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Electronic Supplementary Information

Synthesis and Preclinical Evaluation of a Novel, Selective ^{111}In-labelled Aminoproline-RGD-peptide For Non-invasive Melanoma Tumor Imaging

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CHEMISTRY AND RADIOCHEMISTRY

General aspects and abbreviations

All chemicals were of the highest commercially available quality and were used without further purification. Solvents were dried by standard procedures and reactions requiring anhydrous conditions were performed under nitrogen or argon atmosphere. $^{111}InCl_3$ was purchased from Mallinckrodt Medical BV, H-Gly-2-Cl-Trt resin (loading 0.58 mmol/ g) from Merck and all other reagents from Sigma-Aldrich. Analytical thin layer chromatography (TLC) was performed on silica gel 60 F_{254} pre-coated plates with visualization under short-wavelength UV light and by dipping the plates with molybdate reagent (aqueous H_2SO_4 solution of ceric sulphate/ammonium molybdate) followed by heating. Automated flash column chromatography was carried out with the Biotage Isolera One system using Biotage KP-Sil cartridges. Melting points (mp) were measured with an optical Optiphot2-Pol thermo-microscope and are uncorrected. Optical rotation data were obtained on a digital polarimeter at ambient temperature using a 100 mm cell with a 1 mL capacity and are given in units of 10^{-1} deg cm^2 g^{-1}. Semi-preparative HPLC was performed on a Prostar 210 apparatus (Varian, UV detection) equipped with C_{18}-10 μm columns (Discovery BIO Wide Pore 10 × 250 mm). ^1H and ^{13}C NMR spectra were recorded at 300 K on Bruker Avance 300 or 400 MHz spectrometers. Chemical shifts (δ) are expressed in ppm relative to internal TMS as the standard reference. ESI-mass spectra were recorded on API 150EX apparatus and are reported in the form of *m/z*. HPLC-ESI-MS/MS analyses were carried out on a Thermo Accela UHPLC system equipped with an Accela Open AS autosampler interfaced to a TSQ Quantum Access MAX triple quadrupole mass spectrometer (Thermo Italy, Milan, Italy); a Phenomenex Gemini-NX C_{18}-5 μm column (4.6 × 150 mm) was used for compound separation. High resolution mass analysis (ESI) was performed on LTQ ORBITRAP XL Thermo apparatus. Water used for final HPLC purification and radiolabelling procedures was obtained by Millipore MILLIQ apparatus.

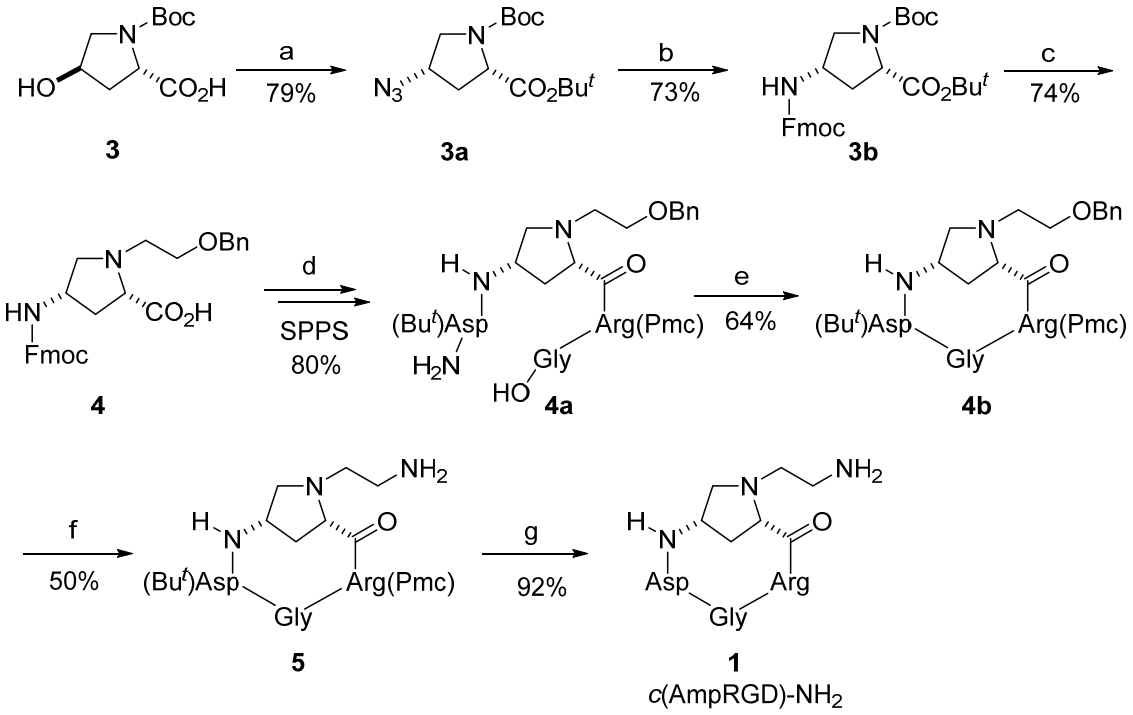
Abbreviations: Amp, 4-amino-L-proline; Bn, benzyl; Boc, *tert*-butoxycarbonyl; Pmc, 2,2,5,7,8-pentamethylchroman-6-sulfonyl; Fmoc, 9-fluorenylmethoxycarbonyl; SPPS, solid phase peptide synthesis; DCE, 1,2-dichloroethane; DCM, dichloromethane; DMF, *N*,*N*-dimethylformamide; DIC, diisopropyl carbodiimide; DIEA, diisopropylethylamine; TFA, trifluoroacetic acid; TFE, trifluoroethanol; MsCl, methanesulfonyl chloride; DEAD, diethyl azodicarboxylate; DPPA, diphenylphosphoryl azide; FmocOSu, 9-fluorenylmethyl-*N*-succinimidyl carbonate; HATU, *O*-(7-azabenzotriazol-1-yl)-*N*,*N*,*N*',*N*'-tetramethyluronium hexafluorophosphate; HOAt, 1-hydroxy-7-azabenzotriazole; HBTU, *O*-(benzotriazol-1-yl)-*N,N,N',N'*-tetramethyluronium hexafluorophosphate; DEPBT, 3-(diethoxyphosphoryloxy)-1,2,3-benzotriazin-4(3*H*)-one; TIS, triisopropylsilane; DOTA, 1,4,7,10-tetraazacyclododecane-1,4,7,10-tetraacetic acid; DOTA(OBu^t)$_3$, 4,7,10-tri-(*tert*-butoxycarbonylmethyl)-1,4,7,10-tetraazacyclododecan-1-yl-acetic acid.

Experimental synthetic procedures and characterization data

Synthesis of *c*(AmpRGD)-NH_2 (1) (Scheme S1)

The synthesis of protected *c*(AmpRGD)-NH_2 (**5**), to be used in the conjugation step with the DOTA derivative **6**, started with protecting groups manipulation of commercially available L-*trans*-hydroxyproline (**3**), followed by installation of the γ-amino group at the C4 position and introduction of the benzyloxyethyl chain at the *N*-α proline function affording scaffold **4** in seven steps (43% overall yield). The γ-amino acid **4** was employed in standard Fmoc-SPPS protocol using the preloaded 2-chlorotrityl-Gly-H resin and the couple DEPBT/DIEA as condensing agents. The Fmoc removal was accomplished by treatment with 20% piperidine/DMF mixture. The protecting groups of the amino acid side chains were *tert*-butyl (Bu^t) for aspartic acid, benzyl (Bn) for the hydroxyethyl chain of γ-aminoproline and 2,2,5,7,8-pentamethylchroman-6-sulfonyl (Pmc) for arginine.

Scheme S1 Synthesis of *c*(AmpRGD)-NH_2 (**1**)[a]



[a]Reagents and conditions: a) DIC, Bu^tOH, CuCl, DCM, 3 days (87%); DEAD, PPh_3, DPPA, THF, 0 °C to rt (91%); b) H_2, Pd/C, MeOH (99%); FmocOSu, aq Na_2CO_3, THF (74%); c) Bu^tOAc, $MeSO_3H$, DCM, 3 days (87%); $CHOCH_2OBn$, $NaBH(OAc)_3$, DCE, rt (93%); TFA, anisole (91%); d) SPPS: 2-Chlorotrityl-Gly-H resin; Fmoc-Arg(Pmc)-OH; Fmoc-Asp(Bu^t)-OH; coupling system: DEPBT, DIEA, DMF; Fmoc removal: 20% piperidine/DMF; cleavage: AcOH/TFE/DCM (1:1:3), (80%); e) HATU, HOAt, collidine, DMF (medium dilution, 3.0 mM) (64%); f) 50 psi H_2, Pd/C, MeOH, HCO_2H, 36 h (80%); MsCl, Et_3N, CH_3CN; then NaN_3, DMF, reflux (70%, 2 steps); H_2, Pd/C, EtOH (89%); g) TFA/TIS/H_2O (92%).

