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Supporting Information

Size-Dependent Cellular Uptake of RGD Peptides

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1 Experimental Section

General Information

Standard chemicals and solvents were purchased from commercial suppliers in highest purity (p.a.). Fmoc-Arg(Pbf)-OH, Fmoc-Asp(*t*Bu)-OH, Fmoc-Gly-OH, Fmoc-Lys(Boc)-OH and Fmoc-Val-OH were purchased from Iris Biotech GmbH or Carbolution Chemicals GmbH. Pyreneboronic acid was purchased from Sigma-Aldrich and PEG's from Iris Biotech GmbH. The water used for reactions, work-ups and purifications was purified by a Millipore Milli-Q bioceldevice. The plasmid vector encoding alcohol dehydrogenase was a kind of Prof. Dr. W. Hummel and the vector encoding the flavin reductase was donated by Prof. Dr. K.-H. van Pée. All final compounds were analysed by analytical RP-HPLC (220 nm) and NMR or MALDI-TOF-MS to confirm a purity of ≤95%.

Analytical RP-HPLC

Analytical RP-HPLC was performed on a Shimadzu Nexera XR UHPLC equipped with a pump LC-20AD, an autosampler SIL-20AXR, a column oven CTO-2CA, a diode array detector SPD-M20A and a communication module CBM-20A using a Phenomenex Luna C_{18} column (3.0 µm, 100×2.0 mm). Measurements were performed with a gradient (in 5.5 min from 5% B to 100% B, back in 0.1 min to 0% B, total run time 9 min) at 40 °C with 0.7 mL/min. Solvent A consists of 99.9% water, and 0.1% TFA, solvent B of 99.9% acetonitrile, and 0.1% TFA.

Analytical LC-MS

Analytical LC-MS and determination of ESI-HRMS (Phenomenex Luna C_{18} column (3.0 µm, 100×2.0 mm)) was performed on an Agilent 6220 TOF-MS (Agilent Technologies) with a dual ESI-source operating with a spray voltage of 2.5 kV, 1200 HPLC system with autosampler, degasser, binary pump, column oven and diode array detector. Nitrogen was generated by a nitrogen generator NGM 11 and served as nebulizer and dry gas. External calibration was performed with ESI-L Tuning Mix (Agilent Technologies). Measurements were performed with a gradient (in 11 min from 0% B to 100% B, back in 0.5 min to 0% B, total run time 15 min) at 40 °C with 0.3 mL/min. Solvent A consists of 94.9% water, 5% acetonitrile, and 0.1% formic acid, solvent B of 94.9% acetonitrile, 5% water, and 0.1% formic acid.

MALDI-TOF-MS

MALDI-TOF-MS was conducted with an Ultraflex (Bruker Daltonik) operated in reflectron positive mode using an LTB nitrogen laser MNL200 (337 nm, 50 Hz, 1000 shots/spectrum, maximum resolution: 20000) using DHB (2,5-dihydroxybenzoic acid) or 4-chloro-α-cyanocinnamic acid (ClCCA) as a matrix. Samples were dissolved in mixtures of acetonitrile and water and calibration was conducted

with PEG 400-1200. FlexControl 3.0 and FlexAnalysis 3.4 (Bruker Daltonik) were used for recording and processing.

Preparative HPLC

Preparative HPLC was performed using a Merck-Hitachi LaChrom HPLC consisting of interface D-7000, pump L-7150, detector L-7420 and a Hypersil Gold C_{18} column (1.9 µm, 250×21.2 mm) at a flow rate of 10 mL/min. Solvent A consist of 94.9% water, 5% acetonitrile, and 0.1% TFA, solvent B of 94.9% acetonitrile, 5% water, and 0.1% TFA.

method 1- halotryptophan

min	**Eluent A [%]**	**Eluent B [%]**
0	100	0
5	100	0
105	0	100

method 2 – Fmoc-Trp(7Br)

min	**Eluent A [%]**	**Eluent B [%]**
0	60	40
60	0	100

method 3 – protected, linear peptides

min	**Eluent A [%]**	**Eluent B [%]**
0	10	90
30	0	100

*method 4 – final purification of peptide **1***

min	**Eluent A [%]**	**Eluent B [%]**
0	90	10
45	0	100

*method 5 – peptide **2** after Boc removal*

min	**Eluent A [%]**	**Eluent B [%]**
0	50	50
50	0	100

*method 6 – peptide **2** after PEGylation*

min	**Eluent A [%]**	**Eluent B [%]**
0	80	20
80	0	100

UV/Vis absorbance spectroscopy

UV-visible spectroscopy was performed on an Agilent 8453 system equipped with a combination of deuterium-discharge lamp and a tungsten lamp to cover the UV/Vis wavelength range from 190-

1100 nm. As detector a photodiode array is used and a tempered 8-position sample holder with temperature control using PT-104A resistance thermometer. Measurements were done at 20 °C.

Fluorescence spectroscopy

Acquisition of fluorescence excitation and emission spectra were performed on a Jasco FP-8300 fluorescence spectrometer equipped with a peltier thermostated 4-position automatic cell changer (10 mm rectangular cells) at 20 °C using a Xe light source.

