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Cyclization of RGD Peptide Sequences via the Macrocyclic Chelator DOTA for Integrin Imaging

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Materials and Methods

1. General procedures

^{1}H- and ^{13}C-NMR spectra were recorded either on a Varian Gemini: Unity plus 200 MHz or a Varian INOVA 400 spectrometer. Data are reported in the following order: chemical shift in ppm (δ); multiplicities are indicated as b (broadened), s (singlet), d (doublet), t (triplet), q (quartet), m (multiplet); coupling constants, J, are reported in Hz; integration is provided. Mass spectra (MS) were measured with a Voyager-DE PRO Biospectrometry Workstation (Applied Biosystems) [matrix-assisted laser desorption/ionization time-of-flight (MALDI-TOF)] operating in reflector mode using α-cyano-4-hydroxycinnamic acid as the matrix. UV-Visible spectra were acquired in a Beckman Coulter DU 730 Life Science UV/VIS spectrophotometer. Analytical and semi-preparative HPLC was performed on a Hewlett Packard Series 1200 system using a Jupiter 5μ C18 300 Å 4.6 mm x 250 mm 5 microns (for analytical) or Jupiter 10μ C18 300 Å 10 mm x 250 mm 5 microns column (for semi-preparative) from Phenomenex. Typical separation procedure was carried out at room temperature with an isocratic or gradient of water/acetonitrile containing 0.1%/0.08% Trifluoroacetic acid (TFA) respectively unless indicated otherwise. Detection was done at 215 and 250 nm and a flow of 1 mL min^{-1} (analytical) or 4.7 mL min^{-1} (semi-preparative) was used.

DOTA-bis-*tert*-butyl ester was prepared according to procedures reported in the literature.[1] Resin and protected L(D)-aminoacids were purchased from Bachem Americas Inc. (Torrance, CA), Novabiochem (San Diego, CA) or Aroz Tech, LLC. (Cincinnati, OH). All other reagents were purchased from Sigma-Aldrich or Fisher Scientific (Pittsburgh, PA). All reagents and solvents were used without further purification unless otherwise noted. NMR spectra were processed in MestReNova v 6.0.2-5475 (Mestrelab Research S.L.) or ACD/HNMR Viewer ACD/Labs 6.00. MS spectra were processed in Data Explorer v 3.5.0.0 (PerSeptive Biosystems). Molecular graphics images were processed using either the UCSF Chimera package from the Resource for Biocomputing, Visualization, and Informatics at the University of California, San Francisco (supported by NIH P41 RR-01081)[2] or HyperChem™ 7.5 (Hypercube, Inc., 1115 NW 4th Street, Gainesville, Florida 32601, USA). Geometry optimization was done in Hyperchem™ 7.5 with the Molecular Mechanics force field Charmm27 using the Polak-Ribiere algorithm until the termination condition of 0.1 Kcal/(Å mol) RMS gradient was met. Copper-64 chloride in 0.1 N HCl was purchased from the University of Wisconsin-Madison. Milli-Q water (18 MX cm) was obtained from a Millipore Gradient Milli-Q water system (Billerica, MA). All aqueous solutions were prepared with Milli-Q water. Light C-18 Sep-Pak cartridges were purchased from Waters

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(Milford, MA). Instant thin-layer chromatography (ITLC-SG) plates were purchased from Pall Life Sciences (East Hills, NY). Small animal PET-CT imaging studies were performed on a Siemens Inveon PET-CT Multimodality System (Siemens Medical Solutions Inc., Knoxville, TN).

2. Molecular Modeling

In the literature one can find several NMR-Molecular Dynamic (NMR-MD) based reports where different criteria related to structure-integrin binding activity relationships of Arg-Gly-Asp (RGD) –containing peptides were investigated. In these reports, it has been proposed that the pseudo-dihedral angle (φ) formed by the Arg Cζ, Arg Cα, Asp Cα and Asp Cγ should be between -45° and 45°, the distance between the charged centers (Arg Cζ - Asp Cγ) should be between 10-15 Å and the distance between beta carbon atoms of Arg and Asp should be between 6.6-9.4 Å.[3, 4] Structure-integrin specificity analysis suggests that cyclic pentapeptides with a kinked RGD backbone and with an aromatic D aminoacid in residue 4 are more specific towards $\alpha_V\beta_3$ integrin.[5] Additionally, a 7-8 Å distance between Arg and Asp beta carbon atoms has been suggested as a prerequisite for $\alpha_V\beta_3$ binding. [6] Further information emerged when the X-ray crystal structure of the $\alpha_V\beta_3$ integrin complexed with the cyclic pentapeptide c(RGDf[NMe]V) was published.[7] c(RGDf[NMe]V) (cilengitide) is the strongest $\alpha_V\beta_3$ binding monomer peptide known up to date (IC50 = 0.6 nM). An structure analysis of the X-ray structure indicates that the main interactions in the $\alpha_V\beta_3$-cilengitide complex are between the positively charged arginine and negatively charged side chains in the α subunit (**D**^{218} and **D**^{150}) and between the anionic aspartic acid and the metal cation (**Mn**) in the MIDAS region of the β subunit. Further stabilization occurs through hydrogen bonds which involve the NH group of Gly-Asp amide bond with the carbonyl group of **R**^{216} residue in the protein as well as between the Asp side chain and **N**^{215}. It is also considered that the aromatic ring of D-phenylalanine is involved in some π-interactions with **Y**^{122}.[8] The remaining residue of the pentapeptide cilengitide points away from the $\alpha_v\beta_3$ binding pocket and seem to play no major role in protein-ligand binding. A summary of the stated interactions between c(RGDf[NMe]V and $\alpha_V\beta_3$ is shown in Figure S1.

The initial model of the c(RGDf[NMe]V)-$\alpha_v\beta_3$ complex was taken from the published crystal structure (PDB ID code 1L5G)[7] and it was processed with UCSF Chimera. c(RGDf[NMe]V) was selected first and then a zone selection >15Å from the selected molecule was performed. Complementary atoms to the current selection were deleted as a way to simplify the structure of $\alpha_v\beta_3$ for further calculations. The simplified model was opened in Hyperchem™ 7.5 as a pdb file. Bonds between c(RGDf[NMe]V) and key interacting distances between residues of $\alpha_v\beta_3$ (indicated in bold) and c(RGDf[NMe]V) were restrained to the values determined from the X-ray structure (values of ^{COO}**D**^{218}-^{NH2CNH}R and **Mn**$^{2+}$ from the MIDAS center-D indicated in the second column of Table S1).[9] c(RGDf[NMe]V) attached to the protein was then selected and the geometry optimized while keeping the $\alpha_v\beta_3$ fragment unchanged (it has been reported that protein structure does not change significantly upon c(RGDf[NMe]V) binding).[8] The structure of Lu- c(DOTA-RGDf) and Lu- c(DOTA-RGD) was optimized so that the LuDOTA motif attained a SAP coordination geometry.[10] Once optimized the bicyclic derivatives were separately merged into the $\alpha_v\beta_3$ substructure without c(RGDf[NMe]V). The DOTA peptides were manually fit into the known binding cavity of the protein and atom lengths of key interacting residues were restrained as stated above. Lu- c(DOTA-RGDf) or Lu- c(DOTA-RGD) were then selected and

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the geometry optimized while keeping the $\alpha_v\beta_3$ fragment fixed. The optimized structures were used as initial structure for the subsequent molecular dynamics (MD) simulation. To survey the conformation space of c(RGDf[NMe]V) and the bicyclic complexes in the presence of the $\alpha_v\beta_3$ fragment a 10 ps MD simulation of the RGD systems was carried out in vacuo while keeping the $\alpha_v\beta_3$ fragment fixed. MD calculation begun with an initial 1 ps heating period from a 10 K starting temperature to a simulation temperature of 300K with a 30 K temperature step and 0.001 ps step size. Data was collected every 5 time steps. The distances and angles that have been reported relevant for cyclic pentapeptides to bind $\alpha_v\beta_3$ were averaged for optimized structures with the lowest potential energy collected between 2 to 11 ps run (Table S1).

