Covalent Attachment of Cyclic TAT Peptides to GFP Results in Protein Delivery into Live Cells with Immediate Bioavailability**

Nicole Nischan, Henry D. Herce, Francesco Natale, Nina Bohlke, Nediljko Budisa, M. Cristina Cardoso,* and Christian P. R. Hackenberger*

anie_201410006_sm_miscellaneous_information.pdf
anie_201410006_sm_movie_mitosis.mov
C

SUPPORTING INFORMATION

Table of Contents

1 Abbreviations used ...................................................................................................................... S2

2 Synthesis Conjugatable Peptides ................................................................................................. S3
  2.1 General Information ................................................................................................................ S3
  2.2 Synthesis of cTAT .................................................................................................................... S3
  2.3 Synthesis of cyclic TAT peptides with peptide linker .......................................................... S4
  2.4 Synthesis of TAT .................................................................................................................... S6

3 Protein Biosynthesis .................................................................................................................... S8
  3.1 Strains and Plasmids ................................................................................................................ S8
  3.2 Protein Biosynthesis and Purification ......................................................................................... S8

4 Protein-Peptide Conjugation ..................................................................................................... S10
  4.1 Optimized Procedure ............................................................................................................... S10
  4.2 Initial efforts on getting access to pure CPP-GFP conjugates ................................................... S10
  4.3 Representative MALDI Spectra of GFP, cTAT-GFP and TAT-GFP ........................................ S12
  4.4 Photophysical Properties of GFP and cTAT-GFP ................................................................. S13

5 Cellular Uptake .......................................................................................................................... S16
  5.1 Cellular Uptake by Transduction Versus Endocytosis .......................................................... S16
  5.2 Live Cell Confocal Microscopy of Transduced Cells ............................................................. S16
  5.3 Fluorescence Recovery After Photobleaching of Internalized GFP ...................................... S17
  5.4 Cellular Uptake Does Not Require Macropinocytosis or Metabolic Energy ......................... S18
  5.5 Quantification of GFP Intracellular Concentration .............................................................. S20
  5.6 Movie of Transduced Cells – Motility and Mitosis ............................................................... S21

6 References ................................................................................................................................... S22

7 Appendix ................................................................................................................................... S23
1 Abbreviations used

AcOH acetic acid  
Alloc allyloxycarbonyl-  
CuAAC Copper(I)-catalyzed Azide-Alkyne Cycloaddition  
DHAP dihydroxyacetone phosphate  
DIC diisopropylcarbodiimide  
DIC differential interference contrast microscopy  
DIPEA N,N-diisopropylethylamine  
DMF dimethylformamide  
DTT dithiothreitol  
EDTA ethylenediaminetetraacetic acid  
Eq. equivalents  
ESI-MS electrospray ionization mass spectrometry  
FA formic acid  
FI fluorescence intensity  
Fmoc fluorenylmethyloxycarbonyl-  
HATU 1-[bis(dimethylamino)methylene]-1H-1,2,3-triazolo[4,5-b]pyridinium 3-oxido hexafluorophosphate  
HBTU N,N,N',N'-Tetramethyl-O-[1H-benzotriazol-1-yl]uronium hexafluorophosphate  
HEPES 2-[4-(2-hydroxyethyl)piperazin-1-yl]ethanesulfonic acid  
HOBt 1-hydroxybenzotriazole  
HPLC high-performance liquid chromatography  
MALDI Matrix-assisted laser desorption/ionization  
MeCN acetonitrile  
NMM 4-methylmorpholine 4-oxide  
OAll Allyloxy-  
PBS phosphate buffered saline  
ROI region of interest  
TFA trifluoroacetic acid  
THPTA Tris(3-hydroxypropyl triazolylmethyl)amine  
TIS triisopropylsilane  
UPLC ultra performance liquid chromatography  
UV ultraviolet
2 Synthesis Conjugatable Peptides

2.1 General Information

Reagents and solvents, unless stated otherwise, were commercially available as reagent grade or HPLC grade and did not require further purification. Amino acids were purchased from IRIS Biotech GmbH (Germany), resins from Novabiochem (Merck KGaA, Germany).

SPPS. Peptides were synthesized with an Activo-P11 Peptide Synthesizer (Activotec SPP Ltd., UK) via standard Fmoc-based conditions using HOBt/HBTU/DIPEA activation and piperidine Fmoc deprotection in DMF.

