MATERIAL AND METHODS

General Methods

All chemicals and solvents were obtained from commercial sources, and used without further purification. P03034 and ^{68}Ga-P03034 were prepared according to previously published procedures (*1*). Balb/c mouse plasma for stability studies was obtained from Innovative Research (Novi, MI). CHO-K1 cell membranes overexpressing the recombinant human B1R were obtained from PerkinElmer (Waltham, MA). B1R-targeting peptides were synthesized using solid phase approach on an Aapptec (Louisville, KY) Endeavor 90 peptide synthesizer. The collected HPLC eluates containing the desired peptide were lyophilized using a Labconco (Kansas City, MO) FreeZone 4.5 Plus freeze-drier. Mass analyses were performed using a Bruker (Billerica, MA) Esquire-LC/MS system with ESI ion source. Purification and quality control of cold and ^{68}Ga-labeled peptides were performed on an Agilent HPLC system equipped with a model 1200 quaternary pump, a model 1200 UV absorbance detector, and a Bioscan (Washington, DC) NaI scintillation detector. The radio-detector was connected to a Bioscan B-FC-1000 Flow-count system, and the output from the Bioscan Flow-count system was fed into an Agilent 35900E Interface which converted the analog signal to digital signal. The operation of the Agilent HPLC system was controlled using the Agilent ChemStation software. The HPLC columns used were a semi-preparative column (Phenomenex C18, 5 μ, 250 × 10 mm) and an analytical

column (Eclipse XOB-C18, 5 μ, 150 × 4 mm). The HPLC solvents were A: H_2O containing 0.1% TFA, and B: CH_3CN containing 0.1% TFA. ^{68}Ga was obtained from an Eckert & Ziegler (Berlin, Germany) IGG100 ^{68}Ga generator, and was purified according to the previously published procedures (*1*) using DGA resin column. Radioactivity of ^{68}Ga-labeled peptides was measured using a Capintec (Ramsey, NJ) CRC®-25R/W dose calibrator, and the radioactivity of mouse tissues collected from biodistribution studies were counted using a Packard (Meriden, CT) Cobra II 5000 Series auto-gamma counter. PET imaging experiments were conducted using a Siemens (Erlangen, Germany) Inveon microPET/CT scanner.

Syntheses of Precursors and Standards

The radiolabeling precursors, DOTA-dPEG2-B9858 and DOTA-dPEG2-B9958, were synthesized via the N^{α}-Fmoc solid-phase peptide synthesis strategy. Fmoc was removed by treating the resin with 20% piperidine (1 × 5 min and 1 × 10 min) in DMF. Side-chain protected amino acids including Fmoc-Lys(Boc)-OH, Fmoc-Arg(Pbf)-OH, Fmoc-Hyp(*t*Bu)-OH, and Fmoc-Ser(*t*Bu)-OH, and the chelator DOTA tri-*t*-butyl ester were used for the synthesis. The coupling was carried out in NMP using Fmoc-protected amino acid (3 equivalents), HBTU (3 equivalents) and DIEA (6 equivalents). At the end of elongation, the peptides were de-protected and simultaneously cleaved from the resin by the treatment of 95/2.5/2.5 TFA/H_2O/TIS for 4 h at room temperature. After filtration, the peptides were precipitated by the addition of cold

diethyl ether to the TFA solution. The precipitated crude peptides were collected by centrifugation, and purified by HPLC using the semi-preparative column. The HPLC eluates containing the desired peptide were collected, pooled, and lyophilized. For DOTA-dPEG2-B9858, the HPLC condition was 72/28 A/B at a flow rate of 4.5 mL/min, and the product retention time was 16.8 min. The yield was 21 %. MS (ESI) calculated for DOTA-dPEG2-B9858 $C_{87}H_{134}N_{20}O_{23}$ 1827.0, found $(M + H)^+$ 1828.4. For DOTA-dPEG2-B9958, the HPLC condition was 77/23 A/B at a flow rate of 4.5 mL/min, and the product retention time was 13.2 min. The yield was 13 %. MS (ESI) calculated for DOTA-dPEG2-B9958 $C_{80}H_{130}N_{20}O_{23}$ 1739.0, found $(M + H)^+$ 1740.4.

