Electronic Supplementary Information

Efficient siRNA-peptide conjugation for specific targeted delivery to tumor cells

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1. General experimental methods

Unless otherwise stated, common chemicals and solvents (HPLC grade or reagent grade quality) were purchased from commercial sources and used without further purification.

Peptide grade DMF was from Scharlau. Fmoc-protected amino acids, resins and coupling reagents for solid phase peptide synthesis were obtained from Novabiochem, Bachem or Iris Biotech. Solid-phase peptide syntheses were performed manually in a polypropylene syringe fitted with a polyethylene disc. Milli-Q water was directly obtained from a Milli-Q system equipped with a 5000-Da ultrafiltration cartridge.
Analytical reversed-phase HPLC analyses of peptides were carried out on a Jupiter Proteo column (250x4.6 mm, 4 mm, flow rate: 1 mL/min), using linear gradients of 0.045% TFA in H_2O (solvent A) and 0.036% TFA in ACN (solvent B). In some cases, small-scale purification was carried out using the same column. Large-scale purification was carried out on a Jupiter Proteo semipreparative column (250 x 10 mm, 10 mm, flow rate: 3 mL/min), using linear gradients of 0.1% TFA in H_2O (solvent A) and 0.1% TFA in ACN (solvent B). After several runs, pure fractions were combined and lyophilized.
ESI mass spectra (ESI-MS) were recorded on a Micromass ZQ instrument with single quadrupole detector coupled to an HPLC.

c(RGDfK) peptide,[1] azido-derivatized c(RGDfK) peptide (**a**)[1] and fluorescein-labeled derivatives of c(RGDfK)[1] and of octreotide[2] were synthesized and characterized as previously described. Tat-AHNP peptide, azido-derivatized Tat-AHNP (**c**) and fluorescein-labeled Tat-AHNP were synthesized by New England Peptide, Inc. (Gardner, MA). Octreotide was purchased from Bachem.

RNA strands that did not contained 5'-terminal octadiyne modification were purchased from Sigma Aldrich.

Reagents for oligonucleotide synthesis including 5'-*O*-DMT-2'-*O*-TBDMS-protected phosphoramidite monomers of A^{Bz}, C^{Ac}, G^{dmf} and U, 5'-*O*-DMT-protected phosphoramidite monomer of dT, the C8-alkyne-5'-*O*-DMT-dT-CE-phosphoramidite, the 5'-deblocking solution (3% TCA in CH_2Cl_2), activator solution (0.3 M 5-benzylthio-1-H-tetrazole in CH_3CN), CAP A solution (acetic anhydride/pyridine/THF), oxidizing solution (0.02 M iodine in tetrahydrofuran/pyridine/water (7:2:1) and 5'-*O*-DMT-dT-3'-

succinyl-LCAA-CPG were purchased from commercial sources and used as received. Reversed-phase-HPLC oligonucleotide purifications were performed on a Jupiter 10 C_{18} column (250x10 mm, flow rate: 3 mL/min), using linear gradients of 0.1 M aqueous TEAA (solvent A) and ACN (solvent B). MALDI-TOF spectra were performed using a Perspective Voyager DETMRP mass spectrometer, equipped with nitrogen laser at 337 nm using a 3ns pulse. The matrix used contained 2,4,6-trihydroxyacetophenone (THAP, 10 mg/mL in CH_3CN/water 1:1) and ammonium citrate (50 mg/mL in water).

2. Synthesis of azido-derivatized octreotide (b)

Solid-phase peptide synthesis was performed on a Rink amide resin-*p*-MBHA (f = 0.34 mmol/g, 100-200 mesh) using standard Fmoc/tBu chemistry following previously described procedures.[2] Briefly, Fmoc-*L*-threoninol *p*-carboxyacetal[2] (1.8 mol equiv) was first coupled using DIPC (1.8 mol equiv) and HOBt (1.8 mol equiv) in anhydrous DMF for 1 h. The following Fmoc-protected amino acids and the Fmoc-linker-OH (8-(9-fluorenylmethyloxycarbonyl-amino)-3,6-dioxaoctanoic acid) (5 mol equiv) were incorporated with DIPC (5 mol equiv) and HOAt (5 mol equiv) in anhydrous DMF. After removal of the Fmoc group from the spacer (20% piperidine in DMF), azido acetic acid (5 mol equiv) was coupled by using DIPC (5 mol equiv) and HOAt (5 mol equiv) in anhydrous DMF. After acid cleavage and deprotection (TFA/H_2O/TIS/EDT 94:2.5:2.5:1, 1 h, RT), the linear peptide was dissolved (final concentration *ca.* 1 mM) in an aqueous ammonium hydrogencarbonate solution (5%, pH 7.8) and stirred at room temperature for 24 h. Finally, the crude was lyophilized and peptide **2** was isolated by HPLC. Overall yield (synthesis + purification): 11%. Characterization: R_t= 18.5 min (analytical gradient: 0 to 100 % in 30 min); ESI MS, positive mode: *m/z* 1247.68 (calcd mass for $C_{57}H_{79}N_{14}O_{14}S_2$ $[M+H]^+$: 1247.53).