1.1 Enzymatic Bromination of Tryptophan

Halogenation on a preparative scale was performed using our previously established ADH-PrnF-RebH-combiCLEA method.[1] Briefly, cells overexpressing halogenase RebH (Uniprot ID: Q8HKZ8) from 1.5 L expression culture were applied for 1 L of reaction buffer consisting of 1 mM D-Trp, 30 mM NaBr, 15 mM Na_2HPO_4, 100 µM NAD^+, 1 µM FADH and 5 mL *i*-PrOH in water. When the conversion stops, solvent is removed under reduced pressure and the residue is first desalted with a manual C_{18} reversed-phase column (25 g, 400-220 mesh) and then purified by RP-HPLC. Fmoc-protection was performed according to our previously published procedure.[1]

1.2 Peptide Synthesis

Linear peptides were synthesized according to Fmoc/^{t}Bu chemistry in a plastic syringe with a polypropylene porous disk at rt on an automated shaker. Peptides were synthesized on 2-chlorotrityl chloride (CTC, 1 mmol/g) resin manually. Fmoc deprotection was performed by treating the resin with 20% piperidine in DMF for 2×20 min. Sequential washing steps were conducted with DMF or NMP (5×). Coupling of standard Fmoc-protected amino acids (5 equiv) was accomplished with *O*-(benzotriazol-1-yl)-*N*,*N*,*N*',*N*'-tetramethyluronium-tetrafluorborate (TBTU, 5 equiv) and DIPEA (10 equiv) in DMF for 2×15 min. Coupling of Fmoc-Trp(7Br)-OH (1.1 equiv) was performed with *O*-(7-azabenzotriazol-1-yl)-*N*,*N*,*N*′,*N*′-tetramethyluronium-hexafluorphosphate (HATU, 1.3 equiv) and 1-hydroxy-7-azabenzotriazole (HOAt, 1.3 equiv) with DIPEA (3 equiv) in NMP for 3 h and subsequent capping was accomplished with Ac_2O (10 equiv) and pyridine (10 equiv) in DMF for 2×20 min. Reaction control was performed by Kaiser-Test and LC-MS. Linear peptides were cleaved with 1% TFA/DCM (10×5 min) from the resin and filtered into *i*-PrOH (100 mL). The crude peptide was purified by RP-HPLC.

Peptide cyclization. For the head-to-tail cyclization between Gly and Asp a syringe was prepared containing the linear, protected peptide (1 equiv) in DMF. A second syringe containing HATU and HOAt (1.5 equiv) in DMF was prepared. A flask was charged with HATU, HOAt (0.1 equiv) and DIPEA (3 equiv) in DMF. Peptide and coupling reagents were added *via* a dual syringe pump with

1.25 mL/h achieving a final concentration of 5 mM for the peptide. Syringes were rinsed subsequently with DMF and the solution stirred for further 0.5 h. If the starting material is not completely converted usually addition of DIPEA (3 equiv) drives the reaction to completion. The solvent was removed under reduced pressure and the crude was applied for cross-coupling without purification.

1.3 Late-stage Modification by SMC

A brown glass vial was charged with the crude, cyclic peptides (1 equiv, 0.13 mmol), 1-pyreneboronic acid (3 equiv), K_3PO_4 (10 equiv), sSPhos (12.5 mol%) and Na_2PdCl_4 (5 mol%) as catalysts. A mixture of DMF/water (2:1, 4 mL) was applied as a solvent and the vial flushed with argon. The suspension was heated in a thermoblock for 1.5 h under stirring at 90 °C and afterwards filtered over a plug of celite and rinsed with methanol (20 mL). The solvent was removed under reduced pressure and for peptide **1** the protecting group was removed with a mixture of TFA/TIS/H_2O (3 mL, 95:2.5:2.5 v/v/v) for 3 h. The solvent was co-evaporated with toluene and the peptide purified by RP-HPLC. For peptide **2** the Boc-protecting group was cleaved with 4 M HCl/dioxane (10 mL) for 1.5 h. The solvent was removed under reduced pressure and the crude was purified by RP-HPLC.[1]

Compound **1**. Compound **1** was obtained as a light-yellow TFA-solid (36.9 mg, 40 µmol, 28%, scale: 140 µmol). HRMS: *m/z* calculated for $C_{44}H_{47}N_9O_7+H^+$ [M+H$^+$] = 814.3671. Found: 814.3707.

1.4 Attachment of PEG-linker

The crude peptide obtained after SMC and Boc deprotection (1 equiv) and MeO-PEG-NHS (1.5 equiv) were dissolved in dry DMF (4-8 mL) and to this DIPEA (3 equiv) was added. The solution was stirred in the dark for 1.5-2 d. Reaction control was performed with a combination of LC-MS and MALDI-TOF-MS. The solvent was removed under reduced pressure and the crude was purified by RP-HPLC. Subsequently, Pbf-protecting group was cleaved with a mixture of TFA/TIS/H_2O (3-6 mL, 95:2.5:2.5 v/v/v) for 2 h. The solvent was reduced *in vacuo* and precipitated with cold diethylether (50-100 mL). The precipitate was cooled for further 20 min at 4 °C and centrifuged (2900×g, 15 min, 4 °C). The supernatant was decanted and the residue dissolved in water/acetonitrile and lyophilized.

Compound **2**. Compound **2** was obtained as a colorless solid (75.9 mg, 12 µmol, 75%). Mass was detected in a range from 5000-7000 *m/z* due to PEG (average MW: 5900 g/mol).

Compound **4**. Compound **4** was obtained as a colorless solid (109.9 mg, 9.6 µmol, 40%). Mass was detected in a range from 10000-13000 *m/z* due to PEG (average MW: 11500 g/mol).