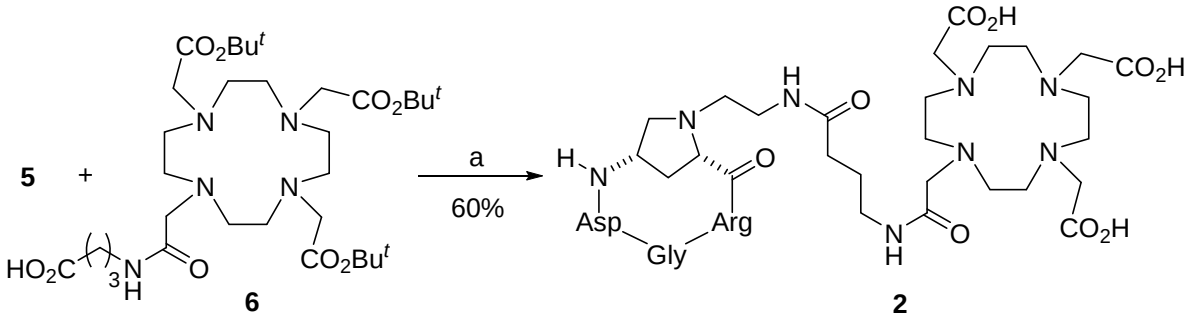
After acidic cleavage from the resin, the linear tetrapeptide **4a** was exposed to HATU/HOAt/collidine system under medium-dilution conditions (3.0 mM) to address the in-solution cyclization. Cyclopeptide **4b** was obtained in 64% yield. An additional four-step sequence was needed to elaborate the benzyloxy functionality into the terminal amino group of

cyclopeptide **5** (50% overall yield). Treatment of **5** in the presence of TFA/TIS/H_2O (95:2.5:2.5) mixture followed by semi-preparative RP-HPLC purification and lyophilisation furnished the deprotected pseudopeptide *c*(AmpRGD)-NH_2 (**1**) ready for in vitro studies.

Synthesis of *c*(AmpRGD)-DOTA (2) (Scheme S2)

Protected DOTA-derivative **6**, previously prepared in two steps from commercial DOTA(Bu^t)$_3CO_2H$ (Macrocyclics, Dallas, TX, USA) and γ-aminobutyric acid, was coupled to the protected RGD cyclopeptide **5** under standard conditions (HBTU/DIEA).

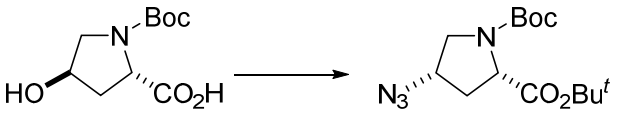
Scheme S2 Synthesis of *c*(AmpRGD)-DOTA **2**[a]



[a]Reagents and conditions: a) HBTU, DIEA, DCM (68%); TFA/TIS/H_2O (89%).

The acidic cleavage of the side chain protecting groups (TFA/TIS/H_2O) followed by RP-HPLC purification afforded conjugate **2** in satisfactory yield (60% over two steps).

(2*S*,4*S*)-1-(*tert*-Butoxycarbonyl)-4-azidoproline *tert*-Butyl Ester (3a)

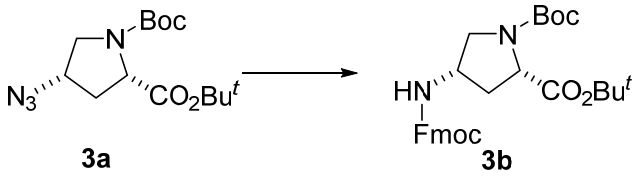


To a solution of DIC (0.84 mL, 5.45 mmol) and Bu^tOH (0.61 mL, 6.36 mmol), CuCl (54 mg, 0.54 mmol) was added and the resulting mixture was stirred at room temperature under argon for 3 days. A solution of *N*-Boc-4-hydroxy-L-proline (**3**) (210 mg, 0.908 mmol) in dry DCM (4 mL) was then added and the solution was stirred for additional 3 days. The reaction was diluted with DCM (6 mL) and filtered through a Celite pad. The crude residue was subjected to flash chromatographic purification (EtOAc/hexanes 70:30) affording a *tert*-butyl ester intermediate (not shown) as a colourless oil (227 mg, 87%): $[\alpha]_D^{25}$ – 54.6 (*c* 6.5; $CHCl_3$); ^1H NMR (400 MHz, $CDCl_3$) δ 4.50 (m, 1H, H4), 4.31 (dd, J = 7.8, 7.8 Hz, 1H, H2), 3.64 (dd, J = 11.5, 4.4 Hz, 1H, H5a), 3.56 (bd, J = 11.5 Hz, 1H, H5b), 2.34-2.25 (m, 1H, H3a), 2.10-2.04 (m, 1H, H3b), 1.48 (s, 9H, Bu^t), 1.46 (s, 9H, Boc).

To an ice-cooled solution of proline intermediate (227 mg, 0.79 mmol) and PPh_3 (414 mg, 1.58 mmol) in dry THF (10 mL) under stirring, 0.72 mL of DEAD (40% solution in toluene, 1.58 mmol) were added dropwise over 30 min, under argon atmosphere. After 10 min, DPPA (0.34 mL, 1.58 mmol) was added dropwise and the reaction was allowed to rise to room temperature. After 24 h, the mixture was concentrated in vacuum and the residue was purified by silica gel

flash chromatography (EtOAc/hexanes 25:75) to afford 4-azidoproline **3a** (224 mg, 91%) as a yellowish oil: $[\alpha]_D^{25}$ – 28.2 (*c* 9.3; $CHCl_3$); ^1H NMR (400 MHz, $CDCl_3$, mixture of rotamers, major isomer) δ 4.22 (dd, *J* = 9.0, 3.5 Hz, 1H, H2), 4.17 (m, 1H, H4), 3.72 (dd, *J* = 11.8, 6.1 Hz, 1H, H5a), 3.49 (dd, *J* = 11.6, 3.3 Hz, 1H, H5b), 2.45 (ddd, *J* = 13.8, 9.1, 5.9 Hz, 1H, H3a), 2.16 (ddd, *J* = 13.6, 3.2, 3.0 Hz, 1H, H3b), 1.49 (s, 9H, Bu^t), 1.45 (s, 9H, Boc). MS (ESI^+) *m/z* 313.2 $[M+H]^+$.

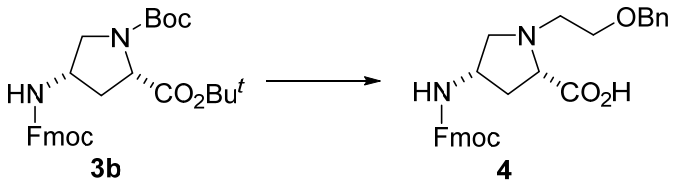
(2*S*,4*S*)-1-(*tert*-Butoxycarbonyl)-4-*N*-(9-fluorenylmethoxycarbonyl)aminoproline *tert*-Butyl Ester (3b)



Azidoproline **3a** (224 mg, 0.72 mmol) was dissolved in MeOH (12 mL) and a catalytic amount of 10% palladium on carbon was added. The reaction vessel was degassed under vacuum and thoroughly purged with hydrogen (three times) and the resulting heterogeneous mixture was stirred under hydrogen for 4 h at room temperature. The catalyst was filtered off and the filtrate was concentrated in vacuum to give crude 4-aminoproline intermediate as a white solid (203 mg, 99%) which was used directly in the following step: $[\alpha]_D^{25}$ – 30.2 (*c* 8.0; MeOH); ^1H NMR (400 MHz, CD_3OD, mixture of rotamers, major isomer) δ 4.16 (dd, *J* = 9.0, 6.0 Hz, 1H, H2), 3.70 (dd, *J* = 10.7, 6.3 Hz, 1H, H5a), 3.44 (m, 1H, H4), 3.20 (dd, *J* = 10.4, 5.7 Hz, 1H, H5b), 2.60-2.46 (m, 1H, H3a), 1.76 (ddd, *J* = 12.9, 6.3, 6.2 Hz, 1H, H3b), 1.52 (s, 9H, Bu^t), 1.45 (s, 9H, Boc).

4-Aminoproline intermediate (203 mg, 0.71 mmol) was dissolved in THF (2 mL), and 10% aqueous Na_2CO_3 solution (1.8 mL) was added. FmocOSu (240 mg, 0.71 mmol) dissolved in THF (2 mL) was then added to the solution pre-cooled to 0 °C. The reaction mixture was stirred for 4 h at room temperature and concentrated in vacuo to leave a residue which was dissolved in EtOAc (5 mL) and treated with saturated aq NH_4Cl solution. The mixture was extracted with EtOAc (4 ×) and the organic layers were collected, dried, filtered, and concentrated to afford a crude residue. Purification by silica gel flash chromatography (EtOAc/hexanes 30:70) afforded protected aminoproline **3b** (265 mg, 74%) as a white foam: $[\alpha]_D^{25}$ + 0.88 (*c* 6.8; $CHCl_3$); ^1H NMR (300 MHz, $CDCl_3$, mixture of rotamers, major isomer) d 7.77 (bd, *J* = 7.7 Hz, 2H, Ar), 7.60 (bd, *J* = 7.3 Hz, 2H, Ar), 7.41 (bdd, *J* = 8.0, 7.3 Hz, 2H, Ar), 7.32 (ddd, *J* = 7.3, 7.3, 2.5 Hz, 2H, Ar), 5.83 (bd, *J* = 8.8 Hz, 1H, NH), 4.48-4.31 (m, 3H, CH_2 Fmoc, H2), 4.30-4.16 (m, 2H, CH Fmoc, H4), 3.68 (dd, *J* = 11.7, 5.3 Hz, 1H, H5a), 3.59 (bd, *J* = 12.0 Hz, 1H, H5b), 2.45 (m, 1H, H3a), 2.02 (bd, *J* = 11.8 Hz, 1H, H3b), 1.48 (s, 9H, Bu^t), 1.47 (s, 9H, Boc). MS (ESI^+) *m/z* 509.4 $[M+H]^+$.