3. Synthesis

3.1 Synthesis of c(DOTA(Arg-Gly-Asp-DPhe-EDA)), (c(DOTA-RGDf) and c(DOTA(Arg-Gly-Asp-EDA)), (c(DOTA-RGD)

The synthesis of the bicyclic DOTA peptides started with the incorporation of arginine onto o-chlorotrityl glycine preloaded resin (Aroz Tech, LLC. Cincinnati, OH, with a 0.56 mmol/g load) at a 0.9 mmol scale by using a single-step coupling of 3 equiv. of Fmoc-Arg(Pbf)-OH, 2.9 equiv HBTU and HOBt, and 6 equiv DIPEA in DMF for 2 hours. The coupling mixture was removed from the resin, the resin was washed with DMF 10X 2mL and DCM 10X 2mL and dried under vacuum. Resin tested negative (yellow color) with Ninhydrin confirming reaction completion. The Fmoc-protecting group was then removed with a 20% solution of piperidine in DMF for 30 minutes followed by washings and drying. Ninhydrin test was positive (blue color) indicating removal of the Fmoc group. Coupling of DOTA-bis-*tert*-butyl ester followed, by adding to the resin a mixture containing 2 equiv. of the ligand and 2 equiv. of HBTU in DMF. Reaction mixture was mixed for 2 hours followed by removal of coupling solution, washing and drying as described above. Ninhydrin test gave a partial positive result (light blue-green color). DOTA-bis-*tert*-butyl ester coupling was repeated by adding 2 equiv. of HBTU to the recovered coupling mixture from the previous step. This solution was added to the resin and the resultant mixture was mixed for 2 hours. After washing and drying the resin, the ninhydrin test was negative. A solution containing HBTU 2 equiv. and DIPEA 2 equiv. was added to the resin and the resultant mixture was stirred for 10 minutes, addition of 20 equiv. of ethylenediamine (EDA) followed with mixing for 2 hours. After washing and drying the resin, the ninhydrin test was positive. Resin was split by half in two batches.

3.1.1 Synthesis of c(DOTA-RGDf)

To one resin batch the following aminoacids were attached by using single-step couplings of 3 equiv. of Fmoc-amino acid, 2.9 equiv HBTU and HOBt, and 6 equiv DIPEA in DMF in the order Fmoc-D-Phe-OH, and Fmoc-Asp(OtBu)-OH by mixing for 2 hours. Fmoc removal and washings were carried out between couplings as stated above.
The linear DOTARGDf-EDA protected peptide was cleaved from the resin without affecting other protecting groups by treating the resin with 5 mL of a mixture of acetic acid, 2,2,2-trifluoroethane (TFE), and DCM (1:1:3) for 1 h at room temperature. The resin was washed twice with 5 mL of the same mixture and then three times with DCM. The eluents were

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combined and solvent removed under high vacuum. Product was purified by HPLC using a gradient of water/acetonitrile containing 0.1% TFA from 80/20 to 10/90 in 35 minutes. Two major fractions were collected (t_r = 25.8 and 28.0 min) and lyophilized giving 41 mg of DOTA bis amide RG MS (MALDI-TOF+) m/z = 1447.17 $[M+H]^+$ (calcld. 1447.76) and 94 mg of the DOTARGDf-EDA, MS (MALDI-TOF+) m/z = 1342.32$[M+H]^+$ (calcld. 1342.65) protected peptides respectively.

The head-to-tail cyclization was performed by slowly adding a solution of the linear protected DOTARGDf-EDA peptide in 5 mL of DCM to a solution of 50% 1-propanephosphonic acid cyclic anhydride in EtOAc (200 µL), DIPEA (200 µL), and DMAP (1 mg) in 5 mL of DCM. Reaction mixture was stirred overnight and reaction completion assessed by MALDI-TOF (MS) until the cyclic compound was observed and no linear unreacted peptide was detected MS (MALDI-TOF+) m/z = 1325.06 $[M+H]^+$ (calcld. 1325.64). Solvent was removed under high vacuum and residue was dissolved in DCM. Organic phase was washed with NaOH 0.1 M two times. Collected organic phase was dried with anhydrous Na_2SO_4 filtered and solvent removed by rotary evaporation. The remaining protecting groups of the above cyclic peptide were removed by stirring the peptide for 2 hours with a mixture a 95% TFA, 5% Milli Q grade water mixture. Acid was removed under a gentle N_2 flow leaving an oily residue which turned into a white solid upon addition of cold ether. The solid in ether was left in the fridge overnight. Solid was decanted and washed with clean fresh ether three times and dried. Solid was further purified by HPLC twice, first with a gradient of water/acetonitrile containing 0.1% TFA from 97/3 to 70/30 in 30 minutes (t_r = 12.3 min) and then with a gradient of water/acetonitrile containing 0.1% TFA from 95/5 to 92/8 in 100 minutes (R_t = 57.2 min). Fraction containing the product was lyophilized giving 23 mg of the peptide. Full synthetic protocol is shown in Scheme S1 and spectral data is shown in Figures S2, S3 and S4.

^{1}H (200 MHz, D_2O) δ 7.26-7.11, (5H, arom.), 4.47 (m), 4.28, 3.85 (m), 3.77, 3.17, 3.07, 2.80, 2.76, 2.74, 2.55, 2.53, 1.85 (2H, m , -C$^{\gamma}$H$_2$-, Arg), 1.50 (2H, m, -C$^{\gamma}$H$_2$-, Arg).

^{13}C NMR (50 MHz, D_2O): δ 174.40, 174.03, 174.00, 173.19, 173.16, 172.45 (C=O) 156.89 ((NH)NH_2C=N), 136.96 (-CH=, arom.), 129.29 (-CH=, arom.), 129.13 (-CH=, arom.), 128.93 (-CH=, arom.), 127.26 (-CH=, arom.), 55.97 (N-*C*H_2CONH), 55.13 (N-*C*H2CO_2H), 54.82 -50.67 (N-CH_2-CH_2-N; -CH-, Phe; -CH-, Arg; -CH-, Asp), 42.77 (-CH_2-, Gly), 40.62 (-C$^{\delta}$H$_2$-, Arg), 38.44 (-C$^{\beta}$H$_2$-, Asp), 36.56-34.86 (-CH_2-, EDA) (-C$^{\beta}$H$_2$-, Phe), 28.11 (-C$^{\beta}$H$_2$-, Arg), 24.80 (-C$^{\gamma}$H$_2$-, Arg).

MS (MALDI-TOF+) m/z = 904.69 (100%) $[M+H]^+$ (calcld. 904.98), 926.65 (35%) $[M+H]^+$ (calcld. 926.45).

3.1.2 Synthesis of c(DOTA-RGD)

To the second resin batch Fmoc-Asp(OtBu)-OH was attached by using a single-step coupling of 3 equiv. of Fmoc-amino acid, 2.9 equiv HBTU and HOBt, and 6 equiv DIPEA in DMF by mixing for 2 hours. The Fmoc-protecting group was then removed with a 20% solution of piperidine in DMF for 30 minutes followed by washings and drying. Ninhydrin test was negative indicating the presence of Fmoc. To fully remove Fmoc the resin was mixed with 20% solution of piperidine in DMF and irradiated in a commercial microwave oven set to deliver 10% power (3 × 20 s) with stirring between pulses. After washings and drying the Ninhydrin test was positive.

