Supporting information for:

Selective intracellular delivery of thiolated cargo to tumor and neovasculature cells using histidine-rich peptides as vectors.

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**Peptide synthesis and characterization**

All peptides were synthesized using an automatic Prelude synthesizer (Protein Technologies Inc.) and standard Fmoc-protected amino acid derivatives (all acquired from Novabiochem). All couplings were done using HCTU (Novabiochem, 8.51012.1000) and diisopropylethylamine (Sigma Aldrich, D125806) in DMF for 1 hr. Fmoc-deprotection reactions were done using 20% piperidine (Sigma Aldrich, 104094) in DMF (5 and 10 mins). After each coupling and deprotection reaction, the resin was washed with DMF.

For amidated peptides Rink amide AM resin (Novabiochem, 8.55130.0025) were used. All histidine-rich vector peptides were acetylated (N-terminus) by treating the resin with 10% acetic anhydride in DMF for 10 min. After the synthesis has been completed the resin was washed with dichloromethane, diethyl ether and allowed to air dry. Peptides were then cleaved and side chains deprotected by treating the resin with 95% trifluoroacetic acid (TFA, VWR, 84578.290), 2.5% triisopropylsilane (TIS, Sigma Aldrich, 233781) and 2.5% water for 3 h. The TFA was removed under vacuum (Rotavapor) and the crude peptide precipitated with diethyl ether, which was subsequently removed by decantation. The crude peptide was left to air dry before UPLC analysis and preparative HPLC purification.

**Thiolated AMPs**

To incorporate a thiol group at the C-terminus a cysteamine 2-chlorotrityl resin (Novabiochem, 01-64-0107) was used as solid support. Peptide cleavage was done by treating the resin with 94% TFA, 2.5% TIS, 2.5% ethanedithiol (EDT, Fluka, 02390) and 1% water for 3 h. Peptide work up was done as with described above.
Npys-modified histidine-rich vector peptides

In order to ensure that the correct (asymmetrical) disulfide bond is formed between the two components of the conjugate, one of the peptides’ cysteine thiol-containing side chain had to be protected/activated. This is routinely achieved by the Npys-group and in this study a slightly modified version of Ghosh and Fan was used. Following the completion of the peptide, the peptidyl-resin was treated with the following a cleavage cocktail composing of 95% TFA, 2.5% water, 2.5% TIS and 7 eq 2,2-dithio-bis(5-nitropyridine) (DTNP, Sigma-Aldrich, 158194) for 3 h.

The cleavage mixture quickly developed a strong reddish color (presumably a 5-nitropyridine-2-thiol adduct). After three hours the TFA was evaporated and the peptide precipitated with ether. Both the precipitate and ether solution had a strong yellow color. The precipitated crude was air dried, dissolved in solvent A, and analyzed using analytical HPLC. A diethyl ether (liquid-liquid) extraction step successfully removed the colored impurity from an aqueous peptide solution, making it possible to purify the Npys-activated peptide to yield a light straw colored material.

HPLC purification

All crude peptides were purified using preparative HPLC (Waters Autoprep: PAD Detector 2998, Binary pump 2545, Sample manager 2767). A XSelect CSH column (C18, 5µm, 19 x 250mm) were used. Both mobile phases (water and acetonitrile) contained 0.1% TFA. The flow rate was 20 mL/min. The minimum purity of all peptides were 95% as determined by UPLC-PDA analyses.

Purity and molecular weight determination
The purity of synthesized peptides was determined using (i) a Waters Acuity H-class UPLC with (ii) a Waters Acquity BEH C18 column 2.1x50 mm with 1.7 µm particles, and (iii) PDA detector with detection set at 200-500 nm, (iv) having a flow rate of 1 mL/min and gradient of 5 - 95 %B over 10 min, and (v) where mobile phase A is water with 0.1% TFA and B is acetonitrile with 0.1% TFA.

