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# *In Vitro* and *In Vivo* Evaluation of a $^{64}\text{Cu}$ -Labeled Polyethylene Glycol-Bombesin Conjugate

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## ABSTRACT

*The goal of this study was to synthesize and evaluate a novel bombesin (BN) analogue containing a polyethylene glycol (PEG) linker that can be radiolabeled with  $^{64}\text{Cu}$  through the DOTA bifunctional chelate. It is hypothesized that PEG linkers would improve the pharmacokinetics of radiolabeled bombesin analogues to optimize their tumor-to-normal tissue ratios for radiotherapy applications. The formation of this conjugate (DOTA-PEG-BN(7-14)) was confirmed by MALDI-TOF mass spectrometry and was radiolabeled with  $^{64}\text{Cu}$  at a specific activity of 2.7 MBq/nmol. DOTA-PEG-BN(7-14) bound specifically to gastrin-releasing peptide receptor (GRPR)-positive PC-3 cells with an  $\text{IC}_{50}$  value of 3.9  $\mu\text{M}$  for displacing  $^{125}\text{I}$ -Tyr<sup>4</sup>-BN. Internalization of  $^{64}\text{Cu}$ -DOTA-PEG-BN(7-14) into PC-3 cells showed that 5.7%, 13.4%, and 21.0% was internalized at 0.5, 2, and 4 hours, respectively. Biodistribution of  $^{64}\text{Cu}$ -DOTA-PEG-BN(7-14) was evaluated in normal, athymic nude mice 2, 4, and 24 hours after i.v. injection. This showed that most of the tissues had a similar uptake and clearance of  $^{64}\text{Cu}$ -DOTA-PEG-BN(7-14) compared to a control peptide with an alkyl linker (DOTA-Aoc-BN(7-14)) at the given time points. There was uptake of 10.8% ID/g of  $^{64}\text{Cu}$ -DOTA-PEG-BN(7-14) 4 hours after i.v. injection in the GRPR-positive pancreas that was inhibited to 2.4% upon injection of an excess of Tyr<sup>4</sup>-BN. These studies demonstrate that BN analogues can be conjugated with PEG linkers, radiolabeled with  $^{64}\text{Cu}$ , and bind to GRPR. Future studies will attempt to increase the affinity of these analogues for GRPR and alter the pharmacokinetics of the  $^{64}\text{Cu}$ -labeled conjugates through the use of various sized PEG linkers.*

**Key words:** copper-64, bombesin, polyethylene glycol, gastrin-releasing peptide receptor

## INTRODUCTION

Radioimmunotherapy is a well-established modality for the treatment of cancer through the specific delivery of radiation. In general, radioactivity has been delivered to the tumor site by attaching it to a monoclonal antibody that is se-

lective for the tumor tissue. One drawback to this approach is that only a limited amount of radioactivity can be delivered to the tumor due to the bone marrow toxicity that is caused by administration of therapeutic doses of the radiolabeled antibody.<sup>1</sup> This toxicity is due to the long serum half-life of antibodies that results in a high radiation dose delivered to bone marrow.<sup>2</sup> To address this limitation, peptides have been used to deliver radiation to the tumor site in place of antibodies. Radiolabeled peptides have the advantage of clearing the serum more rapidly than antibodies, thus lowering the radiation dose de-

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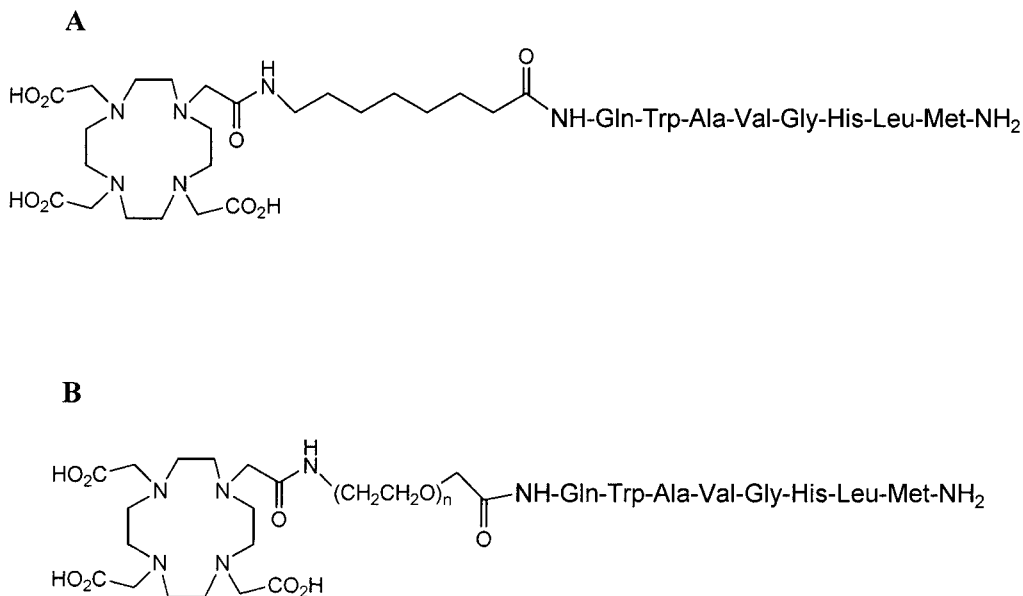
Address reprint requests to: Buck E. Rogers; Department of Radiation Oncology; Washington University in St. Louis; 4511 Forest Park Boulevard, Suite 411; St. Louis, MO 63108; Phone: (314) 362-9787; Fax: (314) 362-9790  
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livered to the bone marrow. In particular, octreotide analogues radiolabeled with indium-111 and yttrium-90 have been evaluated clinically for the treatment of somatostatin receptor subtype 2-positive tumors.<sup>3,4</sup>

