Supplementary Information

Integrin and Matrix Metalloprotease Dual-Targeting with MMP substrate-RGD conjugate

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Table of contents

General chemical procedures	2
Synthesis of cyclopeptide 4	3
Synthesis of cyclopeptide 5	3
Synthesis of peptide 6	4
Synthesis of compound 1	4
RP-HPLC profile and ESI-MS analysis of 5	5
RP-HPLC profile and ESI-MS analysis of 6	6
RP-HPLC profile and ESI-MS analysis of 1	7
RP-HPLC profile and ESI-MS analysis of 2	8
Cell lines and culture conditions	9
Fluospectroscopy	9
Flow cytometry analysis of the integrin $\alpha_v\beta_3$ expression	10
In vivo fluorescence imaging	11

General chemical procedures

Materials

All Fmoc amino acid derivatives and resins were purchased from Advanced ChemTech Europe (Brussels, Belgium), Bachem Biochimie SARL (Voisins-Les-Bretonneux, France) and France Biochem S.A. (Meudon, France). PyBOP was purchased from France Biochem and other reagents were obtained from either Aldrich (Saint Quentin Fallavier, France) or Acros (Noisy-Le-Grand, France). RP-HPLC was performed on Waters system equipped with a Waters 600 controller and a Waters 2487 Dual Absorbance Detector. The purity of peptide derivatives was analyzed on an analytical column (Macherey-Nagel) using the following solvent system: solvent A, water containing 0.09% TFA; solvent B, acetonitrile containing 0.09% TFA and 9.91% H₂O. UV absorbance was monitored at 214 nm and 250 nm simultaneously. Preparative column (Delta-PakTM 100 Å 15 μ m C18 particles, 200x2.5 mm) was used to purify the crude peptides (when necessary) by using an identical solvent system at a flow rate of 22 mL.min⁻¹. ESI mass spectra were recorded on an Esquire 3000 (Bruker) spectrometer. The analysis was performed in the positive mode for peptide derivatives using 50% aqueous acetonitrile as eluent.

Solid-Phase Peptide Synthesis

Assembly of all protected peptides was carried out using the Fmoc/t-Bu strategy manually in a glass reaction vessel fitted with a sintered glass frit or automatically on a peptide synthesizer using 2chlorotritylchloride. Coupling reactions were performed manually by using 2 equiv of N-Fmoc-protected amino acid (relative to the resin loading) activated in situ with 2 equiv of PyBOP and 3-5 equiv of diisopropylethylamine (DIPEA) in DMF (10 mL/g resin) for 30 min except for the first coupling on 2chlorotritylchloride. The coupling efficiency in manual synthesis was assessed by Kaiser and/or TNBS tests. N-Fmoc protecting groups were removed by treatment with a piperidine/DMF solution (1:4) for 10 min (10 mL/g resin). The process was repeated three times and the completeness of deprotection verified by UV absorption of the piperidine washings at 299 nm. Synthetic linear peptides were recovered directly upon acid cleavage. Before cleavage, the resin was washed thoroughly with methylene chloride. The linear peptides were then released from the resin by treatments with a solution of acetic acid/trifluoroethanol/methylene chloride (1:1:8, 10 mL/mg resin, 2x30 min). Hexane (5-10 volumes) was added to the collected filtrates, and the crude peptides were isolated after concentration as white solids. The residue was dissolved in the minimum of methylene chloride and diethyl ether was added to precipitate peptides. Then, they were triturated and washed three times with diethyl ether to obtain crude materials that were used in the next step without further purification.

Synthesis of cyclopeptide 4



Linear peptide was prepared using building blocks as previously described.¹ linear peptide (0.5 mM) was dissolved in DMF and the pH values were adjusted to 8-9 by addition of DIPEA. PyBOP (1 equiv) was added and the solution stirred at room temperature for 1 h. Solvent was removed under reduced pressure and the residue dissolved in the minimum of methylene chloride. Diethyl ether was added to precipitate peptide. Then it was triturated and washed three times with diethyl ether to obtain crude material that was used in the next step without further purification.

Synthesis of cyclopeptide 5



Full deprotection of compound **4** (157.2 mg, 86 μ mol) was carried out using 10 mL of a solution containing TFA/H₂O/TIS (95:2.5:2.5) at room temperature during 3 h. The product was isolated after removal of solvents under reduced pressure and precipitation from diethyl ether to yield deprotected peptide as a white powder in quantitative yield (169 mg, 86 μ mol). In parallel, cyclo[-Arg-Gly-Asp-D Phe-Lys(-CO-CHO)-] was synthesized as previously described.²

To an aqueous solution (2 mL) containing the deprotected peptide (20 mg, 10.2 μ mol) was added freshly prepared cyclo[-Arg-Gly-Asp-D Phe-Lys(-CO-CHO)-] (67.5 mg, 87 μ mol). The reaction was stirred for 1 h at 25° C. The RGD-containing conjugate was isolated after purification by RP-HPLC as a white powder (40.1 mg, 8.8 μ mol, 86%). A serine oxidation of RGD-containing conjugate (20 mg, 4.3 μ mol) by an aqueous solution containing 10 equiv. of NaIO₄ (10.8 mg, 50.4 μ mol) afforded the aldehyde component **5**. The product was directly purified by RP-HPLC to yield compound **5** as a white powder (14.6 mg, 3.3 μ mol, 77%).