1.5 Integrin Binding Assay

An ELISA-like assay using isolated integrins was performed to determine activity. Cilengitide ($\alpha_v\beta_3$: 0.54 nM, $\alpha_5\beta_1$: 15.4 nM) was used as internal standard. All wells of flat-bottom 96-well Immuno Plates (BRAND) were coated overnight at 4 °C with 100 µL protein (*1*, Table 1) in carbonate buffer (15 mM Na_2CO_3, 35 mM $NaHCO_3$, pH 9.6). Each well was then washed with PBS-T-buffer (phosphate-buffered saline/Tween20, 137 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4, 2 mM KH_2PO_4, 0.01% Tween20, pH 7.4; 3x200 µL) and blocked for 1 h at room temperature with TS-B-buffer (Tris-saline/bovine serum albumin (BSA) buffer, 20 mM Tris-HCl, 150 mM NaCl, 1 mM $CaCl_2$, 1 mM $MgCl_2$, 1 mM $MnCl_2$, pH 7.5, 1% BSA; 150 µL/well). A dilution series of the compounds and internal standard was prepared in an extra plate in 1:5 dilution steps (B-E) using TS-B-buffer. After washing the assay plate with PBS-T (3×200 µL), 50 µl of the dilution series was transferred to each well from B-G. For a negative control, well A was filled with TS-B-buffer (100 µL) and for a positive control well H was filled with TS-B-buffer (50 µL). Human integrin (2, 50 µL) in TS-B-buffer were transferred to wells H-B and incubated for 1 h at rt. After washing the plates (3×200 µL) with PBS-T buffer, primary antibody (*3*, 100 µL) was added to all wells and incubated for 1 h at rt. The plate was washed (3×200 µL) with PBS-T buffer and secondary antibody (*4*, 100 µL) was added to all wells and incubated for 1 h at rt. The plate was washed (3×200 µL) with PBS-T buffer and SeramunBlau (Seramun Diagnostic GmbH, Heidesee, 50 µL) was added to all wells. The development was stopped with 3 M H_2SO_4 (50 µL/well) when a blue color gradient from well A to H was visible ($\alpha_v\beta_3$: ~1 min, $\alpha_5\beta_1$: < 1 min). The absorbance was measured with a plate reader at 450 nm (Tecan Spark, 10M). The resulting curves were analyzed with OriginPro 2017G with the inflection point describing the IC_{50} value (sigmoidal fitting: DoseResp). Each compound was tested in triplicates and referenced to the internal standard.[2]

Table 1. Proteins applied in ELISA.

	$\alpha_v\beta_3$	$\alpha_5\beta_1$
(*1*)	1.0 µg/mL human vitronectin, R&D	0.5 µg/mL human fibronectin, R&D
(*2*)	2.0 µg/mL, human $\alpha_v\beta_3$ integrin, R&D	2.0 µg/mL, human $\alpha_5\beta_1$ integrin, R&D
(*3*)	2.0 µg/mL, mouse anti-human CD51/61, BD Bioscience	1.0 µg/mL, mouse anti-human CD49e, BD Bioscience
(*4*)	1.0 µg/mL, anti-mouse IgG-POD goat, Sigma-Aldrich	2.0 µg/mL, anti-mouse IgG-POD goat, Sigma-Aldrich

1.6 Flow Cytometry and Live Cell Imaging

Cell Culture. The human melanoma M21 and M21L cells were kindly provided by Dr. David Cheresh and the Scripps Research Institute (La Jolla, CA, USA). Cells were cultivated in RPMI 1640 supplemented with 10% (v/v) fetal calf serum (FCS), 1% (v/v) L-glutamine and 1% penicillin/streptomycin at 37 °C and 5.3% CO_2. Cells were screened on a regular basis for contamination with mycoplasma.

Flow Cytometry Analysis. M21 and M21L cells were detached using accutase (4 mL) for 10 min at 37 °C. After centrifugation (800×g, 6 min, 24 °C) cells were resuspended in medium and 2×10^6 cells were seeded out in Eppendorf tubes. Cells were spun down and resuspended with 250 µL of peptide (30 µM) in medium without FCS. After incubation for approx. 0, 10 and 30 min at 37 °C and 500 rpm including a delay of ~30 sec due to handling, cells were spun down in a precooled centrifuge (800×g, 6 min, 4 °C) and washed with cold PBS (1×). For flow cytometry measurements cells were resuspended in RPMI without FCS (500 µL). Samples were measured with FCS. Acquisition of 10,000 events for each sample was performed using a Gallios flow cytometer (Beckman Coulter) equipped with a 405 nm laser. Data were analysed using FlowJo software v.10. Negative controls were only incubated with medium. Experiments were conducted as biological duplicates and technical triplicates.

Flow cytometry analysis with primary antibody. Similar to previous experiments, M21 cells were detached and seeded out into Eppendorf tubes (2×10^6). Cells were incubated with mouse anti-human CD51/CD61 antibody at 1 µg/mL in serum-free medium for 15 min on ice. Cells were washed with cold PBS buffer (1×) and incubated with peptide **1** and **2** (30 µM) in serum-free medium for 0 and 10 min at 37 °C. Finally, peptides were washed with PBS buffer (1×) and resuspend in medium for flow cytometry. Negative controls were only incubated with medium.

Temperature dependent flow cytometry. M21 cells were seeded into two 24-well plates (2×10^5) and grown overnight. Cells were washed either with cold or warm PBS buffer (2×) and incubated with peptide (30 µM) for 0 and 10 min at 37 °C or 4 °C. After washing with PBS buffer (4×) cells were detached using accutase and diluted with medium prior to flow cytometry.

