Supporting Information

Strain-promoted Catalyst-free Click Chemistry for Rapid Construction of ^{64}Cu-Labeled PET Imaging Probes

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Experimental Section Pages:

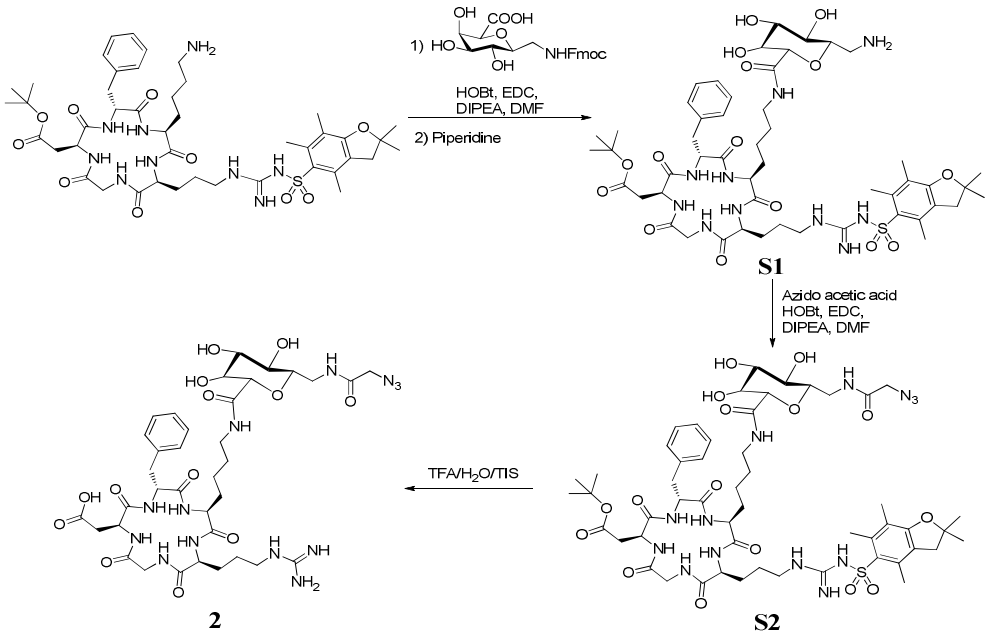
General Materials and Methods

All chemicals and solvents were obtained from Aldrich (Milwaukee, WI, USA) and used without further purification. 1,8-Diamino-3,6,10,13,16,19-hexaazabicyclo[6.6.6]-eicosine was purchased from Areva Med (Dallas, TX). ^{125}I-Echistatin was obtained from Perkin-Elmer (Waltham, MA). ^{1}H and ^{13}C nuclear magnetic resonance (NMR) were recorded on a Varian 400 MHz spectrometer. Mass spectrum was obtained on a ThermoElectron Finnigan LTQ mass spectrometer equipped with an electrospray ionization (ESI) source (Thermo Scientific, USA). HPLC analyses were carried out on an analytical reversed-phase high performance liquid chromatography (HPLC) system equipped with a dual UV absorbance detector (Waters 2487) using a phenomenex C18 RP (250 × 4.6 mm 5 micron). The flow was 1 mL/min, with the mobile phase starting from 100% solvent A (0.1% TFA in water), followed by a gradient mobile phase to 20% solvent A and 80% solvent B (0.1% TFA in acetonitrile) at 30 min. The UV absorbance was monitored at 214 nm and the identification of the peptides was confirmed based on the UV spectrum using a PDA detector. The radioactivity was detected by a model of Ludlum 2200 single-channel radiation detector. No ethylenediaminetetraacetic acid (EDTA) was added to remove the unbound copper-64 for radiolabeling. The stability study was performed under the same HPLC condition. MicroPET scans were performed on a microPET R4 rodent model scanner (Siemens Medical Solutions USA, Inc., Knoxville, TN). The scanner has a computer-controlled bed and 10.8-cm transaxial and 8-cm axial fields of view (FOVs). It has no septa and operates exclusively in the 3-dimensional (3D) list mode. Animals were placed near the center of the FOV of the scanner.