(2*S*,4*S*)-1-[2-Benzyloxyethyl]-4-*N*-(9-fluorenylmethoxycarbonyl)aminoproline (4)



3b **4**

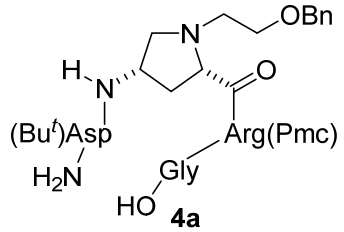
To a solution of protected aminoproline **3b** (265 mg, 0.52 mmol) in dry DCM (0.35 mL) were added Bu^{t}OAc (1.4 mL) and $MeSO_3H$ (84 μL) and the resulting mixture was subjected to sonication for 15 min every 4 h at room temperature. After 3 days, the reaction was treated with aq $NaHCO_3$ solution and extracted with DCM (3 ×) and EtOAc (4 ×). The combined organic layers were dried, filtered and concentrated under reduced pressure. The crude residue was subjected to flash chromatography (EtOAc/MeOH 80:20) affording a N^{α} deprotected aminoproline intermediate (186 mg, 87%) as a white foam: $[\alpha]_D^{25}$ – 8.3 (*c* 5.4; $CHCl_3$); ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.78 (d, *J* = 8.1 Hz, 2H, Ar), 7.61 (d, *J* = 7.6 Hz, 2H, Ar), 7.41 (dd, *J* = 7.3, 7.3 Hz, 2H, Ar), 7.32 (dd, *J* = 7.6, 7.6 Hz, 2H, Ar), 5.37 (d, *J* = 7.1 Hz, 1H, NH Fmoc), 4.46-4.31 (m, 2H, CH_2 Fmoc), 4.30-4.16 (m, 2H, CH Fmoc, H4), 3.73 (dd, *J* = 9.3, 3.5 Hz, 1H, H2), 3.14 (dd, *J* = 11.2, 5.7 Hz, 1H, H5a), 3.04 (bd, *J* = 10.9 Hz, 1H, H5b), 2.45 (bs, 1H, NH), 2.39 (m, 1H, H3a), 1.91 (bd, *J* = 13.4 Hz, 1H, H3b), 1.49 (s, 9H, Bu^t); ^{13}C NMR (75 MHz, $CDCl_3$) δ 174.7 (Cq), 155.5 (Cq), 143.6 (2C, Cq), 141.0 (2C, Cq), 127.4 (2C, CH), 126.7 (2C, CH), 124.8 (2C, CH), 119.7 (2C, CH), 81.5 (Cq), 66.4 (CH_2), 59.0 (CH), 53.3 (CH_2), 51.4 (CH), 46.9 (CH), 37.0 (CH_2), 27.7 (3C, CH_3).

A solution of aminoproline intermediate (186 mg, 0.45 mmol) in 4 mL of dry DCE and a solution of $CHOCH_2OBn$ (82 mg, 0.55 mmol) in 2 mL of dry DCE were simultaneously dropped into a solution of $NaBH(OAc)_3$ (193 mg, 0.91 mmol) in dry DCE (5 mL). The resulting mixture was stirred under argon at room temperature for 7 h, and then quenched with aq $NaHCO_3$ solution. Extraction with DCM (3 ×) and EtOAc (3 ×), drying of the organic layers, filtration and evaporation of the solvent gave a crude residue which was washed with Et_2O affording an *N*-alkylated aminoproline intermediate (230 mg, 93%) as white crystals: $[\alpha]_D^{25}$ – 14.1 (*c* 7.7; $CHCl_3$); mp 94.5–95.5 °C; ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.67 (d, *J* = 7.4 Hz, 2H, Ar), 7.53 (d, *J* = 7.5 Hz, 2H, Ar), 7.31 (dd, *J* = 7.5, 7.5 Hz, 2H, Ar), 7.26 (m, 5H, Ar), 7.22 (dd, *J* = 7.5, 7.5 Hz, 2H, Ar), 5.84 (d, *J* = 9.2 Hz, 1H, NH Fmoc), 4.44 (s, 2H, CH_2 Bn), 4.28 (m, 2H, CH_2 Fmoc), 4.22 (m, 1H, H4), 4.13 (dd, *J* = 7.0, 7.0 Hz, 1H, CH Fmoc), 3.51 (dd, *J* = 5.9, 5.9 Hz, 2H, H2′), 3.18 (dd, *J* = 10.0, 4.0 Hz, 1H, H2), 2.95 (bd, *J* = 9.7 Hz, 1H, H5a), 2.87 (ddd, *J* = 12.9, 5.8, 5.8 Hz, 1H, H1′a), 2.73 (ddd, *J* = 12.9, 5.8, 5.8 Hz, 1H, H1′b), 2.72 (dd, *J* = 9.8, 5.4 Hz, 1H, H5b), 2.35 (ddd, *J* = 13.9, 10.0, 7.2 Hz, 1H, H3a), 1.73 (bd, *J* = 13.9 Hz, 1H, H3b), 1.36 (s, 9H, Bu^t).

To a solution of *N*-alkylated aminoproline (230 mg, 0.42 mmol) in DCM (12 mL) was added anisole (46 μL, 0.42 mmol) and the mixture was cooled to 0° C, then TFA (3 mL) was added dropwise. The mixture was stirred at room temperature for 6 h, then was evaporated under reduced pressure.The crude residue was purified by flash chromatography (EtOAc/MeOH 70:30 to 60:40) to afford aminoproline **4** (188 mg, 91%) as a white powder: ^{1}H NMR (400 MHz, CD_3OD) δ 7.74 (d, *J* = 7.3 Hz, 2H, Ar), 7.59 (d, *J* = 7.0 Hz, 2H, Ar), 7.35 (dd, *J* = 7.3, 7.3 Hz, 2H, Ar), 7.33-7.17 (m, 7H, Ar), 4.49 (m, 2H, CH_2 Bn), 4.33 (m, 2H, CH_2 Fmoc), 4.19 (m, 1H, H4), 4.12 (dd, *J* = 6.6, 6.6 Hz, 1H, CH Fmoc), 3.62 (m, 3H, H2, H2′a,b), 3.45 (bd, *J* =

6.4 Hz, 1H, H5a), 3.34 (m, 1H, H1′a), 3.09 (m, 1H, H5b), 2.93 (m, 1H, H1′b), 2.64 (m, 1H, H3a), 2.00 (m, 1H, H3b); ^{13}C NMR (100 MHz, CD_3OD) δ 174.5 (Cq), 157.0 (Cq), 144.0 (2C, Cq), 141.4 (2C, Cq), 137.7 (Cq), 128.3 (2C, CH), 128.0 (2C, CH), 127.7 (CH), 127.6 (2C, CH), 127.0 (2C, CH), 124.9 (2C, CH), 119.8 (2C, CH), 73.0 (CH_2), 69.4 (CH_2), 66.6 (CH_2) 65.7 (CH), 59.1 (CH_2), 54.5 (CH_2), 49.2 (CH), 47.2 (CH), 35.6 (CH_2). MS (ESI^+) *m/z* 487.7 $[M+H]^+$.

H-Asp(Bu^t)-1-(2-benzyloxyethyl)Amp-Arg(Pmc)-Gly-OH (4a)

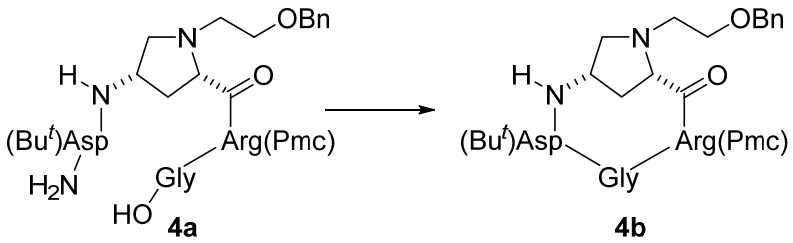


4a

The synthesis of the linear tetrapeptide H-Asp(Bu^t)-Amp(CH_2CH_2OBn)-Arg(Pmc)-Gly-OH (**4a**) was performed using the preloaded 2-chlorotrityl-Gly-H resin (loading 0.58 mmol/g).
Resin swelling: the resin (333 mg, 0.193 mmol) was swollen in a solid phase reaction vessel with dry DMF (10 ml) under mechanical stirring; after 20 min the solvent was drained and the resin was washed with DCM (2 ×) and DMF.
Peptide coupling: a preformed solution of Fmoc-Arg(Pmc)-OH (320 mg, 0.482 mmol) and DEPBT (173 mg, 0.579 mmol) in dry DMF (12 mL), cooled to 0 °C, was treated with DIEA (101 µL, 0.579 mmol) and stirred for 20 min before adding to the resin. The mixture was shaken at room temperature for 3 h. Completion of the reaction was checked with the Kaiser test. The solution was drained and the resin was washed several times with DMF (3 ×), Pr^{i}OH, (3 ×), Et_2O (3 ×), DCM (3 ×). The resin was treated with 20% v/v piperidine in DMF (6 mL) and the mixture was stirred for 40 min. The solution was drained and the resin was washed with DMF (3 ×), Pr^{i}OH, (3 ×), Et_2O (3 ×), DCM (3 ×). The coupling of the Fmoc-Amp(CH_2CH_2OBn)-OH **4** (188 mg, 0.386 mmol) and Fmoc-Asp(Bu^t)-OH (198 mg, 0.482 mmol) was carried out under the same conditions.
Resin cleavage: the resin was treated with 6 mL of a DCM/TFE/glacial AcOH (3:1:1) mixture for 15 min under mechanical stirring at ambient temperature. The solution was recovered and the resin was carefully washed with the above DCM/TFE/AcOH mixture (2 × 6 mL). The combined solution was evaporated under reduced pressure affording linear tetrapeptide **4a** (142 mg, 80%) as a white solid, which was used in the following step without further purification: ^{1}H NMR: (400 MHz, CD_3OD) δ 7.36-7.20 (m, 5H, Ph), 4.48 (s, 2H, CH_2 Bn), 4.33 (m, 1H, H4), 4.23 (dd, *J* = 8.3, 5.3 Hz, 1H, Hα Arg), 4.06 (dd, *J* = 7.4, 5.2 Hz, 1H, Hα Asp), 3.81 (d, *J* = 17.1 Hz, 1H, Hα Gly), 3.67 (d, *J* = 17.5 Hz, 1H, Hα Gly), 3.60 (m, 2H, H2′a,b), 3.29 (m, 1H, H2), 3.20-3.06 (m, 3H, H5a, Hδ Arg), 2.96 (m, 1H, H1′a), 2.88 (dd, *J* = 17.5, 5.2 Hz, 1H, Hβ Asp), 2.84-2.68 (m, 3H, H5b, H1′b, Hβ Asp), 2.64 (dd, *J* = 6.6, 6.6 Hz, 2H, CH_2 Pmc), 2.60 (m, 1H, H3a), 2.57 (s, 3H, CH_3 Pmc), 2.55 (s, 3H, CH_3 Pmc), 2.89 (s, 3H, CH_3 Pmc), 1.87 (m, 1H, H3b), 1.85-1.72 (m, 3H, Hβ Arg, CH_2 Pmc), 1.70-1.47 (m, 3H, Hβ Arg, Hγ Arg), 1.44 (s, 9H, Bu^t), 1.29 (s, 6H, CH_3 Pmc); ^{13}C NMR (100 MHz, CD_3OD) δ 177.2 (Cq), 176.6 (Cq), 174.1 (Cq), 171.5 (Cq), 170.2 (Cq), 155.6 (Cq), 140.3 (2C, Cq), 137.3 (Cq), 136.9 (Cq), 135.6