¹ S. Foillard, M. O. Rasmussen, J. Razkin, D. Boturyn, P. Dumy, J. Org. Chem. 2008, 73, 983.

² D. Boturyn, P. Dumy. *Tetrahedron Lett.*, **2001**, *42*, 2787.

Synthesis of peptide 6

Peptide synthesis was carried out as described in the general procedure. Full deprotection of protected peptide $(Boc)_2NO-CH_2-CO-Gly-Lys(Boc)-Gly-Pro-Gln(Trt)-Gly-Leu-Ala-Gly-Gln(Trt)-Lys(Boc)-Gly-OH (20 mg, 9.8 µmol) was done from a solution of TFA/H₂O/TIS/EDT (90:5:2.5:2.5) during 2 h. The product was directly purified by RP-HPLC to yield compound$ **6**as a white powder (6 mg, 4 µmol, 41%).

Synthesis of compound 1



To a solution containing the derivative **5** (17 mg, 3.8 μ mol) in 400 μ L of H₂0/CH₃CN (1:1) was added the peptide **6** (6 mg, 4 μ mol). The reaction was stirred for 3 h at 25 ° C. MMP-containing conjugate was isolated after purification by RP-HPLC as a white powder (10 mg, 1.7 μ mol, 46%). To a solution containing the MMP-containing conjugate (4 mg, 0.7 μ mol) in 180 μ L of DMF was added 3 μ L DIPEA and Cyanine 5-mono NHS ester (1.7 mg, 1.8 μ mol). The reaction was stirred overnight at room temperature. The product was directly purified by RP-HPLC to yield compound **1** as a blue powder (1.5 mg, 0.22 μ mol, 31%).

RP-HPLC profile of **5** monitored at 214 nm.

Nucleosil column (300Å 6 µm C18 particles, 125 x 3 mm); linear gradient (5 to 100% B in 20 min; flow rate: 1mL/min).



ESI-MS analysis of compound 5



Deconvoluted mass: $M_{found} = 3935.2$; $M_{calc} = 3934.89$

RP-HPLC profile of **6** monitored at 214 nm. Nucleosil column (300Å 6 µm C18 particles, 125 x 3 mm); linear gradient (5 to 100% B in 20 min; flow rate: 1mL/min).





ESI-MS analysis of compound 6



Deconvoluted mass: $M_{found} = 1170.6$; Mcalc = 1169.62

RP-HPLC profile of 1 monitored at 214 nm.

Nucleosil column (100Å 5 µm C18 particles, 250 x 4.6 mm); linear gradient (5 to 100% B in 30 min; flow rate: 2 mL/min).



ESI-MS analysis of compound 1



Deconvoluted mass: $M_{found} = 6364.9$; $M_{calc} = 6364.92$

RP-HPLC profile of MMP cleavage affording peptide fragment **2** monitored at 214 nm. Nucleosil column (100Å 5 μ m C18 particles, 250 x 4.6 mm); linear gradient (5 to 100% B in 30 min; flow rate: 2 mL/min).

 $2 \mu M$ of compound 1 in Tris Buffer (pH 7.8) was incubated with 5 nM of MMP-9 at 37°C for 24 hours, and analyzed by using RP-HPLC. The products observed were afterwards characterized using mass spectrometry.



RT(1) = 17.9 min; RT(2) = 17.4 min

ESI-MS analysis of compound 2



Deconvoluted mass: $M_{found} = 5171.4$; $M_{calc} = 5171.39$

Cell lines and culture conditions

HEK293(β 3), stable transfectants of human β 3 from the human embryonic kidney cell line (kindly provided by J.-F. Gourvest, Aventis, France), HT1080 (human fibrosarcoma cell line) and A375 (human melanoma cell line) are cultured in DMEM supplemented with 1% glutamine, 10% fetal bovine serum (FBS), and in the case of HEK293(β 3) with 700 µg/mL Geneticin (G418 sulfate, Gibco, Paisley, UK). TS/A-pc are mouse mammary cancer cells cultured in RPMI 1640 supplemented with 1% glutamine, 10% fetal bovine serum (FBS), 1200 µg/mL Geneticin and 25 nM 2-mercaptoethanol. All cell lines are cultured at 37°C in a humidified 95% air/5% CO2 atmosphere.

Fluospectroscopy

Fluorescence emission spectra were recorded on a Perkin-Elmer LS 55 Fluorescence Spectrometer with PTP -1 Fluorescence Peltier System equipped with a thermo-stated compartment (37°C) using a 120 V pulsed xenon lamp (excitation wavelength: 640 nm). In parallel the absorption spectra were recorded of each sample using the UV-visible Spectrophotometer Evolution 201 of ThermoScientific.

Fluospectrometric analysis were carried out by using 200 μ L of 0.25 μ M of compound 1 or control Cy-5 containing peptide in 50 mM Tris Buffer (pH 7.5) with 150 mM NaCl, 5mM CaCl₂ and 0.05% Brij-35 at 37°C. Spectra were recorded from 650 to 750 nm.

