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In vitro and in vivo analysis of [^{64}Cu -NO₂A-8-Aoc-BBN(7–14)NH₂]: a site-directed radiopharmaceutical for positron-emission tomography imaging of T-47D human breast cancer tumors*

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Abstract

Introduction—Human breast cancer, from which the T-47D cell line was derived, is known to overexpress the gastrin-releasing peptide receptor (GRPR) in some cases. Bombesin (BBN), an agonist for the GRPR, has been appended with a radionuclide capable of positron-emission tomography (PET) imaging and therapy. ^{64}Cu -NO₂A-8-Aoc-BBN(7–14)NH₂ (NO₂A=1,4,7-triazacyclononane-1,4-diacetate) has produced high-quality microPET images of GRPR-positive breast cancer xenografted tumors in mice.

Methods—The imaging probe was synthesized by solid-phase peptide synthesis followed by manual conjugation of the 1,4,7-triazacyclononane-1,4,7-triacetic acid (NOTA) bifunctional chelator and radiolabeling in aqueous solution. The radiolabeled conjugate was subjected to in vitro and in vivo studies to determine its specificity for the GRPR and its pharmacokinetic profile. A T-47D tumor-bearing mouse was imaged with microPET/CT and microMRI imaging.

Results—The ^{64}Cu -NO₂A-8-Aoc-BBN(7–14)NH₂ targeting vector was determined to specifically localize in GRPR-positive tissue. Accumulation was observed in the tumor in sufficient quantities to allow for identification of tumors in microPET imaging procedures. For example, uptake and retention in T-47D xenografts at 1, 4 and 24 h were determined to be 2.27 ± 0.08 , 1.35 ± 0.14 and 0.28 ± 0.07 % ID/g, respectively.

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Conclusions—The ^{64}Cu -NO₂A-8-Aoc-BBN(7–14)NH₂ produced high-quality microPET images. The pharmacokinetic profile justifies investigation of this bioconjugate as a potentially useful diagnostic/therapeutic agent. Additionally, the bioconjugate would serve as a good starting point for modification and optimization of similar agents to maximize tumor uptake and minimize nontarget accumulation.

Keywords

T-47D; Copper-64; Bombesin; Gastrin-releasing peptide; Site-directed radiopharmaceutical; NOTA

1. Introduction

Cancer continues to be a leading cause of death for women and men in the United States, second only to heart disease [1]. Breast cancer is of particular concern for women. In 2008, it is estimated that the most diagnosed carcinomas in women will be breast cancer (182,460 cases or 26%). Moreover, it is estimated that 15% of female patients dying of cancer in 2008, or 40,480 deaths, will be due to breast cancer [2,3]. When diagnosed at an early stage, 5-year survival rates increase for women diagnosed with breast cancer [2,3]. Clearly, there is a great impetus to improve upon methods for identifying primary and secondary breast cancer tumors.

While a variety of diagnostic imaging techniques exist for identifying breast cancer, low-dose mammography is still considered to be the most accurate and dependable procedure [4]. Still, digital mammography is most accurate in women who are less than 50 years old, with low-density breast and have either not entered or recently passed through menopause [5]. Positron-emission tomography (PET) imaging using ^{18}F -fluorodeoxyglucose (FDG) has been an effective technique for diagnosis of primary breast cancer and benefited from PET imaging devices designed specifically for breast imaging. While FDG-PET was able to identify tumors in the breast, a very high false-positive rate, 87%, is a major limiting factor in its widespread use [6]. Thus there is a need to develop diagnostic modalities with increased specificity for malignant breast tissues and capable of overcoming these limitations. Targeting a unique physiological manifestation of breast malignancy, such as over-expression of an extracellular receptor with a diagnostic radionuclide, might be a procedure capable of imaging tumors of the breast with higher resolution and lower false-positive rates.

The T-47D human breast cancer cell line has been used in a variety of studies to evaluate the therapeutic and diagnostic efficacy of potential drugs. This differentiated cell line, with breast epithelial morphology, was derived from a metastatic pleural effusion originating from an infiltrating ductal carcinoma in a 54-year-old Caucasian patient [7,8]. The cell line is described as “luminal epithelial-like” with markers indicating its premetastatic state according to the epithelial-mesenchymal transition hypothesis for metastatic transformation [7]. The gastrin-releasing peptide receptor (GRPR) has been demonstrated to be overexpressed on the surface of T-47D breast carcinoma cells while being absent from normal breast tissues [9]. This receptor has served as a target for a number of diagnostic and therapeutic strategies [10–16]. T-47D xenografts in mice have been used in preclinical investigations of a variety of diagnostic modalities [12,16–18]. The use of peptide-based bioconjugates in these investigations is common. Typically, a peptide is covalently modified with a bifunctional chelating agent (BFCA) capable of complexing and stabilizing a radionuclide. Pharmacokinetic modifiers can be introduced between the peptide and the BFCA to fine tune the biodistribution of the bioconjugate. The potential of using peptide-based site-directed radiopharmaceuticals for in vivo single-photon emission computed topography and PET imaging has recently become apparent [10–13,19,20].

Copper-64 is one of the few radionuclides possessing physical properties that allow for medical imaging and therapy [21]. The utility of ^{64}Cu [half-life ($t_{1/2}$)=12.7 h] results from its diverse decay profile. Electron capture, with corresponding gamma emission at 1346 keV, accounts for 41% of the decay profile for ^{64}Cu . This is accompanied by emission of a β^- particle (40%, 190 keV) and a positron (β^+ , 19%, 278 keV) [22]. The degree of β^+ decay is sufficient for PET imaging in vivo and has been extensively investigated [22]. Moreover, ^{67}Cu -containing radiopharmaceuticals possess therapeutic potential and provide in vivo pharmacokinetic profiles nearly identical to ^{64}Cu -containing drugs [22]. Copper-67 has ideal physical characteristics that are well suited for radionuclide therapy (β^- , 100%, 121 keV, $t_{1/2}$ = 62 h) [27]. As such, ^{64}Cu can be used as a dose-determinant indicator in ^{67}Cu -based therapeutic regimens.