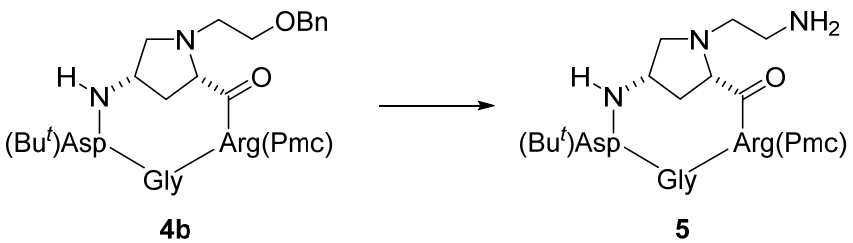
(Cq), 130.3 (2C, CH), 129.8 (2C, CH), 129.6 (CH), 125.8 (Cq), 120.2 (Cq), 84.4 (2C, Cq), 75.7 (CH_2), 75.0 (CH_2), 70.9 (CH_2), 68.2 (CH), 61.0 (CH_2), 56.6 (CH), 55.3 (CH), 52.0 (CH), 51.2 (CH_2), 44.8 (CH_2), 39.4 (CH_2), 38.4 (CH_2), 34.6 (CH_2), 30.6 (CH_2), 29.2 (3C, CH_3), 27.8 (2C, CH_3), 23.2 (CH_2), 22.0 (CH_2), 19.9 (CH_3), 18.8 (CH_3), 13.2 (CH_3). MS (ESI^+) *m/z* 915.7 $[M+H]^+$, 937.7 $[M+Na]^+$.

***cyclo*[Arg(Pmc)-Gly-Asp(Bu^t)-1-(2-benzyloxyethyl)Amp] (4b)**



To a solution of linear tetrapeptide **4a** (142 mg, 0.154 mmol) in dry DMF (51 mL) HATU (176 mg, 0.463 mmol), HOAt (0.6 M in DMF, 772 µL, 0.463 mmol) and 2,4,6-collidine (61 µL, 0.463 mmol). The mixture was degassed and stirred under argon at ambient temperature. After 4 h, the solution was concentrated under vacuum, treated with aq $NaHCO_3$ saturated solution and extracted with EtOAc (4 ×). The combined organic layer was dried, filtered and evaporated under reduced pressure, keeping the temperature under 50 °C. The crude was subjected to flash chromatographic purification (EtOAc/MeOH 80:20) furnishing the cyclic tetrapeptide **4b** (89 mg, 64%) as a colourless glassy solid: $[\alpha]_D^{25}$+ 0.30 (*c* 1.0; MeOH); ^{1}H NMR (400 MHz, CD_3OD) δ 7.33-7.18 (m, 5H, Ph), 4.60 (dd, *J* = 6.6, 5.8 Hz, 1H, Hα Asp), 4.44 (m, 2H, CH_2 Bn), 4.35 (m, 1H, H4), 4.13 (d, *J* = 14.0 Hz, 1H, Hα Gly), 3.85 (dd, *J* = 7.2, 7.2 Hz, 1H, Hα Arg), 3.68 (d, *J* = 9.5 Hz, 1H, H2), 3.59 (m, 2H, H2′a,b), 3.29 (d, *J* = 13.9 Hz, 1H, Hα Gly), 3.23-3.04 (m, 4H, H5a,b, Hδ Arg), 3.03-2.88 (m, 2H, H1′a,b), 2.75-2.60 (m, 4H, Hβ Asp, CH_2 Pmc), 2.57 (s, 3H, CH_3 Pmc), 2.56 (s, 3H, CH_3 Pmc), 2.36 (ddd, *J* = 13.9, 9.7, 6.6 Hz, 1H, H3a), 2.10 (s, 3H, CH_3 Pmc), 1.99 (bd, *J* = 13.6 Hz ,1H, H3b), 1.82 (m, 2H, CH_2 Pmc), 1.61 (m, 1H, Hβ Arg), 1.47 (m, 3H, Hβ Arg, Hγ Arg), 1.44 (s, 9H, Bu^t), 1.30 (s, 6H, CH_3 Pmc); ^{13}C NMR (75 MHz, CD_3OD) δ 177.7 (Cq), 175.3 (Cq), 170.5 (Cq), 170.2 (Cq), 169.9 (Cq), 153.5 (Cq), 138.5 (2C, Cq), 135.3 (Cq), 134.9 (Cq), 133.6 (Cq), 128.2 (2C, CH), 127.5 (2C, CH), 127.4 (CH), 123.8 (Cq), 118.2 (Cq), 82.8 (Cq), 81.0 (Cq), 73.7 (CH_2), 72.9 (CH_2), 69.6 (CH), 63.4 (CH_2), 60.3 (CH_2), 59.6 (CH), 55.1 (CH), 52.5 (CH_2), 49.8 (CH), 44.3 (CH_2), 37.3 (CH_2), 36.2 (CH_2), 32.6 (CH_2), 31.3 (CH_2), 27.1 (3C, CH_3), 25.8 (2C, CH_3), 21.2 (CH_2), 19.7 (CH_2), 17.8 (CH_3), 16.7 (CH_3), 11.2 (CH_3). MS (ESI^+) *m/z* 897.5 $[M+H]^+$, 919.5 $[M+Na]^+$.

***cyclo*[Arg(Pmc)-Gly-Asp(Bu^t)-1-(2-aminoethyl)Amp] (5)**



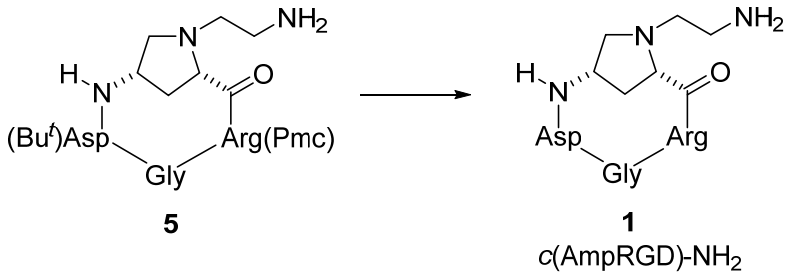
To a solution of cyclotetrapeptide **4b** (178 mg, 0.198 mmol) in MeOH (30 mL) a catalytic amount of Pd/C (10%) and HCO_2H (10 mL) were added. The reaction vessel was degassed under vacuum and thoroughly purged with hydrogen (three times). The mixture was kept under hydrogen pressure (50 psi) in a Parr hydrogenation apparatus for 36 h before being filtered and concentrated under vacuum. The crude hydroxyethyl intermediate (128 mg, 80%) was used as such in the subsequent step: ^{1}H NMR (400 MHz, CD_3OD) δ 4.63 (m, 1H, Hα Asp), 4.35 (dd, J = 5.6, 5.6 Hz, 1H, H4), 4.14 (d, J = 14.0 Hz, 1H, Hα Gly), 4.08 (dd, J = 7.6, 7.6 Hz, 1H, Hα Arg), 3.79 (m, 1H, H2), 3.62 (m, 2H, H2′a,b), 3.33 (d, J = 14.0 Hz, 1H, Hα Gly), 3.28-3.14 (m, 4H, H5a,b, Hδ Arg), 2.94 (m, 2H, H1′a,b), 2.75 (dd, J = 16.4, 7.1 Hz, 1H, Hβ Asp), 2.70-2.60 (m, 3H, Hβ Asp, CH_2 Pmc), 2.56 (s, 3H, CH_3 Pmc), 2.54 (s, 3H, CH_3 Pmc), 2.49 (m, 1H, H3a), 2.12 (bd, J = 13.9 Hz ,1H, H3b), 2.10 (s, 3H, CH_3 Pmc), 1.84 (dd, J = 6.8, 6.8 Hz, 2H, CH_2 Pmc), 1.77-1.69 (m, 2H, Hβ Arg), 1.69-1.47 (m, 2H, Hγ Arg), 1.44 (s, 9H, Bu^t), 1.31 (s, 6H, CH_3 Pmc).