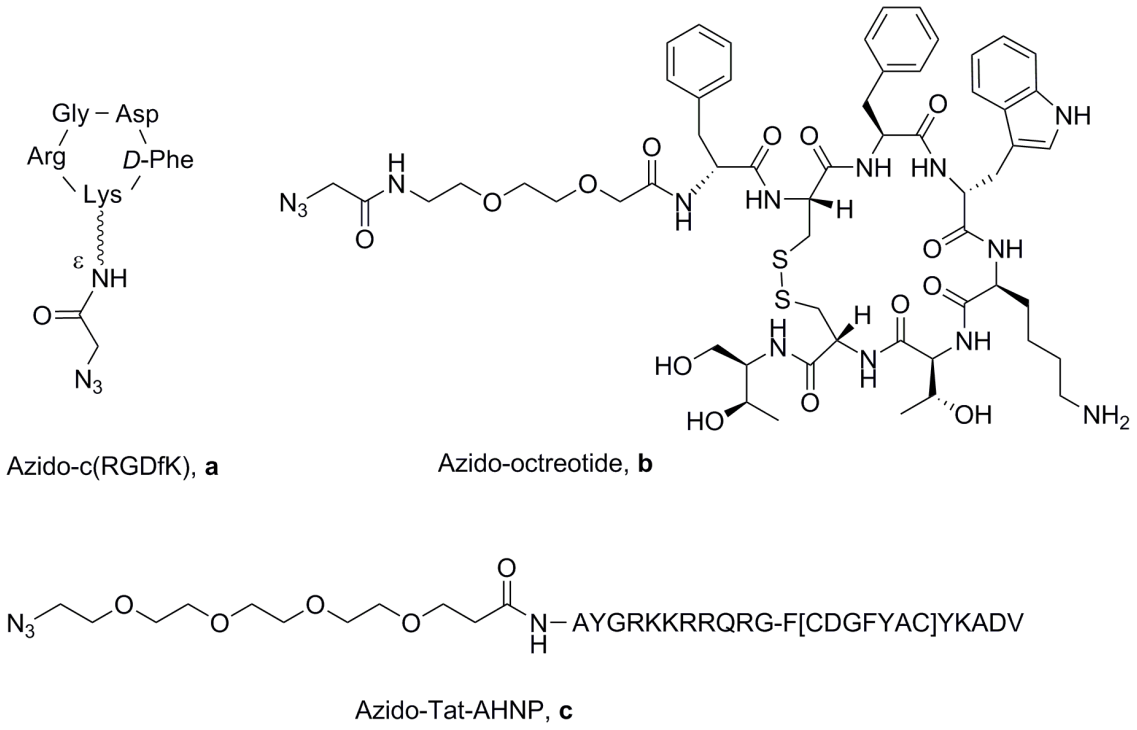

Figure S1. Structure of azido-derivatized peptides used in this work.

3. Synthesis of 5'-alkynyl-modified RNAs

5'-Alkynyl-modified RNAs were synthesized on the 1 μmol scale with a K&A Laborgërate DNA/RNA synthesizer. 2'-*O*-TBDMS-5'-*O*-DMT-protected-CE-phosphoramidites (A^{Bz}, G^{dmf}, C^{Ac} and U), 5'-*O*-DMT-protected dT-CE-phosphoramidite and C8-alkyne-5'-*O*-DMT-protected dT-CE-phosphoramidite were used. The coupling time was 15 min. The coupling yields of natural and modified phosphoramidites were around 95%. Incorporation of the C8-alkyne nucleoside modification did not have a negative effect in the yield. All oligonucleotides were synthesized in DMT-OFF mode.

4. Deprotection and purification of 5'-alkynyl-modified RNAs

After the solid-phase synthesis, the solid support was transferred to a screw-cap vial and incubated at 55 ºC for 1 h with 1.5 mL of NH_3 solution (33%) and 0.5 mL of ethanol. The vial was then cooled on ice and the supernatant was transferred into a 2 mL eppendorf tube. The solid support and vial were rinsed with 50% ethanol (2 x 0.25 mL). The combined solutions were evaporated to dryness using an evaporating centrifuge. The residue that was obtained was dissolved in 1 M TBAF in THF (330 μL) and

incubated at room temperature for 15 h. Then, 1 M triethylammonium acetate (TEEA) (330 μL) and water (330 μL) were added to the solution. The oligonucleotides were desalted on NAP-10 columns using water as the eluent and evaporated to dryness. The oligonucleotides were purified by HPLC (DMT-OFF). Column: Jupiter 10 C_{18} (250 x 10 mm); 30 min linear gradient from 0% to 40% B, flow rate 3 mL/min; solution A was 0.1 M aqueous TEAA and B ACN. The pure fractions were combined and evaporated to dryness. All oligonucleotides were quantified by absorption at 260 nm and confirmed by MALDI mass spectrometry. SiRNAs were prepared by annealing equimolar quantities of complementary oligonucleotides in siRNA buffer (100 mM KOAc, 30 mM HEPES-KOH, 2 mM $MgCl_2$, pH 7.4) by slowly cooling from 96 ºC to r.t.

