Liu, S.; Wang, F.; Chen, X. (18)F, (64)Cu, and (68)Ga labeled RGD-bombesin heterodimeric peptides for PET imaging of breast cancer. *Bioconjug. Chem.* **2009**, *20*, 1016–1025.
- (13) Reubi, J. C.; Maecke, H. R. Peptide-based probes for cancer imaging. *J. Nucl. Med.* **2008**, *49*, 1735–1738.
- (14) Yu, Z.; Carlucci, G.; Ananias, H. J.; Dierckx, R. A.; Liu, S.; Helfrich, W.; Wang, F.; de Jong, I. J.; Elsinga, P. H. Evaluation of a technetium-99m labeled bombesin homodimer for GRPR imaging in prostate cancer. *Amino Acids* **2012**, *12*, 1369–1380.
- (15) Thumshirn, G.; Hersel, U.; Goodman, S. L.; Kessler, H. Multimeric cyclic RGD peptides as potential tools for tumor targeting: solidphase peptide synthesis and chemoselective oxime ligation. *Chem.—Eur. J.* **2003**, *9*, 2717–2725.
- (16) De León-Rodríguez, L. M.; Kovacs, Z. The synthesis and chelation chemistry of DOTA-peptide conjugates. *Bioconjugate Chem.* **2008**, *19*, 391–402.
- (17) Wu, Y.; Zhang, X.; Xiong, Z.; Cheng, Z.; Fisher, D. R.; Liu, S.; Gambhir, S. S.; Chen, X. microPET imaging of glioma integrin $\alpha v \beta 3$ expression using ⁶⁴Cu-labeled tetrameric RGD peptide. *J. Nucl. Med.* **2005**, *46*, 1707–1718.
- (18) Ananias, H. J.; Yu, Z.; Dierckx, R. A.; van de Wiele, C.; Helfrich, W.; Wang, F.; Yan, Y.; Chen, X.; de Jong, I. J.; Elsinga, P. H. (99m)technetium-HYNIC(tricine/TPPTS)-Aca-bombesin(7–14) as a targeted imaging agent with microSPECT in a PC-3 prostate cancer xenograft model. *Mol. Pharmaceutics* **2011**, *8*, 1165–1173.
- (19) Decristoforo, C.; Pickett, R. D.; Verbruggen, A. Feasibility and availability of (68)Ga-labelled peptides. *Eur. J. Nucl. Med. Mol. Imaging* **2012**, *39*, 31–40.
- (20) Kam, B. L.; Teunissen, J. J.; Krenning, E. P.; de Herder, W. W.; Khan, S.; van Vliet, E. I.; Kwekkeboom, D. J. Lutetium-labelled peptides for therapy of neuroendocrine tumours. *Eur. J. Nucl. Med. Mol. Imaging* **2012**, *39*, 103–112.
- (21) Koumariou, E.; Mikołajczak, R.; Pawlak, D.; Zikos, X.; Bouziotis, P.; Garnuszek, P.; Karczmarczyk, U.; Maurin, M.; Archimandritis, S. C. Comparative study on DOTA-derivatized bombesin analog labeled with ⁹⁰Y and ¹⁷⁷Lu: in vitro and in vivo evaluation. *Nucl. Med. Biol.* **2009**, *36*, 591–603.
- (22) Good, S.; Walter, M. A.; Waser, B.; Wang, X.; Muller-Brand, J.; Behe, M. P.; Reubi, J. C.; Maecke, H. R. Macrocyclic chelator-coupled gastrin-based radiopharmaceuticals for targeting of gastrin receptor-expressing tumours. *Eur. J. Nucl. Med. Mol. Imaging* **2008**, *35*, 1868–1877.
- (23) Wagh, N. K.; Zhou, Z.; Ogbomo, S. M.; Shi, W.; Brusnahan, S. K.; Garrison, J. C. Development of hypoxia enhanced (111)In-labeled bombesin conjugates: design, synthesis, and in vitro evaluation in PC-3 human prostate cancer. *Bioconjugate Chem.* **2012**, *23*, 527–537.
- (24) Hoffman, T. J.; Gali, H.; Smith, C. J.; Sieckman, G. L.; Hayes, D. L.; Owen, N. K.; Volkert, W. A. Novel series of ¹¹¹In-labeled bombesin analogs as potential radiopharmaceuticals for specific targeting of gastrin-releasing peptide receptors expressed on human prostate cancer cells. *J. Nucl. Med.* **2003**, *44*, 823–831.
- (25) Zhang, X.; Cai, W.; Cao, F.; Schreiber, E.; Wu, Y.; Wu, J. C.; Xing, L.; Chen, X. 18F-Labeled bombesin analogs for targeting GRP receptor-expressing prostate cancer. *J. Nucl. Med.* **2006**, *47*, 492–501.
- (26) Hoffman, T. J.; Li, N.; Volkert, W. A.; Sieckman, G. L.; Higginbotham, C.; Ochymowycz, L. A. Synthesis and characterization of ¹⁰⁵Rh labeled bombesin analogues: enhancement of GRP receptor binding affinity utilizing aliphatic carbon chain linkers. *J. Labelled Compd. Radiopharm.* **1997**, *40*, 490–493.
- (27) Hoffman, T. J.; Simpson, S. D.; Smith, C. J.; et al. Accumulation and retention of Tc-99m RPS27 by GRP receptor expressing tumors in scid mice [abstract]. *J. Nucl. Med.* **1999**, *40* (Suppl), 104P.
- (28) Hoffman, T. J.; Smith, C. J.; Simpson, S. D.; et al. Optimizing pharmacokinetics of ^{99m}Tc-GRP receptor targeting peptides using multi-amino acid linking groups [abstract]. *J. Nucl. Med.* **2000**, *41* (Suppl), 228P.
- (29) Duncan, J. R.; Welch, M. J. Intracellular metabolism of indium-111-DTPA-labeled receptor targeted proteins. *J. Nucl. Med.* **1993**, *34*, 1728–1738.
- (30) Zhang, H.; Chen, J.; Waldherr, C.; Hinni, K.; Waser, B.; Reubi, J. C.; Maecke, H. R. Synthesis and evaluation of bombesin derivatives on the basis of pan-bombesin peptides labeled with indium-111, lutetium-177, and yttrium-90 for targeting bombesin receptor-expressing tumors. *Cancer Res.* **2004**, *64*, 6707–6715.
- (31) Schroeder, R. P.; Muller, C.; Reneman, S.; Melis, M. L.; Breeman, W. A.; de Blois, E.; Bangma, C. H.; Krenning, E. P.; van

Weerden, W. M.; de Jong, M. A standardised study to compare prostate cancer targeting efficacy of five radiolabelled bombesin analogues. *Eur. J. Nucl. Med. Mol. Imaging* **2010**, *37*, 1386–1396.

(32) Behr, T. M.; Goldenberg, D. M.; Becker, W. Reducing the renal uptake of radiolabeled antibody fragments and peptides for diagnosis and therapy: present status, future prospects and limitations. *Eur. J. Nucl. Med. Mol. Imaging* **1998**, *25*, 201–212.

(33) Schweinsberg, C.; Maes, V.; Brans, L.; Bläuenstein, P.; Tourwé, D. A.; Schubiger, P. A.; Schibli, R.; Garayoa, E. G. Novel glycosylated [^{99m}Tc(CO)₃]-labeled bombesin analogues for improved targeting of gastrin-releasing peptide receptor-positive tumors. *Bioconjugate Chem.* **2008**, *19*, 2432–2439.