One limiting factor in the use of radiolabeled peptides for cancer therapy is that the rapid serum clearance of the peptide can lead to a lower radiation dose delivered to the tumor. Because the peptide clears from serum rapidly, there is little time for the peptide to build a high concentration in the tumor. Thus, the best vehicle for delivery of therapeutic radioisotopes would balance the tumor uptake with the serum clearance. In this regard, several studies have used chemical conjugates or genetic engineering to alter the pharmacokinetics of tumor targeting ligands. Monoclonal antibodies, single-chain Fv proteins (scFv), and chemotherapeutic drugs have been chemically conjugated with dextran, polyethylene glycol (PEG), poly(L-lysine), or N-(2-hydroxypropyl)methacrylamide copolymer (HMPA) as scaffolds to form the chemical links to alter their pharmacokinetics.<sup>5-10</sup> Genetically engineered antibodies that have improved pharmacokinetics compared to intact antibodies, antibody fragments, or scFvs have recently been studied.<sup>11,12</sup>

We hypothesized that the chemical conjugation

of a peptide to a PEG scaffold would improve the pharmacokinetics of the resulting conjugate for delivery of radioisotopes to a tumor site. As a model system, we used the eight C-terminal amino acids of the amphibian tetradecapeptide bombesin (BN), which binds with high affinity to the gastrin-releasing peptide receptor (GRPR) overexpressed on a variety of human carcinomas.<sup>13-17</sup> BN analogues have been evaluated as therapeutic and diagnostic agents in clinical and pre-clinical studies after labeling with a variety of radioactive metals.<sup>18-30</sup> In the present study, we conjugated the N-terminus of BN(7-14) to a 3,500 Da PEG derivative and coupled the resulting conjugate (PEG-BN(7-14)) to 1,4,7,10-tetraazacyclododecane-1,4,7,10-tetraacetic acid (DOTA) via the N-terminus of the PEG moiety for radiolabeling with <sup>64</sup>Cu. The <sup>64</sup>Cu-DOTA-PEG-BN(7-14) was evaluated and compared to a control peptide (<sup>64</sup>Cu-DOTA-Aoc-BN(7-14)) containing an eight carbon spacer in place of PEG *in vitro* using GRPR-expressing PC-3 human prostate cancer cells and *in vivo* in normal athymic nude mice. The <sup>64</sup>Cu-DOTA-Aoc-BN(7-14) was recently evaluated in mice bearing PC-3 tumor xenografts and shown to be useful for microPET imaging.<sup>30</sup> The structures of DOTA-PEG-BN(7-14) and DOTA-Aoc-BN(7-14) are shown in Figure 1.



**Figure 1.** Structures and amino acid sequence of DOTA-Aoc-BN(7-14) (**A**) and DOTA-PEG-BN(7-14) (**B**). The average molecular weight of the polyethylene glycol (PEG) linker in (**B**) was 3,500 Da. Note that the C-terminal methionine in each peptide is amidated.

## MATERIALS AND METHODS

### Synthesis

1,4,7,10-tetraazacyclododecane-1,4,7-tris(acetic acid-*t*-butyl ester)-10-acetic acid (DOTA-tris(*t*-butyl ester)) was purchased from Macro-cyclics, Inc. (Dallas, TX) and 8-amino-octanoic (Aoc) acid was purchased from Advanced ChemTech (Louisville, KY). The DOTA-Aoc-BN(7-14) was synthesized using standard Fmoc chemistry by solid phase peptide synthesis using a Rink amide resin at the University of Alabama at Birmingham Comprehensive Cancer Center Peptide Synthesis and Analysis Shared Facility and shown to be > 98% pure by high performance liquid chromatography (HPLC).

The PEG-BN(7-14) was synthesized in a manner similar to that described before.<sup>8</sup> Briefly, the BN(7-14) (3 mg, 2.8  $\mu$ mol), also synthesized at the UAB Peptide Synthesis and Analysis Shared Facility, was dissolved in 200  $\mu$ L of dry DMF and 5  $\mu$ L of diisopropyl ethylamine (DIEA) were added with stirring. This solution was then added to *N*-hydroxysuccinimidyl *N* $\alpha$ -Fmoc-PEG-carboxylate (9.9 mg, 2.9  $\mu$ mol) (Nektar Therapeutics, Huntsville, AL) in 100  $\mu$ L of the same solvent and the reaction mixture was stirred at 4°C for 2 hours. Piperidine (200  $\mu$ L) was added at 0°C and the solution was stirred at this temperature for 10 minutes after which time the liquids were removed under high vacuum and the H<sub>2</sub>N-PEG-BN(7-14) residue was purified by semi-preparative reversed-phase HPLC.

To a solution of DOTA-tris(*t*-butyl ester) (1.53 mg, 2.7  $\mu$ mol) in 150  $\mu$ L of DMF, was added DIEA (0.5  $\mu$ L) followed by *O*-benzotriazol-1-yl-*N*, *N*, *N*', *N*'-tetramethyluronium hexafluorophosphate (0.97 mg, 2.7  $\mu$ mol). After 20 minutes, a solution of H<sub>2</sub>N-PEG-BN(7-14) in 200  $\mu$ L of the solvent was added and the mixture was stirred at room temperature for 2 hours. The reaction progress was monitored by analytical HPLC. The final product DOTA-PEG-BN(7-14) was purified by semi-preparative HPLC and identified by matrix-assisted laser desorption/ionization time-of-flight mass spectroscopy (MALDI-TOF MS).