Statistical Analysis

Quantitative data were expressed as mean ± SD. Means were compared using one-way ANOVA and student's t-test. P values < 0.05 were considered statistically significant.

Synthesis of Peptide 2



*Preparation of Peptide **S1***

To a solution of (3*R*,4*R*,5*S*,6*S*)-6-((((9*H*-fluoren-9-yl)methoxy) carbonylamino) methyl)-3,4,5-trihydroxytetrahydro-2*H*-pyran-2-carboxylic acid (301 mg, 0.702 mmol, 1.2 equiv) in DMF (5 mL) was added HOBt (95 mg, 0.702 mmol, 1.2 equiv) and EDC (135 mg, 0.702 mmol, 1.2 equiv). The mixture was stirred at room temperature for 2 hrs. The protected c(RGDfK) TFA salt (600 mg, 0.585 mmol, 1.0 equiv) and DIPEA (0.204 mL, 1.169 mmol, 2 equiv) in DMF (5 mL) was added to the reaction dropwise. The mixture was stirred overnight. To the mixture was then added EtOAc (160 mL), ultrasonicated for 30 min, and cooled to 0°C. A white solid precipitated from the solution. The solid was filtered, washed with water (10 mL) and ether (20 mL × 2), and dried. To a round bottom flask containing the solid (650 mg, 0.491 mmol, 1 equiv) was added piperidine (2.15 g, 25.2 mmol). After overnight stirring, the reaction was concentrated under vacuum to remove piperidine. Acetonitrile (20 mL × 3) was added to facilitate co-

evaporation. The residue was dried under vacuum for 2 hrs. The white solid residue was then washed with ether (20 mL × 3) under ultrasound. The residue solid was filtered and dried under vacuum overnight to afford peptide **S1** (560 mg) in a total of 90% yield. ^{1}H NMR (d_6-DMSO, 400 MHz), δ: 8.42 (t, 1H), 8.08-8.06 (m, 2H), 8.02-7.98 (d, 3H), 7.60 (t, 1H), 7.43 (t, 1H), 7.51 (d, 1H), 7.26-7.11 (m, 6H), 4.72 (d, 1H), 4.59 (q, 1H), 4.51 (d, 1H), 4.42 (q, 1H), 4.10 (m, 1H), 4.00 (dd, 1H), 3.95-3.85 (m, 2H), 3.72 (s, 1H), 3.76-3.70 (m, 2H), 3.25-3.20 (m, 2H), 3.05-2.87 (m, 9H), 2.83-2.75 (m, 1H), 2.65-2.55 (m, 3H), 2.47-2.33 (m, 8H), 1.97 (s, 3H), 1.65-1.48 (m, 2H), 1.40-1.20 (m, 20H), 0.97-0.95 (m, 2H). ESI-MS: calculated for $C_{51}H_{76}N_{10}O_{15}S$: 1100.52; found: 1101.4 $[M+H]^+$.

