Published in final edited form as:

Nucl Med Biol. 2014 April ; 41(4): 355–363. doi:10.1016/j.nucmedbio.2014.01.001.

Synthesis and Biological Evaluation of Copper-64 Radiolabeled [DUPA-6-Ahx-(NODAGA)-5-Ava-BBN(7-14)NH₂], a Novel Bivalent Targeting Vector Having Affinity for Two Distinct Biomarkers (GRPr/PSMA) of Prostate Cancer

Rajendra Prasad Bandari^{1,2}, Zongrun Jiang^{1,5}, Tamila Stott Reynolds^{1,6}, Nicole E. Bernskoetter^{1,2}, Ashley F. Szczodroski¹, Kurt J. Bassuner², Daniel L. Kirkpatrick², Tammy L. Rold^{1,4}, Gary L. Sieckman¹, Timothy J. Hoffman^{1,4,5}, James P. Connors², and Charles J. Smith^{1,2,3,*}

¹Research Service, Truman VA, Columbia, Missouri, United States, 65201

²Department of Radiology, University of Missouri School of Medicine, Columbia, Missouri, United States, 65211

³University of Missouri Research Reactor Center, University of Missouri, Columbia, Missouri, United States, 65211

⁴Department of Internal Medicine, University of Missouri School of Medicine, Columbia, Missouri, United States, 65211

⁵Department of Chemistry, University of Missouri, Columbia, Missouri, United States, 65211

⁶Department of Veterinary Pathobiology, University of Missouri College of Veterinary Medicine, Columbia, Missouri, United States, 65211

Abstract

Gastrin-releasing peptide receptors (GRPr) and prostate-specific membrane antigen (PSMA) are two identifying biomarkers expressed in very high numbers on prostate cancer cells and could serve as a useful tool for molecular targeting and diagnosis of disease *via* positron-emission tomography (PET). The aim of this study was to produce the multipurpose, bivalent [DUPA-6-Ahx-(⁶⁴Cu-NODAGA)-5-Ava-BBN(7-14)NH₂] radioligand for prostate cancer imaging, where DUPA = 2-[3-(1,3-Bis-tertbutoxycarbonylpropyl)-ureido]pentanedioic acid, a small-molecule, PSMA-targeting probe, 6Ahx = 6-aminoheptanoic acid, 5-Ava = 5-aminovaleric acid, NODAGA = [2-(4,7-biscarboxymethyl)-1,4,7-(triazonan-1-yl)pentanedioic acid] (a derivative of NOTA (1,4,7-triazacyclononane-1,4,7-triacetic acid)), and BBN(7-14)NH₂ = bombesin or BBN, a GRPr-specific peptide targeting probe.

* Author to whom correspondence should be addressed: Dr. Charles J. Smith, Truman VA, Research Service Room A005, 800 Hospital Drive, Columbia, MO 65201, smithcj@health.missouri.edu, Phone: (573) 814-6000 ext. 53683, Fax: (573) 814-6551.

Publisher's Disclaimer: This is a PDF file of an unedited manuscript that has been accepted for publication. As a service to our customers we are providing this early version of the manuscript. The manuscript will undergo copyediting, typesetting, and review of the resulting proof before it is published in its final citable form. Please note that during the production process errors may be discovered which could affect the content, and all legal disclaimers that apply to the journal pertain.

Methods—The PSMA/GRPr dual targeting ligand precursor [DUPA-6-Ahx-K-5-Ava-BBN(7-14)NH₂], was synthesized by solid-phase and manual peptide synthesis, after which NODAGA was added *via* manual conjugation to the ε-amine of lysine (K). The new bivalent GRPr/PSMA targeting vector was purified by reversed-phase high performance liquid chromatography (RP-HPLC), characterized by electrospray-ionization mass spectrometry (ESI-MS), and metallated with ⁶⁴CuCl₂ and ^{nat}CuCl₂. The receptor binding affinity was evaluated in human, prostate, PC-3 (GRPr-positive) and LNCaP (PSMA-positive) cells and the tumor-targeting efficacy determined in severe combined immunodeficient (SCID) and athymic nude mice bearing PC-3 and LNCaP tumors. Whole-body maximum intensity microPET/CT images of PC-3/LNCaP tumor-bearing mice were obtained 18 h post-injection (p.i.).

Results—Competitive binding assays in PC-3 and LNCaP cells indicated high receptor binding affinity for the [DUPA-6-Ahx-(^{nat}Cu-NODAGA)-5-Ava-BBN(7-14)NH₂] conjugate. MicroPET scintigraphy in PC-3/LNCaP tumor-bearing mice indicated that xenografted tumors were visible at 18 h p.i. with collateral, background radiation also being observed in non-target tissue.

Conclusions—[DUPA-6-Ahx-(⁶⁴Cu-NODAGA)-5-Ava-BBN(7-14)NH₂] targeting vector, as described herein, is the first example of a dual GRPr-/PSMA-targeting radioligand for molecular imaging prostate tumors. Detailed *in vitro* studies and microPET molecular imaging investigations of [DUPA-6-Ahx-(⁶⁴Cu-NODAGA)-5-Ava-BBN(7-14)NH₂] in tumor-bearing mice indicates that further studies are necessary to optimize uptake and retention of tracer in GRPr- and PSMA-positive tissues.

Keywords

gastrin-releasing peptide; prostate-specific membrane antigen; bombesin; prostate cancer; copper-64

1. Introduction

Prostate cancer continues to be the most commonly diagnosed cancer in men in the United States. According to the American Cancer Society, prostate cancer will account for an estimated 238,590 new cases and 29,720 deaths in 2013 [1]. Clinical diagnosis of prostate cancer is often done through digital rectal exam and blood analysis of blood-serum levels of prostate-specific antigen (PSA), and diagnostic anatomical imaging generally with magnetic resonance imaging (MRI), computed tomography (CT), or ultrasound (US) [2, 3]. Elevated PSA levels in blood serum do not correlate well with prostate cancer disease. For example, 20-30% of prostate cancer patients present with PSA levels within the reference range, resulting in a high number of false negatives. Furthermore, false positives resulting in unnecessary biopsies sometimes occur in an additional number of patients who present with elevated PSA levels due to conditions other than prostate cancer, including benign prostatic hyperplasia [4]. The current methods of clinical determination for patients presenting with prostate cancer are considered to be inadequate for early diagnosis. For example, by the time a malignancy has been detected and a clinical diagnosis made, the disease has already metastasized. Clearly, new diagnostic techniques for early detection and staging of primary or metastatic prostate cancer disease are warranted.

Gastrin releasing peptide receptors (GRPr) and prostate-specific membrane antigen (PSMA) are expressed in very high numbers on many types of cancers, making them ideal biomarkers for molecular targeting with radiolabeled peptides or small molecules [4, 5]. The GRPr is present in high concentrations on human cancers such as prostate, breast, pancreatic, and small cell lung carcinoma [6]. Mammalian BBN receptors are G protein-coupled, 7-transmembrane receptors having the capacity to be endocytosed upon binding by an effective agonist ligand. There are four known mammalian bombesin receptor subtypes: BB1 (neuromedin B receptors, NMBR), BB2 (gastrin-releasing peptide receptors, GRPr), and BB3 (BRS-3) [7]. To date, design and development of radiolabeled agents for the GRP receptors have largely focused upon the BB2 receptor subtype. BBN is the 14 amino acid amphibian peptide analog of the 27 amino acid mammalian regulatory peptide GRP. BBN and GRP share a homologous, 7 amino acid amidated C-terminus that is essential for high-affinity binding to GRPr. The ability of GRPr agonists to be rapidly internalized coupled with a high incidence of GRPr expression on various neoplasias has been a driving force for design and development of new diagnostic and therapeutic agents targeting GRP receptor-positive tumors [8]. PSMA, also known as folate hydrolase I or glutamate carboxypeptidase II, is a 750 amino acid integral membrane protein present in the neovasculature of most solid tumors including renal carcinoma, colorectal carcinoma, glioblastoma multiforme, pancreatic duct carcinoma, and breast carcinoma, yet absent in normal, vascular endothelium of non-cancer bearing tissues [9, 10]. PSMA is expressed in prostate tumor epithelium, and to a lesser extent in the vasculature of prostate cancer itself [2, 3, 11]. However, while PSMA is largely limited to the epithelium of prostate cancer cells, upregulation and translocation from internal organelles to the cell surface generally occurs as androgen-independent, metastatic disease progresses [2, 3, 11]. This is a particularly important issue as nearly all prostate cancers progress to the androgen-independent, metastatic stage [2, 3, 11]. For example, homogenous receptor expression has been found in metastatic lesions localized to the lymphatic tissue, bone, and lungs [10]. PSMA is highly expressed on the majority of all prostate cancers and expressed in low numbers or not at all on surrounding collateral tissues. For example, the density per cancer cell is $\sim 10^6$ [3] and is ~ 1000 -fold higher than normal PSMA-expressing tissues such as the kidney, brain, or small intestine [12]. PSMA undergoes internalization *via* clathrin-coated pits and has a tendency to be recycled to the surface of prostate cancer cells for additional internalization events [13, 14]. The ability of PSMA to be rapidly internalized coupled with a high incidence of expression on various tumor neovasculature including prostate cancer has recently led to the design and development of new diagnostic and therapeutic agents targeting PSMA as a clinical biomarker for early detection, staging, and potential treatment of human disease.