A solution of hydroxyethyl intermediate (128 mg, 0.158 mmol) in dry CH_3CN (3 mL) was treated with MsCl (73 μL, 0.950 mmol) and Et_3N (221 μL, 1.58 mmol) and the resulting mixture was stirred at room temperature for 24 h before being evaporated in vacuum. The crude residue was dissolved in EtOAc, washed with aq saturated $NaHCO_3$ solution and extracted. The organic layer was dried, filtered and evaporated leaving an intermediate which was directly dissolved in dry DMF (3 mL) and treated with NaN_3 (107 mg, 1.26 mmol). The resulting mixture was heated to 50 °C and maintained at this temperature for 24 h. After this time the solution was concentrated and the residue was treated with aq $NaHCO_3$ solution and extracted with EtOAc. The organic layer was dried, filtered and evaporated under reduced pressure. The crude was subjected to flash chromatographic purification (EtOAc/MeOH, 90:10) affording an azidoethyl cyclopeptide (92 mg, 70%) as a white solid: ^{1}H NMR (400 MHz, CD_3OD) δ 4.64 (dd, J = 5.6, 5.6 Hz, 1H, Hα Asp), 4.45 (m, 1H, H4), 4.16 (d, J = 13.8 Hz, 1H, Hα Gly), 4.10 (dd, J = 7.1, 7.1 Hz, 1H, Hα Arg), 3.75 (d, J = 8.8 Hz, 1H, H2), 3.46-3.30 (m, 3H, Hα Gly, H2′a,b), 3.21 (m, 2H, H1′a,b), 3.07 (bd, J = 8.7 Hz, 1H, H5a), 2.98 (m, 3H, H5b, Hδ Arg), 2.70 (m, 4H, CH_2 Pmc, Hβ Asp), 2.59 (s, 3H, CH_3 Pmc), 2.58 (s, 3H, CH_3 Pmc), 2.43 (m, 1H, H3a), 2.12 (s, 3H, CH_3 Pmc), 2.02 (d, J = 11.4 Hz, 1H, H3b), 1.86 (dd, J = 6.7, 6.7 Hz, 2H, CH_2 Pmc), 1.77-1.68 (m, 2H, Hβ Arg,), 1.68-1.50 (m, 2H, Hγ Arg), 1.46 (s, 9H, Bu^t), 1.33 (s, 6H, CH_3 Pmc).

Azidoethyl intermediate (92 mg, 0.111 mmol) was dissolved in MeOH (10 mL) and a catalytic amount of 10% palladium on carbon was added. The reaction vessel was degassed under vacuum and thoroughly purged with hydrogen (three times). The resulting heterogeneous mixture was stirred under hydrogen for 4 h at room temperature, then the catalyst was filtered off and the filtrate was concentrated in vacuum. The crude was subjected to flash chromatographic purification (EtOAc/MeOH, 80:20) furnishing aminoethyl cyclopeptide **5** (80 mg, 89%) as a colourless glassy solid: ^{1}H NMR (400 MHz, CD_3OD) δ 4.59 (dd, J = 7.4, 6.0 Hz, 1H, Hα Asp), 4.27 (dd, J = 5.3, 5.3 Hz, 1H, H4), 4.11 (d, J = 13.8 Hz, 1H, Hα Gly), 4.10 (dd, J = 7.6, 7.6 Hz, 1H, Hα Arg), 3.48 (bd, J = 9.1 Hz, 1H, H2), 3.25 (d, J = 13.8 Hz, 1H, Hα Gly), 3.16 (m, 2H, Hδ Arg), 3.04 (bd, J = 9.3 Hz, 1H, H5a), 2.98 (dd, J = 9.2, 5.0 Hz 1H, H5b), 2.94-2.74 (m, 4H, H1′a,b, H2′a,b), 2.74-2.54 (m, 4H, CH_2 Pmc, Hβ Asp), 2.53 (s, 3H, CH_3 Pmc), 2.52 (s, 3H, CH_3 Pmc), 2.46 (m, 1H, H3a), 2.07 (s, 3H, CH_3 Pmc), 1.99 (d, J = 13.2 Hz,

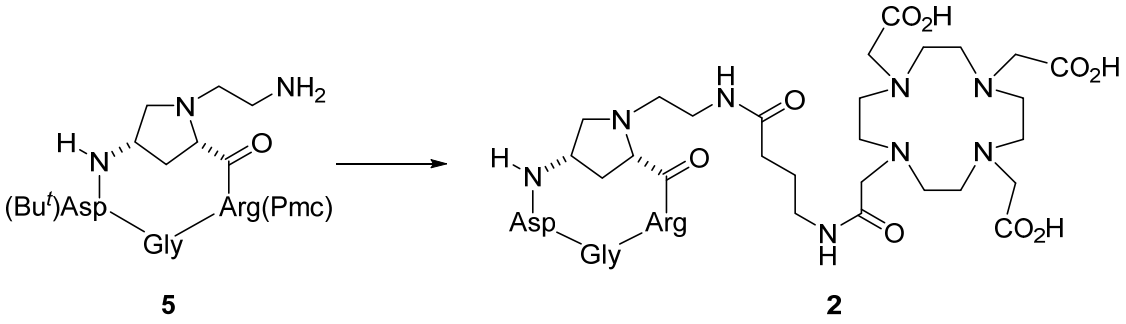
1H, H3b), 1.81 (dd, J = 6.8, 6.8 Hz, 2H, CH_2 Pmc), 1.74-1.61 (m, 2H, Hβ Arg,), 1.60-1.44 (m, 2H, Hγ Arg), 1.40 (s, 9H, Bu^t), 1.28 (s, 6H, CH_3 Pmc). MS (ESI$^+$) *m/z* 806.6 $[M+H]^+$, 828.5 $[M+Na]^+$.

***cyclo*[Arg-Gly-Asp-1-(2-aminoethyl)Amp] (1)**



The protected cyclic tetrapeptide **5** (20 mg, 0.025 mmol) was dissolved in 0.25 mL of a TFA/TIS/H_2O (95:2.5:2.5) mixture and stirred for 4 h at room temperature. Then, the solvent was evaporated and the crude residue was thoroughly washed with Et_2O. Semi-preparative RP-HPLC purification (C_{18}-10 μm column 10 × 250 mm; acetonitrile in H_2O (0.05% TFA); 0-25% linear gradient over 25 min at room temperature; flow rate 4.0 mL/min; detection 220 nm; R_t = 9.7 min) and lyophilisation furnished the deprotected *c*(AmpRGD)-NH_2 **1** (13.6 mg, TFA salt, 91%) as a colourless glassy solid. The ^1H, ^{13}C NMR and mass characterizations of compound **1** fully matched the data reported in the literature.[1]

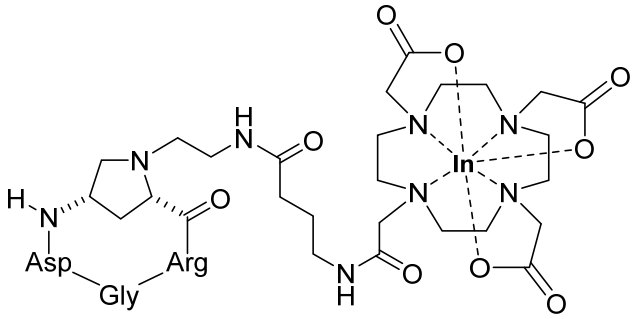
***cyclo*(AmpRGD)-DOTA conjugate (2)**



Protected DOTA-derivative **6** (53 mg, 0.080 mmol) was dissolved in dry DCM (4 mL) under argon and HBTU (42 mg, 0.11 mmol) was added. The resulting mixture was stirred at room temperature for 20 min, then a solution of protected cyclopeptide **5** (60 mg, 0.074 mmol) and DIEA (64 μL, 0.37 mmol) in dry DCM (4 mL) was added. The reaction mixture was stirred overnight and, after reaction completion, the solvent was evaporated under reduced pressure. The crude was dissolved in EtOAc and washed with saturated aqueous NH_4Cl and brine. The organic phase was evaporated and subjected to flash-chromatographic purification on Biotage system eluting with DCM/MeOH (NH_3) from 95:5 to 80:20. Protected intermediate (not shown) was treated with 2.5 mL of a TFA/TIS/H_2O (95:2.5:2.5) mixture at room temperature. After reaction completion, water was removed and the crude was purified by semi-preparative RP-HPLC (C_{18}-10 μm column 10 × 250 mm) using acetonitrile in H_2O (0.05% TFA), 0-25%

linear gradient over 18 min at room temperature, flow rate of 3.0 mL min^{-1} and detection at 220 nm (R_t = 15.0 min). Pure DOTA-conjugate **2** was obtained after lyophilisation as a white solid (47 mg, TFA salt, 60% yield over two steps). The ^{1}H, ^{13}C NMR and mass characterizations fully matched the data reported in the literature.[1]

Synthesis of cold In-DOTA-conjugate (In-2)



$InCl_3$ (1.1 mg, 5.0 μmol) in HCl 0.05 M (700 μL) was added to a solution of DOTA-conjugate **2** (5.4 mg, 5.0 μmol) dissolved in of NH_4OAc buffer (0.2 M, 1.2 mL) in a plastic vial under argon atmosphere. The resulting mixture was heated to 100 °C for 20 min. After reaction completion, the solvent was removed under reduced pressure and the crude was purified by reverse phase HPLC (RP C_{18}-10 μm, 10 × 250 mm column) using acetonitrile in H_2O (0.05% TFA), 0-25% linear gradient over 18 min at room temperature. A flow rate of 3.0 mL min^{-1} was used and detection was at 220 nm. R_t = 14.0 min. After lyophilisation, pure In-DOTA-*c*(AmpRGD) **In-2** was recovered as a white powder (5.8 mg, TFA salt, 99% yield). Formation of the complex was confirmed by MS analysis: MS (ESI^+) *m/z* 534.1 $[M+2H]^{2+}$; 1067.4 $[M+H]^+$.