*Preparation of Peptide **S2***

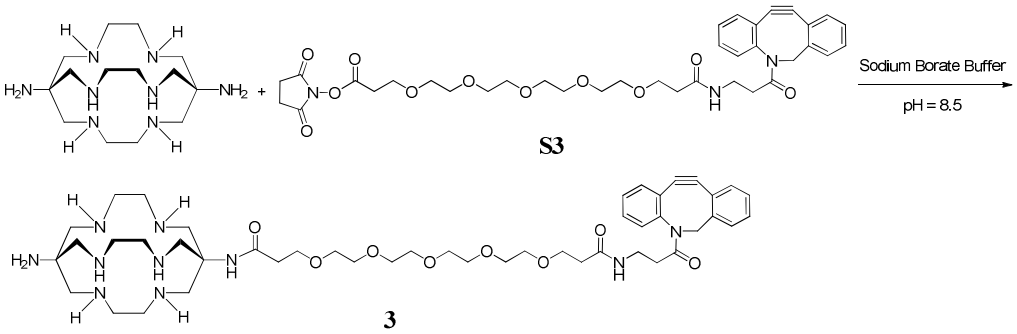
To a solution of azido acetic acid (5.65% solution in DCM/THF, 1.56 g, 0.872 mmol, 2.5 equiv) in DMF (2 mL), was added HOBt (118 mg, 0.872 mmol, 2.5 equiv) and EDC (167 mg, 0.872 mmol, 2.5 equiv). The mixture was stirred at room temperature for 20 min. Peptide **S1** (400 mg, 0.349 mmol, 1 equiv) in DMF (7.5 vol) was added along with DIPEA (0.152 mL, 0.872 mmol, 2.5 equiv) to the above mixture. The reaction was stirred at room temperature for additional 20 min. The reaction was quenched by addition of water (few drops). DMF was removed under vacuum. Acetonitrile (15 mL × 3) was added to facilitate co-evaporation of DMF. To the residue was added water (90 mL) and treated with ultrasound for 30 min. The white solid precipitate was collected by filtration. The cake was washed with mother liquid (20 mL × 1), ether (15 mL × 2), and dried under vacuum with P_2O_5 overnight to afford peptide **S2** (392 mg, 92% yield). ^{1}H NMR (d_6-DMSO, 400 MHz), δ: 8.42 (t, 1H), 8.11-8.05 (m, 2H), 8.00 (d, 2H), 7.50 (d, 1H), 7.43 (t, 1H), 7.26-7.11 (m, 5H), 4.92 (d, 1H), 4.78 (d, 1H), 4.59-4.53 (m, 2H), 4.42 (q, 1H), 4.10 (m, 1H), 4.00 (dd, 1H), 3.95-3.85 (m, 2H), 3.82 (d, 2H), 3.76-3.70 (m, 2H), 3.25-3.20 (m, 2H), 3.05-2.87 (m, 9H), 2.83-2.75 (m, 1H), 2.65-2.55 (m, 1H), 2.47-2.33 (m, 8H), 1.97 (s, 3H), 1.65-1.48 (m, 2H), 1.40-1.20 (m, 20H), 0.97-0.95 (m, 2H). ESI-MS: calculated for $C_{53}H_{77}N_{13}O_{16}$: 1183.53; found: 1184.4 $[M+H]^+$.

*Preparation of Peptide **2***

To peptide **S2** (200 mg, 0.17 mmol, 1 equiv) was added 2 mL of the following solvents TFA: TIS: H_2O = 95 : 2.5 : 2.5. The reaction was stirred at room temperature for 1 h. To the mixture