Our group and many others are interested in ^{64}Cu -radiolabeled peptide analogs for targeting human cancer. ^{64}Cu -labeled radiopharmaceuticals have been of interest due to the ideal nuclear characteristics of ^{64}Cu ; ^{64}Cu [$t_{1/2}=12.7\text{h}$; $E_{\beta^+_{\text{max}}}=0.65\text{MeV}$ (17.9%); $E_{\beta^-_{\text{max}}}=0.57\text{MeV}$ (39%); electron capture (EC) (43.1%)], making it useful for *in vivo* molecular imaging. ^{64}Cu is a cyclotron-produced radionuclide prepared *via* a (p,n) reaction on a highly enriched ^{64}Ni target. The half-life for ^{64}Cu is sufficiently long to allow for drug preparation, quality control, drug incorporation, circulation, and patient imaging [15, 16]. Peptide conjugates containing the chelating agents 1,4,7,10-tetraazacyclododecane-1,4,7,10-

tetraacetic acid (DOTA) and 1,4,8,11-tetraazacyclotetradecane-1,4,8,11-tetraacetic acid (TETA) have been described for production of ^{64}Cu -labeled targeting vectors [17-20]. However, $^{64}\text{Cu}^{2+}$ -complexes of DOTA and TETA are only moderately stable under *in vivo* conditions, resulting in demetallation and accumulation of tracer in non-target tissues such as liver. Cross-bridged, cyclam-based ligand frameworks (CB-TE2A) appended to specific, biologically-active targeting vectors offer improved kinetic stability to *in vivo* transmetallation with various proteins in comparison to DOTA and TETA [17-23]. 1,4,7-triazacyclononane-1,4,7-triacetic acid (NOTA) has been used as a bifunctional chelating agent (BFCA) for divalent copper when conjugated to antibodies [24, 25]. However, NOTA-based peptide conjugates for copper radiometals for production of kinetically inert targeting vectors have been largely unexplored until just recently. NOTA has the capacity to form stable complexes with Cu^{2+} as well as with a host of other di- and trivalent metal centers [24-27]. NODAGA, [2-(4,7-biscarboxymethyl)-1,4,7-(triazonan-1-yl)pentanedioic acid], a derivative of NOTA, also has the capacity to form stable complexes with Cu^{2+} .

The usage of radiolabeled bivalent heterodimers is a relatively new and exciting approach for improved molecular imaging of human cancers [28, 29]. In this research investigation, we report the first radiolabeled dual receptor/biomarker targeting agent based upon the GRPr and PSMA. Each of these two biomarkers have been validated in prostate cancer disease. This article describes synthesis and characterization of [DUPA-6-Ahx-(^{64}Cu -NODAGA)-5-Ava-BBN(7-14) NH_2]. Detailed *in vitro* investigations of this new, dual-targeting PET radiopharmaceutical are described in PC-3 (GRPr-positive; PSMA-negative) and LNCaP (PSMA-positive; GRPr-negative or minimally expressed [30, 31]) prostate cancer cells. We also report on MicroPET molecular imaging investigations of this new bivalent GRPr/PSMA-targeting agent.

2. Materials and Methods

2.1. General

Commercially available chemical reagents were purchased from Fisher Scientific (Waltham, MA) and Sigma Life Science (St. Louis, MO), and used without further purification. $^{64}\text{CuCl}_2$ was obtained from the University of Wisconsin-Madison (Madison, WI) as a 0.1M HCl solution. Amino acid residues and resins for solid-phase and manual peptide synthesis were purchased from either Novabiochem/EMD Biosciences, Inc. (La Jolla, CA), Advanced ChemTech (Louisville, KY), or Chem-Impex International (Wood Dale, IL). Electrospray-ionization mass spectrometry (ESI-MS) for characterization of precursor peptide, conjugate, and metallated conjugate was performed at the MS Facility, Department of Chemistry, University of Missouri (Columbia, MO). PC-3 and LNCaP cells were obtained from American Type Culture Collection and were maintained by the University of Missouri Cell and Immunobiology Core Facility (Columbia, MO).

2.1.1. Reversed-phase High-Performance Liquid Chromatography (RP-HPLC)

—RP-HPLC analyses of radiolabeled and non-radiolabeled compounds were performed on a Shimadzu SCL-10A system (Shimadzu, Kyoto, Japan) equipped with a Shimadzu SPD-10A UV-Vis tunable absorbance detector ($\lambda=280\text{nm}$), an Eppendorf TC-50 column heater (Eppendorf, Hamburg, Germany), and a radiometric, in-line EG&G ORTEC NaI solid

scintillation detector (EG&G, Salem, MA). Purification of [DUPA-6-Ahx-K-5-Ava-BBN(7-14)NH₂] precursor and [DUPA-6-Ahx-(NODAGA)-5-Ava-BBN(7-14)NH₂] conjugate was performed on a semi-preparative, reversed-phase C18 column (Phenomenex Jupiter Proteo, 250×10.00mm, 10μm; Phenomenex, Torrance, CA). Purification of [DUPA-6-Ahx-(^{nat}Cu-NODAGA)-5-Ava-BBN(7-14)NH₂], where ^{nat}Cu = natural copper, and [DUPA-6-Ahx-(⁶⁴Cu-NODAGA)-5-Ava-BBN(7-14)NH₂] conjugates was performed on an analytical, reversed-phase C18 column (Phenomenex Jupiter Proteo, 250×4.60mm, 5μm). The solvent system consisted of ultrapure water containing 0.1% trifluoroacetic acid (Solvent A) and acetonitrile containing 0.1% trifluoroacetic acid (Solvent B). The gradient system began at 75% Solvent A and 25% Solvent B. From 0-15 min, the solvent proportion increased linearly to 65% Solvent A and 35% Solvent B. From 15-17 min, the solvent increased to 5% Solvent A and 95% Solvent B. Flow rate was set at 5mL/min for the semi-preparative and 1.5mL/min for the analytical RP-HPLC.

2.2. Chemistry

2.2.1. Synthesis of 2-[3-(1,3-Bis-tert-butoxycarbonylpropyl)-ureido]pentanedioic acid 1-tert-butyl ester—2-[3-(1,3-Bis-tert-

butoxycarbonylpropyl)-ureido]pentanedioic acid 1-*tert*-butyl ester (DUPA precursor) was prepared according to a published procedure with only slight modification [32]. Triethyl amine (1.0 mL, 8.19 mmol) was added to a solution of L-glutamate di-*tert*-butyl ester hydrochloride (1.0 g, 3.39 mmol) and triphosgene (329.8 mg, 1.12 mmol) in methylene chloride (25.0 mL) and stirred for 2 h at -78 °C under a nitrogen atmosphere. After stirring, a methylene chloride solution (5.0 mL) containing L-GIU(OBn-OtBu) (1.2 g, 3.72 mmol) and triethyl amine (600 mL, 4.91 mmol) was added. The reaction mixture was allowed to come to room temperature over a one hour time period, and the solution continued to stir overnight. The reaction was quenched with 1.0 M HCl, and the organic layer was washed with brine and dried over Na₂SO₄. The crude product was purified using flash chromatography (Hexane:Ethyl Acetate, 1:1) to produce a colorless oil that was recrystallized from a mixture of hexane/methylene chloride. 2-[3-(3-Benzyloxycarbonyl-1-*tert*-butoxycarbonyl-propyl)-ureido]pentanedioic acid di-*tert*-butyl ester was characterized by ¹H and ¹³C nuclear magnetic resonance (NMR) spectroscopy and characterized by ESI-MS (C₃₀H₄₇N₂O₉: Calculated, 579.3282; Found, 579.3289). Deprotection of the benzyl group was performed by hydrogenation. Ten percent palladium on carbon (Pd/C) was added to a solution of 2-[3-(3-Benzyloxycarbonyl-1-*tert*-butoxycarbonyl-propyl)-ureido]pentanedioic acid di-*tert*-butyl ester (1g, 1.73mmol) in methylene chloride. The reaction mixture was hydrogenated at 1 atm for a period of 24 h at room temperature, after which the Pd/C was filtered through a celite pad and washed with methylene chloride. The crude product was purified using flash chromatography (Hexane:Ethyl Acetate, 0.4:0.6) to produce 2-[3-(1,3-Bis-tert-butoxycarbonylpropyl)-ureido]pentanedioic acid 1-*tert*-butyl ester as a colorless oil (676mg, 80.2%). The purified product was characterized by ¹H and ¹³C NMR spectroscopy and characterized by ESI-MS (C₂₃H₄₁N₂O₉: Calculated, 482.2812; Found, 489.2808).