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The linear DOTARGD-EDA protected peptide was cleaved from the resin without affecting other protecting groups by treating the resin with 5 mL of a mixture of acetic acid, 2,2,2-trifluoroethane (TFE), and DCM (1:1:3) for 1 h at room temperature. The resin was washed twice with 5 mL of the same mixture and then three times with DCM. The eluents were combined and solvent removed under high vacuum. Product was purified by HPLC using a gradient of water/acetonitrile containing 0.1% TFA from 65/35 to 50/50 in 35 minutes. Several fractions were collected and analyzed by MS (MALDI-TOF+). Once the desired product was identified (t_r = 6.18 min) MS (MALDI-TOF+) m/z = 1196.68 $[M+H]^+$ (calcld. 1196.47), the fractions containing the product were freeze dried giving 20 mg of DOTARGD-EDA protected peptide.
The head-to-tail cyclization was performed by slowly adding a solution of the linear protected DOTARGD-EDA peptide in 5 mL of DCM to a solution of 50% 1-propanephosphonic acid cyclic anhydride in EtOAc (200 µL), DIPEA (200 µL), and DMAP (1 mg) in 5 mL of DCM. Reaction mixture was stirred overnight and reaction completion assessed by MALDI-TOF (MS) until the cyclic compound was observed and no linear unreacted peptide was detected MS (MALDI-TOF+) m/z = 1178.88 $[M+H]^+$ (calcld. 1178.46). Solvent was removed under high vacuum and residue was purified by HPLC using a gradient of water/acetonitrile containing 0.1% TFA from 75/25 to 60/40 in 40 minutes. The fraction containing the product (t_r = 36.56 min) was lyophilized yielding 9 mg of the cyclic protected peptide. The protecting groups were removed by stirring the peptide for 2 hours with a mixture a 95% TFA, 5% Milli Q grade water mixture. Acid was removed under a gentle N_2 flow leaving an oily residue which turned into a white solid upon addition of cold ether. The solid in ether was left in the fridge overnight. Solid was decanted and washed with clean fresh ether three times and dried. Solid was dissolved in 2 mL of a mixture water/acetonitrile containing 0.1% TFA (90/10), filtered through a 0.2 µm teflon cartridge and lyophilized giving 5.8 mg of the final byciclic peptide. Full synthetic protocol is shown in Scheme S2 and MS spectra is shown in Figure S5.
MS (MALDI-TOF+) m/z = 758.64$[M+H]^+$ (calcld. 758.82).

3.2 Preparation of ^{177}Lu-c(DOTA-RGDf) and ^{177}Lu-c(DOTA-RGD)

A 5-µl aliquot of c(DOTA-RGDf) or c(DOTA-RGD) (1mg/ml) was diluted with 50 µl of 1M NH_4OAc buffer at pH 5, followed by the addition of 7µl of the $^{177}LuCl_3$ (~370 MBq >3 TBq/mg, ITG Isotope Technologies Garching GmbH, Germany) solution. The mixture was incubated at 90 °C in a block heater for 30 min. All solutions were prepared using deionized water. A radiochemical purity >98% was verified by reversed phase HPLC on a C-18 column (m-Bondapack C-18, Waters) using a Waters Millennium system with an in-line radioactivity detector and a gradient of water/acetonitrile containing 0.1% TFA from 100/0 to 50/50 from minute 3 to 20 of the run at 1 ml/min ($^{177}LuCl_3$ t_R = 3 min; ^{177}Lu-c(DOTA-RGDf) t_R = 11.8 min and ^{177}Lu-c(DOTA-RGD) t_R = 11.9 min).

3.3 Preparation of ^{64}Cu-c(DOTA-RGDf) and ^{64}Cu-c(DOTA-RGD)

To a 1.5 mL vial containing 10 µg of c(DOTA-RGDf) or c(DOTA-RGD) in 200 µL of 0.4 M NH_4OAc solution (pH 6.5) was added 74–111 mCi of ^{64}Cu in 0.1 M HCl. The reaction mixture

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was vortexed and then incubated at 37ºC for 30-min. Then 5 µL of 5 mM diethylenetriaminepentaacetic acid (DTPA) was added and the reaction mixture was vortexed and left at room temperature for 5 min. The separation of ^{64}Cu-c(DOTA-RGDf) or ^{64}Cu-c(DOTA-RGD) from ^{64}Cu-DTPA was carried out by passing the reaction mixture through a light C-18 Sep-Pak cartridge. After three times of washing with PBS, the product was eluted with 80% ethanol solution. Radio-TLC analysis was performed on a Rita Star Radioisotope TLC Analyzer (Straubenhardt, Germany) to monitor the radiolabeling reaction using ITLC plates. High performance liquid chromatography HPLC) analysis was conducted to determine the radiochemical purity of the products using a Waters 600 Multisolvent Delivery System equipped with a Waters 2996 Photodiode Array (PDA) detector and an in-line Shell Jr. 2000 radio-detector (Fredericksburg, VA) on a Waters Xterra column (150 x 4.6 mm, 5 µm). The mobile phase was H_2O with 0.1% TFA (solvent A) and acetonitrile with 0.1% TFA (solvent B). The gradient was 5–20% B in 0–5 min and 20–40% B in 5–25 min at 1.0 mL/min flow rate.
The cold references of Cu-c(DOTA-RGDf) and Cu-c(DOTA-RGD) were prepared by reacting 1 mg (0.46 µmol) of c(DOTA-RGDf) or c(DOTA-RGD) with 62 µg (0.46 µmol) of $CuCl_2$ in 1 mL of 0.4 M NH_4OAc solution at 37ºC for 1 h. The products were purified by HPLC using the same condition described above and characterized by MALDI-Mass (MALDI-TOF/MS). The fraction corresponding to Cu-c(DOTA-RGDf) and Cu-c(DOTA-RGD) were lyophilized and stored for use as the cold standard.

3.4 Preparation of Yb(III) and Cu(II) complexes of c(DOTA-RGDf) and c(DOTA-RGD)

A solution of ytterbium(III) chloride hexahydrate (0.026 mmol) in water (5 mL) was added to a solution of the ligand (0.026 mmol) in miliQ water (5 mL). The reaction mixture was stirred at room temperature for 72 hours. Excess metal was removed from the reaction by increasing pH and the precipitated metal was removed by filtration. pH of the final solution was adjusted to 7.0 and solvent was removed under high vacuum. Complex formation was assessed by MS. Copper complexes were prepared similarly using copper(II) chloride dihydrate.

4. Competitive binding assay

The affinity of Lu-c(DOTA-RGDf), Lu-c(DOTA-RGD), Cu-c(DOTA-RGD), Cu-c(DOTA-RGDf), c(RGDfK) and the metal free bicyclic ligands for $\alpha_v\beta_3$ was determined using a solid phase competitive binding assay. For the "cold" labeling of c(DOTA-RGDf) and c(DOTA-RGDf) with Cu^{2+}, each of the peptides (1.5 x 10^{-3} mmol) was dissolved in 1.0 mL of 0.4M ammonium acetate buffer at pH 6, followed by the addition of 100 µL of Copper(II) acetate solution (1.5 x 10^{-3} mmol in 0.1M HCl of $Cu(CH_3COO)_2$, Sigma-Aldrich , St Louis, MO, USA). Each mixture was incubated at 37°C in a block heater for 1 h. For the "cold" labeling of c(DOTA-RGDf) and c(DOTA-RGDf) with Lu^{3+}, each of the peptides (1.5 x 10^{-3} mmol) was dissolved in 1.0 mL of 1 M ammonium acetate buffer at pH 5, followed by the addition of 60 µL of $LuCl_3$ solution (1.5 x 10^{-3} mmol in 1.0 M HCl of $LuCl_3$, Sigma-Aldrich , St Louis, MO, USA). Each mixture was incubated at 100°C in a block heater for 1 h. ^{177} Lu-DOTA-E-[c(RGDfK)]$_2$ (3 MBq/µg) was used as the tracer since it has shown high binding affinity for integrin $\alpha_v\beta_3$ (IC_{50}= 0.7-1.5 nM).[11] Microtiter 96-well vinyl assay plates (Corning, NY, USA) were coated with 100 µl/well of a solution of purified human integrin $\alpha_v\beta_3$ (150 ng/ml, Chemicon-Millipore Corporation, Billerica, MA, USA) in coating buffer (25 mM Tris-HCl, pH 7.4, 150 mM NaCl, 1

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mM $CaCl_2$, 0.5 mM $MgCl_2$ and 1 mM $MnCl_2$) for 17 h at 4°C. The plates were washed twice with binding buffer [0.1% bovine serum albumin (BSA) in coating buffer]. The wells were blocked for 2 h with 200 µL blocking buffer (1% BSA in coating buffer). The plates were washed twice with binding buffer. Then, 100 µl binding buffer containing 10 kBq of ^{177} Lu-DOTA-E-[c(RGDfK)]$_2$ and appropriate dilutions (from 10000 nM to 0.001 nM) of c(DOTA-RGD), c(DOTA-RGDf), Lu-c(DOTA-RGDf), Lu-c(DOTA-RGD), Cu-c(DOTA-RGD) or Cu-c(DOTA-RGDf) in binding buffer were incubated in the wells at 37°C for 1 h. After incubation, the plates were washed three times with binding buffer. The wells were cut out and counted in a gamma counter. IC_{50} values of the RGD peptides were calculated by nonlinear regression analysis. Each data point is the average of five determinations. Data is shown in Figure S6.