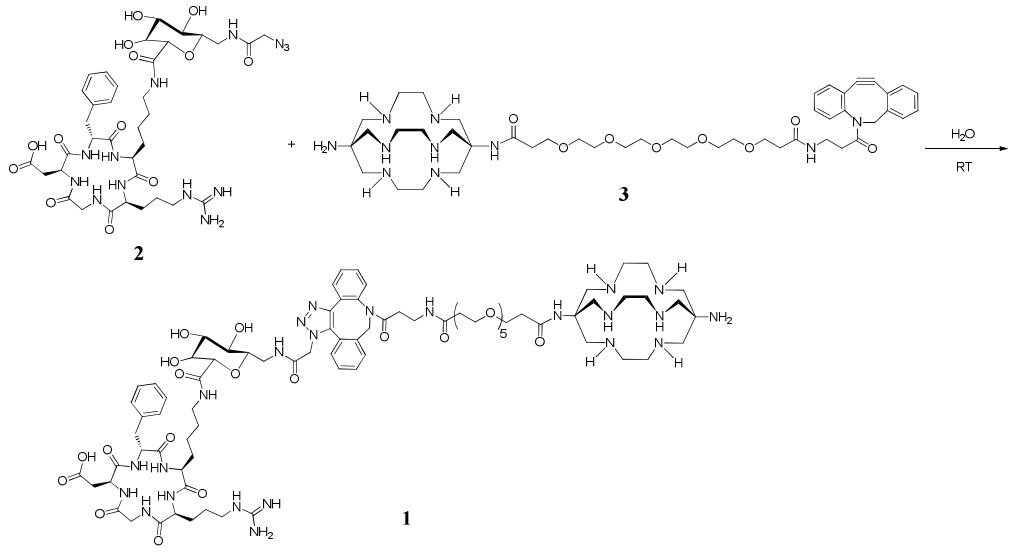
was added cold ether (40 mL). The precipitation was formed and concentrated after centrifugation. The precipitate was filtered and washed with cold ether (3 mL × 3) to afford peptide **2** (110 mg, 65 % yield). ^{1}H NMR (D_2O, 400 MHz), δ: 7.18-7.07 (m, 5H), 4.55 (dd, 1H), 4.45 (dd, 1H), 4.16 (dd, 1H), 4.07 (dd, 1H), 4.01 (d, 1H), 3.95 (s, 1H), 3.86 (s, 2H), 3.71 (dd, 1H), 3.56-3.52 (m, 2H), 3.39-3.26 (m, 4H), 3.04-2.99 (m, 4H), 2.90-2.70 (m, 3H), 2.53 (dd, 1H), 1.71-1.64 (m, 1H), 1.51-1.46 (m, 2H), 1.37-1.32 (m, 3H), 1.27-1.18 (m, 2H), 0.85-0.78 (m, 2H). ^{13}C NMR (D_2O, 100 MHz), δ: 174.7, 174.5, 173.2, 172.9, 171.5, 171.4, 170.9, 170.7, 156.8, 136.1, 129.3, 128.9, 127.4, 78.1, 77.7, 73.5, 69.6, 68.0, 55.5, 55.1, 52.5, 52.1, 49.7, 43.6, 40.6, 40.4, 38.7, 37.1, 34.4, 30.0, 27.8, 27.4, 24.6, 22.6. ESI-MS: calculated for $C_{38}H_{54}F_3N_{13}O_{15}$: 989.38; found: 876.4 $[M\text{-}CF_3COOH+H]^+$.

Synthesis of 3



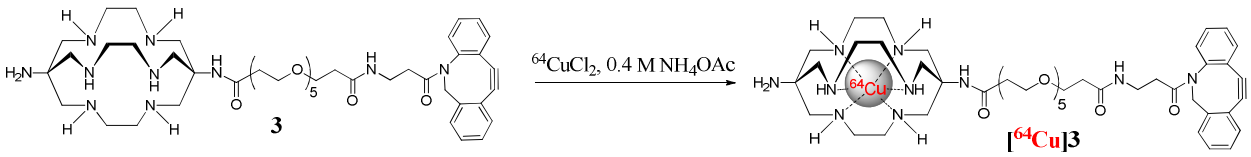
To the solution of 1,8-diamino-3,6,10,13,16,19-hexaazabicyclo[6.6.6]-eicosine (1 mg, 2.47 μmol, 1 equiv) in 200 uL of sodium borate buffer (pH 8.5) was added **S3** (2 mg, 2.88 μmol, 1.17 equiv) in 10 uL of DMSO. The mixture was incubated at room temperature for 2 h and purified by semi-preparative HPLC. The desired peak was collected and concentrated to afford **3** as a white powder (1.0 mg, 45%). ESI-MS: calculated for $C_{46}H_{72}N_{10}O_8$: 893.13; found: 894.0 $[M+H]^+$.

Synthesis of 1



To the solution of **3** (0.9 mg, 1.01 μmol, 1 equiv) in 30 uL of deionized water was added **2** (0.973 mg, 1.11 μmol, 1.1 equiv) in 20 uL of deionized water. The mixture was incubated at room temperature for 1 h and purified by semi-preparative HPLC. The desired peak was collected, concentrated, and lyophilized to afford **1** as a white powder (1.7 mg, 95%). ESI-MS: calculated for $C_{82}H_{125}N_{23}O_{21}$: 1769.01; found: 1770.21 $[M+H]^+$, 885.43 $[M+2H]^{2+}$, 590.90 $[M+3H]^{3+}$.