While the physical properties of copper are well suited for use in radiopharmaceuticals, the metabolism and physiological properties of copper in the human body present a challenge to the development and widespread use of copper-containing radiopharmaceuticals. Ideally, site-directed radiopharmaceuticals accumulate at the target site quickly with little accumulation in nontarget tissues and rapidly clear nontarget tissues after administration. Hence, hepatobiliary clearance of radiopharmaceuticals is much less desirable to renal/urinary clearance. Since the liver is the anatomical location of copper metabolism, the stability of the copper chelate in a ^{64}Cu -containing radiopharmaceutical under physiological conditions is very important [23]. Increasing the stability of copper complexes in vivo has been a major focus in the field of nuclear medicine for some time [22,24–37] as the development of stable copper complexes will allow for a variety of copper-based diagnostic and therapeutic strategies.

Recent work in the field of copper radiochemistry has produced very promising results. A majority of the work has focused on synthesis of new ligand frameworks to chelate copper in a multidentate fashion to prevent loss of the Cu^{2+} ion under in vivo conditions. Cross-bridged TETA (1,4,8,11-tetraazacyclotetradecane-1,4,8,11-tetraacetic acid) derivatives, such as 4,11-bis(carboxymethyl)-1,4,8,11-tetraazabicyclo [6.6.2]hexadecane (CB-TE2A) and 1-*N*-(4-aminobenzyl)-3,6,10,13,16,19-hexa-aza-bicyclo-[6.6.6]eicosane-1,8-diamine (SarAr), have shown increased copper-complex stability in vivo vs. the TETA and DOTA (1,4,7,10-tetraazacyclododecane-1,4,7,10-tetraacetic acid) chelators [11,24,28]. Additionally, derivatives of the triazacyclononane macrocycle have produced improved stability of copper complexes [10,25]. Pippin et al. [29] have shown that complexes of Cu (II) with 1,4,7-triazacyclononane-1,4,7-triacetic acid (NOTA) are more inert towards isotopic exchange with ^{67}Cu than with DOTA or TETA. Recent work by Gasser et al. [25] demonstrated a 2-[4,7-bis(2-pyridylmethyl)-1,4,7-triazacyclononan-1-yl] acetic acid (PMCN) bifunctional chelator containing the ^{64}Cu radionuclide. Recently, we have reported on the superior microPET imaging quality of NOTA bifunctional chelator over that of the DOTA using ^{64}Cu in a GRPR-positive human prostate cancer mouse model [10]. Previous investigations have reported a highly stable complex consisting of NOTA and divalent copper [26,30,31]. Also reported are biomolecules conjugated with NOTA and chelating ^{64}Cu [10,26]. Interestingly, one investigation reported a crystal structure of $[\text{CuCl}(\text{TACNTA})^-]$, or $[\text{CuCl}(\text{NOTA})^-]$, with a pentadentate NOTA where one carboxylate arm, off of the triazacyclononane macrocycle, is not involved in the coordination of copper(II) [32]. This implies that occupation of this nonchelating carboxylate of NOTA, as in our bifunctional NO2A, may not alter the native structure of the Cu–NOTA complex. Herein, we report on findings of our investigations in T-47D cells and xenografted human tumor models in mice with ^{64}Cu -NO2A-8-Aoc-BBN(7–14) NH_2 .

2. Materials and methods

2.1. Reversed-phase high-performance liquid chromatography

All of the compounds synthesized in this report were purified by means of reversed-phase high-performance liquid chromatography (RP-HPLC) employing a binary gradient. Solvent A, 99.9% DI water with 0.01% trifluoroacetic acid (TFA), and solvent B, 99.9% acetonitrile (Fisher Scientific; A998 HPLC grade) with 0.01% TFA, were used with a Shimadzu SCL-10A controller system in conjunction with Shimadzu EZ-Start v. 7.3 software. Samples were observed using an in-line Shimadzu SPD-10A absorption detector ($\lambda=280$ nm) as well as an in-line EG&G Ortec NaI solid crystal scintillation detector. A Phenomenex (Jupiter 4 μ Proteo, 90 Å, 250 $\times$ 4.6 mm) C₁₂ column maintained at 34°C with an Eppendorf CH-50 column heater was used to achieve separation.

Unconjugated peptide was purified on a 95% A/5% B to 30% A/70% B over 25-min gradient while NO₂A-conjugated and radiolabeled peptides were purified on a 25% A/75% B to 35% A/65% B over 15-min gradient. All nonradiolabeled conjugates were lyophilized with a Labconco (Kansas City, MO, USA) Centri-Vap rotary evaporator.

2.2. Synthesis of NO₂A-8-Aoc-BBN(7–14)NH₂

Solid-phase peptide synthesis (SPPS) utilizing standard Fmoc amine protection was used to produce 8-Aoc-BBN(7–14)NH₂, (8-Aminooctanoic Acid)-Gln-Trp-Ala-Val-Gly-His-Leu-Met-NH₂, peptide. Amino acids with appropriate amine and side chain protections were purchased from either Nova BioChem or Advanced ChemTech. Conjugation of NOTA to this peptide resulting in NO₂A-8-Aoc-BBN(7–14) NH₂ took place via an active ester. A solution of 0.1 M 2-[morpholino]ethanesulfonic acid (MES) buffer (pH 4.7), 30 M equivalents of NOTA, 50 equivalents of sulfo-NHS (*N*-hydroxysulfosuccinimide) and 35 equivalents of EDC (1-ethyl-3-[3-dimethylaminopropyl]carbodiimide hydrochloride) (total volume=400 μ l) was prepared with stirring at room temperature. MES, EDC and sulfo-NHS were purchased from Pierce. Three milligrams of peptide, 1 equivalent, was dissolved in 400 μ l of phosphate-buffered solution (pH 7.4) and added to active ester solution in MES buffer. The pH of the resultant solution was slowly adjusted to 7.4 with NaOH. The NO₂A bioconjugate was then isolated by RP-HPLC. Matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI TOF-MS), performed by the Charles W. Gehrke Proteomics Center of the University of Missouri, was used to confirm the identity of the bioconjugate.