Analytical and semi-preparative HPLC used Vydac C18 reversed-phase 4.6  $\times$  10 cm and 10  $\times$  250 cm columns, respectively. A linear gradient of 10% to 100% B in the base solvent A over 30 minutes was used, where A = water/0.1% trifluoroacetic acid and B = acetoni-

trile/0.1% trifluoroacetic acid. Flow rates of 1.0 mL/minute and 2.0 mL/minute were used for the analytical and for semi-preparative samples, respectively. A BioRad model 2800 solvent delivery system, a Bio-Rad model 1806 UV/VIS detector, and a Beckman model 170 radiodetector were used for all HPLC experiments.

### Radiolabeling

Copper-64 was produced on a CS-15 biomedical cyclotron at Washington University in St. Louis School of Medicine, according to a published procedure.<sup>31</sup> <sup>64</sup>CuCl<sub>2</sub> (500  $\mu$ Ci) was diluted with a 10-fold excess 0.1 M ammonium acetate (NH<sub>4</sub>OAc), pH 5.5 and then added to DOTA-Aoc-BN(7-14) (4  $\mu$ g) or DOTA-PEG-BN(7-14) (33  $\mu$ g) in the presence of 2, 5-dihydroxybenzoic acid (4 mg/mL final concentration) (Sigma Chemical Co., St. Louis, MO) to prevent radiolysis. The reaction mixtures were incubated at room temperature for 30 minutes and the radiochemical purity determined by radio-thin layer chromatography (R-TLC) or radioactivity-detecting high performance liquid chromatography (R-HPLC). No further purification was necessary for <sup>64</sup>Cu-DOTA-Aoc-BN(7-14), a sample of the reaction mixture was applied to Whatman MKC18F TLC plates, developed with 10% NH<sub>4</sub>OAc:methanol (30:70, v/v), and analyzed using a BIOSCAN System 200 imaging scanner (Washington, D.C.) to determine the purity. <sup>64</sup>Cu-DOTA-PEG-BN(7-14) was purified by analytical reversed-phase R-HPLC using the gradient described above. The peak that co-eluted with the UV peak for unlabeled DOTA-PEG-BN(7-14) was collected, the solvent was dried, and the residue was resuspended in PBS with 5% EtOH prior to *in vitro* and *in vivo* use.

### *In vitro* Competition Assay

The PC-3 human prostate cancer cell line was obtained from the American Type Culture Collection (Rockville, MD) and cultured in Ham's F12K medium containing 10% fetal bovine serum (FBS) and 1% L-glutamine at 37°C in a humidified atmosphere with 5% CO<sub>2</sub>. For binding assays, the cells were harvested by incubating with 4 mM EDTA/0.05% KCl for 3 minutes, centrifuging and re-suspending in cold PBS at a concentration of 1  $\times$  10<sup>7</sup> cells/mL. The cells (100  $\mu$ L) were then aliquoted into polystyrene tubes in triplicate followed by the addition of 100  $\mu$ L of <sup>125</sup>I-Tyr<sup>4</sup>-BN (0.05 nM final concentration, DuPont/NEN Research Products, Boston, MA). Various amounts of

DOTA-Aoc-BN(7-14), DOTA-PEG-BN(7-14), or Tyr<sup>4</sup>-BN (Sigma Chemical Co.) were added in 10  $\mu$ L such that the final concentrations ranged between 1 pM and 50  $\mu$ M. The cells were incubated for 1 hour at 4°C, then rinsed with PBS, and centrifuged at 1700  $\times$  g for 10 minutes. The supernatant was removed, and the cells were counted in a gamma counter (Packard Auto Gamma 5000 Series, Chicago, IL) to determine the amount of bound radioactivity. The data were analyzed using the GraphPad Prism software (San Diego, CA).

### Internalization Assay

PC-3 cells were harvested and seeded in 6-well plates at 3  $\times$  10<sup>5</sup> cells per well. Twenty-four hours later, <sup>64</sup>Cu-DOTA-Aoc-BN(7-14) or <sup>64</sup>Cu-DOTA-PEG-BN(7-14) were added to the wells such that the final concentration was 1 nM and incubated at 37°C for 30, 120, and 240 minutes. An excess (30  $\mu$ M) of Tyr<sup>4</sup>-BN was added to half of the wells as an inhibitor. At the appropriate time point, a six well plate was removed from the incubator, media was removed, and cells were rinsed with PBS. The cells were then rinsed with Hank's Balanced Salt Solution containing 20 mM NaOAc, pH 4.0 to remove surface-bound radioactivity and the cells were harvested by adding 1 N NaOH. The acid wash and the cells were counted in a gamma counter to determine the amount of surface bound and internalized radioactivity, respectively. These data were normalized to the total amount of radioactivity added to each well.