5. General method for click siRNA-peptide conjugation

Stock solutions of $CuSO_4$ (150 mM) and sodium ascorbate (150 mM), dry peptides and 5-alkynyl-modified RNAs and 0.1 mM Tris·HCl buffer (pH 7.5) were flushed with argon for 15 min prior any further treatment.

To an argon-flushed vial containing 0.3 μmol of azido-peptide, 120 μL of a freshly prepared $CuSO_4$/sodium ascorbate solution (prepared by addition of 15 μL of 150 mM $CuSO_4$ in 0.1 mM Tris·HCl pH 7.5/CH_3CN 8:2 and 15 μL of 150 mM sodium ascorbate in 0.1 mM Tris·HCl pH 7.5/CH_3CN 8:2 to a vial containing 90 μL of 0.1 mM Tris·HCl pH 7.5/CH_3CN 8:2) were added. The resulting pale-blue solution was immediately added to an argon-flushed vial containing 0.15 μmol of dry 5'-alkynyl-bearing RNA. The vial containing the peptide was rinsed with 30 μL of 0.1 mM Tris·HCl pH 7.5/CH_3CN 8:2 and the resulting solution was added to the RNA-peptide mixture. The resulting yellow-coloured solution was throroughly shaken for 30 seconds and allowed to run at r.t. for 90 min.

The reaction was subsequently diluted with Milli-Q water and loaded directly onto HPLC for analysis or purification. Analysis conditions: Jupiter 10 C_{18} column (250 x 4.6 mm), 30 min linear gradient from 0% to 40% B, flow rate 1 mL/min; solution A was 0.1 M aqueous TEAA and B ACN. Purification conditions: Jupiter 10 C_{18} column (250 x 10 mm); 30 min linear gradient from 0% to 40% B, flow rate 3 mL/min; solution A was 0.1 M aqueous TEAA and B ACN.

6. Reversed-phase HPLC analysis of the click siRNA-peptide conjugation reactions

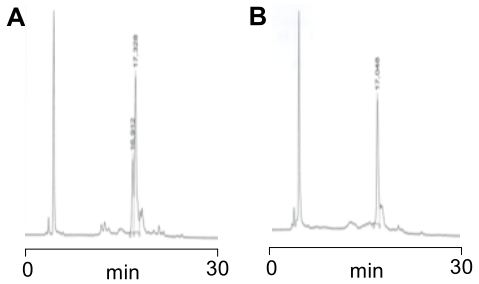

Figure S2. Reversed-phase HPLC analysis of the functionalization of RNA **1** with azido-c(RGDfK) (**a**) in the presence of 15 mol equiv. (A) and 45 mol equiv. (B) of $CuSO_4$ and sodium ascorbate.