Live cell fluorescence microscopy. 2×10^4 (M21) and 4×10^4 (M21L) cells were seeded out in an 8-well µ-slide (Nunc, LabTek) in medium (300 µL) and incubated overnight. Microscopy was performed using an incubation chamber at 37 °C to maintain cell culture conditions. Cells were stained with LysoTracker Red DND-99 (Invitrogen) (35-50 µM) for 5-10 min. Cells were washed with RPMI medium without phenol red (1×) and peptide (**1**:5 µM, **2**:10 µM, **4**:10 µM) was added and incubated for 10 min. Cells were carefully washed (3-5×) especially with semi-adherent M21L cells. Imaging the cells was performed with a 63× objective (LCI Plan-Neofluar 63x/1.3 Imm Korr DIC M27) using immersion oil (Immersol

W (2010), Zeiss) of the inverted laser scanning Zeiss LSM780 microscope. Laser (laser line 405 nm, 561 nm) and detector ranges (418-420 nm (Ch1), 570-620 (Ch2)) were used with corresponding main beam splitters. Image acquisition was performed with the software Zeiss Zen 2011 and image analysis with ImageJ. A macro was used to analyze areas of colocalization between the fluorescence signal of the peptide and the LysoTracker DND-99 signal.

2 Supplemental Figures

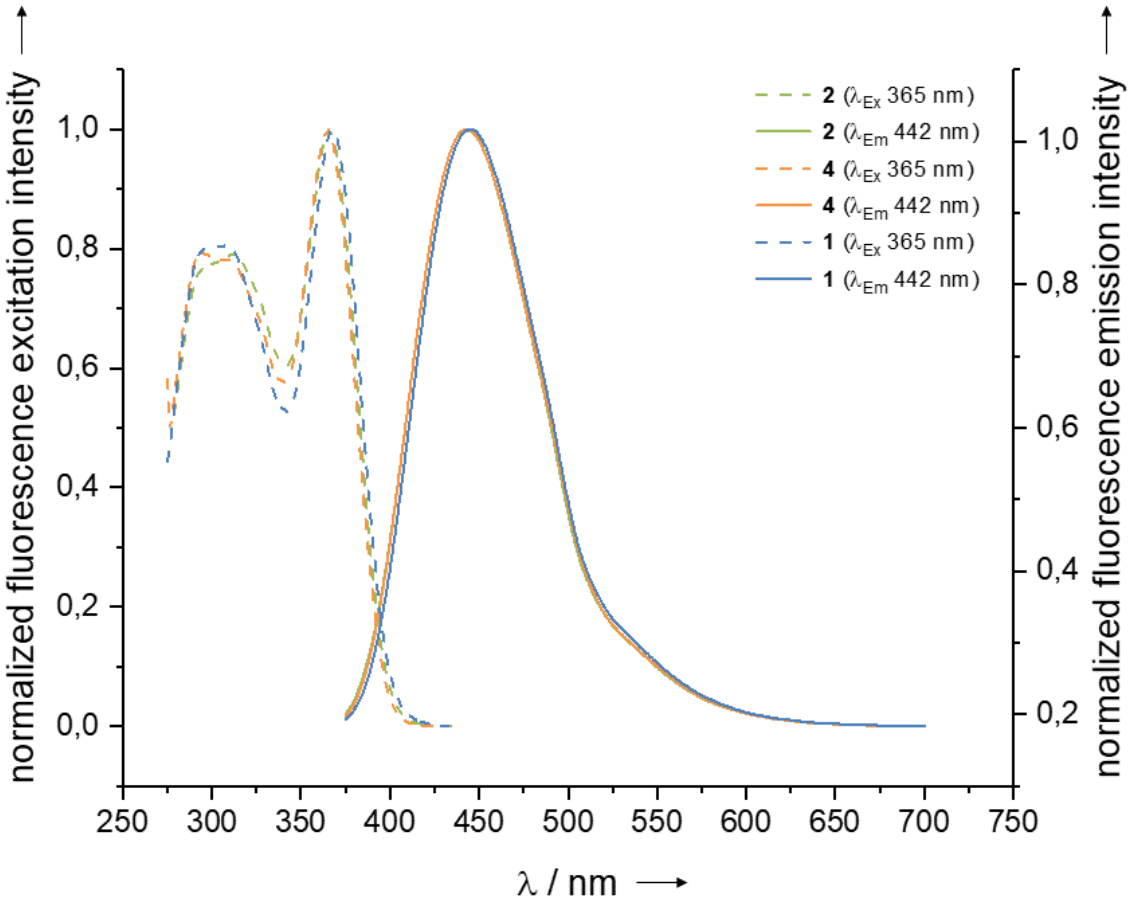


Figure S1. Normalized excitation and emission spectra of peptides **1**,**2**, and **4** in MeOH (0.1 mM) show that further modification by PEGylation does not influence fluorogenic properties.

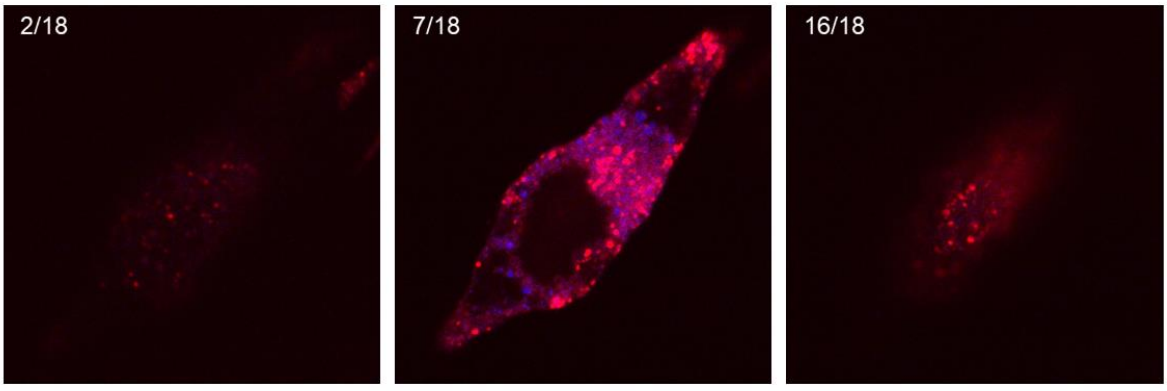
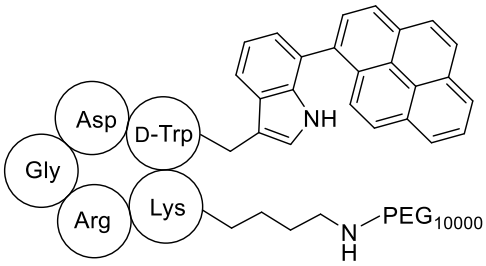


Figure S2. Localization of peptide **2** in M21 cells. A series of XY images along the Z axis (Z-stack) was acquired to determine localization. Three representatives confirm the presence within cells (7/18) and show minor fluorescence at the cell surfaces (2/18 resp. 16/18).