2.2.2. Synthesis of [DUPA-6-Ahx-K-5-Ava-BBN(7-14)NH₂] Precursor—The bivalent [DUPA-6-Ahx-K-5-Ava-BBN(7-14)NH₂] GRPr/PSMA-targeting precursor was

synthesized using a combination of solid-phase and manual peptide synthesis. Briefly, the GRPr/PSMA-targeting precursor was prepared by manual resin-based peptide synthesis employing traditional F-moc chemistry. 6-Ahx and 5-Ava denote the pharmacokinetic modifiers 6-aminohexanoic acid and 5-aminovaleric acid, BBN(7-14)NH₂ = Q-W-A-V-G-H-L-M-NH₂, and DUPA = 2-[3-(1,3-Bis-tert-butoxycarbonylpropyl)-ureido]pentanedioic acid. Briefly, Rink Amide-MBHA resin (0.1 mmol) and F-moc-protected amino acids with appropriate side-chain protections (0.2 mmol) were utilized for synthesis. The resin was swelled using a combination of methylene chloride (3 mL) and dimethyl formamide (DMF, 3 mL). A solution of 20% piperidine in DMF (3 × 3 mL) was added to the resin and nitrogen was bubbled through it for 5 min. The resin was washed with DMF (3 × 3 mL) and isopropyl alcohol (*i*-PrOH, 3 × 3 mL). Formation of the free, N-terminal, primary amine was assessed by the Kaiser Test. Upon swelling the resin once again in DMF, a solution of F-moc-Met-OH (0.2 mmol), O-Benzotriazole-N,N',N'-tetramethyl-uronium-hexafluorophosphate (HBTU) (0.2 mmol), Hydroxybenzotriazole (HOBt) (0.2 mmol) and *N,N*-Diisopropylethylamine (DIPEA) (0.4 mmol) in DMF was added to the resin. The resin was bubbled under nitrogen gas for 2 h, after which it was washed with DMF (3 × 3 mL) and *i*-PrOH, (3 × 3 mL). The coupling efficiency was assessed by the Kaiser Test, and the above coupling procedure was repeated 12 additional times to produce the bivalent ligand precursor. Each coupling procedure took ~2-6 h. A cocktail of water, triisopropylsilane (TIS), and trifluoroacetic acid (TFA) in a ratio of 2.5:2.5:95 was used to cleave the peptide from the resin, followed by peptide precipitation in methyl-*t*-butyl ether. The crude peptide conjugate was obtained in ~80% yield and purified by RP-HPLC. Solvents were removed *in vacuo* using a SpeedVac concentrator (Labconco, Kansas City, MO). The purified peptides were characterized by ESI-MS.

2.2.3. Synthesis of [DUPA-6-Ahx-(NODAGA)-5-Ava-BBN(7-14)NH₂]—NODAGA, [2-(4,7-biscarboxymethyl)-1,4,7-(triazonan-1-yl)pentanedioic acid] (a derivative of NOTA (1,4,7-triazacyclononane-1,4,7-triacetic acid)) was conjugated *via* an active ester onto the ε-amine of lysine (K) on the bivalent peptide precursor [DUPA-6-Ahx-K-5-Ava-BBN(7-14)NH₂] to produce the new NODAGA conjugate, [DUPA-6-Ahx(NODAGA)-5-Ava-BBN(7-14)NH₂], using a modified procedure that has been previously described [33, 34]. Briefly, NODAGA-NHS (27 μmol) was stirred at room temperature in 200 μL of 0.1 M sodium phosphate buffer (pH = 7.0). [DUPA-6-Ahx-K-5-Ava-BBN(7-14)NH₂] (2.7 μmol) was dissolved in 0.1 M sodium phosphate buffer and the pH was adjusted to 7.4 using 10% NaOH. The reaction mixture was allowed to stir for 6 h at 5-10 °C, after which it was allowed to stir overnight at ambient temperature. The bivalent NODAGA conjugate was purified by RP-HPLC. ESI-MS was used to confirm the identity of the new bivalent conjugate.

2.2.4. Metallation of [DUPA-6-Ahx-(NODAGA)-5-Ava-BBN(7-14)NH₂] with ^{nat}Cu and ⁶⁴Cu—[DUPA-6-Ahx-(^{nat}Cu-NODAGA)-5-Ava-BBN(7-14)NH₂] was prepared by addition of 7.4 mM ^{nat}CuCl₂ in 0.05 N HCl (90 nmol) to a plastic tube containing purified [DUPA-6-Ahx-(NODAGA)-5-Ava-BBN(7-14)NH₂] peptide conjugate (80 nmol) and 0.4 M ammonium acetate (250 μL). The pH of the reaction mixture was adjusted to ~7 by the addition of 1% NaOH and then incubated at 70 °C for 1 h. 10 mM DTPA solution (50 μL)

was added to scavenge unbound metal. The metallated conjugate was purified by RP-HPLC and characterized by ESI-MS. The pure product was obtained as a white powder.

Likewise, [DUPA-6-Ahx-(^{64}Cu -NODAGA)-5-Ava-BBN(7-14) NH_2] was prepared by addition of $^{64}\text{CuCl}_2$ (185 MBq, 2.02×10^{-11} mol, 9.16×10^{18} Bq/mol) to a plastic tube containing purified peptide conjugate (50 μg , 25.7 μmol) and 0.4 M ammonium acetate (250 μL). The pH of the reaction mixture was adjusted to ~ 7 by the addition of 1% NaOH and then incubated at 70 $^\circ\text{C}$ for 1 h. 10 mM DTPA solution (50 μL) was added to scavenge unbound radioactivity. Radiolabeled conjugate was purified by RP-HPLC and collected into 1 mg/mL bovine serum albumin (BSA) stabilizing agent (100 μL) and ascorbic acid (25 μg) prior to *in vitro* and *in vivo* evaluation. Acetonitrile was removed from the HPLC-purified tracer under a steady stream of nitrogen gas.

2.3. In Vitro Assays

2.3.1. In Vitro Competitive Displacement Binding Assays—A competitive displacement binding assay (IC_{50}) of [DUPA-6-Ahx-(^{nat}Cu -NODAGA)-5-Ava-BBN(7-14) NH_2] was determined in GRPr-positive PC-3 cells and PSMA-positive LNCaP homogenized cell membranes using [^{125}I -(Tyr 4)-BBN] and [N-acetyl aspartyl ^3H -glutamate] (NAAG) as the radioligands. For the GRPr-positive assay, 3×10^4 PC-3 cells (in D-MEM/F-12K media containing 0.01 M MEM and 2% BSA, pH=5.5) were incubated with 20,000 counts per minute of [^{125}I -(Tyr 4)-BBN] and increasing concentrations (1×10^{-13} – 1×10^{-5} M) of the metallated targeting vector (1 h, 37 $^\circ\text{C}$, 5% CO_2 -enriched atmosphere). After incubation, the reaction medium was aspirated and the cells were rinsed three times with cold media. Cell-associated radioactivity was determined using a Packard Riastar gamma counter. The percent of bound radioligand was plotted against the increasing concentrations of the metallated conjugate to determine the IC_{50} value. IC_{50} values were determined by curve fitting using Prism Software (version 5.0).