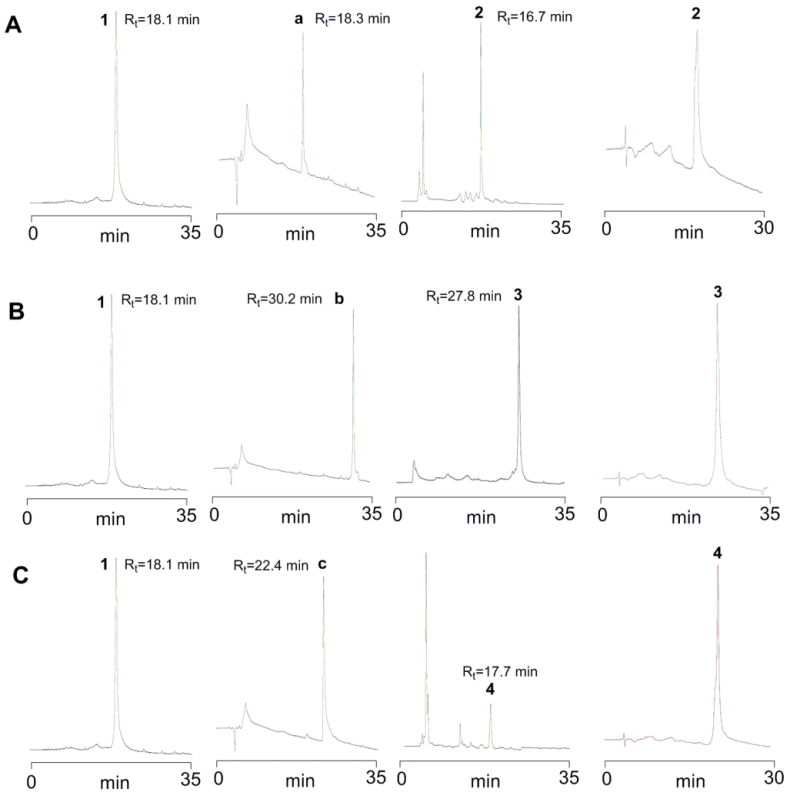

Figure S3. Reversed-phase HPLC analysis of the click functionalization of alkynyl-modified RNA **1** with azido-c(RGDfK) (**a**) (panel A), azido-octreotide (**b**) (panel B) and azido-Tat-AHNP (**c**) (panel B) under the optimized conditions. From left to right: RNA **1**, azido-peptides **a-c**, reaction crude and fully purified RNA-peptide conjugates **2-4**. Detection wavelength: 260 nm for RNA and RNA-peptide conjugates, and 220 nm for azido-peptides.

7. PAGE analysis of the click siRNA-peptide conjugation reactions

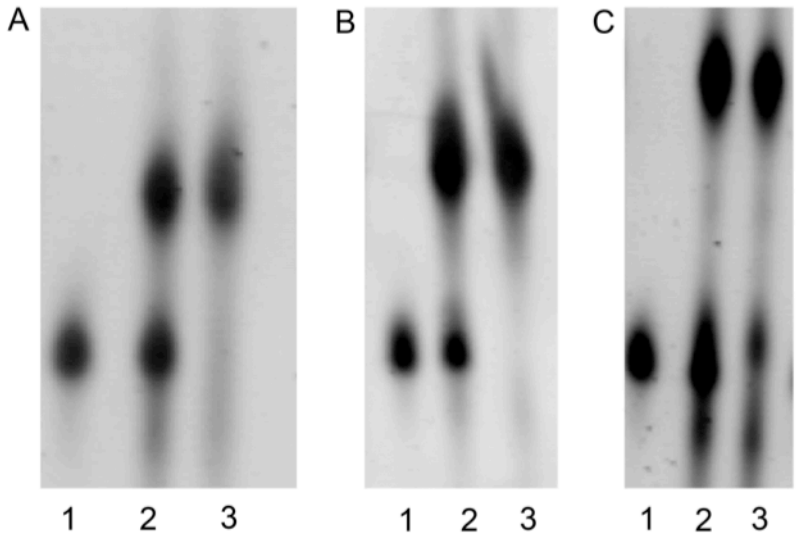

Figure S4. Synthesis of c(RGDfK)-, Octreotide- and Tat-AHNP-sense RNA conjugates **2** (panel A), **3** (panel B) and **4** (panel C) by click reaction between 5'-octadiyne-containing sense strand **1** and azido-peptides **a**, **b** and **c** (c(RGDfK), octreotide and Tat-AHNP, respectively). (A) Synthesis of RGDfk-RNA conjugate **2**; lane 1: 5'-octadiyne-containing sense strand **1**, lane 2: RNA **1** + crude of the reaction mixture at t = 90 min, lane 3: crude of the reaction mixture at t = 90 min; (B) Synthesis of octreotide-RNA conjugate **3**; lane 1: 5'-octadiyne-containing sense strand **1**, lane 2: RNA **1** + crude of the reaction mixture at t = 90 min; lane 3: crude of the reaction mixture at t = 90 min; (C) Synthesis of Tat-AHNP-RNA conjugate **4**; lane 1: 5'-octadiyne-containing sense strand **1**, lane 2: RNA **1** + crude of the reaction mixture at t = 90 min, lane 3: crude of the reaction mixture at t = 90 min. The reaction mixtures were analyzed by 15% denaturing PAGE and visualized with SYBR Gold.

8. Table S1. Mass spectrometry analysis of synthesized RNAs and RNA-peptide conjugates

RNA	Sequence	MW calcd.	MW found
1	5‘-(**5oU**)AAGCCUCACAGAGAUCUUGtt	7046.0	7059.5
2	5‘-(**c(RGDfK)-5oU**)AAGCCUCACAGAGAUCUUGtt	7733.0	7735.9
3	5‘-(**Octreotide-5oU**)AAGCCUCACAGAGAUCUUGtt	8294,0	8305.8
4	5‘-(**Tat-AHNP-5oU**)AAGCCUCACAGAGAUCUUGtt	10106.0 (+Na)	10119.7 (+Na)
	5‘-(**5oU**)AUCUGAAGAAGGAGAAAAAtt	7203.7	7206.3
7	5‘-(**Tat-AHNP)** AUCUGAAGAAGGAGAAAAAtt	10263.7	10261.5