5. Cell culture and animal models

5.1 MCF7 model

Human breast carcinoma cell MCF7 line was originally obtained from the American Type Culture Collection (ATCC, Manassas, VA)). The cells were routinely grown at 37 °C, with 5% CO_2 atmosphere and 100% humidity in RPMI medium supplemented with 10% newborn calf serum and antibiotics (100 μg/mL streptomycin).
Tumor uptake studies in mice were carried out according to the rules and regulations of the Official Mexican Norm 062-ZOO-1999. Athymic male mice (20-22 g) were kept in sterile cages with sterile wood-shavings bed; constant temperature, humidity, and noise; and 12:12 light periods. Water and feed (standard PMI 5001 feed) were given ad libitum. Tumors were induced by subcutaneous injection of MCF7 cells ($1x10^6$) resuspended in 0.2 mL of phosphate-buffered saline, into the backside of 6-7 week old nude mice. The sites of injection were observed at regular intervals for the appearance of tumor formation and progression.

5.2 U87MG model

U87MG cell line was obtained from ATCC. U87MG cells were maintained in RPMI1640 medium (ThermoFisher Scientific, US) supplemented with 10% FBS and 1× Penicillin/Streptomycin at 37 °C in an atmosphere of 5% CO_2 and passaged at 75 % confluence.
All animal studies were performed in compliance with guidelines set by the UT Southwestern Institutional Animal Care and Use Committee. Female SCID mice (6 – 8 weeks of age) were purchased from the UT Southwestern mouse-breeding core (Wakeland Colony). To establish the U87MG tumor model, U87MG cell suspension was injected subcutaneously (2.0×10^6 cells, injection volume 50 µL) into right front flank of animals. After injection, the animals were monitored by general observations.

6. Biodistribution studies of ^{177}*Lu-c(DOTA-RGDf) and* ^{177}*Lu-c(DOTA-RGD)*

Athymic mice with MCF7 tumors (50 – 500 mm^3) received ^{177}Lu-c(DOTA-RGDf) or ^{177}Lu-c(DOTA-RGD) (0.10 ml, ~2MBq) by intravenous tail injection. Mice were sacrificed 3 h (n = 3) after radiopharmaceutical administration. Whole heart, spleen, pancreas, liver, lung kidneys and tumor, and samples of blood, intestines, bone and muscle were saline rinsed, paper blotted and

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placed into preweighed plastic test tubes. The activity was determined in a well-type scintillation detector (Canberra) along with 3 x 0.02 ml aliquots of the diluted standards (^{177}Lu-c(DOTA-RGDf) and ^{177}Lu-c(DOTA-RGD), 0.10 ml, ~1MBq) representing 100% of the injected dose (after correction for the dilution factor) to obtain the activity corrected by decay.. Mean activities were used to obtain the percentage of the injected dose per gram of tissue (% ID/g) or % ID per organ. Blocking studies were performed in three mice with MCF7 induced tumors. One hundred microliters (1.4 mM) of unlabeled c(RGDfK) (Bachem) was intraperitoneally administered (n=3 per agent) 30 min before intravenous tail injection of ^{177}Lu-c(DOTA-RGDf) or ^{177}Lu-c(DOTA-RGD) (0.10 ml, ~2 MBq). Biodistribution data is shown in Table S2.

7. Small Animal PET/CT Imaging

When U87MG tumor size reached the range of 50 – 500 mm^3, the tumor-bearing mice were randomized for the PET-CT imaging with ^{64}Cu labeled conjugates. For blocking studies, c(RGDyK) was co-injected into tumor-bearing mice at the dose of 10 mg/kg. The injected dose was 3.7 MBq of ^{64}Cu-activity in 100 µL of PBS.

Ten minutes prior to imaging, the animal was anesthetized using 3% isofluorane at room temperature until stable vitals were established. Once the animal was sedated, it was placed onto the imaging bed under 2% isofluorane anesthesia for the duration of the imaging. The CT imaging was acquired at 80 kV and 500 µA with a focal spot of 58 µm. The total rotation of the gantry was 360° with 360 rotation steps obtained at an exposure time of approximately 235 ms/frame. The images were attained using a CCD readout of 4096 × 3098 with a bin factor of 4 and an average frame of 1. Under low magnification the effective pixel size was 103.03 µm. Total CT scan time was approximately 6 minutes. CT images were reconstructed with a down sample factor of 2 using Cobra Reconstruction Software. The PET imaging was performed directly after the acquisition of CT data. The PET tracer was injected intravenously via the tail vein. Static PET scans were performed at 1 h, 4 h, and 24 h p.i. for 15 min. After the PET imaging reconstruction, regions of interest were placed in the areas expressing the highest radiotracer activity as determined by visual inspection. The tissues examined include tumors, heart, liver, lung, kidneys, and muscle. The quantitative data were expressed as percent injected dose per gram of tissue (%ID/g) in Table S3. PET images were reconstructed using Fourier Rebinning and Ordered Subsets Expectation Maximization 3D (OSEM3D) algorithm. Reconstructed CT and PET images were fused and analyzed using the Siemens Inveon Research Workplace (IRW) software.

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Tables

Table S1. Calculated structure-binding parameters for RGD peptides bound to $\alpha_v\beta_3$

Peptide	c(RGDf[NMe]V)‡	C(RGDf[NMe]V)*	Lu- c(DOTA-RGDf)*	Lu- c(DOTA-RGD)*
RCζ – DCγ, Å	13.72	13.89	14.12	14.52
RCβ – DCβ, Å	8.87	8.77	8.69	9.04
Φ	62.24°	36.13°	40.25°	-50.07°
^{COO}**D**^{218}-^{NH2CNH}R, Å;	1.93, 1.96;	1.57, 1.54;	3.03, 2.38;	2.86, 2.34;
OONN	-39.74°	-2.10°	-30.23°	-33.45°
^{COO}**D**^{150}- ^{NH2C}R, Å;	3.16;	3.79;	2.80;	2.75;
COH	159.39°	152.43°	149.49°	152.88°
Mn^{2+}-D, Å	2.65	2.98	3.04	3.00
^{NH}**N**^{215}-^{CO}D, Å;	1.81;	1.74;	2.08;	1.82;
NHO	172.37°	165.62°	154.37°	158.39°
^{CO}**R**^{216}-^{NH}D, Å;	2.65;	2.86;	3.04;	3.08;
COH	102.10°	99.78°	100.17°	90.17°
^{C4}**Y**^{122} - ^{C4}F, Å;	6.20;	10.42;	12.50;	-
C^{3}**C**^{5}C^{3}C^{5}	-141.45°	-116.01°	-61.74°	-
π-π interaction with **Y**^{122}	parallel-displaced	none	none	-

Letters in bold refer to residues or atoms within each residue of $\alpha_v\beta_3$. ‡ Values determined form the published X-ray structure.[7] * Values determined in this work.

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Table S2. Biodistribution data of ^{177}Lu-c(DOTA-RGD) and ^{177}Lu-c(DOTA-RGDf) in mice bearing MCF7 tumour at 3 h p.i. Data are presented as %ID/g ± s.d. (n = 3).