Samples were incubated at 37°C and their spectra measured before incubation with the enzyme MatrixMetalloproteinase-9 (human recombinant MMP-9 catalytic domain, EnzoLifescience, Villeurbanne, France). Then, samples were incubated with 5nM of MMP-9 at 37°C, for 2 hours, 24 hours, and 48 hours respectively.

For Plasma assay, 1 μ M of fluorescent compound was incubated with 100 μ L of fresh mouse plasma. Spectra were measured as described above.

For MMP-Inhibition, MMP-9 was pre-incubated with SB-3CT and CTT (specific MMP inhibitor, EnzoLifescience, Villeurbanne, France) before incubation with $1 \mu M$ of compound **1**.

Activity of MMP was controlled by using the fluorogenic substrate Mca-RPPGFSAFK(Dnp) (EnzoLifescience, Villeurbanne, France) which is cleaved by MMP-2 and MMP-9.

Flow cytometry analysis of the integrin $\alpha_{v}\beta_{3}$ expression

For expression analysis, adherent cells are resuspended with trypsin, washed once with cold PBS, and another time with PBS containing 1 mM CaCl₂ and 1 mM MgCl₂ (Ca²⁺/Mg²⁺). One million cells in a final volume of 200 mL are fixed with 2% PFA for 15 minutes, washed with PBS Ca²⁺/Mg²⁺ twice and resuspended in 500µl PBS containing 20 µL of the antibody CD51/CD61 R-Phycoerythrin clone 23C6 mouse anti-human (Pharmingen, BD Bioscience). After 1 hour of incubation at 4°C the volume was adjusted to 5 mL and the samples were centrifuged for 15 min at 4 °C. The solutions are removed and pellets carefully rinsed twice with PBS Ca²⁺/Mg²⁺ (1 mM) at 4 °C. Cells are then rapidly analyzed by flow cytometry (FACS LSRII, BD bioscience, France). The results are reported as DiD fluorescence (FL4) histogram counts.



Cells without antibody

0.2% positive, PE mean 287



3.2% positive, PE mean 325



0.2% positive, PE mean 133

Cells treated with antibody





76.2% positive, PE mean 427



In vivo fluorescence imaging

Female NMRI Nude mice (6–8 weeks old, JANVIER, Le Genest Saint Isle, France) receive a sub-cutaneous xenograft of HEK293(b3) cells (20 x 10^6 per mouse), HT1080 (10 x 10^6 per mouse), A375 (10 x 10^6 per mouse), or TS/A-pc cells (5 x 10^6 per mouse). After tumor growth, mice (n = 3 for each group) are anesthetized (isoflurane/oxygen 4%/3.5% for induction and 2% thereafter) and are injected intravenously in the tail vein with 200 µL of compound 1 or Cy5 control peptide (2 nmol) or 150 µL of MMPSense680 (2 nmol; PerkinElmer's, Boston, USA), then they are illuminated by 633-nm (RAFT-RGD's) or 660-nm (MMPSense) light-emitting diodes equipped with interference filters. Fluorescence images as well as black and white pictures are acquired by a back-thinned CCD camera at -80 °C (ORCAII-BT-512G, Hamamatsu, Massy, France) fitted with high pass filter RG 9 (Schott, Jena, Germany).

All the animal experiments are performed in agreement with the EEC guidelines and the principles of laboratory animal care (NIH Publication No. 86-23, revised 1985). After imaging at different time points after injection (30 minutes, 1 hour, 2 hours, 3 hours, 5 hours and 24 hours), the mice are sacrificed (at either 3 hours or 24 hours after injection) and dissected for imaging organs. Image display and analysis are performed using the Wasabi software (Hamamatsu, Massy, France). The tumor/muscle ratio is calculated by using the semi-quantitative data obtained by drawing ROIs on the tumor and the muscle dissected. The results of organ fluorescence quantifications are expressed as a number of relative light units (RLU) per pixel.

All the data are given as mean \pm standard deviation (SD) of three independent measurements.



Fluorescence images of subcutaneous tumor-bearing Swiss nude mice at 5 hours after intravenous injection of 2 nmol of compound **1** compared to 2 nmol of compound **7** (Red arrow: tumor; white arrow: kidney; exposition Time 500 ms; LUT min to max: A375 1826-15527, TS/A-pc 1826-10148 and HEK293(β 3) 2711-10395).

A.	Cell line	Tumor (3 h)	Cell line	Tumor (24 h)	
	Hek293(β3)	•	Hek293(β3)	۲	
	TS/A-pc	۵	TS/A-pc		
	A375		A375	۲	
	HT1080		HT1080	Ş	

Excised tumor of compound **1** treated subcutaneous tumor bearing mice. The cell lines for xenograft used were Hek293(β 3), TS/A-pc, A375 and HT1080. Left frame shows the tumor after 3 hours of 2 nmol intravenous injection at 500 ms exposition time (LUT min to max: 1832 - 41530), and right frame after 24 hours at 500 ms exposition time (LUT min to max: 1832 - 6779).