Radiosynthesis of [^{64}Cu]3

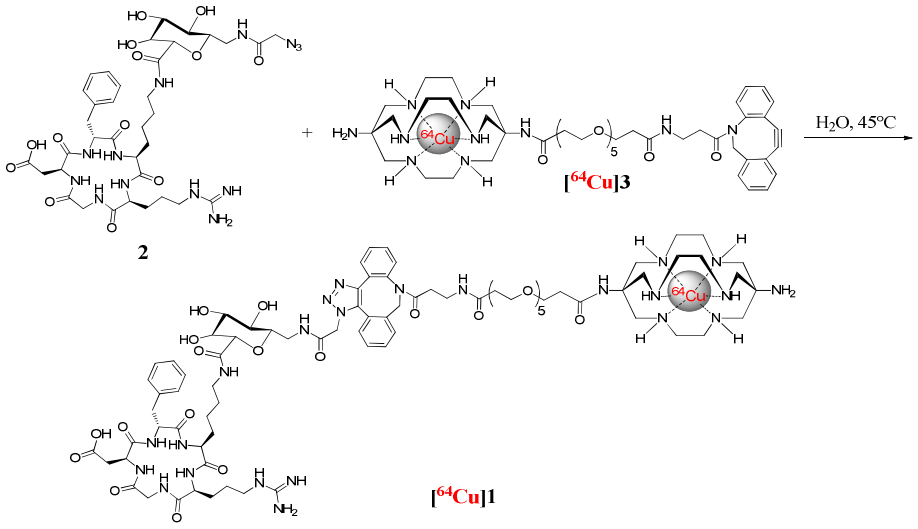


[^{64}Cu]Cu(OAc)$_2$ was prepared by adding 37-111 MBq of [^{64}Cu]CuCl$_2$ in 0.1 N HCl into 300 μL of 0.4 M ammonium acetate buffer (pH = 5.5), followed by mixing and incubating for 15 min at room temperature. The [^{64}Cu]Cu(OAc)$_2$ solution (37–111 MBq) was then added into a solution of **3** (5 μg precursor per mCi ^{64}Cu) dissolved in 0.4 M NH_4OAc (pH = 5.5) solution. The reaction mixture was incubated at 40°C for 30 min. The labeled product was then purified by analytical HPLC. The radioactive peak containing desired product (t_R = 17.67 min) was collected and

concentrated by rotary evaporation to afford [^{64}Cu]**3**. The product was reconstituted in deionized water for use in following experiments. The specific activity of [^{64}Cu]**3** was estimated to be 37 MBq nmol^{-1}.

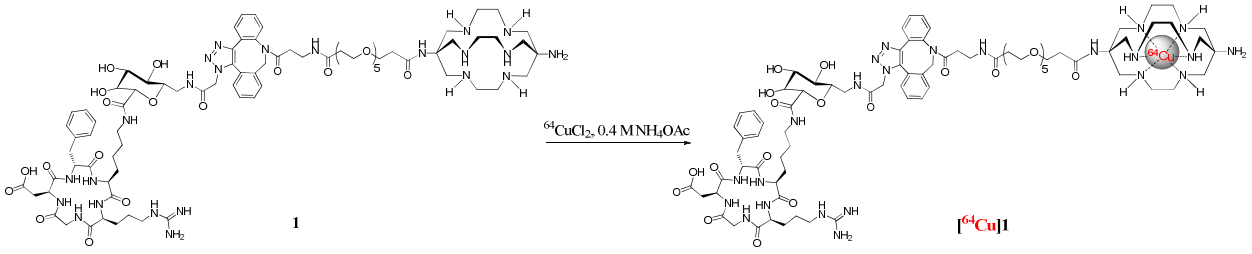
Radiosynthesis of [^{64}Cu]1

*Method A: "Click" Coupling of **2** and [^{64}Cu]**3***



To the solution of [^{64}Cu]**3** (37 MBq) in 50 uL of deionized water was added **2** (1.14 equiv) in 50 uL of deionized water. The reaction mixture was incubated at 45°C for 15 min to afford [^{64}Cu]**1**. The product was then reconstituted in 500-800 µL PBS, and passed through a 0.22-µm Millipore filter into a sterile dose vial for use. The specific activity of [^{64}Cu]**1** was estimated to be 30 MBq nmol^{-1}.