The correct molecular weight of the compounds was determined using (i) a Waters Acuity I-class UPLC with (ii) a Waters Acquity BEH C18 column 2.1x100 mm with 1.7 µm particles column, and (iii) Waters XEVO Q-ToF G2 mass spectrometer, with the tune method set at capillary 0.6 kV, sampling cone 30 V, source temperature 130 °C, desolvation temperature 450 °C, cone gas flow 10.0 L/h and desolvation gas flow 800.0 L/h, the MS method was set at mass 100.00 to 5000.00, scan time as set at 0.50 s and lock mass at 556.2771 (leucine-enkephaline used for lock spray), (iv) the flow rate was 0.5 mL/min, and gradient of 5 - 95 %B over 7 min, and (v) where mobile phase A is water with 0.1 % FA and B is acetonitrile with 0.1% FA.
Figure S1. UPLC and MS analyses of (RW)$_3$-amide (Compound 1).
Figure S2. UPLC and MS analyses of \((RW)_3\text{-thiol}\) (Compound 3).
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Figure S3. UPLC and MS analyses of Ac-Cys(Npys)-Ahx-(GHHPH)$_2$ (Compound 2).
Figure S4. UPLC and MS analyses of Ac-Pen(Npys)-Ahx-(GHHPH)$_2$. 
Figure S5. UPLC and MS analyses of (RW)₃-SS-Cys-Ahx-(GHHPH)₂ (Compound 4).
Figure S6. UPLC and MS analyses of (RW)$_3$-SS-Pen-Ahx-(GHHPH)$_2$. 
Figure S7. UPLC and MS analyses of \((\text{RW})_3\text{-Ahx-(GHPH)}_2\) (Compound 5).
Figure S8. UPLC and MS analyses of (EA)$_3$-SS-Cys-Ahx-(GHHPH)$_2$. 
**In vitro assays**

**Cell viability assay**

Cell viability was determined using the well-established MTT assay.\textsuperscript{1} DU-145 prostate carcinoma cells (ATCC HTB-81) were used in the biological evaluation of the peptides. The cells were cultivated at 37°C in a 5% CO\textsubscript{2} atmosphere (Hera Cell 150 incubator, Thermo Electron Corp.) in Eagle’s Minimal Essential Medium (EMEM) (Sigma Aldrich, M4655) supplemented with 10% FBS (Sigma Aldrich, ). Afterwards, cells were counted (Z1 Coulter Particle Counter, Beckman Coulter) and seeded in 96-well flat-bottomed microtiter plates (Falcon Microtest 96) to a concentration of $1.5 \times 10^5$ cells/mL, and incubated overnight to allow for attachment.

RPMI-1640 medium (Sigma Aldrich, R8758) was used to prepare peptide dilution series (10-500 µg/mL), each concentration was done in triplicate. The pH of the assay-medium was 7.4, and potential changes in pH during the assay was monitored visually by inspecting the color of the assay-medium which should stay red throughout the experiment. Each experiment was done at least in duplicate. To simulate elevated zinc levels found at angiogenic sites the media was adjusted with 50 µM ZnCl\textsubscript{2}. For negative controls the same media without peptide was used. For positive controls 1% Triton X-100 (Sigma Aldrich, T8787) was added to the media. A pilot study showed that incubation with ZnCl\textsubscript{2} is not toxic to DU-145 cells within the concentrations and incubation times used.

Table S1. Effect of ZnCl\textsubscript{2} concentration on cell viability.
Extracellular thiols were blocked by preincubating cells for 1 h with RPMI-1640 containing 2.5 mM 5,5′-Dithiobis(2-nitrobenzoic acid), or DTNB, (Sigma Aldrich, D8130). Following the preincubation step the DTNB solution was removed and the cells washed once with RPMI-1640 media. To ensure a thiol free environment during the whole incubation period the peptide dilution series were supplemented with 1 mM DTNB. A pilot study showed that incubation with DTNB is not toxic to DU-145 cells within the concentrations and incubation times used.