Stability of conjugate In-2

Pooled rat and human plasma employed for stability experiments was supplied by Tebu-Bio (Tebu-Bio Srl, Milan, Italy) and stored at −70 °C until use. In vitro stability of conjugate **In-2** was evaluated in 80% v/v rat and human plasma buffered with 10 mM phosphate buffered saline (PBS, pH 7.4) and analysis was performed by liquid chromatography coupled to mass spectrometry (HPLC-ESI-MS/MS). Briefly, rat and human plasma were pre-incubated at 37 °C for 10 min before adding **In-2**, reaching a 5 μM final concentration. At different time points (30 min, 1, 2, 4, 6 hours), aliquots of the incubation mixture were withdrawn, treated with two volumes of acetonitrile and centrifuged (14000 g, 10 min, 4 °C). An aliquot of the resulting supernatant was evaporated to dryness, reconstituted in HPLC eluent and analyzed by HPLC-ESI-MS/MS to determine the percentage of unmodified complex. A Thermo Accela UHPLC system equipped with an Accela Open AS autosampler interfaced to a TSQ Quantum Access MAX triple quadrupole mass spectrometer (Thermo Italy, Milan, Italy) with an heated electrospray ionization (H-ESI) ion source was employed for data acquisition. Mass spectrometric analyses were done in positive ion mode. H-ESI interface parameters were set as follows: probe, middle (D) position; capillary temperature, 270 ° C; spray voltage, 3.0 kV.

Nitrogen was used as nebulizing gas at the following pressure: sheath gas, 35 psi; auxiliary gas, 15 arbitrary units (a.u.). Argon was used as the collision gas at a pressure of approximately 1.5 mtorr. For quantitative analysis, the following multiple parent ion → product ions transitions were selected: **In-2** (cold conjugate): *m/z* = 534.1 $[M+2H]^{2+}$→ *m/z* = 346.1, 627.1, 694.1 (tube lens 92 V; collision energy 26, 28, 28 eV); **2** (ligand): *m/z* = 497.2 $[M+2H]^{2+}$→ *m/z* = 346.1 (tube lens 107 V; collision energy 24 eV). A Phenomenex Gemini-NX C18 column (150 × 4.6 mm; 5 µm particle size) was used for compound separation following a gradient elution. Flow rate was set at 1000 µL min^{-1}. Solvent A: water and solvent B: acetonitrile both additioned of 0.1% v/v formic acid. HPLC gradient was as follows: t(0 min): A: 100%; B: 0%; t(1 min): A: 100%; B: 0%; t(11 min): A: 60%; B: 40%; t(13 min): A: 5%; B: 95%; t(15 min): A: 5%; B: 95%; t(16 min): A: 100%; B: 0% followed by 10 min of column reconditioning. Retention times were: 4.1 min for **In-2** and 5.2 min for **2**. Data acquisition and processing were performed employing Thermo Xcalibur software (version 2.1).

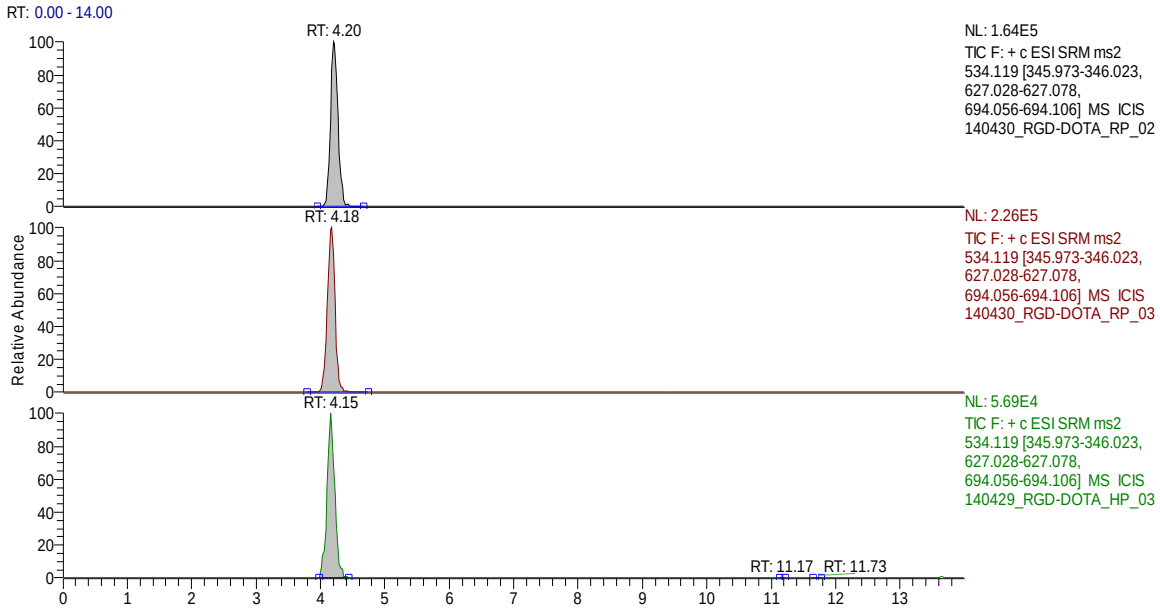


Fig. S1 HPLC-ESI-MS/MS chromatograms showing the peaks at the MRM transitions (m/z 534.1 → m/z 346.1; 627.1; 694.1) corresponding to cold **In-2** in buffer PBS (first row), in 80% v/v rat (second row) and human (third row) plasma after 6 h incubation at 37° C.

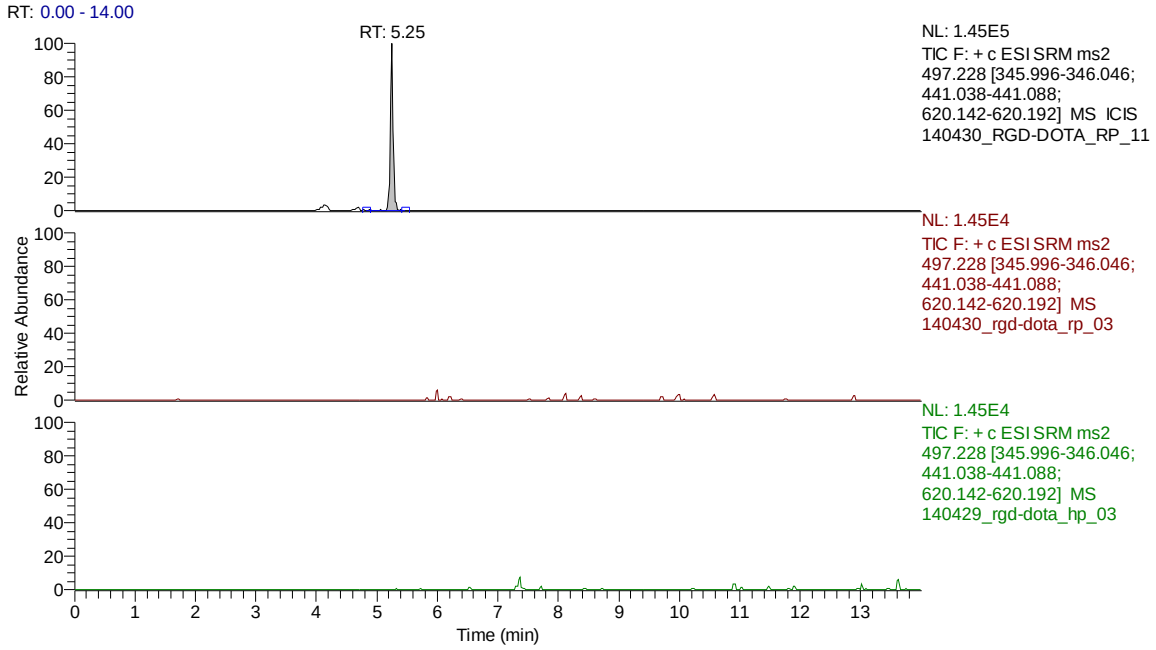


Fig. S2 HPLC-ESI-MS/MS chromatograms showing the peak corresponding to ligand **2** (R_t: 5.25 min) in buffer PBS and the absence of the same peak in the 6 h incubations in rat (second row) and human plasma (third row).

Determination of distribution coefficient (Log $D_{oct,7.4}$)

Distribution coefficient (*D*) at physiological pH was determined for **In-2** by the shake-flask method at room temperature (21.0±0.5 °C), employing *n*-octanol and 50 mM morpholino propan-3-sulphonic acid (MOPS) buffer (pH 7.4), 0.15 M KCl ionic strength, mutually saturated by overnight stirring. A weighted amount (0.3-0.5 mg) of compound was dissolved in 0.5 mL of buffer, water-saturated *n*-octanol was added (0.3-0.5 mL) and the sample was stirred for 4 h to reach partition equilibrium. Partition phases were then centrifuged (9000 g, 10 min, 20 °C), manually separated, diluted with methanol and dosed in HPLC-MS/MS, employing the elution conditions described for stability assays. Reported Log $D_{oct,7.4}$ is the mean ± SD (n=3).

