Chemical and Supplies:

Unless otherwise noted all solvents were used without further purification. Deionized water was purified by a Millipore (U.S.) Mili-Q-Biocel. Acetonitrile, formic acid, *N, N*-diisopropylethylamine (DIEA), *N*,*N*-dimethylformamide (DMF), dichloromethane (DCM), *N,N'*-dicyclohexylcarbodiimide (DCC) , N-methylpyrrolidone (NMP), thioanisol, sodium hydroxide, L-ascorbic acid, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES), bovine serum albumin (BSA) and sodium dodecyl sulfate (SDS) were purchased from Fisher Scientific (U.S.). 2-nitroimidazole was purchased from Amfinecom (U.S.). O-Benzotriazole-N,N,N',N'-tetramethyl-uronium-hexafluoro-phosphate (HBTU), Fmoc-protected natural amino acids and the appropriate Rink Amide resins were purchased from Nova Biochem (U.S.). Fmoc-8-AOC was purchased from *CreoSalus* (U.S). Roswell Park Memorial Institute (RPMI) 1640 media, phosphate buffered saline (PBS), and mammalian protein extraction reagent (M-PER) were purchased from Thermo Scientific (U.S.). Indium-111 chloride ($^{111}InCl_3$) was purchased from MDS Nordion (Canada). Naturally abundant indium chloride ($^{nat}InCl_3$), triisopropyl silane and 3, 6-dioxa-1, 8-octanedithiol were purchased from Sigma-Aldrich (U.S.). The [^{125}I-Tyr$_4$]-Bombesin was purchased from Perkin Elmer (U.S.). Prostate cancer (PC-3) cell lines were obtained from American Type Culture Collection (U.S.) and cultured under vendor recommended conditions. TrypLE^{TM} Express was purchased from Invitrogen (U.S.). The separation of macromolecules and small molecules was performed using Amicon Ultra (Ireland) Ultracel-30K centrifugal filters in protein association analysis. Four week-old Institute of Cancer Research severely combined immunodeficient (ICR SCID) mice were obtained from Charles River Laboratories (Wilmington, MA). Food and water were given ad libitum. Each animal was kept in individual cage with an air filter cover under light- (12 h light/dark cycle) and

temperature-controlled (22±1°C) environment. The animals were treated in accordance to the Principles of Animal Care outlined by National Institutes of Health and approved by the Institutional Animal Care and Use Committee of the University of Nebraska Medical Center.

Equipment:

The peptides were synthesized on a Liberty microwave peptide synthesizer from CEM (U.S.). HPLC/MS analyses were performed on a Waters (U.S.) e2695 system equipped with a Waters 2489 absorption detector and a Waters Qtof Micro electrospray ionization mass spectrometer. Evaluation and purification of radiolabeled conjugates was performed on a Waters 1515 binary pump equipped with a Waters 2489 absorption detector and a Bioscan (U.S.) Flow Count radiometric detector system. A Phenomenex (U.S.) Jupiter 10μ Proteo 250 × 10 mm semiprep column was used for the purification of bulk amounts of peptides. The peptides were concentrated on a Centrivap (U.S.) Centrifugal Concentrator. For the purification of conjugates, ^{nat}In-conjugates, and ^{111}In-radioconjugates a Phenomenex Jupiter 10μ Proteo 250 × 4.60 mm analytical column was employed. Solid phase extraction was performed using Empore (U.S.) C_{18} 10 mm high performance extraction disks. Hypoxic PC-3 cells were incubated in hypoxic glove box with temperature, CO_2 and humidity controller (Coy Laboratory Products INC, Grass Lake, MI). Gamma decay detection of ^{111}In and ^{125}I for the *in vitro* binding, receptor saturation, efflux studies and protein binding fractionation studies was accomplished using a LTI (U.S.) Multi-Wiper nuclear medicine gamma counter. Gas anesthesia was administered at a vaporizer setting of 5% isoflurane (Halocarbon Corp, River Edge, NJ) with 0.5 L/min oxygen using an E-ZAnesthesia apparatus (EUTHANEX Corp, Palmer, PA). Biodistribution radiation measurements were made with a NaI (Tl) well detector constructed by AlphaSpectra, Inc. (U.S.).

Small animal SPECT/CT data acquisition is achieved with the dual Flex Triumph CT/SPECT instrument (GE Healthcare, Gamma-Medica Ideas, Northridge, CA).

Solid-Phase Peptide Synthesis (SPPS)

Peptide synthesis was performed using an automated solid-phase peptide synthesizer employing traditional Fmoc chemistry and using a Rink Amide resin. Briefly, the resin (100 μmol of the resin substituted peptide anchors) was deprotected using piperidine, resulting in the formation of a primary amine from which the C-terminus of the growing peptide was anchored. The Fmoc protected amino acids (300 μmol) with appropriate orthogonal protection were activated with HBTU and sequentially added to the resin. The resulting peptide was orthogonally deprotected and cleaved from the resin using a cocktail consisting of triisopropyl silane (0.1 mL), water (0.1 mL), 3,6-dioxa-1,8-octanedithiol (0.1 mL) trifluoroacetic acid (4.625 mL) and thioanisole (0.075 mL), respectively. The cleaved peptide was subsequently precipitated and washed using cold (0 °C) methyl-*tert*-butyl ether (10 mL × 3). The crude conjugate was dried by a centrivap concentrator and weighed. ES-MS was used to determine the molecular mass of the prepared peptides. All conjugates were peak purified to ≥ 95% purity and quantified by RP-HPLC prior to *in vitro/in vivo* investigations.