HPLC-UV purity of peptides was determined at 220 nm using a system consisting of a Waters 600S controller, a Waters 616 pump, a Waters 717plus autosampler and a Waters 2489 UV/Visible detector (all Waters Corporation, US) on a C18-column (Eclipse, Agilent Technologies, USA) at constant flow of 1.0 mL/min of 0 % to 50 % acetonitrile in water (with 0.1 % TFA) over 30 min.

High-resolution mass spectra (HRMS) were either collected with a Waters Acquity UPLC coupled to an LCT Premier Micromass Technologies System using as an eluent water and MeCN containing 0.1 % formic acid at a flow rate of 0.2 mL/min on a C18 column. The data was processed using Mass Lynx Software (all Waters Corporation, US). Alternatively, ESI-MS was measured on an Agilent 6210 ToF LC-MS system (Agilent Technologies, US) at constant flow of 0.2 mL/min with water and MeCN containing 1 % FA to give high resolution mass spectra.

2.2 Synthesis of cTAT

Scheme S1. Synthesis of conjugatable cyclic TAT peptide (cTAT).

First, a linear peptide of the sequence (Fmoc)(Alloc)KrRrGrKkRr(OAll)Q was synthesized on a Wang resin (0.25 mmol, 0.22 mmol/g). Upper case letters correspond to L-, lower case letters to D-amino acids. The first amino acid, Fmoc-(allyl)glutamic acid, was loaded manually. Other couplings were performed using double couplings of 10 eq. of amino acid The N-terminal Fmoc was left on the peptide.

The Alloc and OAll protecting groups were removed using Pd(PPh₃)$_4$ (270 mg, 0.5 eq.) in a mixture of CHCl₃/AcOH/NMM in a ratio of 37/2/1 for two hours at room temperature under Argon bubble mixture. To remove the Palladium catalyst afterwards, it was additionally washed with 0.2 M DIPEA in DMF. Then the peptide was cyclized using two equivalent of HATU/DIPEA in DMF for two hours, followed by capping. After Fmoc removal a linker consisting of two repeats of [2-[2-aminoethoxy]ethoxy]acetic acid was introduced and azidobutanoic acid was coupled to the N-terminus of this linker. These couplings were each performed twice using 5 eq. of HOBt/DIC activated acid in DMF.
After washing and drying the peptide was cleaved from the solid support (two hours in 8 mL of mixture 95 % TFA, 2 % TIS, 2 % DTT, 1 % MeSPh) and precipitated in 100 mL of diethylether. The peptide was isolated using preparative HPLC (gradient 0 to 40 % in 100 min of B in A, A being 100 % water, B being 100 % MeCN, each containing 0.1 % TFA on a RP-C18 column (Nucleodur C18 H Tec (Machenerey Nagel, Germany), 100 Å, 5 μm, 250 mm x 32 mm, 17 mL/min)) to yield in a white trifluoroacetate (48.92 mg, 17.35μmol, yield 6.9 %, molar mass (peptide) = 1.91 kDa, molar mass (TFA salt) = 2.82 kDa) in good purity. HRMS: m/z: 382.6376 [M+5H]^5+ (calcd. m/z: 382.6375, ∆ 0.3 ppm), see appendix for spectra.

Figure S1. HPLC-UV purity of cTAT.

2.3 Synthesis of cyclic TAT peptides with peptide linker

Cyclic TAT with GKGNG-linker functionalized with azidobenzoic acid

Scheme S2. Synthesis of conjugatable cyclic TAT peptide with GKGNG-linker functionalized with azidobenzoic acid (cTAT A).

Wang resin (0.1 mmol, 0.20 mmol/g) was loaded with Fmoc-(allyl)glutamic acid and applied to SPPS using double couplings and capping with HOBT/HBTU/DIPEA in NMP on the Applied Biosystem synthesizer for the first nine standard amino acids (sequence = rRrGrKkRr) and Alloc-protected lysine. After that, Allyl-protected glutamic acid and Alloc-protected lysine were deprotected using Pd(PPh₃)₄ in a mixture of CHCl₃/ AcOH/ NMM in a ratio of 37/ 2/ 1 for two hours at room temperature.
Afterwards the peptide was cyclized on the solid support using one equivalent of HATU and two equivalents of DIPEA. Then the linker (GKGNG) was attached to the peptide via SPPS using double couplings with HOBT/DIC in DMF manually. At the N-terminus, para-azidobenzoic acid was coupled (10 eq., 2 x 2h, RT, HOBT and DIC in DMF). After washing and drying, the peptide was cleaved from the solid support (2 h in 2 ml of mixture 95 % TFA, 2 % TIS, 2 % DTT, 1 % MeSPh) and purified by semi-preparative HPLC (0 to 50 % of MeCN in Water, containing 0.1 % TFA on RP-C18 column) to yield in a white trifluoroacetate (9.83 mg, 3.18 μmol, yield 3.2 %, molar mass (peptide) = 2.06 kDa, molar mass (TFA$_4$ salt) = 3.09 kDa) in good purity. HRMS: m/z: 517.3029 [M+4H]$^{4+}$ (calcd. m/z: 517.3042), see appendix for spectra.