2.3. Synthesis of ⁶⁴Cu-NO₂A-8-Aoc-BBN(7–14)NH₂

Copper radionuclide was purchased from either MDS Nordion (Vancouver, BC, Canada) or Trace Life Sciences (Denton, TX, USA). Specific activity of free metal was ~25,000 Ci/g. Radiolabeling of the NO₂A-8-Aoc-BBN(7–14)NH₂ conjugate was similar to a previously published procedure [10,11]. To 100 μ g NO₂A-8-Aoc-BBN(7–14) NH₂ peptide in 250 μ l 0.4 M ammonium acetate (pH 7.1) was added ⁶⁴CuCl₂ in 0.05 N HCl solution (~50 μ l). The resulting solution was heated at 70°C, pH=7.1. After 30 min of heating time, a small aliquot of reaction mixture was analyzed by RP-HPLC. After 1 h of total heating time, 100 μ l of 10 nM diethylene triamine pentaacetic acid (DTPA) was added to the reaction mixture and the final product, ⁶⁴Cu-NO₂A-8-Aoc-BBN(7–14)NH₂, was purified by RP-HPLC. Purified conjugate was collected into 100 μ g of bovine serum albumin (BSA) and 25 mg of ascorbic acid in 100 μ l of Millipore-filtered water. The acetonitrile was removed from the solution using a stream of nitrogen gas. Samples used for in vitro or in vivo studies were subjected to quality control RP-HPLC analysis. Nonradioactive Cu-NO₂A-8-Aoc-BBN(7–14)NH₂ conjugate was prepared according to similar procedures by reaction of NO₂A-8-Aoc-BBN(7–14)NH₂ peptide with CuCl₂. The solution was heated for 30 min at 70°C, pH=7.1, and purified as previously described.

The ^{64}Cu -NO₂A-8-Aoc-BBN(7–14)NH₂ conjugate was also able to be synthesized under mild reaction conditions. For example, an identical radiolabeling procedure was prepared (pH 7.1) and placed on a heating mantle set to 34°C for a total of 1 h.

2.4. In vitro receptor binding affinity study

The affinity of NO₂A-8-Aoc-BBN(7–14)NH₂ and Cu-NO₂A-8-Aoc-BBN(7–14)NH₂ for the GRPR was determined using a competitive displacement/binding bioassay in human, breast cancer T-47D cells. ^{125}I -[Tyr⁴]-BBN, purchased from Perkin Elmer Life Sciences (Waltham, MA, USA), was used as the radiolabeled competitive binding ligand. Cells were contained in media consisting of RPMI 1640 plus L-glutamine, 4-(2-hydroxyethyl)-1-piperazine-ethanesulfonic acid (HEPES) 4.9 g/L and 2% BSA maintained at pH 7.4. Approximately 300,000 T-47D cells were incubated at 37°C for 40 h with 20,000 cpm ^{125}I -[Tyr⁴]-BBN and NO₂A-8-Aoc-BBN(7–14)NH₂ or Cu-NO₂A-8-Aoc-BBN(7–14)NH₂.

Increasing concentrations from 0.33 pM NO₂A-8-Aoc-BBN(7–14)NH₂ or Cu-NO₂A-8-Aoc-BBN(7–14)NH₂ were used to compare receptor affinity against that of the radioligand. Incubation was followed by aspiration of the media and three washes of the cells with fresh media. The cell-associated activity was counted using a Packard Riastar multiwell gamma scintillation detector. The inhibitory concentration 50% (IC₅₀) was determined. Study was run in triplicate and IC₅₀ derived from the average of the three experiments.

2.5. In vitro internalization/efflux study

Internalization and efflux data for ^{64}Cu -NO₂A-8-Aoc-BBN(7–14)NH₂ were obtained in T-47D cells. Approximately 300,000 T-47D cells were incubated for 15, 30, 45, 60, 90 and 120 min at 37°C with 20,000 cpm ^{64}Cu -NO₂A-8-Aoc-BBN(7–14)NH₂. After incubation, the media was aspirated and the cells were washed three times with media. Surface-bound activity (S) was removed with a wash of 0.2 N acetic acid/0.5 M NaCl at pH 2.5. The remaining cell-associated activity (I) and (S) were counted and internalization percentage calculated to be I/(S+I). Activities were counted using a Packard Riastar multiwell gamma scintillation detector.

Efflux of ^{64}Cu -NO₂A-8-Aoc-BBN(7–14)NH₂ from T-47D cells was measured by incubation of the cells in a 20,000-cpm solution of conjugate for 45 min followed by a secondary incubation period. At 0-, 15-, 30-, 45-, 60- and 90-min periods, the media was aspirated and the cells were washed three times with acetic acid/saline (pH=2.5, 4°C). The degree of internalization, I/(S+I), was determined in the same manner as for the internalization analysis.