9. Copper determination by ICP-MS

Peptide-modified siRNAs (0.375 nmol) were dissolved in 500 µL of concentrated 72% v/v nitric acid, and the samples were then transferred into wheaton v-vials (Sigma-Aldrich) and heated in an oven at 373 K for 18 h. The vials were then allowed to cool, and each sample solution was transferred into a volumetric tube and combined with washings with Milli-Q water (1.5 mL). Digested samples were diluted 5 times with Milli-Q to obtain a final HNO_3 concentration of approximately 3.6% v/v. Copper content was analyzed on an ICP-MS Agilent 7500CE model instrument at the Centres Científics i Tecnològics of the Universitat de Barcelona. The solvent used for all ICP-MS experiments was Milli-Q water with 4% HNO_3. The copper standard was purchased from High-Purity Standards (1000 µg/mL $\pm$ 5 µg/mL in 5% HNO_3). The concentrations used for the calibration curve were in all cases 0, 2, 4, 10 and 20 ppb. The isotope detected was ^{63}Cu and readings were made in triplicate. Rhodium was added as an internal standard in all samples.

10. UV-monitored thermal denaturation studies

Absorbance versus temperature curves of siRNA duplexes **I-IV** were measured at 1 μM strand concentration in 10 mM phosphate buffer (pH 7.0) containing 5 mM EDTA. Experiments were performed in Teflon-stoppered 1 cm path length quartz cells on a Varian-Cary-100 spectrophotometer equipped with thermoprogrammer. The samples were heated to 95ºC, allowed to slowly cool to 20ºC, and then warmed during the denaturation experiments at a rate of 0.5◦C/min to 95ºC, monitoring absorbance at 260 nm. The data were analyzed by the denaturation curve processing program, MeltWin v. 3.0. Melting temperatures (T_m) were determined by computer fit of the first derivative of absorbance with respect to 1/T.

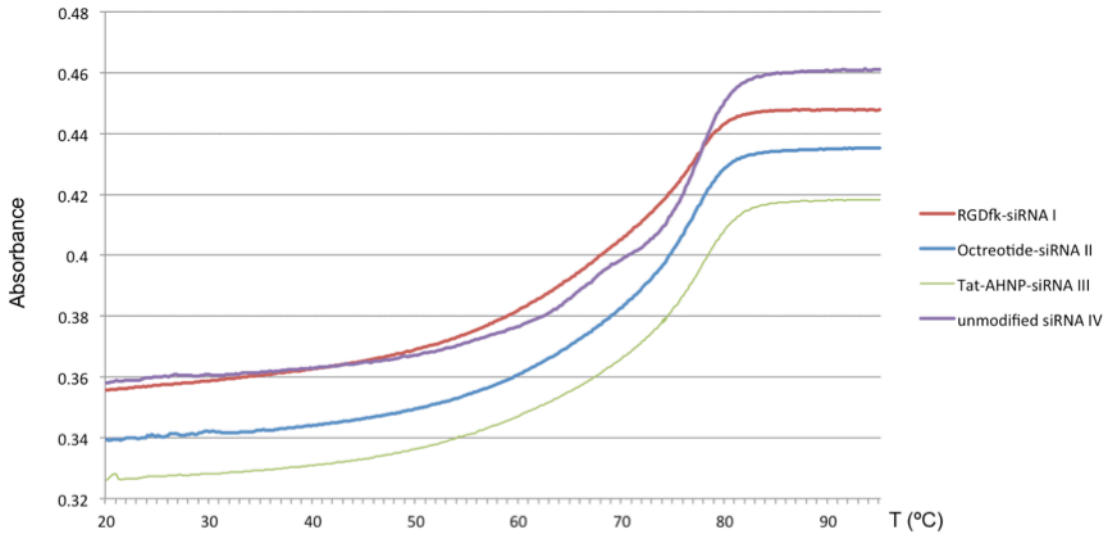

Figure S5. Thermal denaturation of siRNA-peptide conjugates **I-III** and of the unmodified siRNA **IV**.

11. Cell culture

All cell lines (SK-BR-3, BT-474 and SK-MEL-28) were mantained at 37 ºC in a humidified atmosphere with 5% CO_2. BT-474 and SK-MEL-28 cells were cultured in Dulbecco's modified Eagle's medium (DMEM; GIBCO) supplemented with fetal bovine serum (FBS, 10%), penicillin (100 U mL^{-1}), and streptomycin (100 µg mL^{-1}). SK-BR-3 cells were cultured in McCoy's modified medium (GIBCO) supplemented with fetal bovine serum (FBS, 10%), penicillin (100 U mL^{-1}), and streptomycin (100 µg mL^{-1}).

12. Flow cytometry expression analysis of $\alpha_V\beta_3$ integrin, HER2 and somatostatin subtype-2 receptors on SK-BR-3, BT-474 and SK-MEL-28 cells.