For the PSMA-positive assay, the binding affinity was measured using the N-acetylated-a-linked acidic dipeptidase (NAALADase) assay with only minor modification [35]. Briefly, [DUPA-6-Ahx-(^{nat}Cu -NODAGA)-5-Ava-BBN(7-14) NH_2] (1×10^{-13} – 1×10^{-5} M) in 50 μL of Tris-HCl buffer (50 mM, pH = 7.4) was incubated with LNCaP tissue culture homogenized cell membranes for a period of 45 min. Then, [N-acetyl aspartyl ^3H -glutamate] was added to the reaction mixture and the solution was allowed to incubate for an additional 15 min at 37 $^\circ\text{C}$. The enzymatic reaction was stopped by addition of 50 μL of cold sodium phosphate buffer (0.1 M, pH = 7.4). [N-acetyl aspartyl ^3H -glutamate] and [^3H -glutamate] were resolved by cation exchange chromatography using AG 50WX8 columns (200–400 mesh). Columns were pre-equilibrated with 0.2 M HCl prior to loading of the reaction mixture. Fractions containing [^3H -glutamate] were eluted using 6 mL of 2 M HCl. Scintillation cocktail was added to each fraction and the amount of radioactivity in each was determined by liquid scintillation counting. The percent of bound radioligand was plotted against the increasing concentrations of the metallated conjugate to determine the IC_{50} value. IC_{50} values were determined by curve fitting using Prism Software (version 5.0).

2.3.2. In Vitro Internalization Assays of [DUPA-6-Ahx-(Rhodamine)-5-Ava-BBN(7-14)NH₂]

—To assess the cell targeting ability and endocytosis of the new radioligand, a fluorescent rhodamine B isothiocyanate (Rhodamine-B) derivative of the [DUPA-6-AhxK-5-Ava-BBN(7-14)NH₂] targeting vector was prepared. Briefly, 7.2 mg of Rhodamine-B isothiocyanate (31 μmol) was added to a solution of [DUPA-6-Ahx-K-5-Ava-BBN(7-14)NH₂] (3 mg, 2.7 μmol, 0.1 M sodium phosphate buffer) in 200 μL of DMF, followed by addition of four equivalents of DIPEA (N,N-Diisopropylethylamine). The reaction was allowed to stir overnight (20 h) under zero-light conditions, after which purification by semi-preparative RP-HPLC afforded the new fluoroprobe in high yield (~70%). The new conjugate was characterized by ESI-MS. Internalization and blocking assays of the [DUPA-6-Ahx-(Rhodamine)-5-Ava-BBN(7-14)NH₂] targeting vector were performed by incubation of conjugate in the presence or absence of blocking agent with either PC-3 (GRPr-positive) or LNCaP (PSMA-positive) human, prostate cancer cells for 45 min, followed by an acid wash using 0.2 N acetic acid/0.5 M NaCl (pH=2.5). Cells were seeded into poly-D-lysine microwell Petri dishes and allowed to form monolayers over a 24 h period. Spent medium was replaced with fresh medium along with [DUPA-6-Ahx(Rhodamine)-5-Ava-BBN(7-14)NH₂] (100 nM), and the cells were allowed to incubate for a 45 min time period at 37 °C. The cells were washed three times with phosphate-buffered saline (PBS) to removed unbound [DUPA-6-Ahx-(Rhodamine)-5-Ava-BBN(7-14)NH₂] from the cellular suspension. For blocking assays, cells were incubated with either 100 μM of commercially available BBN(1-14) (GRPr blocking agent) or 2-(Phosphonomethyl)-pentanedioic acid (PMPA, PSMA blocking agent) 15 min prior to addition of the Rhodamine probe. Cell-associated fluorescence was affirmed using an automated upright, microscope (Leica DM4000B, Wetzlar, Germany) set to 590 nm.

2.4. In Vivo Assays

2.4.1. MicroPET Scintigraphy of [DUPA-6-Ahx-(⁶⁴Cu-NODAGA)-5-Ava-BBN(7-14)NH₂] in GRPr-positive and PSMA-positive Tumors

—All animal studies were conducted in compliance with the NIH guide for Care and Use of Laboratory Animals and the Policy and Procedures for Animal Research at the Harry S. Truman Memorial Veterans' Hospital. ICR SCID (Institute of Cancer Research Severe Combined Immunodeficient) female mice (4-5 weeks of age) and male athymic nude-*FoxI^{nu}* mice were supplied from Taconic Farms (Germantown, NY) or Harlan Laboratories (Madison, WI). The mice were housed in sterile micro isolator cages in a temperature- and humidity-controlled room with a 12 h light/12 h dark schedule. The animals were fed autoclaved rodent chow (Ralston Purina Company, St. Louis, MO) and water *ad libitum*. SCID mice were anesthetized for injections with isoflurane (Baxter Healthcare Corp., Deerfield, IL) at a rate of 2.5% with 0.4 L oxygen through a nonbreathing anesthesia vaporizer. PC-3 cells were injected bilaterally into the subcutaneous flank regions, with ~5×10⁶ cells in a suspension of 100 μL normal sterile saline per injection site. Tumors were allowed to grow 2-5 weeks post inoculation, developing tumors ranging in mass from ~0.2-0.4 g. Athymic nude mice were also anesthetized for injections with isoflurane (Baxter Healthcare Corp., Deerfield, IL) at a rate of 2.5% with 0.4 L oxygen through a non-rebreathing anesthesia vaporizer. LNCaP cells were injected bilaterally into the subcutaneous flank regions, with ~5×10⁶ cells in a suspension of 100 μL normal sterile saline per injection site. Tumors were

allowed to grow 2-5 weeks post inoculation, developing tumors ranging in mass from ~0.2-0.4 g.

Maximum intensity micro-positron emission tomography (microPET) coronal images were obtained on a Siemens INVEON small-animal, dedicated PET unit (Siemens, Nashville, TN, USA). The unit has a transverse field of view (FOV) of 10 cm and an axial length of 12.7 cm. The scanner operates in a three-dimensional (3D) volume imaging acquisition mode. Mice were laser aligned at the center of the scanner FOV for subsequent imaging. Mice bearing xenografted human prostate PC-3 or LNCaP tumors were administered ~25 MBq (675 μ Ci) of [DUPA-6-Ahx-(64 Cu-NODAGA)-5-Ava-BBN(7-14)NH₂] in 100 μ L of isotonic saline *via* tail vein injection. At 15 h p.i., the mice were euthanized by CO₂ administration. MicroPET image reconstruction was obtained with an ordered subset expectation maximization (OSEM) algorithm with 3D resolution recovery and the absence of tissue attenuation correction. The microPET data was filtered with a Gaussian full width at half maximum filter. Additionally, the micro-computed tomography (microCT) coronal images were also obtained on the Siemens INVEON small-animal CT unit (Nashville, TN, USA) immediately after microPET for the purpose of anatomic/molecular data fusion. The microCT images were acquired for ~8 min, and concurrent image reconstruction was achieved using a conebeam (Feldkamp) filtered backprojection algorithm. The reconstructed (raw) microPET datasets (with a matrix size of 512 $\times$ 512 $\times$ 159) were imported into the INVEON Research Workstation software for subsequent image fusion with microCT image data and 3D visualization.

3. Results

3.1. Chemistry and Radiochemistry

2-[3-(1,3-Bis-*tert*-butoxycarbonylpropyl)-ureido]pentanedioic acid 1-*tert*-butyl ester (DUPA precursor) was prepared according to a published procedure, purified by flash chromatography, and characterized by ESI-MS and 1 H/ 13 C NMR spectroscopy [32]. Deprotection by hydrogenation afforded 2-[3-(1,3-Bis-*tert*-butoxycarbonylpropyl)-ureido]pentanedioic acid 1-*tert*-butyl ester, which was also characterized by ESI-MS and 1 H/ 13 C NMR spectroscopy. The PSMA/GRPr dual targeting ligand precursor [DUPA-6-Ahx-K-5-Ava-BBN(7-14)NH₂], was synthesized by solid-phase peptide synthesis (SPPS). Amino acid and 2-[3-(1,3-Bis-*tert*-butoxycarbonylpropyl)-ureido]pentanedioic acid 1-*tert*-butyl ester stepwise addition, however, was performed manually, followed by deprotection using a cocktail of water, triisopropylsilane (TIS), and TFA. NODAGA was also added *via* manual conjugation to the ϵ -amine of lysine (K) to produce [DUPA-6-Ahx-(NODAGA)-5-Ava-BBN(7-14)NH₂] in high yield (~65%). The new, bivalent GRPr/PSMA targeting vector was purified by RP-HPLC and characterized by ESI-MS and the new peptide conjugate was metallated with 64 CuCl₂ and nat CuCl₂ to produce [DUPA-6-Ahx-($^{64}/^{nat}$ Cu-NODAGA)-5-Ava-BBN(7-14)NH₂] (Figure 1). The RP-HPLC chromatographic profile of [DUPA-6-Ahx-(nat Cu-NODAGA)-5-Ava-BBN(7-14)NH₂] has a retention time (t_R) of 11.6 min and is shown in Figure 2. The 64 Cu- and nat Cu-conjugate co-eluted on RP-HPLC, which indicated structural similarity between the tracer and macroscopic molecules. Radiolabeling efficiency to produce [DUPA-6-Ahx-($^{64}/^{nat}$ Cu-NODAGA)-5-Ava-BBN(7-14)NH₂] was

95%. Table 1 is a list of ESI-MS observed and calculated data and t_R for the metallated, unmetallated, and fluorescent conjugates.