4

IC50 $\alpha_v\beta_3$ = 0.88±0.03 nM
IC50 $\alpha_5\beta_1$ = 172±42 nM

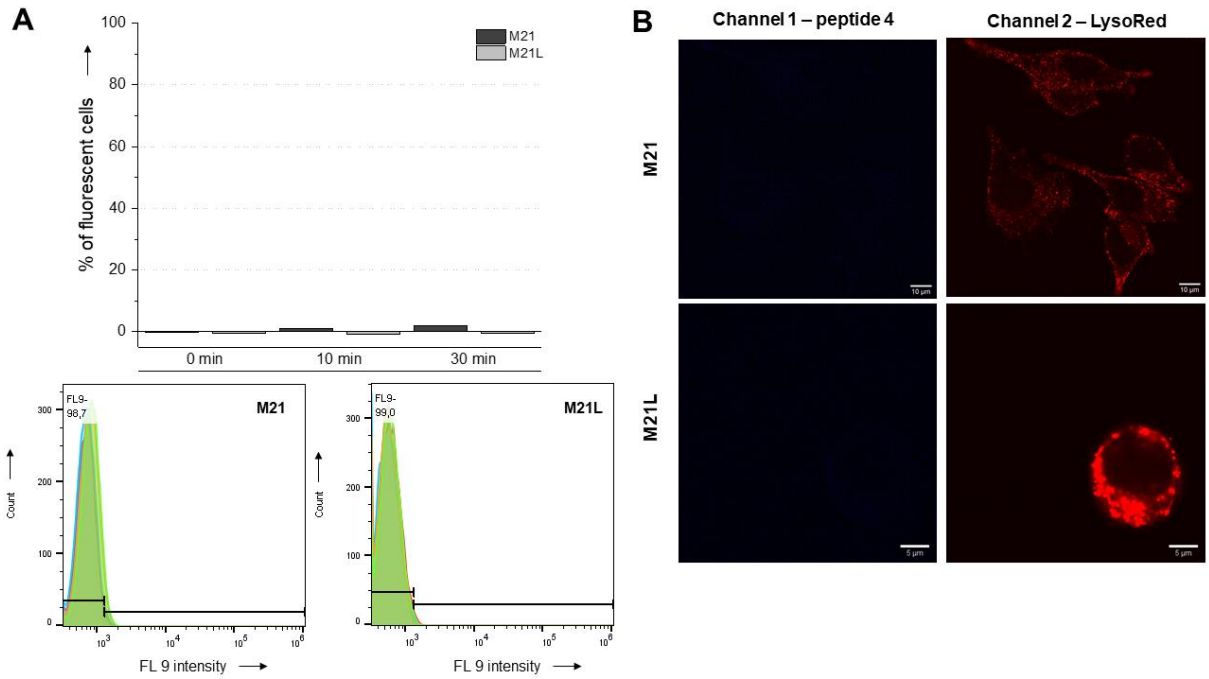


Figure S3. Uptake studies with peptide **4**. Flow cytometry analysis was performed after incubation of peptide **4** (30 μM) with M21 or M21L cells for 0 min (blue), 10 min (orange) and 30 min (green). All signals overlap with the negative control (red, no addition of peptide) and show no change in fluorescence (**A**). M21 and M21L cells were stained with LysoTracker Red DND-99 and incubated for 10 min with peptide **4** (10 μM) prior to fluorescence microscopy (**B**). Settings are identical to peptide **2**. Similar to flow cytometry, no fluorescence was visible for peptide **4** confirming its absence. This could be caused by binding of PEG to residual serum proteins from cultivation of cells with fetal calf serum. Alternatively, the increased PEG size might prevent an interaction of RGD moiety and integrin. Thus, peptide **4** is removed by washing steps and no fluorescence can be detected by flow cytometry.