Radiolabelling procedures

DOTA-conjugate **2** (2.28 µg, 2.39 nmol) was dissolved in degassed NH_4OAc buffer (11.5 µL, 0.2 M, pH 5.5), and $^{111}InCl_3$ solution (100 µL, 40 MBq in 0.05 M HCl) was added to a final ligand concentration of 0.02 mM and 1:6.2 In:ligand ratio. The pH of final solution was 4.0 and the mixture was warmed to 100 °C in a reactor for 15 min under argon atmosphere; then labelling was confirmed by radio-TLC on paper strips (sodium citrate 0.2 N, pH 2 as eluent). The thin layer chromatograms were analyzed with a Raytest miniGITA TLC scanner, the radiochemical purity (RCP) of ^{111}In-DOTA conjugate **^{111}In-2** resulted >99% without further purification (Figure S3). Aiming at further lowering the In:ligand ratio to 1:3.1, $^{111}InCl_3$ (100 µL, 40 MBq in 0.05 M HCl) was added to compound **2** (1.14 µg, 1.19 nmol) dissolved in NH_4OAc buffer reaching final pH 4.0. The mixture was warmed to 100 °C for 15 min affording

a good >93% RCP (specific activity 33.6 MBq/nmol) without further purification (Figure S4), which was judged viable for following in vivo studies. Saline solution (1 mL) was added to reach a final radioactivity concentration of 35.9 MBq/mL before injection to mice.

Radiochemical purity of ^{111}In-2

Radio-TLC used Whatman paper strips and sodium citrate 0.2 N (pH 2) as eluent. Using this method, the ^{111}In-labelled AmpRGD ligand **^{111}In-2** remained at the origin, while the free **^{111}In** migrates to the solvent front.

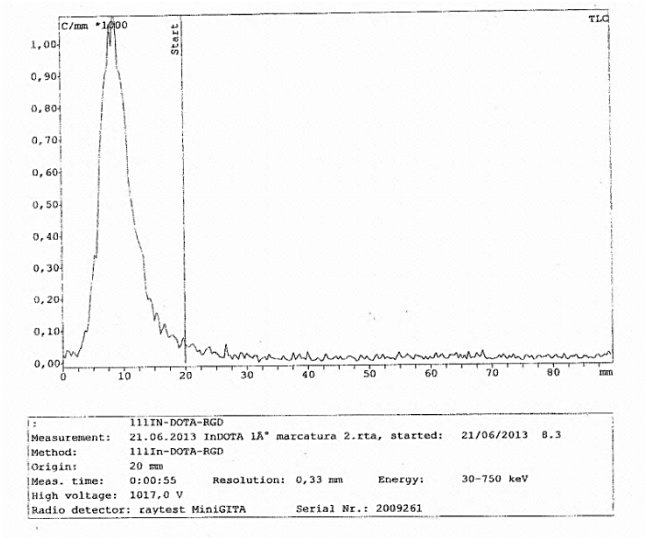


Fig. S3 Radiochromatogram (radio-TLC) of **^{111}In-2** (1:6.2 In:ligand ratio, pH 4.0).

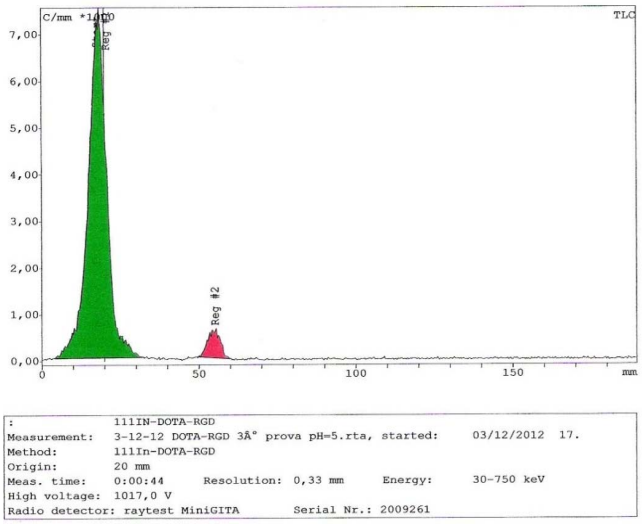


Fig. S4 Radiochromatogram (radio-TLC) of **^{111}In-2** (1:3.1 In:ligand ratio, pH 4.0).

BIOLOGY

Solid phase receptor binding assay

Purified $\alpha v\beta_3$ receptor (Millipore, Temecula, CA, USA) was diluted to 0.5 μg/mL in coating buffer containing 20 mM Tris-HCl (pH 7.4), 150 mM NaCl, 1 mM $MnCl_2$, 2 mM $CaCl_2$ and 1 mM $MgCl_2$. An aliquot of diluted receptor (100 μL/well) was added to 96-well microtiter plates (NUNC MW 96F Maxisorp Straight) and incubated overnight at 4 °C. The plates were then incubated with blocking solution (coating buffer plus 1% bovine serum albumin) for additional 2 h at room temperature to block nonspecific binding, followed by 3 h incubation at room temperature with various concentrations (10^{-12}–10^{-5} M) of test compounds in the presence of 1 μg/mL biotinylated VN (Molecular Innovations, Inc., Novi, MI, USA). Biotinylation was performed using an EZ-Link Sulfo-NHS-Biotinylation kit (Pierce, Rockford, IL, USA). After washing, the plates were incubated for 1 h at room temperature with biotinylated streptavidin–peroxidase complex (Amersham Biosciences, Uppsala, Sweden) followed by 30 min incubation with 100 μL/well substrate reagent solution (R&D Systems, Minneapolis, MN, USA) before stopping the reaction by addition of 50 μL/well 2 N H_2SO_4. Absorbance at 415 nm was read in a Synergy™ HT Multi-Detection Microplate Reader (BioTek Instruments, Inc.). Each data point represents the average of triplicate wells; data analysis was carried out by nonlinear regression analysis with Prism GraphPad program. For the $\alpha_5\beta_1$ binding assay, NUNC Maxisorp 96-wells plate were coated with $\alpha_5\beta_1$ receptor (R&D Systems, Inc., Minneapolis, MN, USA) diluted to 0.5 μg/mL in coating buffer and blocked as described for $\alpha v\beta_3$. The plates were then incubated with various concentrations (10^{-12}–10^{-5} M) of test compounds in the presence of 1 μg/mL biotinylated FN (Sigma, St. Luis MO, USA) obtained as described for VN and visualized as described above.

Flow cytometric determination of integrin expression

Melanoma and EPC cells were detached by gentle treatment with Accutase in 0.5 mM EDTA solution, washed, and incubated for 1 h at 4 °C in the presence of anti-$\alpha v\beta_3$ and anti-β_1 monoclonal antibody (1 μg/50 μL, anti-integrin $\alpha v\beta_3$, clone LM609, Millipore; anti-CD29, β1 subunit, Immunotools). Cells were then washed and incubated for 1 h at 4 °C with a secondary antibody, 5 μg/mL goat antimouse IgG conjugated with FITC (Santa Cruz Biotechnology, Inc., Santa Cruz, CA). Cells were analyzed at 488 nm on the flow cytometer FACScan system (BD-FACS Canto). EPC cells were also characterized for specific marker expression: anti-Ulex (Ulex europaeus I lectin), anti-CD44, anti-CD31, anti-KDR (data not shown).

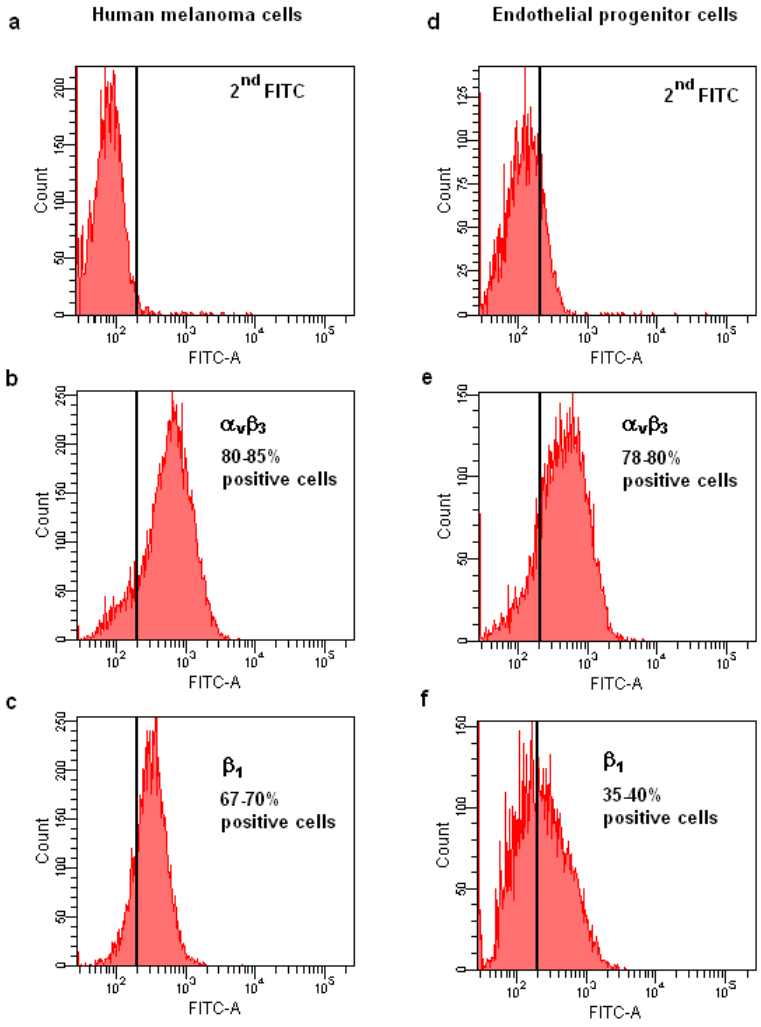


Fig. S5 Flow cytometric determination of integrin subunit expression. Representative analysis of $\alpha_V\beta_3$ and β_1-positive A375M human melanoma cells (panels a, b and c). Representative analysis of $\alpha_V\beta_3$ and β_1-positive human endothelial progenitor cells (panels d, e and f). The levels of integrin subunit expression are reported as percentage of positive cells relative to cells exposed to a secondary antibody (2^{nd} FITC).