Coupling of 2-NIAA to Bombesin Conjugates

The 2-NIAA was manually coupled to ε-amino group of the lysine residue for peptides **2*, 3*** and **4*** (9.2, 29.3 and 16.4 mg respectively) using standard amidation chemistry. Briefly, for peptides **2*** and **4*,** the 2-NIAA (3.6 and 12.1 mg, respectively)/NHS (3.6 and 6.1mg, respectively) mixtures were dissolved in HBTU/DMF (0.5 M, 83.4 and 142.4 μl) solution followed by addition of DIEA/NMP solution (5.7 M, 18.1 and 30.9μl). For peptide **3*,** the 2-NIAA (16.6 mg)/NHS (11.2 mg) mixtures were dissolved in DCC/DMF (0.9 M, 141.2 μl)

solution followed by addition of DIEA/NMP solution (5.7 M, 56.3 μl). These solutions were allowed to stand for 2 h in an ice bath before addition of the conjugate in DMF (200 μL). The reaction mixture was stirred overnight at room temperature and subsequently evaporated to dryness. The residue was re-dissolved in water : acetonitrile : formic acid (8 : 2 : 0.05), peak purified by RP-HPLC and characterized by mass spectrometry.

Labeling with $^{nat}InCl_3$

For the convenient characterization of the ^{111}In-Bombesin conjugates, naturally abundant ^{nat}In was used to substitute for ^{111}In in the ES-MS and *in vitro* binding studies. A sample of conjugates (0.5 mg) was dissolved in ammonium acetate buffer (1 M, 200 μL, pH 5.5) and mixed with a solution of $^{nat}InCl_3$ (5.5 mg, 50 μmol). The solution was heated for 60 min at 50 °C. After cooling to room temperature, ^{nat}In-conjugates were then peak purified by RP-HPLC. All ^{nat}In-conjugates were ≥ 95% purity before mass spectrometric characterization and *in vitro* binding studies were performed.

Radiolabeling with $^{111}InCl_3$

Radiolabeling was performed on all conjugates by mixing 100 μg samples with 37 MBq $^{111}InCl_3$ in ammonium acetate buffer (1 M, 200 μL, pH 5.5). The solution was heated for 60 min at 90 °C. The resulting specific radioactivities were 0.64, 0.71, 0.78 and 0.86 MBq/nmol for **^{111}In-1**, **^{111}In-2**, **^{111}In-3** and **^{111}In-4.** In order to separate radiolabeled peptides from unlabeled peptides on HPLC, 4-5 mg $CoCl_2$ were then added and incubated for 5 min at 90 °C to increase the hydrophobicity of unlabeled conjugates. Retention time of ^{111}In-1-4 and ^{59}Co-1-4 are listed in Supplemental Table 1. The resulting radioconjugates were allowed to cool to room temperature and peak purified using RP-HPLC (≥95%) and concentrated using C_{18} extraction disk. Elution of the extraction disk with ethanol/sterile saline solution (6 : 4, 150 μL × 2) delivered the

radioconjugate in high purity. L-ascorbic acid (~20 mg) was added to all radioconjugates to reduce radiolysis. The specific activities for all peak-purified ^{111}In-conjugates are essentially the theoretical maximum of 1725 MBq/nmol.

HPLC Purification and Analysis Methodology

When necessary, bulk sample purification was performed using a semi-preparative Proteo column with a flow rate of 5.0 mL/min. Sample purification for *in vitro/in vivo* studies was performed on analytical Proteo column with a flow rate 1.5 mL/min. HPLC solvents consisted of H_2O containing 0.1% formic acid (solvent A) and acetonitrile containing 0.1% formic acid (solvent B). For all unlabeled and $^{111/nat}$In-conjugates, purification was achieved using an initial gradient of 85 % A : 15 % B which linearly decreased to 75 % A : 25 % B over a 15 minute time period. At the end of the run time, the column was flushed with the gradient 5 % A : 95 % B and re-equilibrated to the starting gradient.

***In Vitro* Competitive Cell-Binding Studies**

Briefly, the PC-3 cells (~3×10^4) were suspended in RPMI 1640 media (pH 7.4, 4.8 mg/mL HEPES, and 2 mg/mL BSA) and incubated at 4°C for 45 min in the presence of radiolabeled [^{125}I-Tyr$_4$]-Bombesin and various concentrations of the ^{nat}In-conjugate. At the end of the incubation period, the cells were centrifuged, aspirated and washed with media a total of four times. The cell associated activity was measured using a gamma counter and the IC_{50} values determined by nonlinear regression using the one-binding site model of Graphpad PRISM 5 (U.S.).