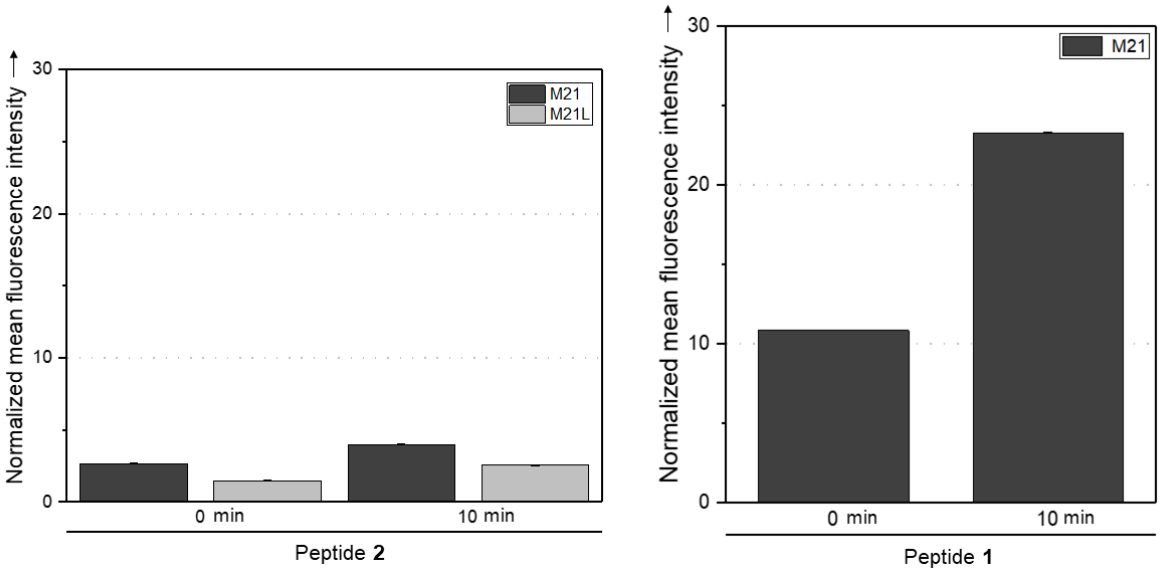


Figure S4. Flow cytometry results after blocking integrins with anti-$\alpha_v\beta_3$ antibody prior to incubating with peptides. Peptide **2** shows receptor-mediated endocytosis and blocking of integrins leads to reduced endocytosis. Uptake of peptide **1** is fluid-phase dependent and thus, not hindered.

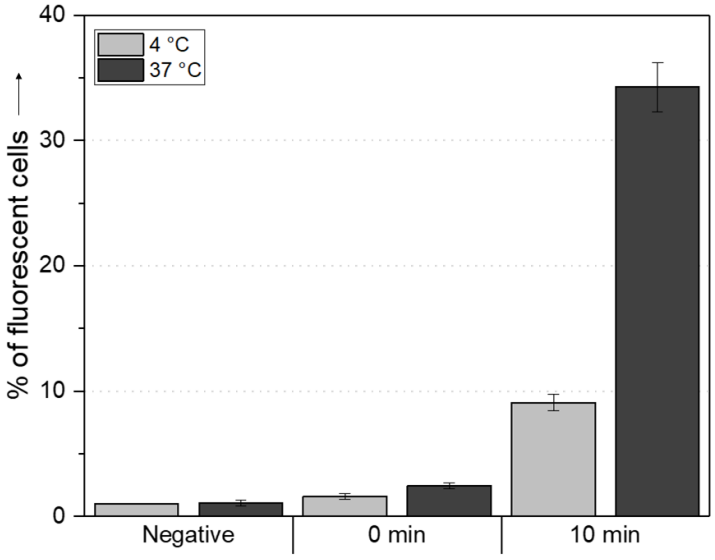


Figure S5. Temperature-dependent internalization of peptide **2** with M21 cells at 37 °C and 4 °C. Peptide **2** shows a significant reduced internalization at 4 °C. The negative control was only incubated with medium.

3 Analytical Data

Peptide 1

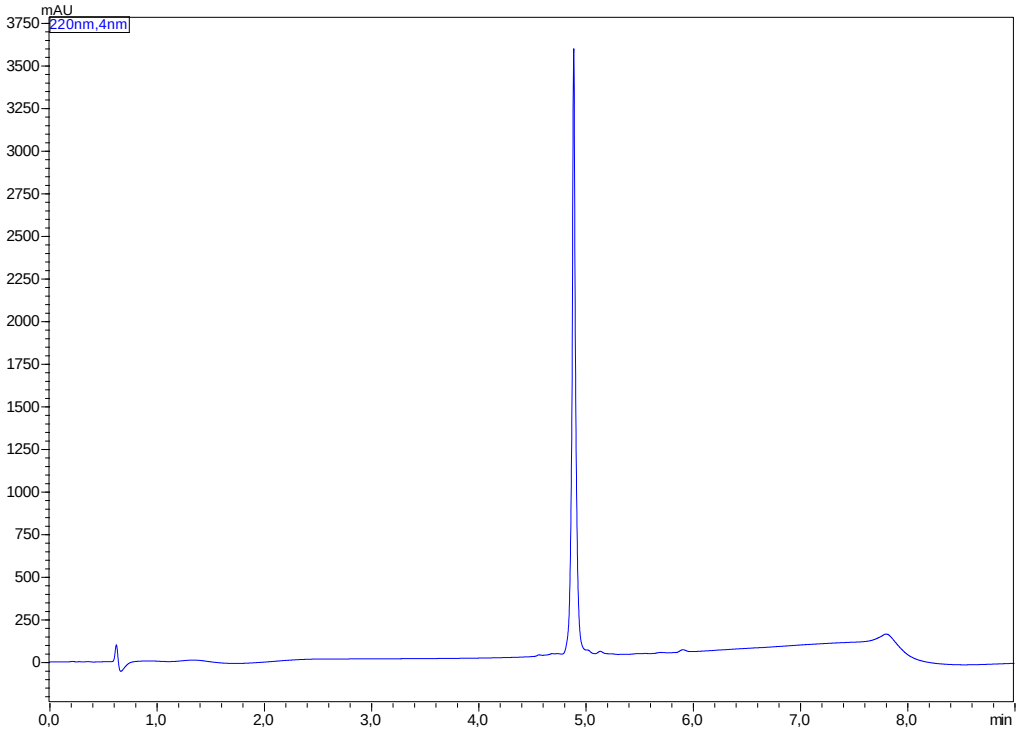


Figure S5. RP-HPLC of purified peptide **1** at 220 nm provides purity of >95%.