**Figure S2.** HPLC-UV purity of cTAT A.

**Cyclic TAT with GKGNG-linker functionalized with azidobutanoic acid**

**Scheme S3.** Synthesis of conjugatable cyclic TAT peptide with GKGNG-linker functionalized with azidobutanoic acid (cTAT B).

Wang resin (0.05 mmol, 0.22 mmol/g) was loaded with Fmoc-(allyl)glutamic acid and applied to SPPS using double couplings and capping with HOBT/HBTU/DIPEA in NMP on the Applied Biosystem synthesizer for the first nine standard amino acids (sequence = rRrGrKkRr) and Alloc-protected lysine. After that, allyl-protected glutamic acid and Alloc-protected lysine were deprotected using Pd(PPh$_3$)$_4$ in a mixture of CHCl$_3$/ AcOH/ NMM in a ratio of 37/ 2/ 1 for two hours at room temperature. Afterwards the peptide was cyclized on the solid support using one equivalent of HATU and two equivalents of DIPEA. Then the linker (GKGNG) was attached to the peptide via SPPS using double couplings with HOBT/DIC in DMF manually. At the N-terminus, para-azidobenzoic acid was coupled (10 eq., 2 x 2h, RT, HOBT and DIC in DMF). After washing and drying, the peptide was cleaved from the solid support (2 h in 2 ml of mixture 95 % TFA, 2 % TIS, 2 % DTT, 1 % MeSPh) and purified by semi-preparative HPLC (0 to 50 % of MeCN in Water, containing 0.1 % TFA on RP-C18 column) to yield in a white trifluoroacetate (9.83 mg, 3.18 μmol, yield 3.2 %, molar mass (peptide) = 2.06 kDa, molar mass (TFA$_4$ salt) = 3.09 kDa) in good purity. HRMS: m/z: 517.3029 [M+4H]$^{4+}$ (calcd. m/z: 517.3042), see appendix for spectra.
couplings with HOBt/DIC in DMF manually. At the N-terminus, azidobutanoic acid was coupled (20 eq., 2h, RT, HATU and DIPEA in DMF). After washing and drying, the peptide was cleaved from the solid support (2 h in 3 ml of mixture 95 % TFA, 2 % TIS, 2 % DTT, 1 % MeSPh) and purified by semi-preparative HPLC (0.8 to 32 % of MeCN in Water containing 0.1 % TFA over 70 min on a RP-C18 column (100 Å 5 μ, 21 mm x 250 mm) at a flow of 5 mL/min) to yield in a white trifluoroacetate (5.94 mg, 1.94 μmol, yield 3.9 %, molar mass (peptide) = 2.03 kDa, molar mass (TFA salt) = 3.06 kDa) in good purity. HRMS: m/z: 678.0753 [M+3H]³⁺ (calcd. m/z: 678.0748), see appendix for spectra.

Figure S3. HPLC-UV purity of cTAT B.

2.4 Synthesis of TAT

Scheme S4. Synthesis of conjugatable linear TAT peptide (TAT).

The linear control peptide ArRrGrKkRrQ was synthesized on Rink amide resin (0.1 mmol, 0.71 mmol/g) using double couplings of 5 eq. of amino acid. Upper case letters correspond to L-, lower case letters to D-amino acids. To the N-terminus, a linker consisting of two repeats of [2-[2-aminoethoxy]ethoxy]acetic acid and finally azidobutanoic acid was coupled. These couplings were each performed twice using 5 eq. of HOBt/DIC activated acid in DMF. After washing and drying the peptide was cleaved from the solid support (two hours in 3 ml of mixture 95 % TFA, 2 % TIS, 2 % DTT, 1 % MeSPh), precipitated in 100 mL of diethylether. The peptide was isolated using preparative HPLC (5 to 50 % of B in A, A being 100 % water, B being 80 % MeCN in water, each containing 0.1 % TFA on a RP-C18 column (100 Å, 5 μm, 250 mm x 20 mm, 5 mL/min) to yield in a white trifluoroacetate (25.06 mg, 9.02 μmol, yield 9.0 %, molar mass (peptide) = 1.87 kDa, molar mass (TFA salt) = 2.78 kDa) in good purity. HRMS: m/z: 374.6313 [M+5H]³⁺ (calcd. m/z: 374.6309, ∆ 1.1 ppm), see appendix for spectra.