2.6. In vivo pharmacokinetic studies in normal mice

The biodistribution of ^{64}Cu -NO₂A-8-Aoc-BBN(7–14)NH₂ in normal mice was determined using CF-1 mice at five per time point. The National Institutes of Health Guide for Care and Use of Laboratory Animals and the Policy and Procedures for Animal Research at the Harry S. Truman Memorial Veterans' Hospital were strictly adhered to during all animal studies. The mice were administered approximately 7 μCi of ^{64}Cu -NO₂A-8-Aoc-BBN(7–14)NH₂ in 100 μl of isotonic saline via intravenous injection in the tail vein. At 1, 4 and 24 h postinjection (pi) time points, the mice were sacrificed and the tissues and organs excised, weighed and counted in a NaI well counter in order to calculate the percent of injected dose (% ID) and percent of injected dose per gram (% ID/g). The % ID/g in blood was estimated by assuming a 6.5% of whole body mass for the total blood volume. The % ID for urine was estimated by measuring urine, bladder and cage paper-associated radioactivity. A kidney blocking experiment study was conducted by intravenous administration of 15 mg D-Lysine (Lys), purchased from Sigma-Aldrich (St. Louis, MO, USA), in 75 μl phosphate buffered solution (PBS) immediately before administration of ^{64}Cu -NO₂A-8-Aoc-BBN(7–14)NH₂ and followed by sacrifice at a 1-h time point.

2.7. In vivo pharmacokinetic studies in tumor-bearing mice

The biodistribution of ^{64}Cu -NO2A-8-Aoc-BBN(7–14) NH_2 in tumor-bearing mice was determined using severely compromised immunodeficient (SCID) mice bearing T-47D human breast cancer-derived xenografts. Four- to 5-week-old female Institute of Cancer Research (ICR) SCID outbred mice were purchased from Taconic (Germantown, NY, USA). The mice were fed autoclaved water and manufacturer-irradiated rodent chow from Ralston Purina (St. Louis, MO, USA) ad libitum. Animals were housed five to a sterile, micro-isolator cage in a humidity-controlled environment with a 12-h light/dark cycle. Estradiol pellets, 0.50 mg/90-day release pellets (Innovative Research of America, Sarasota, FL, USA), were placed under the skin between the shoulder blades of each mouse 6 to 10 days before tumor cell inoculation. Approximately 5×10^6 human T-47D breast cancer cells, in a 100- μl solution of sterile saline and Matrigel (1:1) (BD Biosciences, San Jose, CA, USA), were injected bilaterally into the subcutaneous flanks of each mouse while under anesthesia. Isoflurane purchased from Baxter Healthcare (Deerfield, IL, USA) at a rate of 3.5% was administered with 0.4 L of oxygen through a nonbreathing anesthesia vaporizer. The xenografts were allowed to grow for 6–8 weeks following inoculation. The pharmacokinetic profile of ^{64}Cu -NO2A-8-Aoc-BBN(7–14) NH_2 was determined in the same manner as for the CF-1 mice (*vide supra*). The % ID/g of tumor tissue is reported as the average and standard deviation of the two individual bilateral xenografts. The blocking study was conducted by intravenous administration of 100 μg BBN (1–14) NH_2 (bombesin acetate hydrate), purchased from Sigma-Aldrich, in 100 μl PBS 15 minutes prior to administration of ^{64}Cu -NO2A-8-Aoc-BBN(7–14) NH_2 and followed by sacrifice at a 1-h time point.

2.8. Multimodal image acquisition and analysis of ^{64}Cu -NO2A-8-Aoc-BBN(7–14) NH_2 in tumor-bearing mice

MicroPET tissue data imaging analysis was accomplished utilizing a MOSAIC small animal PET unit (Philips, USA). The unit has a gantry diameter of 21 cm, a transverse field of view (FOV) of 12.8 cm and an axial length of 11.6 cm. The scanner operates in a 3D volume imaging acquisition mode. Small animals are frequently laser aligned at the center of the scanner FOV for subsequent imaging. A mouse bearing xenografted human T-47D breast cancer tumors was administered a 5.1-mCi dose of ^{64}Cu -NO2A-8-Aoc-BBN(7–14) NH_2 in 150 μl sterile saline via intravenous injection into the tail vein. At 24 h pi, the mouse was placed in a prone position on a custom-built cradle. The cradle was prepared with image fusion markers that served as reference points for succeeding image co-registration of PET/computed tomography (CT)/magnetic resonance imaging (MRI). The breast tumor-bearing mouse was imaged and data were collected using an emission microPET scan protocol. MicroPET image reconstruction was performed with the use of a 3D row action maximum likelihood algorithm (RAMLA) without tissue attenuation correction. The microPET data were filtered with a 1.1-mm Gaussian FWHM filter.

The Micro-CT unit (Siemens, TN, USA) consists of a CCD X-ray detector and an 80-kVp micro-focus X-ray source (40 μm focal spot). MicroCT imaging was performed immediately following microPET imaging for the purpose of anatomic/molecular data fusion. Micro-CT imaging was performed and concurrent image reconstruction was achieved with the use of a Fanbeam (Feldkamp) filtered back projection algorithm. Co-registration, visualization and image analysis of PET/CT/MRI data were achieved with the use of the Amira 3.1 software package (TGS, Germany).

Magnetic resonance imaging of the tumor-bearing animals was performed on a 7-T/210-mm Varian Unity Inova MRI system equipped with a quadrature-driven birdcage coil (38 mm ID) (Varian, Inc., Palo Alto, CA, USA) in order to correlate the relative degree of uptake to the anatomical features of the mice. Coronal anatomical images were collected using a proton

density-weighted (pW) fast spin-echo multislice sequence with repetition time (TR)/echo time (TE)=2000 ms/12 ms. Both T2-weighted (T2W) and diffusion-weighted (DW) spin-echo multislice (TR/TE=200 ms/37 ms) images were collected so as to obtain transverse images in order to show detailed tumor heterogeneities including tumor necrosis. Image slice thickness was 1 mm, in-plane resolution was $59 \times 59 \mu\text{m}^2$ in the final images and typically 11 to 17 slices were collected to cover the entire tumors.