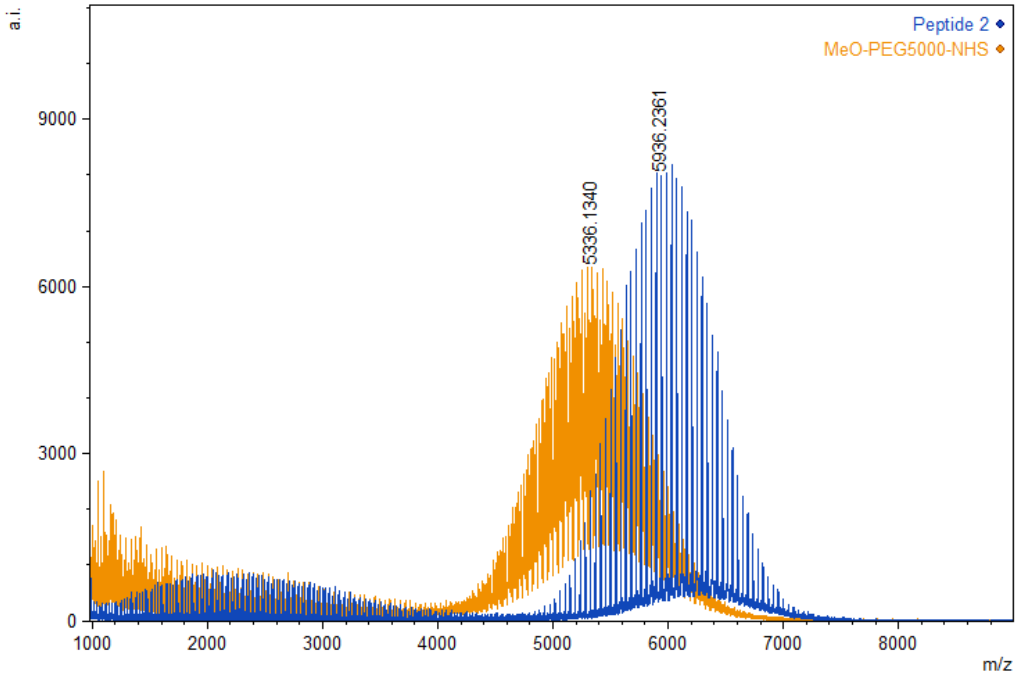


Figure S6. MALDI-TOF-MS spectrum of purified peptide **2** and MeO-PEG$_{5000}$-NHS starting material were overlaid demonstrating no contamination of the desired product with UV-invisible PEG.

Peptide 2

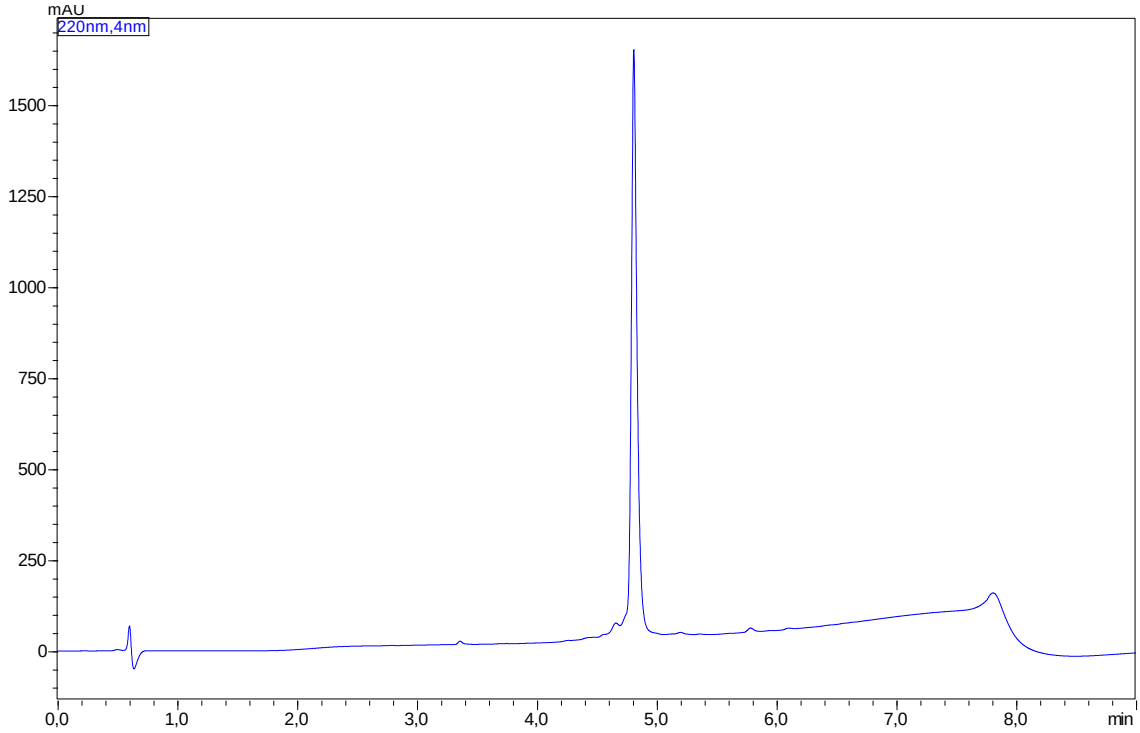


Figure S7. RP-HPLC of purified peptide **2** at 220 nm provides purity of >95% in combination with MALDI-TOF-MS.