Cell biology assays

Cell lines and growth conditions The A375M human melanoma cell line was obtained from the American Type Culture Collection (ATCC, Rockville, MD, USA) and grown in Dulbecco's Modified Eagle Medium, containing 4500 mg/L glucose (DMEM 4500, GIBCO) supplemented with 10% fetal calf serum (FCS) at 37 °C in a humidified incubator containing 10% CO_2. Then 5.0×10^5 melanoma cells were seeded in 100 mm dishes and propagated every 3 days by incubation with a trypsin-EDTA solution. Cultures were periodically monitored for mycoplasma contamination. Primary cultures of human EPC, isolated from the umbilical vein as previously described,[2] were grown in Endothelial cell Growth Medium-2 (EGM-2) containing endothelial cell growth supplements, and were subcultivated using a trypsin-EDTA solution, 1:3 split ratio. Cells between passages 2 and 6 grown to confluence in plates coated with 1% bovine gelatin were used in the experiments.

In vitro cell adhesion assay Plates (96 wells) were coated with FN (1 μg/mL), or VN (10 μg/mL) by overnight incubation at 4 °C and then incubated at 37 °C for 1 h with PBS−1% BSA. Melanoma cells or EPC were counted, resuspended in serum free medium, and exposed to compounds **1**, **2** and In-labelled **In-2** (final concentrations 0.01, 0.1, 1, or 10 μM) at 37 °C for 30 min to allow the ligand-receptor equilibrium to be reached. Cell adhesion assays were performed in the presence or in the absence of 2 mmol/L $MnCl_2$. Cells were plated ($4-5 \times 10^4$ cells/well) and incubated at 37 °C for 2 h. Non-adherent cells were removed with PBS, and adherent cells were stained with 0.5% crystal violet solution in 20% methanol. After 2 h of incubation at 4 °C, plates were examined at 540 nm in a counter ELX800 (Biotek Instruments). Experiments were conducted in triplicate and were repeated at least three times. Data are presented as means ± SD from three independent experiments.

In vitro capillary network formation Matrigel (10 μg/μL, BD Bioscience, San Jose, CA) precoated 96-well plates were used, and EPC (20.000 cells/well) were seeded in the presence of compounds **1**, **2** and **In-2** (final concentration 10 μM). Cells were incubated at 37 °C for 16-18 h in the presence of a serum free medium, and capillary-like tube structures were examined and photographed by using a Nikon inverted microscope. The experiments were repeated in triplicate.

IN VIVO STUDIES

All experimental procedures involving animals were performed in accordance with national guidelines, approved by the Ethical committee of the Animal Welfare Office of the Italian Work Ministry and conformed to the legal mandates and Italian guidelines for the care and maintenance of laboratory animals.

Human melanoma xenogratfs Male CD1 nu/nu mice (6-8 weeks old; Charles River Laboratories International) were fed with a regular chow diet (Harlan Laboratories) and water ad libitum. The animals (n=3) were injected subcutaneously with 100 μL of PBS containing 1×10^6 A375M cells. Tumor growth was monitored daily.

Biodistribution in healthy mice

Biodistribution studies were performed in male CD1 nu/nu healthy mice (n=3) to evaluate radioactivity distribution at 30, 120 min and 4 h post injection using a Micro PET/SPECT/CT for small animals Trifoil FLEX™ Triumph. Radiolabelled DOTA-conjugate **^{111}In-2** (2.7-3.0 MBq) was diluted with 200 μL physiological saline solution and injected in the tail vein of healthy mice kept under isoflurane anaesthesia. CT scan was performed 10 min p.i. followed by SPECT tomography at 30, 120 min and 4 h p.i. with animals being kept under anaesthesia on a warmed bed and monitoring the breath frequency. A further acquisition was taken at 48 h p.i. to ascertain the complete clearance of the radiotracer (Figure S6). The CT scan was acquired with 50 kV and 320 μA tube settings. The magnification factor was set to 2 resulting in an axial field of view of 59.2 mm. 512 Projections, 500 μsec each, were acquired on a circular orbit in 256 sec with an acquisition matrix of 1184×1120, 115.6 μm pixel size. Having the animal bed

in the same position, 64 SPECT projections (20 sec each) were acquired on a circular orbit with radius of rotation of 40 mm and a 5-pinhole high-resolution collimator (1 mm hole diameter, 75 mm focal distance) for a total acquisition time of about 22 min. The acquisition matrix was 80×80, 1.5 mm pixel size. The photopeak window width was set to 10% of both ^{111}In photopeak (171 and 245 keV). Once the scanning session was complete, animals were recovered in a housing unit and monitored until awake.

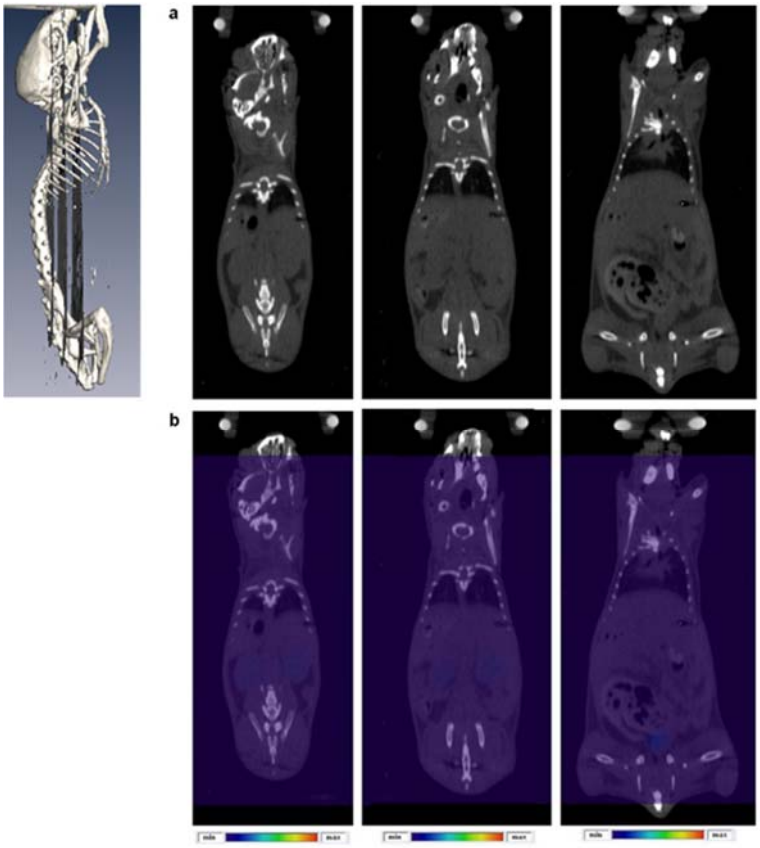


Fig. S6 Biodistribution of **^{111}In-2** in healthy mice at 48h p.i.. CT images (panel a) and SPECT/CT images (panel b) taken at different levels of mouse body. Radioactivity in different organs was undetectable.

Human melanoma xenografts uptake

Saturation experiments (SE) and displacement experiments (DE) were performed in CD1 immunodeficient mice bearing A375M human melanoma xenografts. SE were performed by the injection of **^{111}In-2** (3.55 MBq) and the radioligand tumor uptake was evaluated at 30, 60 and 120 min p.i.. DE were performed two days after the SE and preceded by SPECT analysis to exclude the presence of residual radioactivity in xenografted mice; DE were performed by i.p. injection of compound **2** (30 μg dissolved in 200 μL of physiological saline solution) 10 min before administration of radiolabelled compound to 400:1 final ratio (1.6 mg/kg). Radiotracer uptake was measured in tumor tissue at 30, 60, 120 min p.i.. Data were normalized to muscular tissue, corrected for background activity and expressed as mean *nACt* ± SD (n=3).

Data analysis of small-animal SPECT/CT studies The CT image data set was reconstructed using Filtered Back Projection (FBP) algorithm implementing an apodization low pass filter devoted to statistical noise minimization. The reconstruction matrix was 512×512×512, 180 μm pixel size. SPECT data were reconstructed with an Ordered Subset Expectation Maximization (OSEM) algorithm (5 iterations, 8 subsets), reconstruction matrix 60×60×60, pixel size 1.5 mm. Fused images were visually analyzed to detect regional tracer activities and quantitatively analyzed using the software Vivid (Gamma Medica-Ideas, Northridge, CA). For biodistribution studies in healthy animals, absolute activity concentration in the target organ (*AACt*) was analyzed in heart, lungs, liver, gallbladder, spleen, pancreas, stomach, small and large intestine, kidneys, and urinary bladder; in particular for the intestine *AACt* was assessed at the site showing the highest tracer uptake. Elliptical Regions Of Interest (ROIs) were hand-drawn delineated corresponding to a volume of approximately 0.5 mL for each organ; as reference tissue, contralateral muscle tissue was selected (*AACr*).[3] Data were decay-corrected to the injection time and administered activity was corrected for residual activity in the syringe. Final *AACt* data for each organ were normalized (*nACt*) to muscle activity as background activity according to the formula:

$$nACt = \frac{AACt - AACr}{AACr}$$

The biodistribution data expressed as *nACt* ratios are reported as mean plus SD based on the results from three animals at each time point. Displacement experiments data are shown as mean plus SD from three animals. The level of significance was set at $p<0.05$.

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