Efflux Studies

Efflux studies were performed using PC-3 cells under normoxic (95% air, 5% CO_2) and hypoxic (94.9% N_2, 0.1% O_2, 5% CO_2) conditions. PC-3 cells were incubated in six-well plates (0.5×10^6

/ well) under hypoxic conditions overnight in RPMI 1640 media (pH 7.4, 4.8 mg/mL HEPES, and 2 mg/mL BSA). On the day of experiment, the cells medium were changed and incubated for 2 h under normoxic and hypoxic conditions, respectively. The cells were then pre-incubated for 2 h at 37°C in the presence of 100,000 cpm of each ^{111}In-radioconjugate. Upon completion of the incubation, cells were washed thrice with media to discard the unbound peptide. At time points 0, 2, 4 and 8 h, the media was harvested for quantitative analysis as the effluxed ligand. Surface bound radioactivity was collected by washing the cells twice with an acid wash (50 mM glycine-HCl/0.1 M NaCl buffer, pH 2.8). The cells were then lysed at 37°C using a 10 % aqueous SDS solution and the lyses were collected as internalized ligand. The radioactivity of the effluxed, surface bounded and internalized fractions for each radioconjugate was determined using a Multi Viper gamma counter. Statistical analyses were performed by two-way analysis of variance (ANOVA) using Graphpad PRISM 5 (U.S.).

Cellular Protein Analysis

For the cellular fractionation studies, the procedure for the preparation of normoxic and hypoxic PC-3 cells (2.5×10^5 / well) was carried out as outlined in the efflux studies above. On the day of the experiment, the medium was removed, and the cells were washed with medium and incubated at 37°C in the presence of 100,000 cpm of each ^{111}In-radioconjugate. At time points 2, 4 and 8 h, the cells were washed thrice with medium to remove the extracellular radioconjugates and then lysed using 1mL of M-PER at 37°C. The cellular debris was centrifuged down at 13300×g and the supernatant was then transferred to an Amicon Ultracel 30kDa filter device with extra PBS (1mL). The samples were centrifuged at 4000×g for 10 minutes and washed with

PBS (1mL×2). The radioactivity associated with the molecular weight fractions was collected and determined using a gamma counter.

Pharmacokinetic Studies of ^{111}In-Radioconjugates in PC-3 Xenograft SCID Mice.

Pharmacokinetic studies were carried out using PC-3 tumor bearing SCID mice. The mice were inoculated with PC-3 cells, and the tumors were allowed to grow ranging from 0.1 to 1 g (4-6 weeks post-inoculation), before the mice were utilized in pharmacokinetic studies. Each mouse (average weight, 20 g) received an intravenous bolus via the tail vein of 7.5 μCi (277.5 kBq) of the radio-RP-HPLC peak purified ^{111}In-radioconjugate (**^{111}In-1, ^{111}In-2 or ^{111}In-4**) in 100 μL of saline. The mice were sacrificed and their tissues were excised at 1, 4, 24, 48 and 72 h time points post injection. The excised tissues were weighed, the radioactivity in each tissue was measured, and the %ID or %ID/g was calculated for each tissue. Blocking studies were also investigated on ^{111}In-4 by coinjection with 300 μg of unlabeled conjugate 4 (n=3).

Small Animal SPECT/CT Imaging Studies.

The SPECT g-camera incorporates an array of 5-by-5 solid state cadmium/zinc/telluride (CZT) modules, each with 1.5-mm (0.06-inch) pixel array of 16 x 16, providing a total of 80 x 80 pixel array within a field of view of 12.7-cm by 12.7-cm (5-inch by 5-inch). The X-ray detector is a complementary metal oxide semiconductor (CMOS)-based device, having a 2240 X 2368 pixel matrix with a 50 mm pitch to yield a 12 mm x 12 mm (4.72 inch x 4.72 inch) field of view. The mice were administered 4~11MBq (0.108~0.300 mCi) of the desired BB2r-targeted peptide in 100 - 200 μL of saline via tail vein injection. At 1, 24, 48 and 72 h post injection, mice were anesthetized with 1~1.5% isoflurane delivered in a 2:1 mixture of nitrous oxide/oxygen. Image acquisition was accomplished using a FLEX Triumph X-ray computed tomography/single photon emission computed tomography system (CT/SPECT) and software (Gamma Medica,

Inc., Northridge, CA) fitted with a 5-pinhole (1.0 mm/pinhole) N5F75A10 collimator. 64 SPECT projections (30 to 90-s acquisition time per projection based on the actual counts) for each image were acquired using Triumph_SPECT and reconstructed using SpectReconstructionApp. 512 CT projections for each image were acquired and reconstructed using Triumph X-O 4.1. Co-registration of anatomical CT images and functional SPECT was performed using 3D image visualization and analysis software VIVID, which is based on Amira 4.1.

Supplemental Table 1

Retention time of ^{111}In-1-4 and ^{59}Co-1-4

Analogue	HPLC retention time (min)
^{111}In-1	7.08
^{111}In-2	9.48
^{111}In-3	10.63
^{111}In-4	12.52
^{59}Co-1	8.43
^{59}Co-2	10.95
^{59}Co-3	12.56
^{59}Co-4	14.35