The expression of $\alpha_V\beta_3$ integrin, HER2 and somatostatin subtype-2 (SSTR2) receptors on the cell surface was analyzed by double immuno-fluorescence. The cells were incubated for 30 min at 4˚C with monoclonal antibodies against human $\alpha_V\beta_3$ (clone LM609) (Millipore, Temecula, CA), HER2 (c-neu(Ab-5) (Calbiochem, EMD Millipore, Darmstadt, German) and SSTR2 (R&D systems, Minneapolis, USA) or medium alone as negative control. Prior SSTR2 staining, cells were fixed for 30 min at 4 ºC with 1.5% formaldehyde in PBS and permeabilized for 20 min at 37ºC with 0.2% Tween 20 (Sigma) in PBS. After washing with phosphate-buffered saline (PBS) (Gibco-BRL), cells were incubated for additional 30 min at 4˚C with the Alexa-Fluor 488-conjugated goat anti-mouse IgG antibody (Invitrogen, Carlsbad, CA, USA). Next, the cell fluorescence was analyzed using a FACSCalibur flow cytometer (Becton Dickinson Immunocytometry Systems, San Jose, CA, USA) equipped with CellQuest™ software (Becton Dickinson). Ten thousand cells were analysed in each experiment. Fluorescence intensity was represented on a four orders of magnitude log scale (1-10.000).

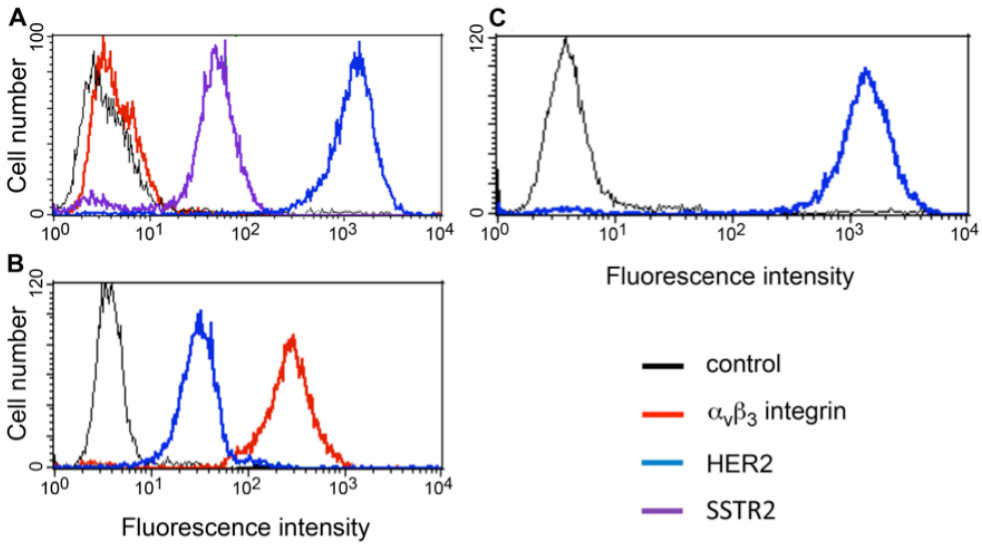

Figure S6. Expression of $\alpha_V\beta_3$ integrin, SSTR2 and HER2 in SK-BR-3 cell line (A), $\alpha_V\beta_3$ integrin and HER2 in SK-MEL-28 cell line (B) and HER2 in BT-474 cell line (C). Representative flow cytometry histograms obtained after indirect immune-fluorescence staining. Colored lines represent the fluorescence intensity of the cells after incubation with monoclonal antibodies against $\alpha_V\beta_3$ integrin, SSTR2 and HER2 followed by incubation with secondary antibody conjugated to Alexa-Fluor 488. Black lines indicate the background staining with the secondary antibody alone.

13. Uptake experiments

The uptake efficiency of the carrier peptides Tat-AHNP, octreotide and c(RGDfK) by SK-BR-3 cells (in the cases of Tat-AHNP and octreotide) and SK-MEL-28 cells [in the case of c(RGDfK)][1] was quantified by flow cytometry. To this end, peptides were labelled with fluorescein (FITC). Aliquots of 50000 cells were seeded in 24 well-plates and allowed to attach for 24 h. Next, cells were treated with the compounds (previously heated to 95 ºC for 1 minute and allowed to cool down to r.t. or without previous heat treatment) at 5 μM for 3 h at 37 °C. The cells were harvested by trypsinization and gently washed with 1% FBS in cold PBS. The fluorescence of the cells, corresponding to the cellular uptake of the fluorescein-labelled peptides, was analysed using FACSCalibur (Becton Dickinson Immunocytometry Systems, San Jose, CA) equipped with the CellQuestTM software (Becton Dickinson). Ten thousand cells were analyzed

in each experiment. Mean fluorescence intensity was represented on a four orders of magnitude log scale (1-10,000).