3.2. In Vitro Assays

3.2.1. In Vitro Competitive Displacement Binding Assays—A competitive displacement binding assay (IC_{50}) of [DUPA-6-Ahx-(^{nat}Cu -NODAGA)-5-Ava-BBN(7-14)NH₂] was determined in GRPr-positive PC-3 and PSMA-positive LNCaP cells using [^{125}I -(Tyr⁴)-BBN] and [N-acetyl aspartyl 3H -glutamate] (NAAG) as the radioligands. The new, bivalent conjugate showed very high affinity for biomarkers expressed on PSMA-/GRPr-expressing cells (Figures 3 and 4) and demonstrates the utility of the multipurpose ligand as a targeting vector for diagnosis of human prostate cancer *via* PET scintigraphy. [DUPA-6-Ahx-(^{nat}Cu -NODAGA)-5-AvaBBN(7-14)NH₂] conjugate showed an IC_{50} of 11.1 ± 0.46 nM in GRPr-expressing and 1.16 ± 1.35 nM in PSMA-expressing cells.

3.2.2. In Vitro Internalization Assays of [DUPA-6-Ahx-(Rhodamine)-5-Ava-BBN(7-14)NH₂]—[DUPA-6-Ahx-(Rhodamine)-5-Ava-BBN(7-14)NH₂] was prepared in high yield (~70%) by addition of Rhodamine-B isothiocyanate to a solution of [DUPA-6-Ahx-K-5-Ava-BBN(7-14)NH₂]. The new fluoroprobe was purified by semi-preparative RP-HPLC and characterized by ESI-MS (Table 1). Internalization and blocking assays of the [DUPA-6-Ahx-(Rhodamine)-5-Ava-BBN(7-14)NH₂] targeting vector were performed by incubation of conjugate in the presence or absence of blocking agent (BBN(1-14) for GRPr; PMPA for PSMA), with either PC-3 or LNCaP human prostate cancer cells followed by fluorescence microscopy at 590 nm. Results from these studies are shown in Figure 5. Panels A and B demonstrate internalization and blocking assays In PC-3 (GRPr-expressing) cells while panels C and D show uptake and internalization with effective blocking in the LNCaP (PSMA-expressing) prostate cancer cell line. The fluorescence profiles indicated in panels A and C show a high degree of conjugate throughout the cell cytoplasm which is indicative of receptor-mediated uptake and internalization of the fluoroprobe. Blocking assays (Panels B and D) show only minimal fluorescence-associated uptake of conjugate at the 45 min time point. These results clearly show the effective targeting ability and receptor/biomarker specificity of the new multipurpose, bivalent targeting vector for both GRPr- and PSMA-expressing cells.

3.3. In Vivo Assays

3.3.1. MicroPET Scintigraphy of [DUPA-6-Ahx-(^{64}Cu -NODAGA)-5-Ava-BBN(7-14)NH₂] in GRPr-positive and PSMA-positive Tumors—Maximum intensity microPET tumor and microCT skeletal fusion coronal images of PC-3 and LNCaP tumor-bearing SCID and nu/nu mice were obtained after 18 h, following tail-vein injection of [DUPA-6-Ahx-(^{64}Cu -NODAGA)-5-Ava-BBN(7-14)NH₂]. The two xenografted PC-3 tumors on the left and right flanks are clearly identifiable in all microPET/CT images (Figure 6). Significant uptake and retention of tracer in the abdomen is presumably due to metabolic profile and excretion parameters of a hydrophobic molecule. Although much of the excretion of tracer is *via* the renal-urinary pathway, biodistribution analyses clearly indicate retention of tracer in liver, spleen, and small intestine at the 24 h time point. Furthermore, receptor-mediated accumulation and retention of tracer in PSMA-positive

kidney also accounts for a very large percentage of the abdominal radioactivity. Figure 7 represents maximum intensity, whole-body, microPET tumor and microCT skeletal fusion coronal images of [DUPA-6-Ahx-(⁶⁴Cu-NODAGA)-5-Ava-BBN(7-14)NH₂] in a LNCaP tumor-bearing nude mouse model. Only a single xenografted LNCaP tumor is identifiable by microPET/CT, with significant uptake and retention of tracer in the abdomen in organs such as the liver, small intestine and kidneys.

4. Discussion

It is important to note that the clinical utility of monomeric radiolabeled peptides can be limited by a number of factors, including receptor density, binding affinity, and pharmacokinetics [36]. Heterodimeric or multivalent regulatory peptide probes have recently become a new and exciting approach for diagnostic molecular imaging of tumors expressing either singly- or multi-targetable receptors [28, 29, 37]. GRPr/ $\alpha_v\beta_3$ dual receptor targeting probes have been pioneered by others and more recently investigated by our own research group [36]. The $\alpha_v\beta_3$ integrin is known to be expressed in very high numbers in many tumor cell types including lung carcinomas, osteosarcomas, breast cancer, and glioblastomas but only moderately expressed on the surfaces of some prostate tumors. For example, the $\alpha_v\beta_3$ integrin receptor density per cell is approximately 2.8×10^3 on PC-3 prostate cancer cells [38]. The GRPr density per cell (PC-3), on the other hand, is approximately 2.7×10^5 [16, 39]. While each of these two receptor subtypes is expressed minimally on normal human tissue, the ability to design radiolabeled monovalent or bivalent BBN/RGD (Arginine-Glycine-Aspartic Acid) derivatives that exhibit high affinity for GRP/ $\alpha_v\beta_3$ receptor subtypes on human prostate cancers provides only limited opportunities. For example, the ability to specifically target primary or early stage prostatic cancer as well as metastatic disease could limit the utility of these radioligands. On the other hand, a high incidence of expression of GRPr and PSMA on either primary or metastatic prostate cancer disease provides impetus for development of new and innovative bivalent radioligands to tailor receptor-specific uptake, optimize localization in cancerous tissues, and minimize uptake in normal tissues to produce high-quality, high-contrast PET images for early diagnosis and staging of human prostate cancer. Agents of this type would allow the nuclear medicine physician to capture all stages of disease, a requirement which could be severely limited by monovalent GRPr/PSMA-targeting or bivalent GRP/ $\alpha_v\beta_3$ -targeting ligands alone, where one or the other molecular targets is expressed in moderate to low numbers.

In recent years, many of the research investigations in our laboratory and many others have focused upon monovalent GRPr-targeting ligand frameworks. Recent studies, however, have shown that the usage of radiolabeled bivalent, heterodimeric GRPr/ $\alpha_v\beta_3$ -targeting ligands provide superior results in biodistribution data and microPET molecular imaging investigations as compared to the monomers [28, 29, 37, 40]. The binding mechanism for bivalent peptide probes of this general type has been previously postulated for bivalent GRPr/ $\alpha_v\beta_3$ -targeting ligands. We believe the GRPr/PSMA-targeting ligands described herein behave much the same way. First, superiority in bivalent probe development arises from the ability to target either the GRPr or PSMA biomarker. The total number of effective binding sites for the radioligand is the sum of all receptors combined. Second, small, peptide-based bivalent ligands bind to the receptor in a monovalent capacity and are not

antibody-like in their binding mechanism. In other words, there is no “cross-linking” of receptors. Constrained structural features inherent to the targeting vector make simultaneous binding of the BBN and DUPA binding sequences to their appropriate receptor highly unlikely [41]. Third, any dissociation of targeting vector from the receptor could result in rapid rebinding of the targeting vector to either GRPr or PSMA biomarker, hence providing for an overall lower dissociation rate of radioligand binding. Last of all, size, charge density, and hydrophobicity/hydrophilicity all play a pivotal role in production of a favorable/unfavorable pharmacokinetic profile for ligands of this type [41].