	^{177}Lu-c(DOTA-RGD)		**^{177}Lu-c(DOTA-RGDf)**	
	Unblocked	Blocked**	Unblocked	Blocked**
Blood	0.012 ± 0.006	0.023 ± 0.013	0.007 ± 0.005	0.004 ± 0.0003
Heart	0.035 ± 0.034	0.056 ± 0.031	0.036 ± 0.032	0.020 ± 0.005
Lung	0.135 ± 0.078	0.091 ± 0.075	0.229 ± 0.227	0.120 ± 0.011
Liver	0.706 ± 0.407	0.532 ± 0.328	0.204 ± 0.115	0.167 ± 0.048
Pancreas	0.096 ± 0.001	0.050 ± 0.002	0.009 ± 0.001	0.038 ± 0.025
Spleen	0.387 ± 0.261	0.159 ± 0.137	0.162 ± 0.157	0.125 ± 0.012
Kidneys	2.498 ± 1.496	1.882 ± 1.182	2.277 ± 1.349	2.058 ± 0.371
Intestine	0.237 ± 0.264	0.073 ± 0.032	1.888 ± 0.002	0.051 ± 0.018
Muscle	0.053 ± 0.011	0.038 ± 0.023	0.043 ± 0.046	0.014 ± 0.016
Bone	0.277 ± 0.044	0.303 ± 0.158	0.080 ± 0.020	0.076 ± 0.084
Brain	0.012 ± 0.007	0.010 ± 0.003	0.012 ± 0.005	0.003 ± 0.004
Tumor	1.370 ± 0.183	0.243 ± 0.003	0.284 ± 0.248	0.159 ± 0.180

Blocking was done using c(RGDfK)
**Significant statistical difference ($p < 0.05$) between blocked and unblocked

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Table S3. Quantitative PET data of ^{64}Cu-c(DOTA-RGD), ^{64}Cu-c(DOTA-RGDf), and ^{64}Cu-DOTA-c(RGDyK) in U87MG tumour bearing mice at 1 h, 4 h and 24 h p.i. Data are presented as %ID/g ± s.d. (n = 3)..

		non-blocking			**Blocking**	
	Tissues	1h	4h	24h	1h	4h
^{64}Cu-c(DOTA-RGD)	Tumor	0.89 ± 0.16	0.85 ± 0.09	0.46 ± 0.03	/	/
	Heart	0.35 ± 0.07	0.16 ± 0.01	0.13 ± 0.02	/	/
	Lung	0.25 ± 0.04	0.20 ± 0.02	0.13 ± 0.03	/	/
	Liver	1.47 ± 0.21	1.40 ± 0.17	1.17 ± 0.21	/	/
	Kidney	1.90 ± 0.17	1.43 ± 0.06	0.67 ± 0.14	/	/
	Muscle	0.04 ± 0.02	0.05 ± 0.00	0.04 ± 0.03	/	/
^{64}Cu-c(DOTA-RGDf)	tumor	1.73 ± 0.70	2.13 ± 0.91	1.15 ± 0.19	1.30 ± 0.10	1.63 ± 0.29
	Heart	0.68 ± 0.17	0.49 ± 0.15	0.47 ± 0.10	0.42 ± 0.06	0.50 ± 0.15
	Lung	0.70 ± 0.19	0.43 ± 0.14	0.46 ± 0.15	0.48 ± 0.06	0.37 ± 0.02
	Liver	3.47 ± 0.67	3.43 ± 0.29	3.37 ± 0.70	2.90 ± 0.36	2.87 ± 0.31
	Kidney	3.50 ± 0.26	2.80 ± 0.52	1.27 ± 0.31	2.10 ± 0.46	1.93 ± 0.51
	Muscle	0.36 ± 0.08	0.17 ± 0.02	0.12 ± 0.02	0.30 ± 0.05	0.11 ± 0.09
^{64}Cu-DOTA-c(RGDyK)	tumor	4.33 ± 0.68	4.17 ± 0.90	2.63 ± 0.68	1.67 ± 0.12	1.60 ± 0.14
	Heart	0.94 ± 0.31	0.74 ± 0.11	0.73 ± 0.23	0.59 ± 0.26	0.66 ± 0.49
	Lung	0.87 ± 0.28	0.82 ± 0.07	0.89 ± 0.26	0.43 ± 0.20	0.45 ± 0.37
	Liver	5.60 ± 1.61	5.93 ± 1.96	5.00 ± 1.06	4.63 ± 3.56	4.80 ± 3.54
	Kidney	5.17 ± 2.20	3.47 ± 1.75	2.20 ± 0.69	3.03 ± 1.53	2.25 ± 0.78
	Muscle	0.74 ± 0.30	0.34 ± 0.08	0.30 ± 0.11	0.20 ± 0.04	0.14 ± 0.05

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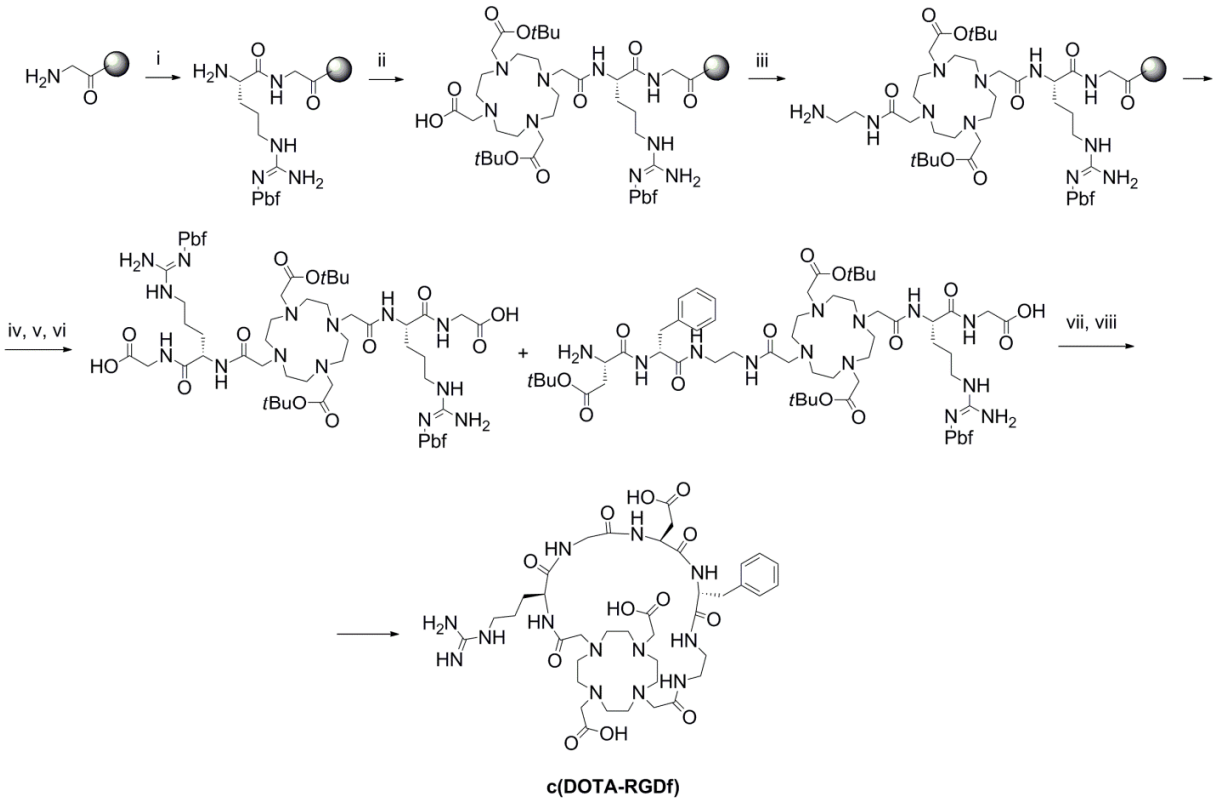
Schemes and Figures



Scheme S1. i) Fmoc-Arg(Pbf)-OH, HBTU, HOBt, DIPEA/DMF; Piperidine/DMF, ii) DOTA-bis-*tert*-butyl ester, HBTU, DIPEA/DMF, iii) HBTU, EDA/DMF, iv) Fmoc-DPhe-OH, HBTU, HOBt, DIPEA/DMF; Piperidine/DMF, v) Fmoc-Asp(OtBu)-OH, HBTU, HOBt, DIPEA/DMF; Piperidine/DMF, vi) AcOH, TFE, DCM, vii) 1-propanephosphonic acid cyclic anhydride/ EtOAc, DIPEA, DMAP/DCM viii) TFA/H_2O.