S6
Figure S4. UPLC-UV purity of TAT was determined at 220 nm using an Acquity UPLC H-Class operated with Empower 3 software (all Waters Corporation, US) on a C18-column (Acquity UPLC BEH C18 1.7 μm, 2.1 x 50 mm) at constant flow of 0.6 mL/min of 2 % to 100 % acetonitrile in water (with 0.1 % TFA) over 15 min.
3 Protein Biosynthesis

3.1 Strains and Plasmids

The plasmid pET30b_GFPhs1-RM encoding a mutated variant of GFP containing only the N-terminal methionine (Met) (GFPhs1-RM, 245 amino acids, 27.6 kDa) was kindly provided by Soundrarajan Nagarasundarapandian from the group of Prof. Sun Gu Lee (Pusan University, South Korea). A Tobacco Etch Virus (TEV) protease recognition site was introduced for removal of the C-terminal His-tag. Glutamine at the N-terminus ensures that the N-terminal Met or homopropargylglycine (HPG), respectively, is not cleaved.

For expression of GFPhs1-RM_TEV, the Met auxotrophic E. coli strain B834 (DE3) (B→B834[2]→ B834[3]) was used.

3.2 Protein Biosynthesis and Purification

Protein biosynthesis was performed using Met auxotrophic E. coli strain B834 (DE3).[3] Cells were transformed with pET30b_GFPhs1-RM_TEV. An overnight culture in LB media was used to inoculate the new minimal media expression culture (1:1000).[4] The expression culture containing 45 µM Met as natural substrate was grown at 30°C overnight until depletion of Met and an optical density at 600 nm between 0.6 and 0.7. Defined concentration of the canonical amino acid allows the production of cell mass up to an OD₆₀₀ value of 0.6-0.8. The next day, the Met analogue homopropargylglycine (HPG, Chiralix B. V., Netherlands) was added to a final concentration of 50 mg/ml, and target gene expression was induced by addition of 0.5 mM isopropyl β-D-1-thiogalactopyranoside. For protein production cells were incubated at 28 °C for four hours with vigorous shaking at 160 RPM. After cell harvest, cells were lysed with sonication and the lysate cleared by high-speed centrifugation (18,000 g, 4 °C, 30 minutes). Soluble target protein was purified from the supernatant using a Ni-NTA column (GE Healthcare, UK) equilibrated with sodium dihydrogenphosphate buffer (50 mM, pH 8) containing 300 mM NaCl, 20 mM imidazole and 5 % glycerol (buffer NA). Unspecifically bound proteins were washed out with 30 mM imidazole and the desired protein was eluted with 500 mM imidazole. Afterwards, the buffer was exchanged via dialysis to TEV reaction buffer (50 mM NaH₂PO₄, 100 mM NaCl, 0.5 mM EDTA, 1mM DTT, pH 8). The C-terminal His-tag was cleaved using TEV protease which was added to the protein and incubated at room temperature overnight. Next, the protein was dialyzed into buffer NA again and His-tagged TEV protease and not-cleaved protein were separated from cleaved protein via a Ni-NTA column. The flow through containing the cleaved protein was collected and dialyzed against storage buffer (50 mM NaH₂PO₄, 100 mM NaCl, 5 % glycerol, pH 8). Protein purity was analyzed by SDS-PAGE and Coomassie staining, whereby protein concentration was determined by measuring the absorption at 280 nm. Incorporation of HPG in GFPhs1-RM was confirmed via ESI-MS (Exactive ESI-Orbitrap-MS, Thermo Fisher Scientific, US). The purified proteins were stored at -80 °C in aliquots until usage.
**Figure S5.** ESI-MS analysis of GFPs1-RM_TEV with HPG incorporated at the N-terminus. Calculated mass: 27,543 Da, Measured mass: 27,542 Da.
4 Protein-Peptide Conjugation