Table S2. Effect of DTNB concentration on cell viability.

| Incubation time | 2 h | 4 h |
|-----------------|-----|-----|
| Conc. in µM     |     |     |
| 10              |     |     |
| 0.4681          | 0.4806 | 0.4844 |
| 0.4263          | 0.4764 | 0.4893 |
| 0.4409          | 0.4918 | 0.5111 |
| 0.4336          | 0.4737 | 0.4886 |
| 0.4774          | 0.5267 | 0.5046 |
| 0.4577          | 0.5027 | 0.4991 |
| 0.478           | 0.5075 | 0.5166 |
| 0.4739          | 0.4896 | 0.4931 |
| **% live cells**| 92  | 101  |

| Conc. in µM     |     |     |
| 25              |     |     |
| 0.455671        | **0.493626** | 0.496183 |
| 0.48845         | 0.043688 |

| Conc. in µM     |     |     |
| 50              |     |     |
| 0.4382          | **0.449525** | **0.452925** |
| 0.471475        | 0.0454 |

| Conc. in µM     | NC  | PC  |
|-----------------|-----|-----|
| 10              |     |     |
| 0.4663          | 0.0446 |
| 0.4773          | 0.0439 |
| 0.4912          | 0.0439 |
| 0.4827          | 0.0444 |
| 0.5086          | 0.0434 |
| 0.5016          | 0.0434 |
| 0.5166          | 0.0427 |
| 0.4931          | 0.0436 |
| **% live cells**| 92  | 102  |

| Conc. in µM     | NC  | PC  |
|-----------------|-----|-----|
| 25              |     |     |
| 0.481           | 0.0443 |
| 0.4922          | 0.0421 |
| 0.4857          | 0.0404 |
| 0.4872          | 0.0429 |
| 0.495           | 0.0424 |
| 0.4584          | 0.0581 |
| 0.4407          | 0.042 |
| 0.4316          | 0.042 |
| **% live cells**| 92  | 95  |

| Conc. in µM     | NC  | PC  |
|-----------------|-----|-----|
| 50              |     |     |
| 0.448          | 0.0443 |
| 0.4922         | 0.0421 |
| 0.4857         | 0.0404 |
| 0.4872         | 0.0429 |
| 0.495         | 0.0424 |
| 0.4584         | 0.0581 |
| 0.4407         | 0.042 |
| 0.4316         | 0.042 |
| **% live cells**| 92  | 95  |
Following a 24 h peptide treatment, 10 µL of 12 mM MTT (Sigma Aldrich, M5665) in PBS was added and incubated for 4 h. Subsequently, 70 µL of each well were carefully removed. The formazan crystals were dissolved by adding 100 µL acidic isopropanol (40 mM HCl) and shaking the plate for 30 min. Cell viability was expressed as percent of control after measuring absorbance at 590 nm VersaMax™ plate reader (Molecular Devices, Sunnyvale, CA, USA). All IC₅₀ values reported are averages obtained from two independent experiments done in triplicate.

All peptides were prepared as TFA salts and since the peptides contain a high number of basic amino acids with TFA counterions, TFA content were taken into account when determining IC₅₀ values. Although net peptide content was not empirically determined it is expected that the freeze dried material will contain a significant amount of TFA, see Table S1. Therefore, MW values, adjusted for theoretical TFA content, were used in calculating IC₅₀ values.
Table S3. Peptides synthesized and tested in this study.