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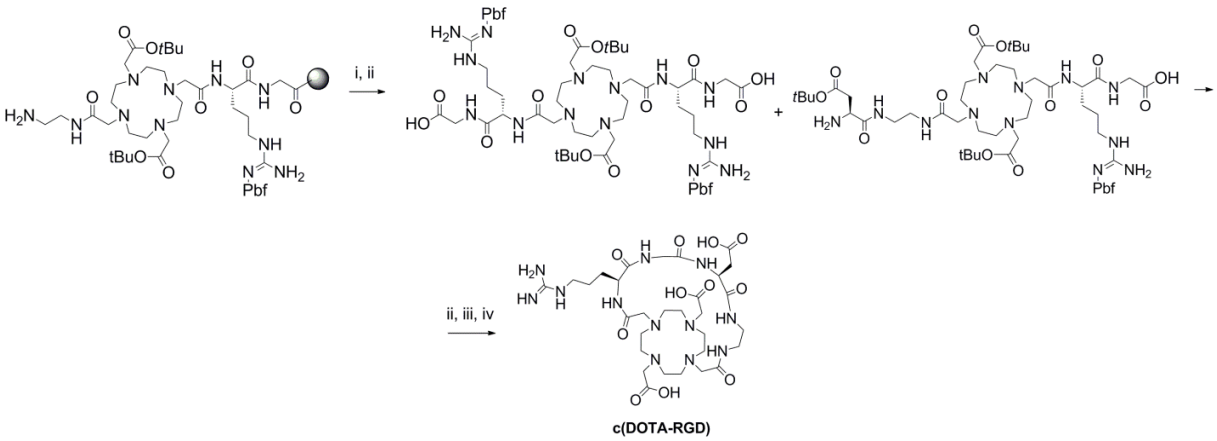

Scheme S2. i) Fmoc-Asp(OtBu)-OH, HBTU, HOBt, DIPEA/DMF; Piperidine/DMF, ii) AcOH, TFE, DCM, iii) 1-propanephosphonic acid cyclic anhydride/ EtOAc, DIPEA, DMAP/DCM.

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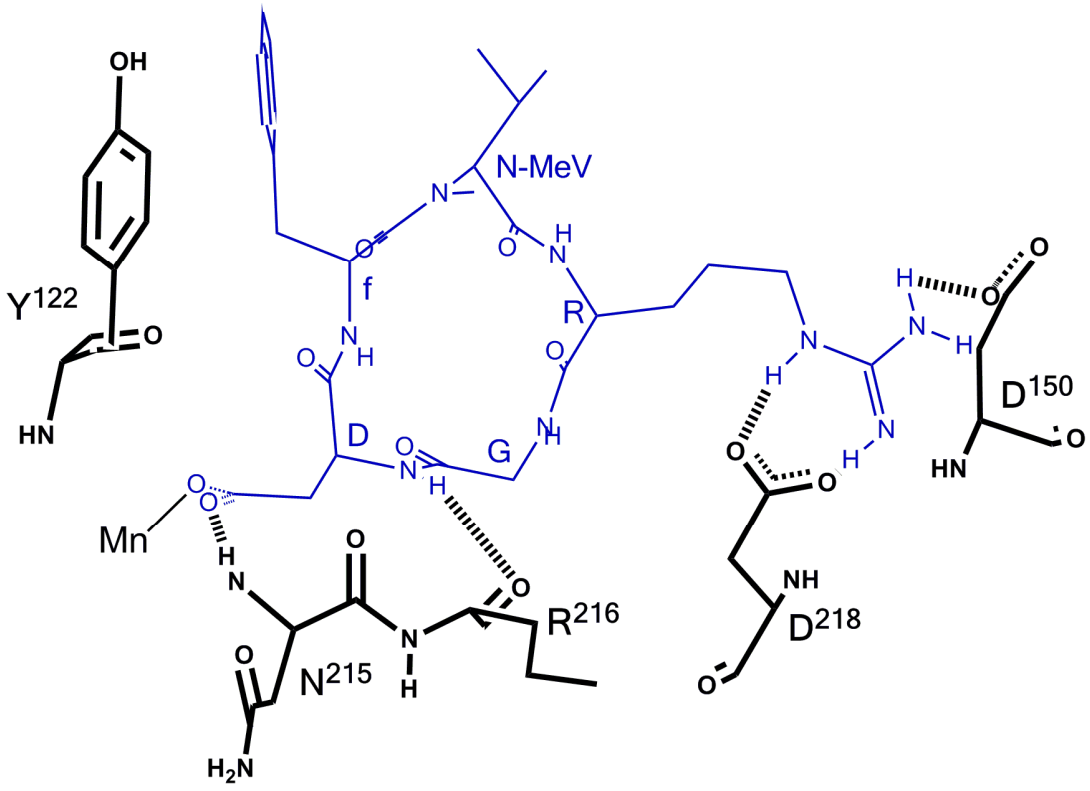

Figure S1. Graphical representation of the interaction of c(RGDf[NMe]V) (structure in blue) with key residues of the $\alpha_v\beta_3$ integrin (residues in black).

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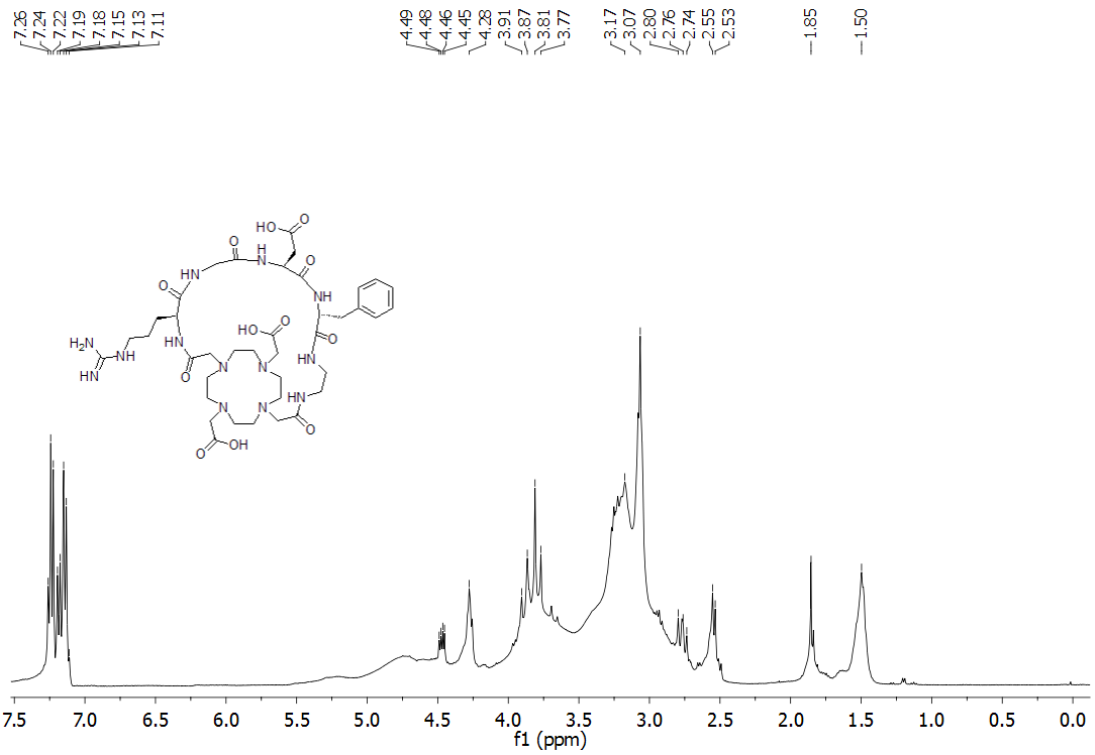

Figure S2. ^{1}H (200 MHz, D_2O) NMR spectrum of c(DOTA-RGDf)