3. Results

3.1. Synthesis and characterization of ^{64}Cu -NO2A-8-Aoc-BBN(7–14)NH₂

The site-directed radiopharmaceutical, ^{64}Cu -NO2A-8-Aoc-BBN(7–14)NH₂, was prepared via a multistep synthetic approach. The unconjugated peptide precursor, 8-Aoc-BBN(7–14)NH₂, was synthesized by automated SPPS and subsequently purified using RP-HPLC. 8-Aoc-BBN(7–14)NH₂ was found to have a retention time of 7.7 min. The NO2A bifunctional chelator was conjugated manually and found to have a retention time of 9.5 min and a monoisotopic mass [M+H]⁺ of 1366.68 (calculated, 1366.73). Cu-NO2A-8-Aoc-BBN(7–14)NH₂ conjugate was found to have a retention time of 8.8 min and a monoisotopic mass [M+H]⁺ of 1426.66 (calculated, 1426.63). Radiolabeling of NO2A-8-Aoc-BBN(7–14)NH₂ produced a signal in the chromatographic profile at 8.7 min. Radiochemical yields for ^{64}Cu -NO2A-8-Aoc-BBN(7–14)NH₂ (Fig. 1) were greater than 90% when synthesized at 70°C. The final pH of the reaction mixture was determined to be 7.1. For the purpose of consistency, all of the in vitro and in vivo studies reported in this paper used the conjugate synthesized at 70°C. Alternatively, radiolabeling at 34°C for 1 h proved to be remarkably efficient in spite of the more mild reaction conditions. Free $^{64}\text{CuCl}_2$ had a t_R of 2 min and was not observed in the crude reaction mixture's RP-HPLC radiochromatogram (Fig. 2). Moreover, a very strong signal was observed at 8.7 min, corresponding to ^{64}Cu -NO2A-8-Aoc-BBN(7–14)NH₂ with a radiochemical yield greater than 95%.

3.2. In vitro evaluation of ^{64}Cu -NO2A-8-Aoc-BBN(7–14)NH₂

A number of in vitro assays were used to evaluate the uptake and affinity of ^{64}Cu -NO2A-8-Aoc-BBN(7–14)NH₂ for T-47D human breast cancer cells. Competitive binding displacement assays using ^{125}I -[Tyr⁴]-BBN, NO2A-8-Aoc-BBN(7–14)NH₂ and Cu-NO2A-8-Aoc-BBN(7–14)NH₂ were used to determine the affinity of NO2A-8-Aoc-BBN(7–14)NH₂ and Cu-NO2A-8-Aoc-BBN(7–14)NH₂ (Fig. 3) for T-47D cells. The concentration of NO2A-8-Aoc-BBN(7–14)NH₂ needed to inhibit ^{125}I -[Tyr⁴]-BBN binding by 50% (IC₅₀) was determined to be 5.99 ± 0.04 nM. Similarly, the concentration of Cu-NO2A-8-Aoc-BBN(7–14)NH₂ needed to inhibit ^{125}I -[Tyr⁴]-BBN binding by 50% was determined to be 7.56 ± 2.23 nM. Therefore, the presence of metal, hence the change in structure, does little to alter the receptor binding affinity in this particular cell line. The rate at which cell-associated radioactivity was internalized within the T-47D cells was also measured (Fig. 4). After incubating T-47D cells in media containing ^{64}Cu -NO2A-8-Aoc-BBN(7–14)NH₂ for 45 min, nearly 90% of all cell-associated radioactivity had internalized. This remained nearly constant for subsequent time points. The efflux of ^{64}Cu -NO2A-8-Aoc-BBN(7–14)NH₂ from T-47D cells was measured by removing cells from conjugate-containing media after 45 min and washing them to remove all surface-bound radioactivity (Fig. 5). No significant efflux of radiotracer was observed over the 90-min period.

3.3. In vivo evaluation of ^{64}Cu -NO2A-8-Aoc-BBN(7–14)NH₂ in normal mice

The biodistribution of ^{64}Cu -NO2A-8-Aoc-BBN(7–14)NH₂ in normal, CF-1 mice is shown in Fig. 6. A number of tissues and organs (heart, lung, spleen, stomach, muscle, bone, brain and blood tissues) retained very little of the accumulated conjugate through most or all time points. At 1, 4 and 24 h pi, accumulation and retention in renal tissue were measured to be 2.92 ± 0.64 ,

0.72±0.07 and 0.42±0.05 % ID/g, respectively. The small intestine showed uptake values of 10.8±1.1, 1.87±0.51 and 0.37±0.06 % ID/g, while the large intestine accumulated 2.14±0.35, 15.1±3.9 and 0.53±0.16 % ID/g at these time points. Uptake and retention of conjugate in hepatic tissue were determined to be 1.54±0.59, 1.07±0.10 and 0.73±0.09%ID/g. Pancreatic accumulation and retention of conjugate were determined to be 27.0±3.2, 9.1±1.4, and 1.42±0.13 % ID/g at 1, 4 and 24 h pi, respectively. At these time points, urinary excretion was determined to be 63±51, 76.5±6.4 and 80.8±4.0 % ID.

The Lys blocking study in CF-1 mice showed a 1-h biodistribution profile largely similar to that of the unblocked 1-h study (Fig. 6). Uptake and retention of conjugate in all of the tissues were very much similar to the 1-h biodistribution study in unblocked normal mice. Urine was found to contain 59±42 % ID of the administered radioactivity.

3.4. In vivo evaluation of ^{64}Cu -NO2A-8-Aoc-BBN(7–14)NH₂ in tumor-bearing mice

The biodistribution of ^{64}Cu -NO2A-8-Aoc-BBN(7–14)NH₂ in SCID mice bearing T-47D xenografts was very similar to that of the CF-1 biodistribution (Fig. 7). Heart, lung, spleen, stomach, muscle, bone, brain and blood tissues retained little accumulation of conjugate through all of the time points. At 1, 4 and 24 h pi, uptake and retention of conjugate in renal tissue were measured to be 2.62±0.43, 0.43±0.12 and 0.26±0.06 % ID/g, respectively. The small intestine had uptake and retention values of 7.70±0.60, 1.65±0.52 and 0.23±0.05 % ID/g, while the large intestine had accumulations of 1.76±0.19, 11.4±1.8 and 0.31±0.06 % ID/g at these time points. Accumulation and retention of ^{64}Cu -NO2A-8-Aoc-BBN(7–14)NH₂ in hepatic tissue at 1, 4 and 24 h pi were determined to be 0.87±0.13, 0.64±0.25 and 0.36±0.11 % ID/g. Receptor-mediated pancreatic uptake and retention were 19.8±1.5, 4.23±0.80 and 1.60±0.54 % ID/g at 1, 4 and 24 h pi, respectively. The average uptake and retention of conjugate in T-47D xenografted tumors at these time points were 2.27±0.08, 1.35±0.14 and 0.28±0.07 % ID/g, while the urine was found to contain 66.6±1.5, 82±17 and 84.8±0.9 % ID.