*Method B: Direct ^{64}Cu labeling of **1***



[^{64}Cu]Cu(OAc)$_2$ was prepared by adding 37-74 MBq of [^{64}Cu]CuCl$_2$ in 0.1 N HCl into 300 μL of 0.4 M ammonium acetate buffer (pH = 5.5), followed by mixing and incubating for 15 min at room temperature. The [^{64}Cu]Cu(OAc)$_2$ solution (37-74 MBq) was then added into a solution of **3** (5 µg precursor per mCi ^{64}Cu) dissolved in 0.4 M NH_4OAc (pH = 5.5) solution. The reaction

mixture was incubated at 40°C for 30 min. The labeled product was then purified by analytical HPLC. The radioactive peak containing desired product (t_R = 15.05 min) was collected and concentrated by rotary evaporation to afford [^{64}Cu]**1**. The specific activity of [^{64}Cu]**1** was estimated to be 30-37 MBq nmol^{-1}.

Partition Coefficient

The partition coefficient value was expressed as log *P*. Log *P* of [^{64}Cu]**1** was determined by measuring the distribution of radioactivity in 1-octanol and PBS. Approximately 111 kBq of [^{64}Cu]**1** in 2 μL of PBS (pH = 7.4) was added to a vial containing 0.5 mL 1-octanol and 0.5 mL of PBS (pH = 7.4). After vigorously vortexing for 10 min, the vial was centrifuged at 12,500 rpm for 5 min to ensure the complete separation of layers. 200 μL of each layer was pipetted into test tubes, and radioactivity was measured using a gamma counter (Perkin-Elmer Packard Cobra). The mean value was calculated from triplicate experiments. The log *P* value of [^{64}Cu]**1** was determined to be -1.94 ± 0.10.

Probe Stability Determination

The stability of [^{64}Cu]**1** was tested in PBS and mouse serum. In brief, 3.7 MBq of the [^{64}Cu]**1** was pipetted into 0.5 mL of the PBS, and incubated in PBS at room temperature or mouse serum at 37°C with gentle shaking at 300 rpm. For PBS study, at various time points (1, 6, and 24 h), an aliquot of the solution was directly taken and the radiochemical purity was determined by reverse-phase HPLC under identical conditions. The parent [^{64}Cu]**1** was determined to be >99%, 98%, and 96% at 1, 6, and 24 h, respectively. For mouse serum study, at various time points (1, 6, and 24 h), trifluoroacetic acid was added, and the soluble fraction was clarified with a 0.22-mm filter. An aliquot of the solution was then taken and the radiochemical purity was determined by reverse-phase HPLC under identical conditions. The parent [^{64}Cu]**1** was determined to be >98%, 97%, and 96% at 1, 6, and 24 h, respectively.

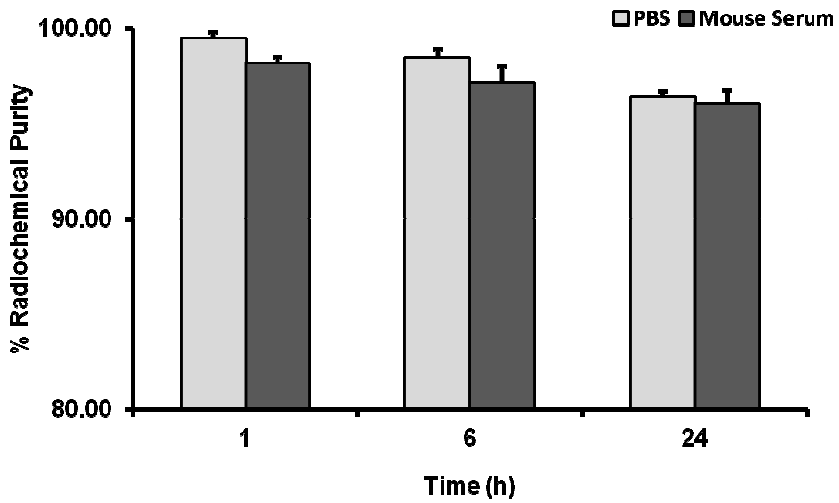


Figure S1. Stability of [^{64}Cu]**1** in PBS (pH = 7.4) at room temperature (light grey color) and mouse serum at 37°C (dark grey color) for 1, 6, and 24 h.