Much of the research for targeting PSMA-expressing prostate tumors has focused upon radiohalogenated or radiometallated small molecule derivatives based upon urea [3]. The structural binding features of PSMA include a more-hydrophobic region as well as a highly-glycosylated region that interacts with the urea-functionalized radioligands [10, 12]. Foss and co-workers have described ^{11}C - and ^{125}I -PSMA inhibitors for molecular imaging of PSMA-positive cancers *in vivo* [11]. However, the usage of ^{11}C necessitates the presence of an on-site cyclotron due to a 22-minute half-life, limiting its clinical usefulness. ^{125}I also suffers from a clinical perspective in that its physical half-life is too long (60 days). While $^{123/124}\text{I}$ can be substituted for ^{125}I , these isotopes are often expensive and can suffer from lack of availability [3]. Kularatne et al., have described $^{99\text{m}}\text{Tc}$ -labeled DUPA (2-[3-(1,3-dicarboxypropyl)-ureido]pentanedioic acid) PSMA inhibitors having high uptake and retention in PSMA-positive, human prostate LNCaP cancer cells ($\sim 10\%$ ID/g, 1 h p.i.). However, uptake in PSMA-positive murine kidney tissue was also markedly high for the $^{99\text{m}}\text{Tc}$ - N_2S_2 -DUPA derivatives [13, 14]. In order to overcome many of the inherent difficulties associated with PSMA inhibitors, Banergee et al., and Eder et al., have recently described ^{68}Ga -radiolabeled, urea-based small molecules for targeting PSMA expression. Each of these studies resulted in moderately high tumor accumulation and retention as well as markedly elevated kidney uptake [3, 12]. Low expression of PSMA in human kidneys, however, continues to make small-molecule derivatives of this type a plausible targeting strategy for PSMA-expression, and two agents are currently being evaluated in human clinical trials.

In this study, we have described synthesis, characterization, *in vitro*, *in vivo*, and microPET investigations using the new bivalent GRPr/PSMA-targeting radioconjugate, [DUPA-6-Ahx-(^{64}Cu -NODAGA)-5-Ava-BBN(7-14) NH_2]. To our knowledge, this is the first example of a radioligand of this type to be reported in the literature. Fluorescence microscopy of the Rhodamine-B derivative of [DUPA-6-Ahx-K-5-Ava-BBN(7-14) NH_2] at 590 nm demonstrated the effectiveness of targeting the GRPr and PSMA biomarker with a single radioligand. MicroPET molecular images for [DUPA-6-Ahx-(^{64}Cu -NODAGA)-5-Ava-BBN(7-14) NH_2] clearly demonstrate the effectiveness of using a multivalent GRPr/PSMA-targeting radioligand to possibly capture all stages of prostate cancer disease. The microPET images reported herein in GRPr/PSMA-positive human prostate cancer xenografted rodent models, however, were altogether unremarkable and not superior to other tracers we have evaluated in our laboratory. Preliminary biodistribution analyses for this new conjugate in PC-3, tumor-bearing mice indicated the primary mechanism of excretion to be the renal-urinary pathway. However, retention of [DUPA-6-Ahx-(^{64}Cu -NODAGA)-5-Ava-

BBN(7-14)NH₂] in liver, spleen, and small intestine at the 24 h time point was also apparent and is indicative of a larger molecular weight, hydrophobic tracer. Furthermore, receptor-mediated accumulation and retention of tracer in PSMA-positive kidney also accounts for a very large percentage of the abdominal radioactivity shown in the microPET image and was also supported by biodistribution studies.

Significant accumulation and retention of tracer in the gut could limit the usefulness of target primary and metastatic disease of the lower abdomen. An alternative towards improving the tumor targeting ability and reducing background collateral uptake of tracer in non-target tissues is currently underway in our laboratory. For example, recent studies suggest the superiority of using antagonist ligand frameworks based upon BBN for selective targeting of the GRPr. For example, reports by Nock et al., show compelling evidence that radiopharmaceutical design and development based upon antagonist-type ligand frameworks bear reexamination [42]. Antagonist ligands are presumably not internalized, and therefore are not expected to residualize as effectively in tumor tissue when compared to agonist-based ligand frameworks. Studies using ^{99m}Tc-Demobesin1, ([^{99m}Tc-N₄⁰⁻¹,bzlg⁰,D-Phe⁶,Leu-NHt¹³,des-Met¹⁴]BBN(6-14)), demonstrated very high affinity and selectivity for the GRPr with pronounced accumulation and retention of radioactivity in human tumor xenografts in rodents [42]. Maecke and co-workers have begun investigating another antagonist-like targeting vector, [D-Phe-Gln-Trp-Ala-Val-Gly-His-Sta-Leu-NH₂] (AR), having high selectivity for the GRPr [43]. Studies of this ligand framework conjugated to numerous complexing agents for radiolabeling with ⁶⁴Cu, ⁶⁸Ga, and ^{99m}Tc showed very high tumor-to-background ratios in a PC-3 tumor-bearing nude mouse model at later time-points, presumably due to the fast washout of tracer from normal tissues [44]. MicroPET/SPECT images at ~12 h p.i. showed very high tumor accumulation and retention with minimal radioactivity in surrounding abdominal tissues. The encouraging results for ⁶⁴Cu-CB-TE2A-AR shown in this study have led to clinical investigations in patients with primary prostate cancer and suggest the possibility of using a bivalent PSMA/GRPr-targeting ligand based upon DUPA-AR for improved targeting of primary and metastatic prostate cancer disease. The investigations described herein, however, collectively support our hypothesis of targeting both GRPr and PSMA, which are expressed in high numbers on the surfaces of nearly all prostate cancer cells, using only a single, multipurpose, radioligand.

5. Conclusion

The ability to target the GRPr and PSMA using a single, universal molecular imaging agent for prostate cancer disease could provide a fundamental tool for early detection and determination of disease burden, promote evaluation of tumor progression as a function of disease stage, allow for a means to evaluate possible recurrence of disease, and allow for monitoring the effects of therapeutic intervention, ultimately improving patient outcomes. Herein we have described synthesis, characterization, *in vitro*, *in vivo*, and microPET investigations using a new bivalent GRPr-/PSMA-targeting radioconjugate. *In vitro* and *in vivo* investigations validate the targeting ability for each biomarker. Clearly, the microPET images that we have obtained for [DUPA-6-Ahx-(⁶⁴Cu-NODAGA)-5-Ava-BBN(7-14)NH₂] in PC-3 (GRPr-positive) and LNCaP (PSMA-positive) prostate cancer cells are not superior

to similar monovalent or bivalent constructs that we and others have previously reported [36, 45-47]. Nonetheless, this study demonstrates the ability to design and develop a new family of receptor-targeting radioligands with a propensity for PET molecular imaging of human prostate cancer.

Acknowledgments

This material was the result of work supported with resources and the use of facilities at the Truman VA, Columbia, MO, 65201 and the University of Missouri School of Medicine, Columbia, MO 65211, USA. This work was funded in part by The United States Department of Veterans' Affairs, VA Merit Bridge Funding Award Mechanism.