### Biodistribution

Animal experiments were reviewed and approved by the Institutional Animal Care and Use Committee at the University of Alabama at Birmingham. Experiments were performed in 4–5-week-old athymic nude mice (National Cancer Institute Frederick Research Laboratory, Frederick, MD). The mice were injected with 185 kBq (5  $\mu$ Ci; 26 pmol) of <sup>64</sup>Cu-DOTA-Aoc-BN(7-14) or 111 kBq (3  $\mu$ Ci; 41 pmol) of <sup>64</sup>Cu-DOTA-PEG-BN(7-14) via the tail vein. Biodistribution was performed with groups of 5 mice sacrificed 2, 4, and 24 hours post-injection of the radiolabeled ligands. Another group of mice were co-injected with <sup>64</sup>Cu-DOTA-Aoc-BN(7-14) (n = 5) or <sup>64</sup>Cu-DOTA-PEG-BN(7-14) (n = 3) and 100  $\mu$ g of Tyr<sup>4</sup>-BN as an inhibitor and sacrificed 4 hours post-injection. The blood, liver, small intestine, spleen, kidney, muscle (thigh), bone (femur), and

pancreas were removed and weighed, and the radioactivity was counted in a gamma counter to determine the percent-injected dose per gram of tissue (% ID/g).

## RESULTS

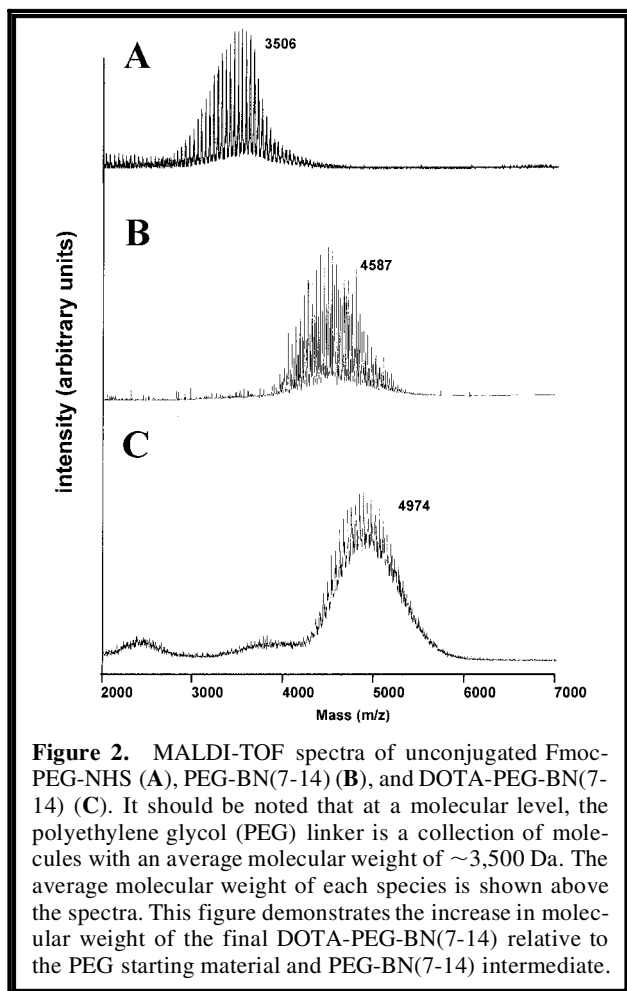
### Synthesis and Radiolabeling

The synthesis of the DOTA-PEG-BN(7-14) conjugate was carried out through an active ester protocol in 16% overall yield. The synthesis was similar to that previously described for a paclitaxel-PEG-BN(7-13) conjugate.<sup>8</sup> The progress of the synthesis was monitored by direct molecular weight (MW) measurements using MALDI-TOF MS, which has proved to be a valuable tool for direct MW monitoring in large-molecule proteins and polymeric material.<sup>8,32</sup> Under the controlled reaction conditions of this synthesis, the increases in MWs clearly indicated the formation of intermediate and final-product compounds, in a 1:1:1 molar ratio with respect to the DOTA, PEG, and BN(7-14) (Fig. 2).

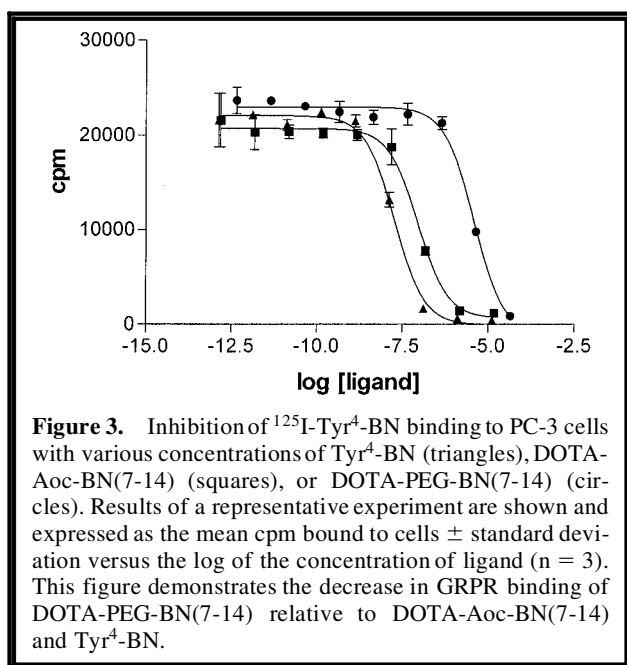
DOTA-Aoc-BN(7-14) was radiolabeled with <sup>64</sup>Cu at a specific activity of 7 MBq/nmol (190  $\mu$ Ci/nmol) in >98% radiochemical purity as determined by radio-TLC. The resulting <sup>64</sup>Cu-DOTA-Aoc-BN(7-14) did not require further purification and was used immediately for both *in vitro* and *in vivo* assays. DOTA-PEG-BN(7-14) was radiolabeled with <sup>64</sup>Cu at a specific activity of 2.7 MBq/nmol (73.5  $\mu$ Ci/nmol). The <sup>64</sup>Cu-DOTA-PEG-BN(7-14) was purified by HPLC and co-eluted with the UV trace for DOTA-PEG-BN(7-14) at 29.9 minutes. It was assumed that the retention time of <sup>64</sup>Cu-DOTA-PEG-BN(7-14) would not significantly differ from the retention time of the large, 5000 Da DOTA-PEG-BN(7-14) and therefore, the radioactive peak corresponding to the retention time of DOTA-PEG-BN(7-14) was collected, concentrated and re-suspended for *in vitro* and *in vivo* evaluation.