Cell Culture

U87MG human glioblastoma cell line was obtained from the American Type Culture Collection (ATCC, Manassas, VA). U87MG glioma cells were grown in Dulbecco's modified medium (USC Cell Culture Core, Los Angeles, CA) supplemented with 10% fetal bovine serum (FBS) at 37°C in humidified atmosphere containing 5% CO_2.

Integrin $\alpha_v\beta_3$ Receptor Binding Assay

U87MG cells were suspended with binding buffer [25 mM 2-amino-2-(hydroxymethyl)-1,3-propanediol, hydrochloride (Tris-HCl), pH 7.4, 150 mM NaCl, 1 mM $CaCl_2$, 0.5 mM $MgCl_2$, 1 mM $MnCl_2$, and 0.1% bovine serum albumin (BSA)]. Incubation was conducted in a 96-well plate with a total volume of 200 μL in each well containing 2×10^5 cells, 0.02 μCi (0.74 kBq) ^{125}I-echistatin (Perkin-Elmer), and 0-5000 nM of c-(RGDyK) or peptide **1** for 3 h on a shaker at room temperature. After incubation, cells were washed three times with cold phosphate buffered saline (PBS) with 0.1% BSA. Thereafter, the plate was heated to 40°C and dried. The dried filter membranes were punched off from the wells and collected in polystyrene culture test tubes. Cell bound radioactivity was then measured using a gamma counter (Perkin-Elmer Packard Cobra). The IC_{50} values were calculated by nonlinear regression analysis using the GraphPad Prism

computer-fitting program (GraphPad Software, Inc., San Diego, CA). Each data point is a result of the average of duplicate wells.

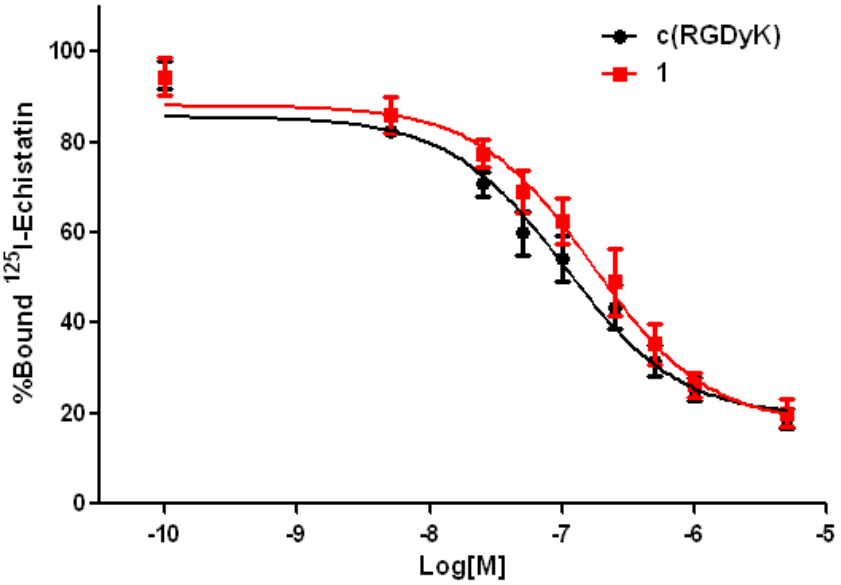


Figure S2. In vitro inhibition of ^{125}I-echistatin bound to integrin $\alpha_v\beta_3$ on U87MG glioma cells by the non-modified peptide c(RGDyK) and peptide **1**. The IC_{50} values of c(RGDyK) and peptide 1 were calculated to be 105 ± 5 nM and 170 ± 3 nM, respectively.