The receptor blocking study in T-47D tumor-bearing mice showed a significant decrease in the accumulation of ^{64}Cu -NO2A-8-Aoc-BBN(7–14)NH₂ in a number of tissues (Fig. 7). The accumulation of radiotracer in heart, lung, spleen, stomach, muscle, bone, brain, blood, renal and hepatic tissues was very much similar to the 1-h biodistribution in tumor-bearing mice. Tissues with significant decreases in accumulation of conjugate included the small intestine (3.05±0.66 % ID/g), large intestine (0.18±0.05 % ID/g), pancreas (0.79±0.06 % ID/g) and tumor (0.58±0.10 % ID/g). In the blocking assay, the degree of urinary excretion increased to 82±18 % ID.

3.5. Multimodal imaging analyses

A SCID mouse bearing a T-47D xenograft underwent microMR and microPET/CT imaging 24 h following injection with ^{64}Cu -NO2A-8-Aoc-BBN(7–14)NH₂. The animal was immobilized in a cradle that allows for the correct alignment and coregistration of various imaging modalities (Fig. 8). Most visible in the microPET image appears to be the pancreas. Also identifiable is the liver and, to a lesser extent, the kidneys. The two xenografted tumors can be seen in the right and left flank. MR imaging aided in identifying these tissues. For example, liver lobes, kidneys, muscle and tumor show superior contrast in the microMRI slices (Fig. 8C). These anatomical structures were correlated to the microPET/CT images. Tumor volumes were determined to be 0.67 and 1.33 ml for the left and right tumors, respectively, using the transverse microMRI slices. Also identifiable in the microMRI are low proton-density and necrotic areas in the central region of the xenografted tumors. The low proton-density areas show dark signals in the pW and T2W MRI. The necrotic tissues of the tumor core regions show high water diffusion in the DW imaging and the apparent diffusion coefficient MRI of the tumors (data not shown). The necrotic tissues and the low proton-density areas in the tumors

appear as low-intensity regions, which are distinguishable from the solid tumor tissues in the microPET images. Fused PET/CT/MRI coronal and axial slices correlated precisely, demonstrating that photopenic tumor regions, as seen via microPET imaging, were associated with low proton-density regions as visualized via CT and MRI.

As anticipated, upon multimodality molecular and anatomical coregistration, tumor heterogeneity was visualized. Image histogram and region of interest analysis revealed that target-to-nontarget (tumor to liver) ratios were comparable for the compound analyzed.

4. Discussion

The GRPR has been targeted with bombesin-like peptides radiolabeled with a variety of diagnostic radionuclides for imaging of a number of neoplastic tissues, including breast cancers [12,33,34]. In fact, a number of ^{64}Cu -labeled bombesin conjugates have been developed for use as PET imaging probes of human cancer xenografted tumors [10–13,25]. Here, we have evaluated the efficacy of ^{64}Cu -NO2A-8-Aoc-BBN(7–14) NH_2 as a microPET imaging probe for T-47D human breast cancer xenografts in SCID mice. High radiochemical yields were obtained and the separation of NO2A-8-Aoc-BBN(7–14) NH_2 from the labeled species allowed for production of ^{64}Cu -NO2A-8-Aoc-BBN(7–14) NH_2 with very high specific activity. Of interest to us is the ability of ^{64}Cu -NO2A-8-Aoc-BBN(7–14) NH_2 to target GRPR-positive tissues *in vivo*, the particular routes of clearance and the extent of retention in radiosensitive tissues.

A number of tissues in mice have demonstrated expression of the GRPR including small intestine, large intestine and pancreas [35]. Administration of 100 μg of BBN(1–14) NH_2 15 min prior to injection of the radiolabel significantly decreased the accumulation of conjugate in these tissues. It appears that the accumulation of ^{64}Cu -NO2A-8-Aoc-BBN(7–14) NH_2 in these tissues is at least in part GRPR mediated. The exact translational relevance of this accumulation is not evident. A number of tissues in humans have demonstrated mRNA expression of the GRPR, including small intestine, large intestine, pancreas and stomach [36]. Additionally, the GRPR has been implicated in a variety of nongastrointestinal physiological processes in rodents, such as perception of the itch sensation in mice, localized in the spinal cord [37], and consolidation of memory in rats, localized in the basolateral complex of the amygdala [38]. Although expression of the GRPR has been observed in a number of locations, the extent of expression and accessibility of the receptors in humans is unknown.