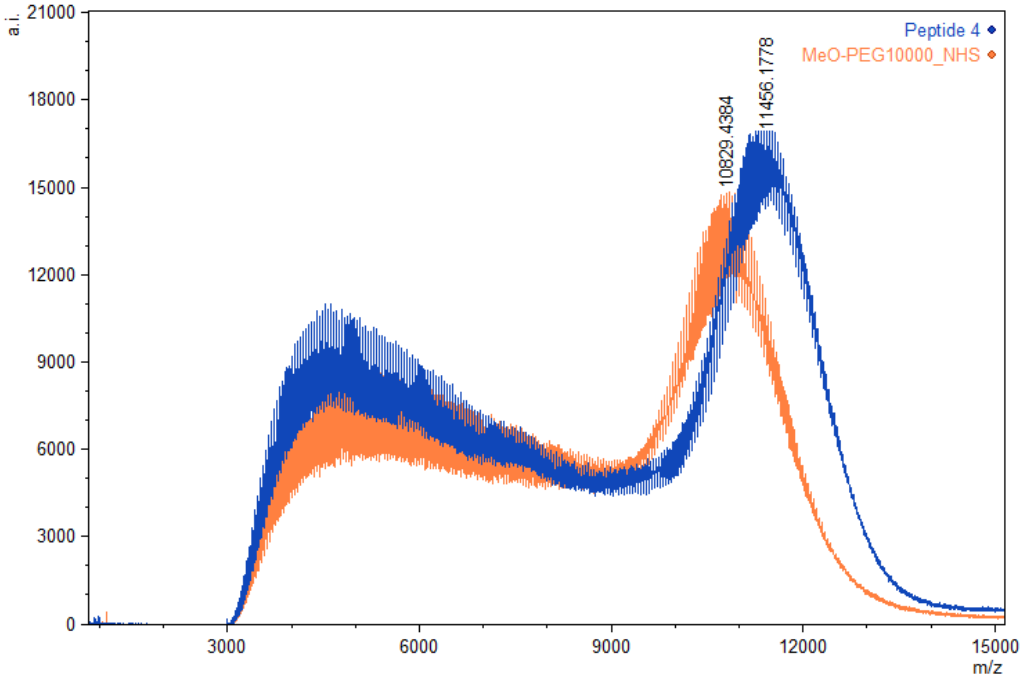


Figure S8. MALDI-TOF-MS spectrum of purified peptide **4** and MeO-PEG$_{10000}$-NHS starting material were overlaid demonstrating no contamination of the desired product with UV-invisible PEG.

Peptide 4

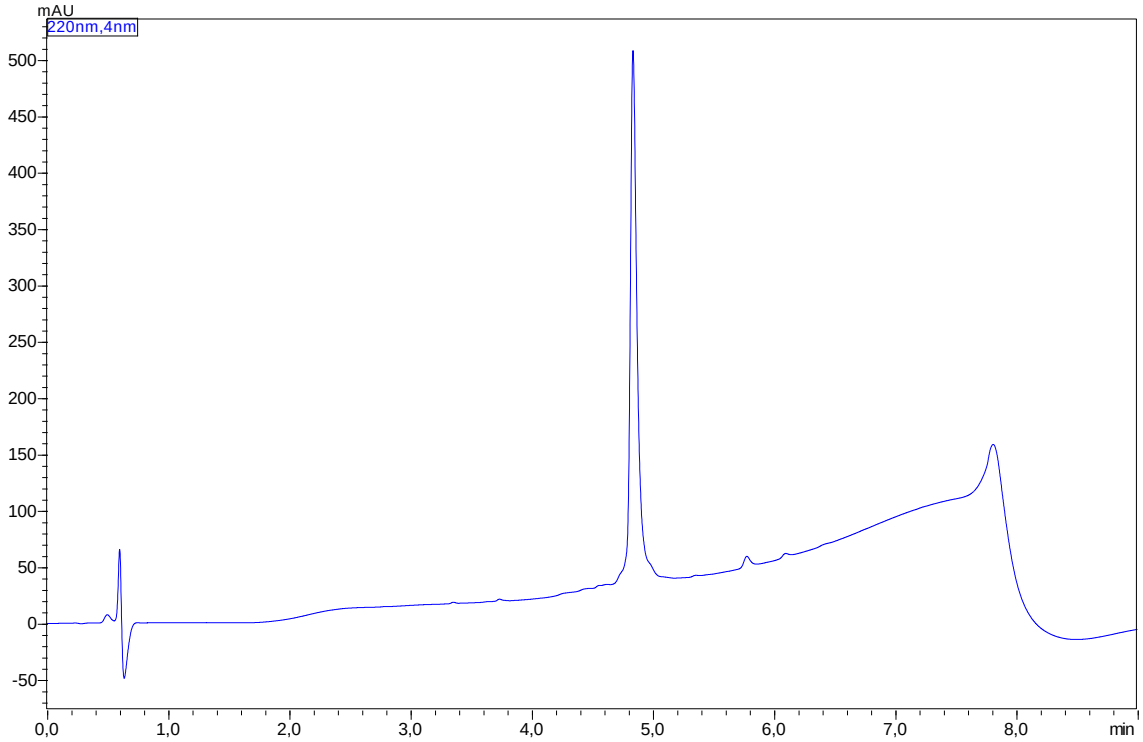


Figure S9. RP-HPLC of purified peptide **4** at 220 nm provides purity of >95% in combination with MALDI-TOF-MS.

4 References

[1] I. Kemker, C. Schnepel, D. C. C. Schröder, A. Marion, N. Sewald, *J. Med. Chem.* **2019**, *62*, 7417-7430.

[2] T. G. Kapp, F. Rechenmacher, S. Neubauer, O. V. Maltsev, E. A. Cavalcanti-Adam, R. Zarka, U. Reuning, J. Notni, H.-J. Wester, C. Mas-Moruno, J. Spatz, B. Geiger, H. Kessler, *Sci. Rep.* **2017**, *7*.