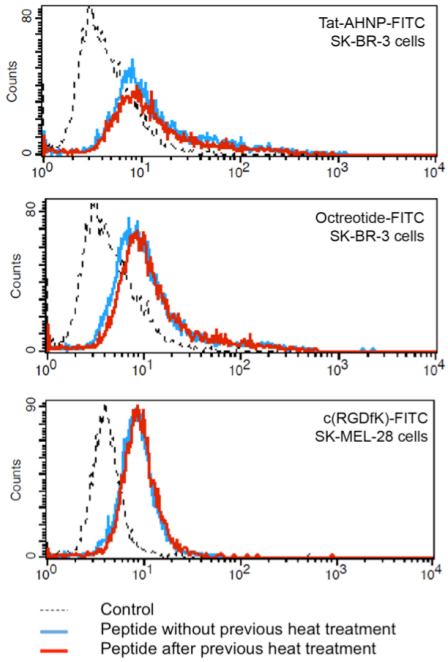

Figure S7. Flow cytometry histograms of SK-BR-3 and SK-MEL-28 cells treated with FITC-labelled c(RGDfK), octreotide and Tat-AHNP peptides at 5 μM concentration. Experiments were performed without previous heat treatment of the peptide (blue lines) and with previous heat treatment (red lines).

14. Analysis of HER2 knockdown by Western blot

SK-BR-3, BT-474 and SK-MEL-28 cells were seeded 24 h before transfection in 24-well plates at a density of 150000 cells/well in the corresponding medium containing 10% FBS. Following overnight culture, siRNA duplexes were added (in the presence or in the absence of Lipofectamine 2000) to each dish with a final volume of 500 μL. After a 48-h incubation time, the cells were harvested with PBS and lysed by incubation in RIPA buffer containing protease inhibitors (Roche) at 4 ºC for 1 h. Cell debris were removed by centrifugation at 8000 x g for 20 min at 4 ºC, and protein concentration was determined using the BCA assay (Pierce). 20 μg of protein were resolved by SDS electrophoresis and transferred to a poly(vinylidene difluoride) membrane (Immobilon-P, Millipore). The membrane was blocked with 5% skim milk in TBS containing 0.1% Tween for 1 h at r.t. and subsequently probed with anti-ERBB2 monoclonal antibody (Santa Cruz Biotechnology) (diluted 1:1500 in blocking buffer in the case of SK-BR-3 and BT-474 cells and 1:200 in blocking buffer in the case of SK-MEL-28 cells) overnight at 4 ºC. β-Actin was selected as internal control and was detected by incubation with anti-actin monoclonal antibody (Sigma-Aldrich) (at a dilution of 1:3500 in blocking buffer) for 1 h at r.t. Horseradish peroxidase-labeled polyclonal goat anti-mouse secondary antibody (Thermo Scientific, Rockford, IL) was incubated at 1:1000 dilution in the blocking solution for 1 h at r.t. The intensities of the bands were analyzed using ImageJ 1.45 software (Rasband, W.S., ImageJ, U. S. National Institutes of Health, Bethesda, Maryland, USA, http://imagej.nih.gov/ij/, 1997-2011).

15. Western blot analysis of HER2 expression in SK-BR-3 and HEK-293 cell lines

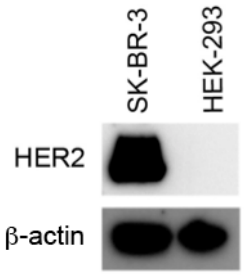

Figure S8. Western blot analysis of HER2 indicates that the protein is overexpressed in the SK-BR-3 breast cancer cell line compared with the non-malignant cell line HEK-293. β-actin serves as a loading control.

16. Western blot analysis of HER2 expression in cells treated with peptides alone

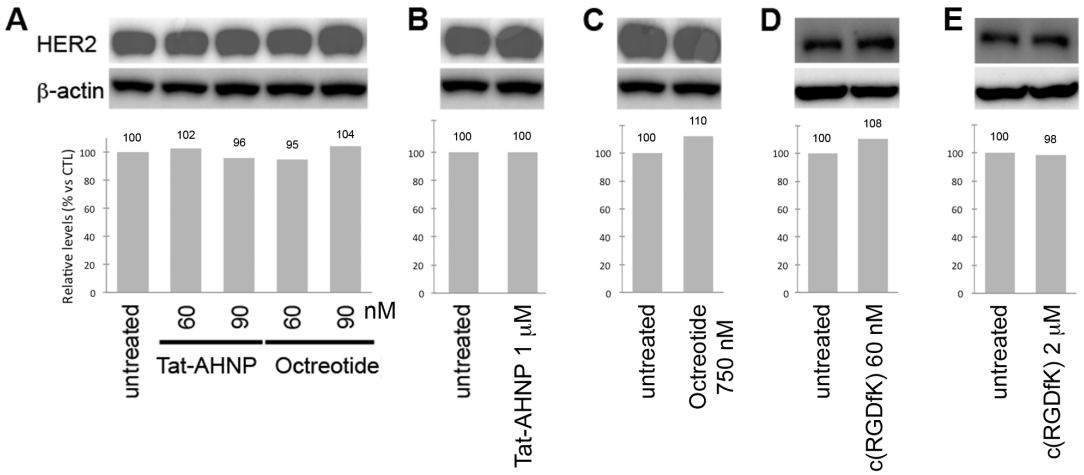

Figure S9. Western blot analysis of cells treated with peptides [c(RGDfK), octreotide and Tat-AHNP] alone in the presence (A and D) and in the absence of transfecting agent (B, C and E). Panels A-C: SK-BR-3 cells; Panels D, E: SK-MEL-28 cells. β-actin was used as loading control. Untreated cells: cells treated with cell medium alone.