Investigations of targeting the human breast cancer T-47D cell line using ^{64}Cu and BBN targeting vector are very limited. However, a study of the T-47D cell line using the DOTA chelator and 8-Aoc pharmacokinetic modifier has been conducted by Parry et al. [12]. They have reported that DOTA-Aoc-BN(7–14) had an IC_{50} of 6.7 ± 1.1 nM for T-47D cells in competition assays with the radioligand ^{125}I -[Tyr⁴]-BBN [12]. This is very similar to our observed 5.99 ± 0.04 nM IC_{50} for NO2A-8-Aoc-BBN(7–14) NH_2 , suggesting that the two conjugates have near identical affinity for the GRPR. Thus, differences in the biodistribution profiles of the two radiolabeled conjugates should be a result of differences in their pharmacokinetics and stability of the ^{64}Cu -chelate complex. Uptake and retention of ^{64}Cu -DOTA-Aoc-BN(7–14) [12] in nearly all tissues are higher than what we observed with ^{64}Cu -NO2A-8-Aoc-BBN(7–14) NH_2 in T-47D tumor-bearing SCID mice. Comparison of the (tumor % ID/g)/(tissue % ID/g) ratios for radioactivity accumulation in tumors compared to accumulation in various tissues is therefore deemed appropriate. Radiotracer accumulation for ^{64}Cu -DOTA-Aoc-BN(7–14) in muscle, blood, kidney, liver and pancreas compared to T-47D tumors is shown in Table 1 [12]. Tumor-to-tissue ratios for the accumulation of small and large intestines have not been reported [12]. Tumor-to-tissue ratios of the uptake and retention of ^{64}Cu -NO2A-8-Aoc-BBN(7–14) NH_2 in these tissues are also reported in Table

1. ^{64}Cu -NO2A-8-Aoc-BBN(7–14) NH_2 had greater relative accumulation in tumor for nearly all tissues at all three time points. This is especially pertinent in the liver, the major site of copper metabolism [23]. The only difference between the two ^{64}Cu -conjugates is the BFCA. The DOTA conjugate has a net -1 charge, while the NO2A conjugate is electronically neutral. Therefore, the NO2A conjugate would be expected to be more hydrophobic than the corresponding DOTA derivative. Increased accumulation in liver reported for ^{64}Cu -DOTA-Aoc-BN(7–14) is likely to be a result of loss of ^{64}Cu from the DOTA chelator rather than a function of the conjugate's hydrophobicity and subsequent clearance via the hepatobiliary system [39].

Two types of blocking studies were performed at 1 h pi to determine the contribution of the ^{64}Cu -NO2A complex and the GRPR-binding component (BBN(7–14) NH_2) of ^{64}Cu -NO2A-8-Aoc-BBN(7–14) NH_2 to the pharmacokinetic profile. Blocking studies in which BBN(1–14) NH_2 was administered 15 min prior to targeting vector produced no significant change in accumulation in the liver. Additionally, the accumulations in the small and large intestines were significantly decreased during the blocking study. This suggests that the radioactivity detected in the intestines was at least partially the result of a receptor-mediated process located along the lower gastrointestinal tract and not solely a function of hepatobiliary excretion. Additionally, accumulation of radiotracer in pancreatic tissue and tumors was significantly decreased in this blocking experiment. This suggests the ^{64}Cu -NO2A-8-Aoc-BBN(7–14) NH_2 conjugate is intact and that GRPR-mediated binding is facilitating uptake in the tissues.

The Lys-based and BBN-based blocking studies failed to significantly lower accumulation of ^{64}Cu in the kidneys at 1 h pi, in CF-1 and T-47D xenograft-bearing mice, respectively. The BBN-based blocking study indicates that the receptor-binding portion of ^{64}Cu -NO2A-8-Aoc-BBN(7–14) NH_2 , BBN(7–14) NH_2 , is not responsible for any significant retention in the kidneys. Additionally, the Lys-based blocking study failed to significantly reduce renal uptake of the radiotracer. In a previous study, lysine demonstrated the inability to significantly reduce renal uptake of a neutral radiolabeled BBN derivative [40]. These studies suggest that the neutral ^{64}Cu -NO2A complex of ^{64}Cu -NO2A-8-Aoc-BBN(7–14) NH_2 does not significantly contribute to retention of radioactivity in the kidneys and that the observed accumulation is the result of nonspecific accumulation. Both cationic and anionic trapping mechanisms have been implicated in the retention of radiolabeled peptides in the kidneys [40]. This suggests that the net negative charge found on bifunctionalized ^{64}Cu -DOTA and ^{64}Cu -TETA complexes or the net positive charge found on bifunctionalized ^{64}Cu -CB-TE2A, ^{64}Cu -SarAr and ^{64}Cu -PMCN complexes may contribute to retention of ^{64}Cu in the kidneys. Kidneys are known to be particularly sensitive to radiation and can be a limiting factor in nuclear medicine-based therapy [40,41]. This indicates the ^{64}Cu -NO2A complex may be well suited for therapeutic regimens based on ^{64}Cu , a radionuclide known to have radiotoxicity in kidneys [40,41]. The liver is also known to be a radiosensitive organ [41]. Low accumulation of ^{64}Cu -NO2A-8-Aoc-BBN(7–14) NH_2 in these abdominal tissues is justification for pursuit of ^{64}Cu -or ^{67}Cu -NO2A-based therapies, while at the same time allowing for high-resolution and highly informative diagnostic microPET images to be obtained.

As a result of optimal uptake and retention of ^{64}Cu -NO2A-8-Aoc-BBN(7–14) NH_2 in T-47D xenografted tumor, microPET/MicroCT/microMRI multimodal imaging experiments were also evaluated in tumor-bearing mice. Anatomical structures observed via microPET imaging are consistent with pharmacokinetic data produced in in vivo experiments. For example, tumors, liver and kidneys, to a lesser extent, are clearly identifiable via microPET imaging analysis. Analysis of MRI data indicated small necrotic regions in the xenografted tumors as evident by increased water diffusivity.

Observable in the microPET data were regions of reduced intensity that correlated well to these necrotic regions of the tumors. Additionally, the liver is observed to lack a uniform intensity. The drop in intensity correlates to the separation between the median and left lobes of the liver. The identification of such fine structures in the microPET data is a goal of diagnostic nuclear medicine. High-resolution imaging may aid in identifying structural boundaries as well as possible malignant sites.