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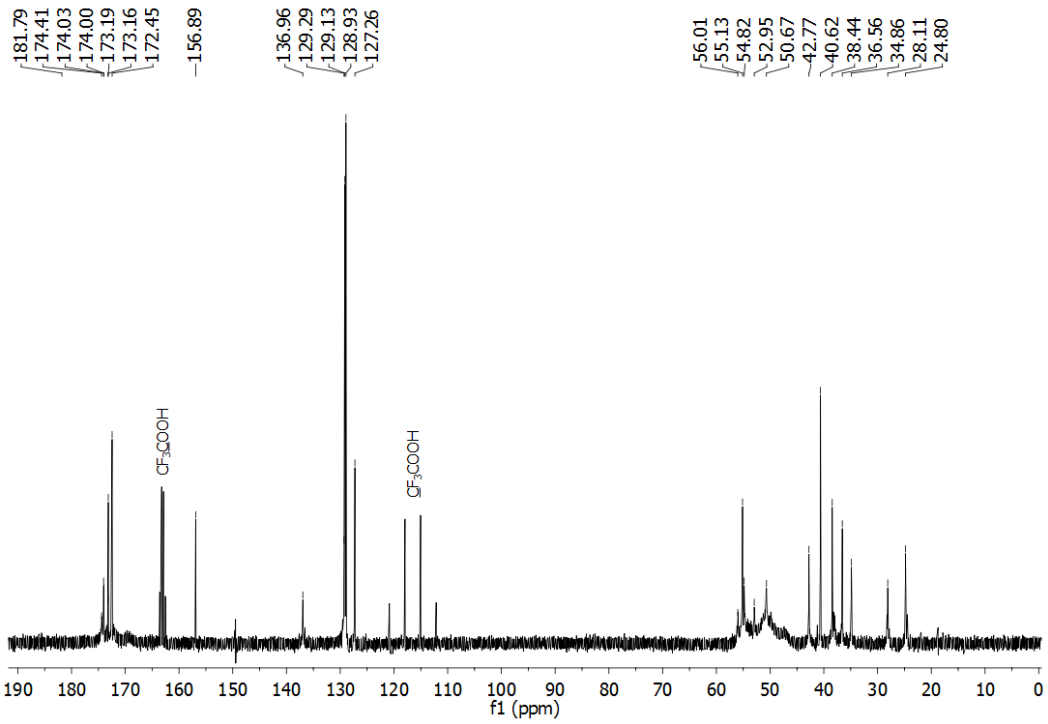

Figure S3. $^{13}C\{^{1}H\}$ (50 MHz, D_2O) NMR spectrum of c(DOTA-RGDf)

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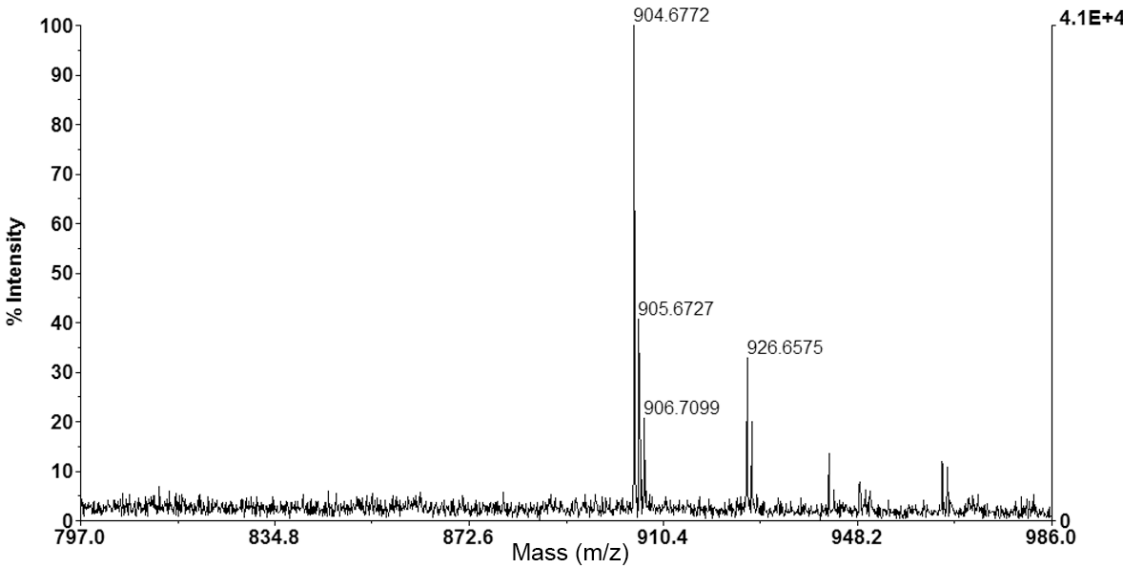

Figure S4. MS (MALDI-TOF+) spectrum of c(DOTA-RGDf)

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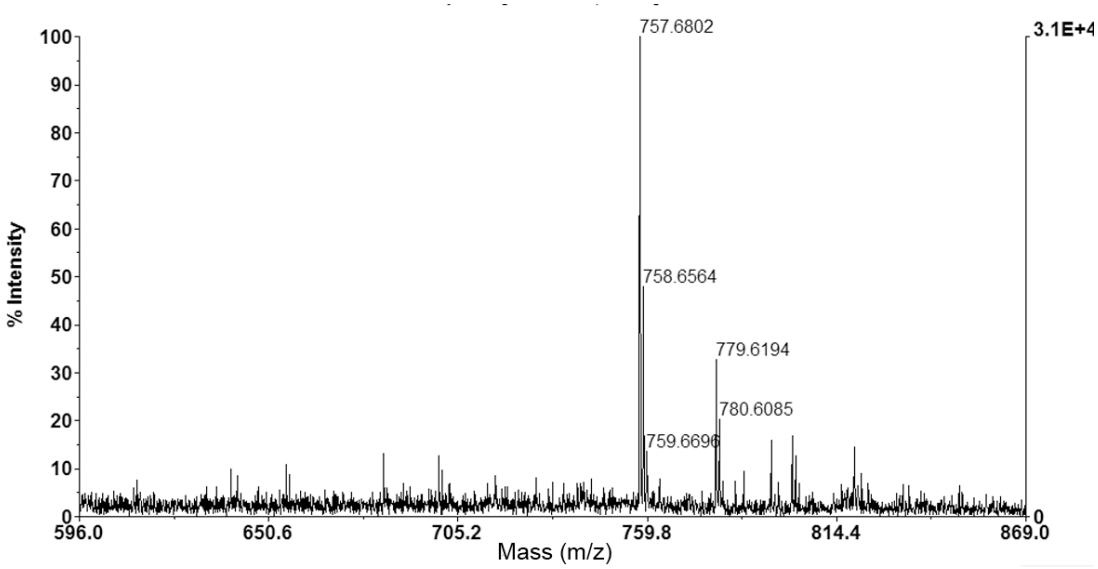

Figure S5. MS (MALDI-TOF+) spectrum of c(DOTA-RGD)

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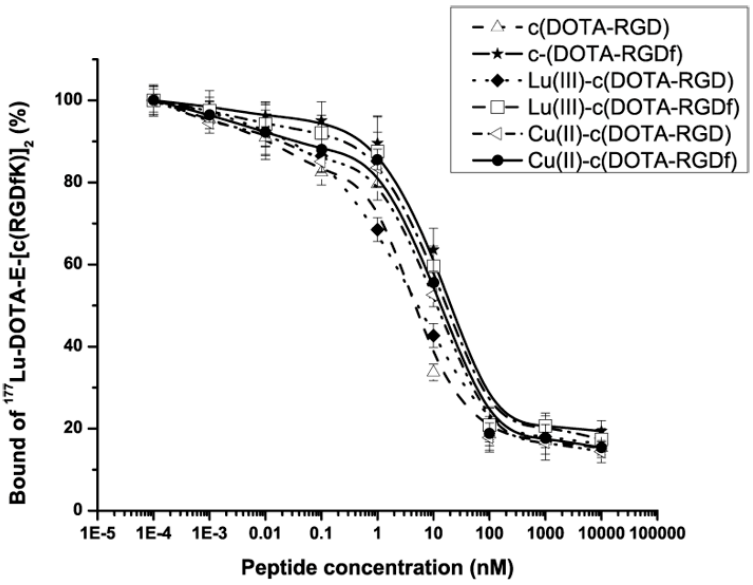

Figure S6. $\alpha_V\beta_3$ competition binding assay data.