### *In vitro* Evaluation

A representative competitive binding assay is shown in Figure 3. The binding of <sup>125</sup>I-Tyr<sup>4</sup>-BN to PC-3 cells was inhibited by various concentrations of Tyr<sup>4</sup>-BN, DOTA-Aoc-BN(7-14), or DOTA-PEG-BN(7-14). The IC<sub>50</sub> values for Tyr<sup>4</sup>-BN, DOTA-Aoc-BN(7-14) and DOTA-PEG-BN(7-14) were 18.8  $\pm$  2.3 nM, 90.5  $\pm$  22.0 nM, and 3.9  $\pm$  0.6  $\mu$ M, respectively. Thus, 4.8-fold



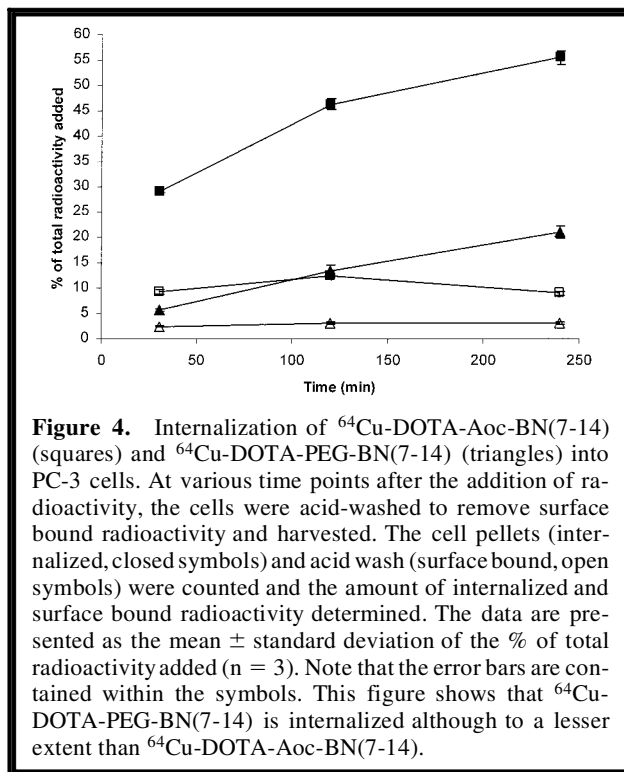
**Figure 2.** MALDI-TOF spectra of unconjugated Fmoc-PEG-NHS (A), PEG-BN(7-14) (B), and DOTA-PEG-BN(7-14) (C). It should be noted that at a molecular level, the polyethylene glycol (PEG) linker is a collection of molecules with an average molecular weight of  $\sim 3,500$  Da. The average molecular weight of each species is shown above the spectra. This figure demonstrates the increase in molecular weight of the final DOTA-PEG-BN(7-14) relative to the PEG starting material and PEG-BN(7-14) intermediate.