References

1. American Cancer Society. Cancer Facts & Figures. 2013
2. Chen Y, Dhara S, Banerjee SR, Byun Y, Pullambhatla M, Mease RC, et al. A low molecular weight PSMA-based fluorescent imaging agent for cancer. *Biochemical and Biophysical Res Comm*. 2009; 390:624–9.
3. Banerjee SR, Foss CA, Castanares M, Mease RC, Byun Y, Fox JJ, et al. Synthesis and evaluation of technetium-99m- and rhenium-labeled inhibitors of the prostate-specific membrane antigen (PSMA). *J Medicinal Chem*. 2008; 51:4504–17.
4. Hillier SM, Maresca KP, Femia FJ, Marquis JC, Foss CA, Nguyen N, et al. Preclinical evaluation of novel glutamate-urea-lysine analogues that target prostate-specific membrane antigen as molecular imaging pharmaceuticals for prostate cancer. *Cancer Res*. 2009; 69:6932–40. [PubMed: 19706750]
5. Lane SR, Nanda P, Rold TL, Sieckman GL, Figueroa SD, Hoffman TJ, et al. Optimization, biological evaluation and microPET imaging of copper-64-labeled bombesin agonists, [⁶⁴Cu-NO₂A-(X)-BBN(7-14)NH₂], in a prostate tumor xenografted mouse model. *Nucl Med Biol*. 2010; 37:751–61. [PubMed: 20870150]
6. Cescato R, Maina T, Nock B, Nikolopoulou A, Charalambidis D, Piccand V, et al. Bombesin receptor antagonists may be preferable to agonists for tumor targeting. *J Nuclear Med*. 2008; 49:318–26.
7. Jensen RT, Battey JF, Spindel ER, Benya RV. International union of pharmacology. LXVIII. Mammalian bombesin receptors: Nomenclature, distribution, pharmacology, signaling, and functions in normal and disease states. *Pharmacological Reviews*. 2008; 60:1–42. [PubMed: 18055507]
8. Thomas R, Chen J, Roudier MM, Vessella RL, Lantry LE, Nunn AD. In vitro binding evaluation of ¹⁷⁷Lu-AMBA, a novel ¹⁷⁷Lu-labeled GRP-R agonist for systemic radiotherapy in human tissues. *Clinical and Experimental Metastasis*. 2009; 26:105–19. [PubMed: 18975117]
9. Chang SS, Reuter VE, Heston WDW, Bander NH, Grauer LS, Gaudin PB. Five different anti-prostate-specific membrane antigen (PSMA) antibodies confirm PSMA expression in tumor-associated neovasculature. *Cancer Res*. 1999; 59:3192–8. [PubMed: 10397265]
10. Rajasekaran AK, Anilkumar G, Christiansen JJ. Is prostate-specific membrane antigen a multifunctional protein? *Am J Physiol- Cell*. 2005; 288:C975–C81.
11. Foss CA, Mease RC, Fan H, Wang Y, Ravert HT, Dannals RF, et al. Radiolabeled small-molecule ligands for prostate-specific membrane antigen: In vivo imaging in experimental models of prostate cancer. *Clin Cancer Res*. 2005; 11:4022–8. [PubMed: 15930336]
12. Eder M, Schäfer M, Bauder-Wüst U, Hull WE, Wängler C, Mier W, et al. ⁶⁸Ga-complex lipophilicity and the targeting property of a urea-based PSMA inhibitor for PET imaging. *Bioconjugate Chem*. 2012; 23:688–97.
13. Kularatne SA, Wang K, Santhapuram HKR, Low PS. Prostate-specific membrane antigen targeted imaging and therapy of prostate cancer using a PSMA inhibitor as a homing ligand. *Molecular Pharmaceutics*. 2009; 6:780–9. [PubMed: 19361233]
14. Kularatne SA, Zhou Z, Yang J, Post CB, Low PS. Design, synthesis, and preclinical evaluation of prostate-specific membrane antigen targeted ^{99m}Tc radioimaging agents. *Molecular Pharmaceutics*. 2009; 6:790–800. [PubMed: 19361232]

15. Anderson CJ, Welch MJ. Radiometal-labeled agents (non-technetium) for diagnostic imaging. *Chemical Reviews*. 1999; 99:2219–34. [PubMed: 11749480]
16. Prasanphanich AF, Nanda PK, Rold TL, Ma L, Lewis MR, Garrison JC, et al. [^{64}Cu NOTA-8-Aoc-BBN(7-14) NH_2] targeting vector for positron-emission tomography imaging of gastrin-releasing peptide receptor-expressing tissues. *PNAS*. 2007; 104:12462–7. [PubMed: 17626788]
17. Bass LA, Wang M, Welch MJ, Anderson CJ. In vivo transchelation of copper-64 from TETA-octreotide to superoxide dismutase in rat liver. *Bioconjugate Chem*. 2000; 11:527–32.
18. Chen X, Park R, Hou Y, Tohme M, Shahinian AH, Bading JR, et al. MicroPET and autoradiographic imaging of GRP receptor expression with ^{64}Cu -DOTA-[Lys³]bombesin in human prostate adenocarcinoma xenografts. *J Nucl Med*. 2004; 45:1390–7. [PubMed: 15299066]
19. Rogers BE, Bigott HM, McCarthy DW, Della Manna D, Kim J, Sharp TL, et al. MicroPET imaging of a gastrin-releasing peptide receptor-positive tumor in a mouse model of human prostate cancer using a ^{64}Cu -labeled bombesin analogue. *Bioconjugate Chem*. 2003; 14:756–63.
20. Rogers BE, Manna DD, Safavy A. In Vitro and In Vivo Evaluation of a ^{64}Cu -Labeled Polyethylene Glycol-Bombesin Conjugate. *Cancer Biother Radio*. 2004; 19:25–34.
21. Boswell CA, Sun X, Niu W, Weisman GR, Wong EH, Rheingold AL, et al. Comparative in Vivo Stability of Copper-64-Labeled Cross-Bridged and Conventional Tetraazamacrocyclic Complexes. *J Med Chem*. 2004; 47:1465–74. [PubMed: 14998334]
22. Sprague JE, Peng Y, Sun X, Weisman GR, Wong EH, Achilefu S, et al. Preparation and biological evaluation of copper-64-labeled Tyr³-octreotate using a cross-bridged macrocyclic chelator. *Clin Cancer Res*. 2004; 10:8674–82. [PubMed: 15623652]
23. Sun X, Wuest M, Weisman GR, Wong EH, Reed DP, Boswell CA, et al. Radiolabeling and in vivo behavior of copper-64-labeled cross-bridged cyclam ligands. *J Med Chem*. 2002; 45:469–77. [PubMed: 11784151]
24. Kukis DL, Diril H, Greiner DP, DeNardo SJ, DeNardo GL, Salako QA, et al. A comparative study of copper-67 radiolabeling and kinetic stabilities of antibody-macrocyclic chelate conjugates. *Cancer*. 1994; 73:779–86. [PubMed: 8306260]
25. Kukis DL, Li M, Meares CF. Selectivity of antibody-chelate conjugates for binding copper in the presence of competing metals. *Inorg Chem*. 1993; 32:3981–2.
26. Delgado R, Sun Y, Motekaitis RJ, Martell AE. Stabilities of divalent and trivalent metal ion complexes of macrocyclic triazatriacetic acids. *Inorg Chem*. 1993; 32:3320–6.
27. Wiegardt K, Bossek U, Chaudhuri P, Herrmann W, Menke BC, Weiss J. 1,4,7-Triazacyclononane- $\text{N},\text{N}',\text{N}''$ -Triacetate (TCTA), a hexadentate ligand for divalent and trivalent metal ions. Crystal structures of $[\text{CrIII}(\text{TCTA})]$, $[\text{FeIII}(\text{TCTA})]$, and $\text{Na}[\text{CuII}(\text{TCTA})]\cdot 2\text{NaBr}\cdot 8\text{H}_2\text{O}$. *Inorg Chem*. 1982; 21:4308–14.
28. Liu Z, Li ZB, Cao Q, Liu S, Wang F, Chen X. Small-animal PET of tumors with ^{64}Cu -labeled RGD-bombesin heterodimer. *J Nucl Med*. 2009; 50:1168–77. [PubMed: 19525469]
29. Liu Z, Niu G, Wang F, Chen X. ^{68}Ga -labeled NOTA-RGD-BBN peptide for dual integrin and GRPR-targeted tumor imaging. *E J Nucl Med and Mol Imag*. 2009; 36:1483–94.
30. Wen X, Chao C, Ives K, Hellmich MR. Regulation of bombesin-stimulated cyclooxygenase-2 expression in prostate cancer cells. *BMC Mol Biol*. 2011; 12:29. [PubMed: 21745389]
31. Reile H, Armatis P, Schally A. Characterization of high-affinity receptors for bombesin/gastrin releasing peptide on the human prostate cancer cell lines PC-3 and DU-145: internalization of receptor bound 125I-(Tyr⁴) bombesin by tumor cells. *Prostate*. 1994; 25:29–38. [PubMed: 8022709]
32. Kozikowski AP, Zhang J, Nan F, Petukhov PA, Grajkowska E, Wroblewski JT, et al. Synthesis of Urea-Based Inhibitors as Active Site Probes of Glutamate Carboxypeptidase II: Efficacy as Analgesic Agents. *Journal of Medicinal Chemistry*. 2004; 47:1729–38. [PubMed: 15027864]
33. Prasanphanich AF, Nanda PK, Rold TL, Ma L, Lewis MR, Garrison JC, et al. [^{64}Cu NOTA-8-Aoc-BBN(7-14) NH_2] targeting vector for positron-emission tomography imaging of gastrin-releasing peptide receptor-expressing tissues. *PNAS*. 2007; 104:12462–7. [PubMed: 17626788]
34. Prasanphanich AF, Retzlaff L, Lane SR, Nanda PK, Sieckman GL, Rold TL, et al. *In vitro* and *in vivo* analysis of [^{64}Cu -NO₂A-8-Aoc-BBN(7-14) NH_2]: a site-directed radiopharmaceutical for