^{64}Cu -based PET imaging has great potential to overcome many of the limitations of current diagnostic breast-imaging procedures. These findings suggest that ^{64}Cu -NO2A-8-Aoc-BBN(7–14) NH_2 would be a good starting point. Moreover, the investigations of ^{64}Cu -NO2A-8-Aoc-BBN(7–14) NH_2 have made apparent a number of desirable qualities of the ^{64}Cu -NO2A complex. For example, the very mild conditions required to form ^{64}Cu -NO2A complex will, very likely, allow for ^{64}Cu -NO2A-labeled antibodies to be synthesized without fear of denaturing of the antibodies. When compared to other chelators, the ^{64}Cu -NO2A-8-Aoc-BBN(7–14) NH_2 has decreased accumulation in the liver while still retaining affinity for GRPR-positive xenografts. A more detailed, direct comparison of the various copper chelators is needed to identify a candidate producing ideal chemical and biological properties for clinical applications. Nonetheless, high-resolution microPET images revealing hyperfine anatomical structure were obtained using the ^{64}Cu -NO2A-8-Aoc-BBN(7–14) NH_2 conjugate.

5. Conclusion

In this report, we evaluated the ability of ^{64}Cu -NO2A-8-Aoc-BBN(7–14) NH_2 to specifically target T-47D xenografts in SCID mice via the GRPR. Following intravenous administration of conjugate, hepatic and renal accumulation and retention of radioactivity were very low, even at the 24-h time point. As a result of these studies, we were able to observe the accumulation and retention of ^{64}Cu -NO2A-8-Aoc-BBN(7–14) NH_2 in human breast cancer xenografted tumors in mice. The biodistribution profile of conjugate at 24 h pi was such that microPET imaging revealed major sites of radioactivity uptake and retention with relatively high resolution. Lobes of the liver as well as necrotic regions of the tumors were able to be discriminated in the microPET image, demonstrating the ability to image very fine structure. As such, the ^{64}Cu -NO2A complex may yield high-quality PET images when conjugated to other peptides or antibodies used in site-directed imaging studies. Complete optimization of the ^{64}Cu -NO2A-bombesin system for targeting of GRPR-positive tumors may be possible through selection of an ideal pharmacokinetic modifier. Studies are currently underway to fine tune the specific targeting of GRPR-positive xenografted human tumors. Furthermore, analysis of the pharmacokinetic profile of ^{64}Cu -NO2A-8-Aoc-BBN(7–14) NH_2 in normal mice and tumor-bearing mice justifies the investigation of the efficacy of ^{67}Cu -NO2A-8-Aoc-BBN(7–14) NH_2 as a therapeutic agent for mice bearing GRPR-positive xenografted tumors.

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Fig. 1.
Structure of ^{64}Cu -NO2A-8-Aoc-BBN(7-14) NH_2 .

Fig. 2.
RP-HPLC chromatographic profile of the crude reaction mixture of ^{64}Cu -NO₂A-8-Aoc-BBN
(7–14) NH_2 synthesized at 34°C for 1 h at pH 7.1.

Fig. 3. IC₅₀ data of Cu-NO₂A-8-Aoc-BBN(7–14)NH₂ in T-47D cells. The IC₅₀ was determined to be 7.56 ± 2.23 nM.

Fig. 4. Internalization data of ^{64}Cu -NO₂A-8-Aoc-BBN(7-14)NH₂ in T-47D cells at selected time points.

Fig. 5.
Efflux data of ^{64}Cu -NO₂A-8-Aoc-BBN(7-14)NH₂ from T-47D cells at selected time points.

Fig. 6. Pharmacokinetic data of ^{64}Cu -NO₂A-8-Aoc-BBN(7-14)NH₂ in CF-1 normal mice at 1, 4 and 24 h pi time points with a 1-h blocking study where D-Lysine was administered immediately prior to 7 μCi intravenous tail vein injection of ^{64}Cu -NO₂A-8-Aoc-BBN(7-14)NH₂.

Fig. 7. Pharmacokinetic data of ^{64}Cu -NO₂A-8-Aoc-BBN(7-14)NH₂ in T-47D tumor-bearing SCID mice at 1, 4 and 24 h pi time points with a 1-h blocking study where BBN(1-14)NH₂ was administered 15 min prior to 7 μCi intravenous tail vein injection of ^{64}Cu -NO₂A-8-Aoc-BBN(7-14)NH₂.

Fig. 8.

In vivo microPET/CT and microMRI images of a T-47D tumor-bearing mouse 24 h after 5.1 mCi tail vein injection of ^{64}Cu -NO₂A-8-Aoc-BBN(7-14)NH₂ conjugate. (A) MicroPET/MRI fusion coronal image. (B) MicroPET/CT fused coronal image showing specific tumor uptake of ^{64}Cu -NO₂A-8-Aoc-BBN(7-14)NH₂ conjugate. (C) Proton density-weighted microMRI coronal image. (D) An axial cross section of microPET/CT images showing functional image of tumor uptake. (E) The corresponding high-resolution T₂-weighted microMRI axial image correlating anatomical features of the xenografted tumors to (D).

Table 1
The ratio, (tumor % ID/g)/(tissue % ID/g), of ⁶⁴Cu-NO2A-8-Aoc-BBN(7-14)NH₂ and ⁶⁴Cu-DOTA-Aoc-BN(7-14) [12] in selected tissues compared to T-47D xenografted tumors at selected time points

Tissue	⁶⁴ Cu-NO2A-8-Aoc-BBN(7-14)NH ₂			⁶⁴ Cu-DOTA-Aoc-BN(7-14) [12]		
	1 h	4 h	24 h	1 h	4 h	24 h
Muscle	9.5	7.5	5.6	4.5	6.5	4.5
Blood	4.9	11.3	7.0	3.2	3.1	2.0
Kidney	0.9	3.1	1.1	0.7	0.8	0.4
Small intestine	0.3	0.8	1.2	–	–	–
Large intestine	1.3	0.1	0.9	–	–	–
Liver	2.6	2.1	0.8	0.6	0.5	0.2
Pancreas	0.1	0.3	0.2	0.2	0.2	0.4