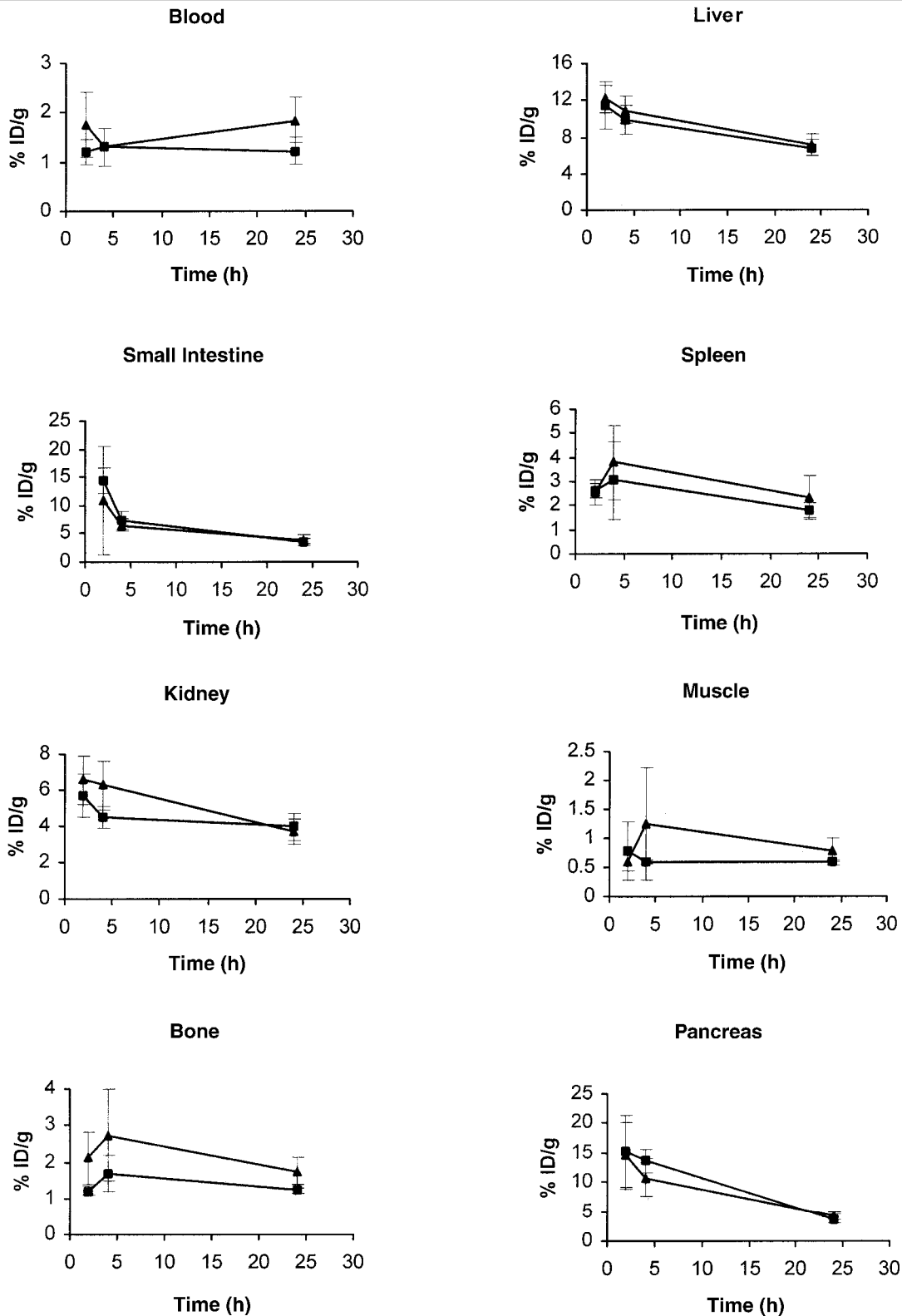
**Figure 3.** Inhibition of  $^{125}\text{I}$ -Tyr<sup>4</sup>-BN binding to PC-3 cells with various concentrations of Tyr<sup>4</sup>-BN (triangles), DOTA-Aoc-BN(7-14) (squares), or DOTA-PEG-BN(7-14) (circles). Results of a representative experiment are shown and expressed as the mean cpm bound to cells  $\pm$  standard deviation versus the log of the concentration of ligand ( $n = 3$ ). This figure demonstrates the decrease in GRPR binding of DOTA-PEG-BN(7-14) relative to DOTA-Aoc-BN(7-14) and Tyr<sup>4</sup>-BN.

more DOTA-Aoc-BN(7-14) was needed than Tyr<sup>4</sup>-BN to inhibit  $^{125}\text{I}$ -Tyr<sup>4</sup>-BN binding, while DOTA-PEG-BN(7-14) required 207-fold more than Tyr<sup>4</sup>-BN. This demonstrates that the substitution of the Aoc linker with the PEG linker had a dramatic effect on the affinity of BN(7-14) for GRPR.

The internalization of  $^{64}\text{Cu}$ -DOTA-PEG-BN(7-14) and  $^{64}\text{Cu}$ -DOTA-Aoc-BN(7-14) into PC-3 cells is shown in Figure 4. The amount of surface bound radioactivity did not increase over time for both  $^{64}\text{Cu}$ -DOTA-PEG-BN(7-14) and  $^{64}\text{Cu}$ -DOTA-Aoc-BN(7-14). The amount of surface bound radioactivity for  $^{64}\text{Cu}$ -DOTA-Aoc-BN(7-14) ranged from 9.3–12.4% for all time points and was significantly greater ( $p < 0.0001$ ) than the surface bound radioactivity for  $^{64}\text{Cu}$ -DOTA-PEG-BN(7-14) that ranged from 2.4–3.1%. When the Tyr<sup>4</sup>-BN inhibitor was present, there was less than 0.2% of the radioactivity either surface bound or internalized at all time points. There was a significant increase ( $p < 0.002$ ) in the amount of internalized radioactivity for both compounds over the course of the internalization assay. As with the surface bound radioactivity, the amount of internalized  $^{64}\text{Cu}$ -DOTA-Aoc-BN(7-14) was significantly greater ( $p < 0.0001$ ) than the internalization of  $^{64}\text{Cu}$ -DOTA-PEG-BN(7-14).



**Figure 4.** Internalization of  $^{64}\text{Cu}$ -DOTA-Aoc-BN(7-14) (squares) and  $^{64}\text{Cu}$ -DOTA-PEG-BN(7-14) (triangles) into PC-3 cells. At various time points after the addition of radioactivity, the cells were acid-washed to remove surface bound radioactivity and harvested. The cell pellets (internalized, closed symbols) and acid wash (surface bound, open symbols) were counted and the amount of internalized and surface bound radioactivity determined. The data are presented as the mean  $\pm$  standard deviation of the % of total radioactivity added ( $n = 3$ ). Note that the error bars are contained within the symbols. This figure shows that  $^{64}\text{Cu}$ -DOTA-PEG-BN(7-14) is internalized although to a lesser extent than  $^{64}\text{Cu}$ -DOTA-Aoc-BN(7-14).



**Figure 5.** Biodistribution of  $^{64}\text{Cu}$ -DOTA-Aoc-BN(7-14) (squares) and  $^{64}\text{Cu}$ -DOTA-PEG-BN(7-14) (triangles) in normal, athymic nude mice. The data are expressed as the % ID/g for the mean  $\pm$  standard deviation of 5 mice per time point. This figure shows the comparison of the tissue uptake of  $^{64}\text{Cu}$ -DOTA-PEG-BN(7-14) compared to  $^{64}\text{Cu}$ -DOTA-Aoc-BN(7-14) at the indicated time points.