To prepare P04158 and Z02090, ~ 2 μmol of their respective precursor (DOTA-dPEG2-B9858 or DOTA-dPEG2-B9958) was incubated with $GaCl_3$ (5 equivalents) in 0.1M NaOAc buffer (500 μL, pH 4.2) at 80 °C for 15 min. The reaction mixture was purified by HPLC using the semi-preparative column, and the HPLC eluates containing the desired peptide were collected, pooled, and lyophilized. For P04158, the HPLC condition was 72/28 A/B at a flow rate of 4.5 mL/min. The product retention time was 19.3 min. The yield was 99%. MS (ESI) calculated for P04158 $C_{87}H_{131}GaN_{20}O_{23}$ 1893.9, found $(M + 2H)^{2+}$ 947.6. For Z02090, the HPLC condition was 77/23 A/B at a flow rate of 4.5 mL/min. The product retention time was 17.0 min. The yield was 89%. MS (ESI) calculated for Ga-DOTA-dPEG2-B9958 $C_{80}H_{127}GaN_{20}O_{23}$ 1805.9, found $(M + H)^+$ 1805.5.

***In Vitro* Competition Binding Assays**

The affinity of the peptides for B1R was measured using competition binding assays as reported previously (*1*). CHO-K1 cell membranes overexpressing the recombinant hB1R were used for those assays. Briefly, 96-wells MultiScreen plates with glass fiber filter and PVDF support were pre-soaked with 0.5% of cold polyethyleneimine solution for 30 minutes. Afterward, wells were washed once with Tris-HCl buffer (50 mM, pH 7.4). The wells were loaded with the assay buffer (pH 7.4) containing 50 mM of Tris-HCl and 5 mM of $MgCl_2$. Varying concentration of peptides of interest were added in the presence of 4.8 nM of [^{3}H]-[Leu^9,des-Arg^{10}]kallidin. B1R membranes were added to each well to a final protein concentration of 50 µg/well. The MultiScreen plate was incubated at 27 ^{o}C for 15 min with gentle agitation at 300 rpm. The assay was stopped by aspirating the reaction solution through the PVDF membrane filter, followed by washing with ice-cold 50 mM Tris-HCl (pH 7.4). The filter membranes were dried prior to adding scintillation liquid, and the activity in the plates was measured using a 1450 MicroBeta Counter (PerkinElmer). Data analysis was performed with GraphPad Prism 5, using a one-site competitive binding model.

Radiochemistry

Purified ^{68}Ga in 0.5 mL water was added into a 4-mL glass vial preloaded with 0.7 mL of HEPES buffer (2 M, pH 5.0) and 100 µg DOTA-dPEG2-B9858 (or 40 µg DOTA-dPEG2-B9958). The radiolabeling reaction was carried out under microwave heating for 1 min. The reaction mixture was purified by HPLC using the semi-preparative column

eluted with 69/31 A/B for ^{68}Ga-P04158 or 79/21 TEA-Phosphate Buffer (pH 7.3)/CH_3CN for ^{68}Ga-Z02090 at a flow rate of 4.5 mL/min. The retention times of ^{68}Ga-P04158 and ^{68}Ga-Z02090 were 14.1 and 12.8 min, respectively. The eluate fraction containing the radiolabeled product was collected, diluted with water (50 mL), and passed through a C18 Sep-Pak cartridge that was pre-washed with ethanol (10 mL) and water (10 mL). After washing the C18 Sep-Pak cartridge with water (10 mL) the ^{68}Ga-labeled product was eluted off the cartridge with ethanol (0.4 mL), and diluted with saline (4 mL) for plasma stability and imaging/biodistribution studies.

$LogD_{7.4}$ Measurements

Aliquots (2 μL) of the ^{68}Ga-labeled peptides were added to a vial containing 3 mL of octanol and 3 mL of 0.1 M phosphate buffer (pH 7.4). The mixture was vortexed for 1 min and then centrifuged for 10 min. Samples of the octanol (1 mL) and buffer (1 mL) layers were taken and counted. $LogD_{7.4}$ was calculated using the following equation: $LogD_{7.4} = \log_{10}[(\text{counts in octanol phase})/(\text{counts in buffer phase})]$.

Stability in Mouse Plasma

Aliquots (100 μL) of the ^{68}Ga-labeled peptide (P04158 and Z02090) were incubated with 400 μL of mouse plasma for 5, 15, 30, and 60 minutes at 37 °C. At the end of each incubation period, samples were quenched with 500 μL 70% CH_3CN and centrifuged for 20 min. The metabolites were measured using a semi-preparative

HPLC system with the same HPLC conditions as described for the preparation of ^{68}Ga-labeled P04158 and Z02090.

REFERENCE

1. Lin KS, Pan J, Amouroux G, et al. In vivo radioimaging of bradykinin receptor B1, a widely overexpressed molecule in human cancer. *Cancer Res.* 2015;75:387-393.