Animal Model

All animal studies were approved by the University of Southern California Institutional Animal Care and Use Committee. Female athymic nude mice (about 4–6 weeks old, with a body weight of 20-25 g) were obtained from Harlan Laboratories (Livermore, CA). The U87MG human glioma xenograft model was generated by subcutaneous injection of 5 × 10^6 U87MG human glioma cells into the front flank of female athymic nude mice. The tumors were allowed to grow 3-5 weeks until 200-500 mm^3 in volume. Tumor growth was followed by caliper measurements of the perpendicular dimensions.

MicroPET Imaging and Blocking Experiment

MicroPET scans and imaging analysis were performed using a rodent scanner (microPET R4 scanner; Siemens Medical Solutions). About 7.4 MBq of [^{64}Cu]**1** (0.25 nmol) was intravenously injected into each mouse (n = 5) under isoflurane anesthesia. Five-minute static scans were

acquired at 2, 4, and 20 h pi. The images were reconstructed by a two-dimensional ordered-subsets expectation maximum (OSEM) algorithm. For each microPET scan, regions of interest (ROIs) were drawn over the tumor, normal tissue, and major organs on the decay-corrected whole-body coronal images. The radioactivity concentrations (accumulation) within the tumor, muscle, liver, and kidneys were obtained from mean pixel values within the multiple ROI volumes, which were converted to counts/min/mL by using the calibration constant. Assuming a tissue density of 1 g/mL, the ROIs were then converted to counts/min/g, and then divided by the administered radioactivity to obtain an imaging ROI-derived percentage administered radioactivity per gram of tissue (%ID/g). For the blocking experiment, mice bearing U87MG tumors were scanned (5 min static) at 2, 4, and 20 h after the co-injection of 7.4 MBq of [^{64}Cu]**1** with 10 mg/kg c(RGDyK) peptide (about 400 nmol) per mouse.

Biodistribution Studies

The U87MG tumor bearing mice (n = 5) were injected with 7.4 MBq of [^{64}Cu]**1**. At 20 h after intravenous injection of [^{64}Cu]**1**, mice were sacrificed and dissected. Blood, U87MG tumor, major organs, and tissues were collected and weighed wet. The radioactivity in the tissues was measured using a gamma counter (Perkin-Elmer Packard Cobra). The results were presented as percentage injected dose per gram of tissue (%ID/g). For each mouse, the radioactivity of the tissue samples was calibrated with a known aliquot of the injected activity. Mean uptake (%ID/g) for a group of animals was calculated with standard deviations.

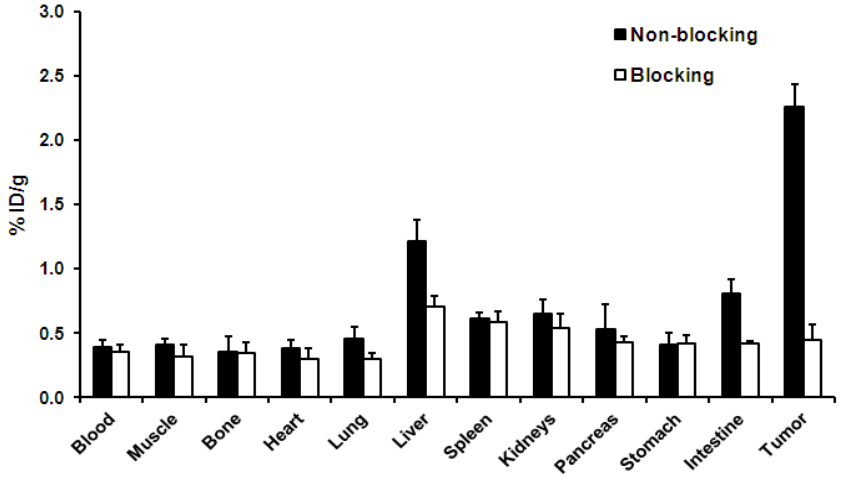


Figure S3. Biodistribution of [^{64}Cu]**1** in U87MG tumor bearing athymic nude mice at 20 h with and without co-injection of 10 mg/kg of c(RGDyK) as a blocking agent (n = 5/group).