## In vivo Evaluation

The biodistributions of  $^{64}\text{Cu}$ -DOTA-PEG-BN(7-14) and  $^{64}\text{Cu}$ -DOTA-Aoc-BN(7-14) at 2, 4, and 24 hours in normal, athymic nude mice are shown in Figure 5. This shows that the only significant differences in the uptake and retention between  $^{64}\text{Cu}$ -DOTA-PEG-BN(7-14) and  $^{64}\text{Cu}$ -DOTA-Aoc-BN(7-14) were in the bone at 2 hours ( $p < 0.03$ ) and 24 hours ( $p < 0.02$ ), the kidneys at 4 hours ( $p < 0.03$ ), and the blood at 24 hours ( $p < 0.04$ ). Specific GRPR uptake for  $^{64}\text{Cu}$ -DOTA-PEG-BN(7-14) and  $^{64}\text{Cu}$ -DOTA-Aoc-BN(7-14) is only observed in the pancreas as shown in Table 1. The pancreas is the only tissue that shows a significant reduction ( $p < 0.005$ ) 4 hours after the injection of  $^{64}\text{Cu}$ -DOTA-PEG-BN(7-14) or  $^{64}\text{Cu}$ -DOTA-Aoc-BN(7-14) upon co-injection of an excess of Tyr<sup>4</sup>-BN.

## DISCUSSION

The N-terminus of BN(7-14) was used for conjugation because the C-terminal amide is necessary for receptor binding and the amide moiety is not as amenable to conjugation as the amine on the N-terminus. The overall yield of 16% for DOTA-PEG-BN(7-14) was adequate for these initial studies, but will need to be improved in future studies requiring large amounts of product. It should be noted that at a molecular level, the PEG linker is a collection of molecules with an average molecular weight of  $\sim 3,500$  Da, thereby the broad MALDI peaks of Figure 2. This variation in PEG molecular weight, however, would be expected to have little effect on

*in vitro* binding, internalization, or *in vivo* pharmacokinetics.

The *in vitro* binding of DOTA-PEG-BN(7-14) to GRPR was lower than the binding of DOTA-Aoc-BN(7-14) (Fig. 3). It is not unexpected that the relatively large PEG moiety had an adverse effect on the binding of BN(7-14) to GRPR. For example, Wen et al. showed that conjugation of PEG to a monoclonal antibody interfered with the binding activity of the antibody.<sup>10</sup> This example shows that even when the PEG is small relative to the targeting molecule ( $\sim 4,000$  Da PEG to  $\sim 150$  kDa antibody) a negative effect on binding can occur. Of course, there were multiple PEGs conjugated to the antibody, which also contributes to the decrease in binding. To overcome this problem, it may be necessary to develop PEG linkers that can incorporate several BN analogues in order to increase the valency and the affinity of the conjugate. Lee et al. demonstrated that PEG-sFv conjugates that contained multiple sFvs had a higher affinity than conjugates that contained a single sFv.<sup>9</sup> Similarly, DeNardo et al. evaluated PEG moieties that contained eight lymphoma specific binding peptides and demonstrated that there was not a difference in cell binding or affinity when the size of the PEG was increased.<sup>33</sup> Although the IC<sub>50</sub> value was 43-times lower for DOTA-PEG-BN(7-14) than for DOTA-Aoc-BN(7-14), radiolabeling with  $^{64}\text{Cu}$  was performed for further *in vitro* and *in vivo* evaluation.

The internalization assay (Fig. 4) showed a decrease in surface bound and internalized radioactivity for  $^{64}\text{Cu}$ -DOTA-PEG-BN(7-14) compared to  $^{64}\text{Cu}$ -DOTA-Aoc-BN(7-14) that is likely due to the lower affinity of  $^{64}\text{Cu}$ -DOTA-PEG-BN(7-14) for

**Table 1.** Biodistribution of  $^{64}\text{Cu}$ -DOTA-Aoc BN(7-14) (DOTA-BN) (n = 5) and  $^{64}\text{Cu}$ -DOTA-PEG-BN(7-14) (DOTA-PEG-BN) (n = 3) in normal, athymic nude mice at 4 hours with and without coinjection of 100  $\mu\text{g}$  of Tyr<sup>4</sup>-BN blocking agent. The data are expressed as %ID/g with standard deviation in parentheses.

Tissue	DOTA-BN	DOTA-BN Block	DOTA-PEG-BN	DOTA-PEG-BN block
Blood	1.31 (0.38)	1.49 (0.24)	1.30 (0.39)	1.61 (1.12)
Liver	9.92 (1.57)	13.26 (4.32)	10.93 (1.49)	10.69 (3.76)
Sm Int	7.31 (1.11)	7.38 (2.22)	6.50 (1.51)	5.61 (2.51)
Spleen	3.04 (1.60)	2.23 (0.49)	3.79 (1.53)	2.03 (0.86)
Kidney	4.50 (0.59)	4.84 (0.89)	6.26 (1.35)	5.85 (0.80)
Muscle	0.59 (0.03)	0.57 (0.11)	1.25 (0.96)	0.79 (0.50)
Bone	1.67 (0.50)	1.32 (0.27)	2.73 (1.27)	2.00 (1.01)
Panceas	13.69 (1.99)	2.52 (0.52)	10.80 (3.11)	2.42 (1.29)