17. Luciferase siRNA assays

HEK-293 cells were regularly passaged to maintain exponential growth. The cells were seeded one day prior to the experiment in a 24-well plate at a density of 150000 cells/well in complete DMEM containing 10% FBS (500 µL per well). Following overnight culture, the cells were treated with siRNAs and *Renilla* and Firefly luciferase vectors as follows:

PROTOCOL **A** (transfection of siRNAs using Lipofectamine 2000). *Renilla* and Firefly luciferase vectors (0.1 μg and 1.0 μg per well, respectively) and Tat-AHNP-bearing and unmodified siRNAs (**V** and **VI** respectively; 90 nM per well) were transfected into the cells using Lipofectamine 2000 (Invitrogen) as described by the manufacturer for adherent cell lines. The final volume was 500 μL per well. The cells were harvested 48 hours after transfection, and lysed using passive lysis buffer (100 μL per well) according to the instructions of the Dual-Luciferase Reporter Assay System (Promega). The luciferase activities of the samples were measured using a MicroLuma*Plus* LB 96V Luminometer (Berthold Technologies) with a delay time of 2 s and an integrate time of 10 s. The following volumes were used: 20 μL of sample and 30 μL of each reagent (Luciferase Assay Reagent II and Stop and Glo Reagent). The inhibitory effects generated by antisense oligonucleotides were expressed as normalized ratios between the reporter GL3 (Firefly) luciferase gene and the control RL (*Renilla*) luciferase gene.

PROTOCOL **B** (transfection of siRNAs in the absence of Lipofectamine 2000). *Renilla* and Firefly luciferase vectors (0.1 μg and 1.0 μg per well, respectively) were transfected into the cells using Lipofectamine 2000. Cells were incubated with the plasmids for 3 hours. Medium was discarded and the cells were washed with PBS. Then, 475 μL of fresh medium without antibiotics were added to each well. Tat-AHNP-bearing and unmodified siRNAs (**V** and **VI** respectively) were prepared at a

concentration of 20 μM in HEPES buffer, and 25 μL of oligonucleotide solution were added to each well (1 μM per well). 48 hours after transfection cell lysates were prepared and analyzed using the Dual-Luciferase Reporter Assay System as described above.

18. Cell viability assay

Interference with *in vitro* growth rate of SK-BR-3 and SK-MEL-28 cells by the peptides [c(RGDfK), octreotide and Tat-AHNP] was measured using crystal violet. 150.000 SK-BR-3 or SK-MEL-28 cells were plated in 24-well plates. Twenty-four hours after plating (0 hrs), cells were separately treated (in parallel) with peptides [c(RGDfK), octreotide and Tat-AHNP] (2 μM, 750 nM and 1 μM, respectively). At different time points (48 h or 72 h), cells were fixed with 4% formalin for 10 minutes, then washed twice with distilled water and stained with 0.1% freshly prepared crystal violet for 30 minutes. After washing, the stain was dissolved with 10% acetic acid and subsequently quantified by absorbance at 570 nM.

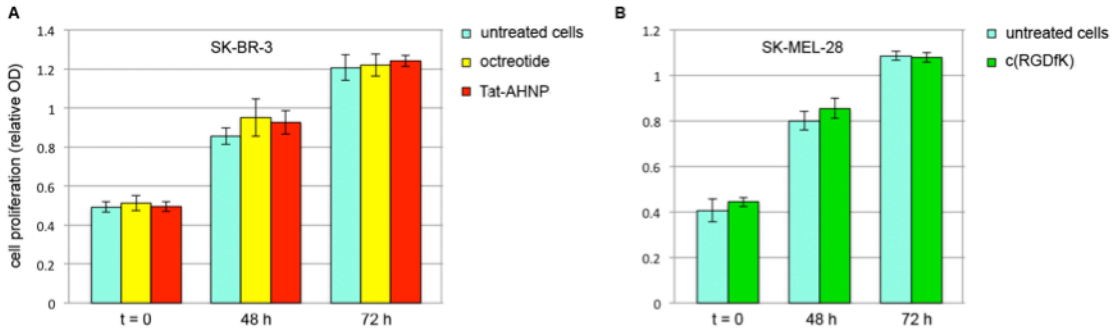

Figure S10. Cell viability assay after treatment of SK-BR-3 (A) and SK-MEL-28 (B) cells with the peptides [octreotide (A), Tat-AHNP (A) and c(RGDfK) (B). The growth of cells were assessed using grystal violet assay.

19. References

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