- positron-emission tomography imaging of T-47D human breast cancer tumors. *Nucl Med Biol.* 2009; 36:171–81. [PubMed: 19217529]
35. Graham K, Lesche R, Gromov AV, Boehnke N, Schaefer M, Hassfeld J, et al. Radiofluorinated Derivatives of 2-(Phosphonomethyl)pentanedioic Acid as Inhibitors of Prostate Specific Membrane Antigen (PSMA) for the Imaging of Prostate Cancer. *J Med Chem.* 2012
36. Jackson AB, Nanda PK, Rold TL, Sieckman GL, Szczodroski AF, Hoffman TJ, et al. ^{64}Cu -NO₂A-RGD-Glu-6-Ahx-BBN(7-14)NH₂: A heterodimeric targeting vector for positron emission tomography imaging of prostate cancer. *Nucl Med Biol.* 2012; 39:377–87. [PubMed: 22226021]
37. Liu Z, Yan Y, Chin FT, Wang F, Chen X. Dual integrin and gastrin-releasing peptide receptor targeted tumor imaging using ^{18}F -Labeled PEGylated RGD-bombesin heterodimer ^{18}F -FB-PEG3-Glu-RGD-BBN. *Journal of Medicinal Chemistry.* 2009; 52:425–32. [PubMed: 19113865]
38. Shi J, Kim YS, Zhai S, Liu Z, Chen X, Liu S. Improving tumor uptake and pharmacokinetics of ^{64}Cu -labeled cyclic RGD peptide dimers with Gly3 and PEG4 linkers. *Bioconjugate Chem.* 2009; 20:750–9.
39. Maddalena ME, Fox J, Chen J, Feng W, Cagnolini A, Linder KE, et al. ^{177}Lu -AMBA biodistribution, radiotherapeutic efficacy, imaging, and autoradiography in prostate cancer models with low GRP-R expression. *Journal of Nuclear Medicine.* 2009; 50:2017–24. [PubMed: 19910427]
40. 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. *Bioconjugate Chem.* 2009; 20:1016–25.
41. Chen K, Conti PS. Target-specific delivery of peptide-based probes for PET imaging. *Advanced Drug Delivery Reviews.* 2010; 62:1005–22. [PubMed: 20851156]
42. Nock BA, Nikolopoulou A, Galanis A, Cordopatis P, Waser B, Reubi JC, et al. Potent bombesin-like peptides for GRP-receptor targeting of tumors with $^{99\text{m}}\text{Tc}$: A preclinical study. *J Med Chem.* 2005; 48:100–10. [PubMed: 15634004]
43. Mansi R, Wang X, Forrer F, Waser B, Cescato R, Graham K, et al. Development of a potent DOTA-conjugated bombesin antagonist for targeting GRPr-positive tumours. *E J Nucl Med and Mol Imag.* 2011; 38:97–107.
44. Abiraj K, Mansi R, Tamma ML, Fani M, Forrer F, Nicolas G, et al. Bombesin Antagonist-Based Radioligands for Translational Nuclear Imaging of Gastrin-Releasing Peptide Receptor-Positive Tumors. *Journal of Nuclear Medicine.* 2011; 52:1970–8. [PubMed: 22080443]
45. Abd-Elgaliel WR, Gallazzi F, Garrison JC, Rold TL, Sieckman GL, Figueroa SD, et al. Design, synthesis, and biological evaluation of an antagonist-bombesin analogue as targeting vector. *Bioconjugate Chemistry.* 2008; 19:2040–8. [PubMed: 18808168]
46. Nanda PK, Pandey U, Bottenus BN, Rold TL, Sieckman GL, Szczodroski AF, et al. Bombesin analogues for gastrin-releasing peptide receptor imaging. *Nucl Med Biol.* 2012; 39:461–71. [PubMed: 22261143]
47. Nanda PK, Rold TL, Sieckman GL, Szczodroski AF, Hoffman TJ, Rogers BE, et al. Positron-emission Tomography (PET) Imaging Agents for Diagnosis of Human Prostate Cancer: Agonist Versus Antagonist Ligands. *In Vivo.* 2012; 26:583–92. [PubMed: 22773572]

Figure 1.

Chemical structure of [DUPA-6-Ahx-(^{64}Cu -NODAGA)-5-Ava-BBN(7-14) NH_2].

Figure 2.
HPLC chromatographic profiles of [DUPA-6-Ahx-(^{64}Cu -NODAGA)-5-AvaBBN(7-14) NH_2] ($t_R = 11.6$ min) and ESI mass spectrum ($M + H^+ = 2001.87$) for [DUPA-6-Ahx-($^{\text{nat}}\text{Cu}$ -NODAGA)-5-Ava-BBN(7-14) NH_2].

1

Figure 3.

Inhibitory concentration half maximum (IC_{50}) of [DUPA-6-Ahx-(^{nat}Cu-NODAGA)-5-Ava-BBN(7-14)NH₂] ($IC_{50} = 3.09 \pm 0.34$ nM) in human, prostate, PC-3 cells.

Figure 4.
Inhibitory concentration half maximum (IC_{50}) of [DUPA-6-Ahx-(^{nat}Cu-NODAGA)-5-Ava-BBN(7-14)NH₂] ($IC_{50} = 1.16 \pm 1.35$ nM) in human, prostate, LNCaP cells.

Figure 5.

A. Uptake/Internalization of [DUPA-6-Ahx-(K-Rhodamine)-5-Ava-BBN(7-14)NH₂] targeting vector in PC-3 cells. B. Blocking of [DUPA-6-Ahx-(K-Rhodamine)-5-Ava-BBN(7-14)NH₂] targeting vector in PC-3 cells. C. Uptake/Internalization of [DUPA-6-Ahx-(K-Rhodamine)-5-Ava-BBN(7-14)NH₂] targeting vector in LNCaP cells. D. Blocking of [DUPA-6-Ahx-(K-Rhodamine)-5-Ava-BBN(7-14)NH₂] targeting vector in LNCaP cells.

Figure 6. Maximum intensity microPET tumor and microCT skeletal fusion coronal whole-body image of a PC-3 tumor bearing SCID mouse at 18 h after tail vein injection of [DUPA-6-Ahx-(^{64}Cu -NODAGA)-5-Ava-BBN(7-14) NH_2].

Figure 7. Maximum intensity microPET tumor and microCT skeletal fusion coronal whole-body image of a LNCaP tumor bearing nude mouse at 18 h after tail vein injection of [DUPA-6-Ahx-(^{64}Cu -NODAGA)-5-Ava-BBN(7-14) NH_2].

Table 1ESI-MS and HPLC retention times (t_R) for [DUPA-6-Ahx-K-5-Ava-BBN(7-14)NH₂] derivatives.

Analog	Mass Spectrometry			HPLC t_R (min)
	MF	Calculated	Observed	
[DUPA-6-Ahx-K-5-Ava-BBN(7-14)NH ₂]	C ₇₁ H ₁₁₁ N ₁₉ O ₂₀ S	1581.80	1582.64	11.3
[DUPA-6-Ahx-(NODAGA)-5-Ava-BBN(7-14)NH ₂]	C ₈₆ H ₁₃₄ N ₂₂ O ₂₇ S	1940.02	1941.03	12.2
[DUPA-6-Ahx-(^{nat} Cu-NODAGA)-5-Ava-BBN(7-14)NH ₂]	C ₈₆ H ₁₃₂ N ₂₂ O ₂₇ SCu	2001.09	2001.87	11.6
[DUPA-6-Ahx-(Rhodamine)-5-Ava-BBN(7-14)NH ₂]	C ₁₀₀ H ₁₄₁ N ₂₂ O ₂₃ S ₂	2082.00	2083.45	18.3