| Peptide       | Sequence                         | #TFA\(^{(a)}\) | Monoisotopic Mass | % TFA\(^{(c)}\) |
|---------------|----------------------------------|----------------|------------------|-----------------|
|               |                                  |                |                  |                 |
| (RW\(_3\))-amide | H\(_2\)N-RWRWRW-amide           | 4              | 1043.57          | 1499.54         | 30.4            |
| (RW\(_3\))-thiol | H\(_2\)N-RWRWRW-cysteamine      | 4              | 1103.57          | 1559.54         | 29.2            |
| (RW\(_3\))-SS-Cys(GHHPH)\(_2\) | H\(_2\)N-RWRWRW-(Ac)CAhxGHHPHGHHPH-amide | 10         | 2507.19          | 3647.12         | 31.3            |
| (RW\(_3\))-SS-Pen(GHHPH)\(_2\) | H\(_2\)N-RWRWRW-(Ac)PenAhxGHHPHGHHPH-amide | 10         | 2535.22          | 3675.15         | 31.0            |
| (RW\(_3\))-Ahx-(GHHPH)\(_2\) | H\(_2\)N-RWRWRWAhxGHHPHGHHPH-amide     | 10         | 2287.15          | 3427.08         | 33.3            |
| (EA\(_3\))-SS-Cys(GHHPH)\(_2\) | H\(_2\)N-EAEAEA-SS-(Ac)CAhxGHHPHGHHPH-amide | 7          | 2080.89          | 2878.84         | 27.7            |

\(^{(a)}\) Number of TFA counterions possible (underlined basic amino acids and N-terminus).

\(^{(b)}\) The monoisotopic mass for the TFA salt of the peptide. This value is used in IC50 calculations.

\(^{(c)}\) Minimum theoretical TFA content of freeze dried peptide. Net peptide content was not empirically determined.

H\(_2\)N- denotes a free N-terminal amine; (Ac) denotes the acetylated N-terminus of the vector peptides; SS denotes the disulfide bond between the vector and cargo peptides.

**Linker reduction assay**

The stability of two disulfide peptides toward reduced glutathione (GSH) was determined in a kinetic assay. The peptides were dissolved in water to yield a stock solution of 1 mg/mL. Two different targetide:GSH ratios were tested to simulate GSH concentrations found in plasma (1:10) and the cytosol (1:100). The peptides were diluted with 100 mM Tris (pH 7.6) to 50 µM working solutions right before the start of the reaction. A 10 mM GSH (Sigma-Aldrich, G4251) solution was prepared in 100 mM Tris (pH 7.6) and added to the peptide solution. The reaction was followed with UPLC and samples were taken every 15 min.
and the reaction was monitored until all of the original disulfide targetide disappeared. New peaks representing reduction byproducts were identified (MS) and quantified (UV). The stability of the linkers were expressed as LR$_{50}$ values, which can be defined as the amount of time needed for the reductive cleavage of 50% of the disulfide conjugates present in a sample. Reported LR$_{50}$ values are averages obtained from duplicate experiments.

**Transmission electron microscopy**

DU-145 1.5 x 10$^5$ cells/mL were suspended in serum free RPMI-1640 medium and transferred in 6-well flat-bottomed plates (Falcon multiwell, Corning). Peptide concentration and incubation time were chosen according to MTT assay and determined IC$_{50}$ values. All cells were pre-fixed with Karnovsky's cacodylate-buffered (pH 7.2) formaldehyde-glutaraldehyde fixative and removed from wells with a cell scraper. The cells were spun down and the supernatant replaced with fixative. The fixed cells were stored at 4 °C overnight. Cell samples were prepared for transmission electron microscopy as described earlier.$^2$
Figure S9. TEM micrographs of DU-145 cells in overview (A, C, E; scale bar represents 5 µm) and higher magnifications of indicated locations (B, D, F; scale bar represents 0.5 µm).

Reference List

(1) Mosmann, T. Rapid colorimetric assay for cellular growth and survival: application to proliferation and cytotoxicity assays. J. Immunol. Methods 1983, 65, 55-63.

(2) Hanski, L.; Ausbacher, D.; Tiirola, T.; Strøm, M.B.; Vuorela, P.M. Amphipathic β2,2-Amino Acid Derivatives Suppress Infectivity and Disrupt the Intracellular Replication Cycle of Chlamydia Pneumoniae. PLoS ONE 2016, 11(6).