GRPR. Interestingly, although the affinity of  $^{64}\text{Cu}$ -DOTA-PEG-BN(7-14) for GRPR is low, it still has significant internalization that is decreased only 2.6-fold relative to  $^{64}\text{Cu}$ -DOTA-Aoc-BN(7-14) at 4 hours. This relatively small difference in internalization may be due to the fact that the assay was performed with an approximately 10-fold excess of the radiolabeled ligands over the number of receptors. Studies using various concentrations of the radiolabeled ligands may demonstrate more significant differences that correlate with the differences in  $\text{IC}_{50}$ . An efflux assay of  $^{64}\text{Cu}$ -DOTA-Aoc-BN(7-14) from PC-3 cells demonstrated a 62% decrease in cell-associated activity from 4–18 hours (data not shown). This is similar to the 72% decrease of  $^{64}\text{Cu}$ -DOTA-Aoc-BN(7-14) in the pancreas from 4–24 hours in Figure 5. This indicates that  $^{64}\text{Cu}$  is not well residualized in GRPR expressing tissues.

The biodistributions of  $^{64}\text{Cu}$ -DOTA-PEG-BN(7-14) and  $^{64}\text{Cu}$ -DOTA-Aoc-BN(7-14) are shown in Figure 5. We hypothesized that  $^{64}\text{Cu}$ -DOTA-PEG-BN(7-14) would have a longer serum half-life than  $^{64}\text{Cu}$ -DOTA-Aoc-BN(7-14) and would thus have a higher blood concentration at the early time points (2 hours and possibly 4 hours). In addition, we anticipated that this longer serum half-life would have an effect on the uptake and clearance in other tissues. However, the differences in blood clearance were not observed as the  $^{64}\text{Cu}$ -DOTA-PEG-BN(7-14) cleared more rapidly than expected. The reason for the rapid blood clearance (< 2% ID/g remaining at 2 hours) of  $^{64}\text{Cu}$ -DOTA-PEG-BN(7-14) is likely due to the fact that the size of the PEG linker was not large enough to avoid rapid renal clearance. DeNardo et al. evaluated lymphoma specific peptides conjugated to PEGs ranging in molecular weight from 40 kDa to 150 kDa.<sup>33</sup> This study showed that the  $t_{1/2}$  in blood increased from 5.4 hours using a 40 kDa PEG conjugate to 17.7 hours for the 150 kDa PEG conjugate. This difference is likely due to the fact that proteins with molecular weights greater than 69 kDa are generally unable to filter through the glomerular membrane, while a protein of 30 kDa has a permeability of 50 percent.<sup>34</sup> Thus, future conjugates will consist of PEG linkers in the 30–80 kDa range. It is anticipated that conjugates within this range of molecular weights will increase their initial blood concentration and thus alter their uptake and clearance in other tissues (including tumors). In this regard, DeNardo et al. also showed that the

tumor uptake increased as the molecular weight of the PEG increased.<sup>33</sup> We hypothesize that conjugates > 80 kDa would have serum half-lives that are too long to have optimal tissue-to-blood ratios. These studies will need to be conducted to determine overall blood clearance and effect on tissue-to-blood ratios. Of course, as mentioned above, it may be necessary to incorporate several BN analogues into the PEG linker to optimize the affinity of the conjugate for GRPR.

It is interesting that the pancreatic uptake of  $^{64}\text{Cu}$ -DOTA-Aoc-BN(7-14) is only 1.3-fold higher than the uptake of  $^{64}\text{Cu}$ -DOTA-PEG-BN(7-14) due to the lower affinity of DOTA-PEG-BN(7-14) for GRPR. It has been shown by others and by our group that the mouse pancreas has a relatively low number of GRPR.<sup>30</sup> We found that the concentration of GRPR on the mouse pancreas is 27 fmol/mg,<sup>30</sup> while Fanger et al. reported a similar level of 75 fmol/mg.<sup>35</sup> Therefore, due to the low specific activity of  $^{64}\text{Cu}$ -DOTA-PEG-BN(7-14) and  $^{64}\text{Cu}$ -DOTA-Aoc-BN(7-14) a relatively large amount of the peptides were injected (41 pmol and 26 pmol, respectively) and no difference in pancreatic uptake was observed. A biodistribution study conducted with higher specific activity  $^{64}\text{Cu}$ -DOTA-Aoc-BN(7-14) (108 MBq/nmol; 2924  $\mu\text{Ci/nmol}$ ), and thus a lower amount of peptide administered (1.7 pmol vs. 26 pmol), showed an increase in pancreatic uptake at 2 hours from 15.2% ID/g to 30.9% ID/g (data not shown). It is anticipated that a higher specific activity for  $^{64}\text{Cu}$ -DOTA-PEG-BN(7-14), allowing the injection of less peptide, would not result in higher pancreatic uptake due to the low affinity for GRPR, although this will need to be demonstrated.

## CONCLUSION

This study demonstrates that a peptide-PEG conjugate can be synthesized and radiolabeled with  $^{64}\text{Cu}$ . In particular, DOTA-PEG-BN(7-14) was synthesized and shown to bind to GRPR-expressing PC-3 human prostate cancer cells.  $^{64}\text{Cu}$ -DOTA-PEG-BN(7-14) was internalized into PC-3 cells and demonstrated GRPR-specific uptake in mouse pancreas after intravenous injection. Future studies will evaluate the conjugation of BN analogues with various molecular weight PEG linkers to determine how the size of the PEG linker alters the *in vivo* pharmacokinetics. In addition, the conjugation of several BN analogues

to a PEG linker will be evaluated to determine if this increases the affinity of the conjugate for GRPR.

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