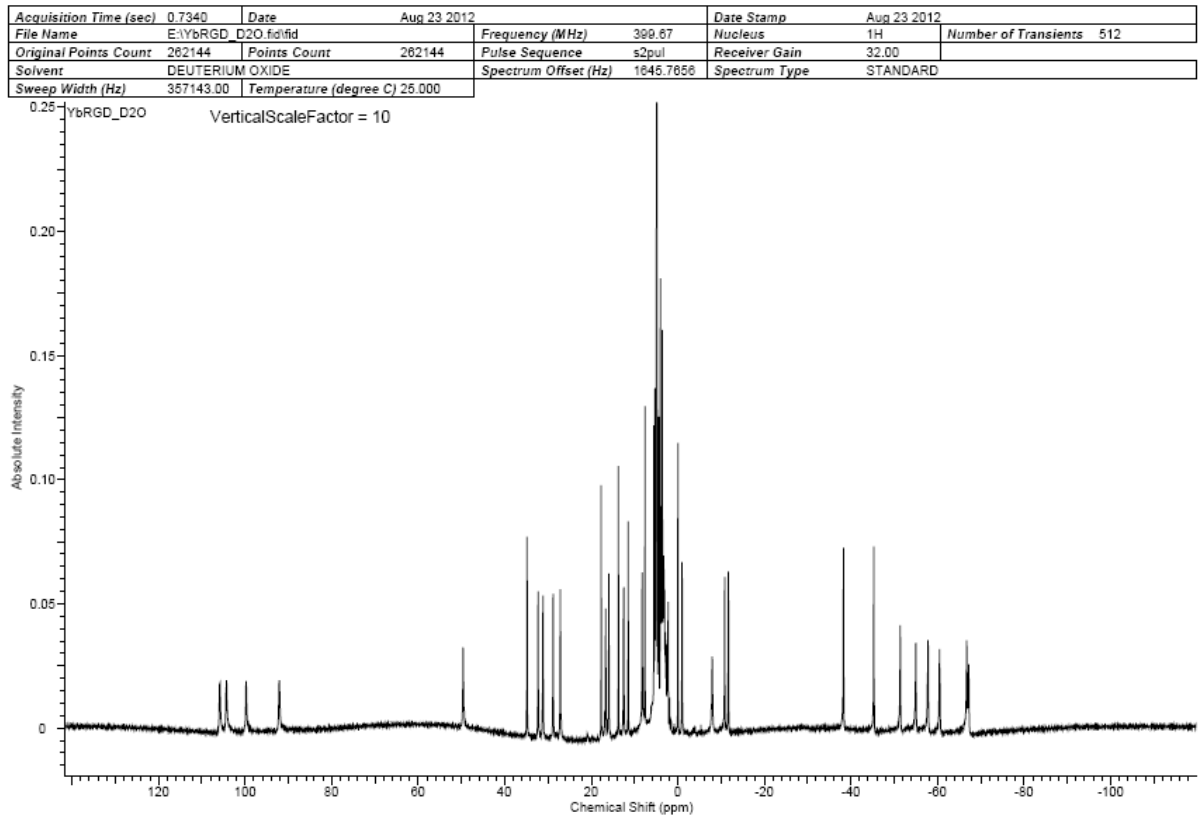

Figure S7. ^{1}H (400 MHz, D_2O) NMR spectrum of Yb-c(DOTA-RGD).

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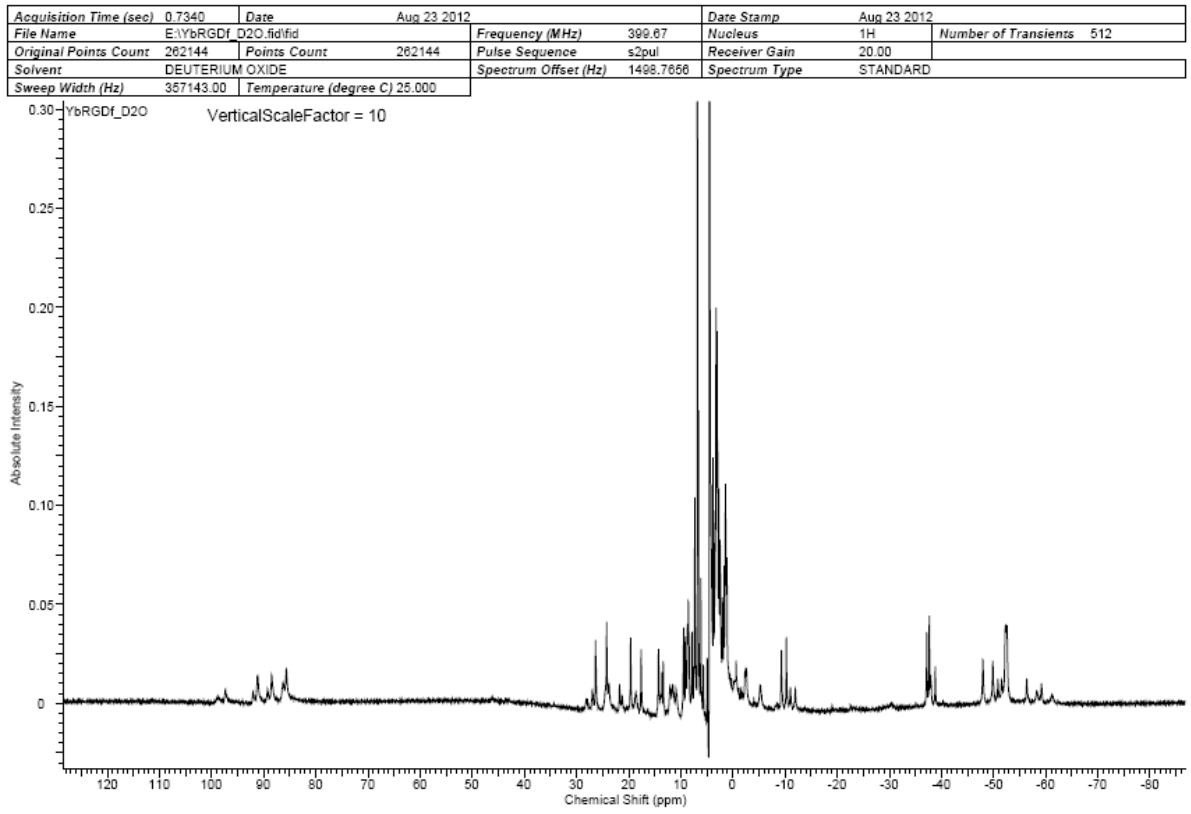

Figure S8. ^{1}H (400 MHz, D_2O) NMR spectrum of Yb-c(DOTA-RGDf).

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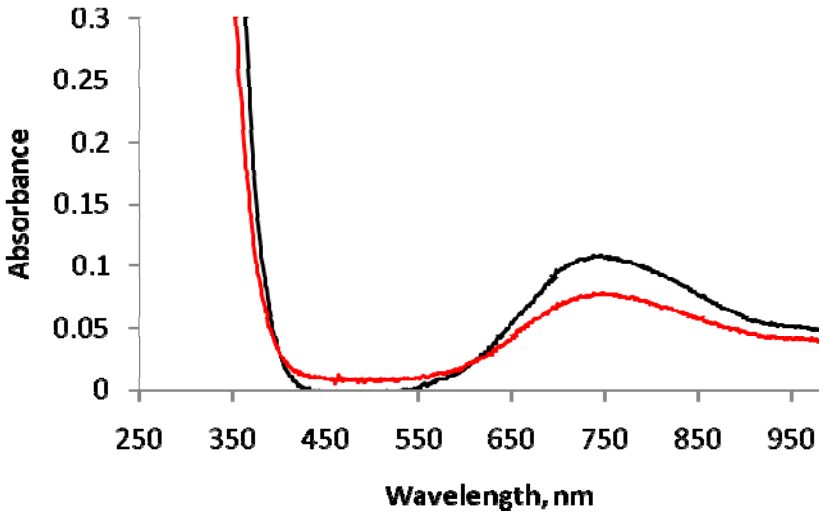

Figure S9. UV-Visible spectum of 2 mg/mL aqueous solutions of Cu-c(DOTA-RGD) (black) and Cu-c(DOTA-RGDf) (red).

Electronic Supplementary Material (ESI) for Dalton Transactions
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