4.1 Optimized Procedure

Mutant GFP was conjugated with cyclic or linear TAT peptides using CuAAC. Optimized reaction conditions include shaking with 1,000 turns per minute at 15 °C for 20 h in Ca/Mg free Dulbecco’s PBS at final concentrations of protein 20 µM, azide peptide 500 µM, CuSO₄ 200 µM, THPTA ligand 1,000 µM and aminoguanidine hydrochloride and sodium ascorbate 10 mM (added stepwise at the start of the reaction and after two hours). For screening of conditions and different azido peptides, the batch size was 250 µL, conjugation for cell experiments was done at batch size 500 µL. Then, the reaction was quenched with 50 µL of 5 mM EDTA. Copper was removed via dialysis against 2.5 mM EDTA in Ca/Mg free Dulbecco’s PBS (three times 400 µL), then EDTA was removed via dialysis against HEPES buffer (three times 400 µL). This was done using centrifugal filters (Amicon Ultra 0.5 mL 10K MWCO regenerated cellulose, Merck Millipore, Merck KGaA, Germany). Full conversion and formation of product was verified using an AB SCIEX TOF/TOF 5800 (Applied Biosystems, Life Technologies, US). For this, 2 µL of the concentrated product were precipitated in 8 µL of acetone. The precipitate was then dissolved in 2 µL mQ water, 2 µL of each DHAP matrix and 2.0 % TFA were added and spotted on the target.

Table S1. Optimized reaction setup with final concentrations and equivalents used.

<table>
<thead>
<tr>
<th>Reagents added in this order (stock concentration)</th>
<th>Volume / µL</th>
<th>Conc. / µM</th>
<th>Eq.</th>
</tr>
</thead>
<tbody>
<tr>
<td>HPG-GFP (50µM)</td>
<td>100</td>
<td>20</td>
<td>1</td>
</tr>
<tr>
<td>PBS 1x without Ca,Mg</td>
<td>90</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Azido-peptide (50 mM)</td>
<td>2.5</td>
<td>5</td>
<td>500</td>
</tr>
<tr>
<td>Premixed CuSO₄ (20 mM) : THPTA (50 mM) = 1:2</td>
<td>7.5</td>
<td>15</td>
<td>200/1,000</td>
</tr>
<tr>
<td>Aminoguanidine HCl (100 mM)</td>
<td>25</td>
<td>50</td>
<td>10</td>
</tr>
<tr>
<td>Sodium Ascorbate (100 mM)</td>
<td>15+10</td>
<td>25+25</td>
<td>10</td>
</tr>
</tbody>
</table>

4.2 Initial efforts on getting access to pure CPP-GFP conjugates

Initially, we aimed at conjugating a cyclic peptide containing a linker of five amino acids and introduced an azido benzoic acid as reaction handle (cTAT A). CuAAC of cTAT A and GFP at optimized conditions (testreaction, 15 °C, 20 h, Table S1) lead to 50 percent of conversion (Figure S6). Addition of more copper led to degradation. Alternatively, we installed azido butanoic acid instead (cTAT B) which lead to essentially similar results (Figure S7).

For protein mixtures, plenty of purification methods are available. We rationalized that the charge is the major difference between starting material GFP (pl = 6.3) and conjugation product CPP-GFP (pl = 8.8) which is why we put effort into cation and anion exchange chromatography. Albeit trying different conditions of pH and ionic strength we could only recover traces of protein from the column. Also, we found it impossible to achieve separation with size exclusion chromatography and reversed phase chromatography.

Accordingly, we aimed at a CPP with a thin and flexible linker, which is why we designed the cyclic CPP with a PEG linker (cTAT) as reported in this paper. CuAAC with cTAT lead to a conversion of 90 to 100 percent (Figure S8 and S10).
Figure S6. MALDI of test reaction of HPG-GFP and cTAT A at conditions given in table S1.

Figure S7. MALDI of test reaction of HPG-GFP and cTAT B at conditions given in table S1.
Figure S8. MALDI of test reaction of HPG-GFP and cTAT at conditions given in table S1.

4.3 Representative MALDI Spectra of GFP, cTAT-GFP and TAT-GFP

Figure S9. MALDI of GFP, expected: 27,543 Da (M+H\(^+\)), 13,772 (M+2H\(^+\)), 9,182 (M+3H\(^+\)), found 27,487 (M+H\(^+\)), 13,766 (M+2H\(^+\)), 9,175 (M+3H\(^+\)).
4.4 Photophysical Properties of GFP and cTAT-GFP

Similar aliquots of HPG-GFP were subjected to either optimized reaction conditions (cTAT-GFP), reaction conditions without azide (GFP mock 1) or without any reagents (GFP mock 2), a control stayed frozen (GFP). Then all samples were subjected to identical workup procedure consisting of dialysis against EDTA/Dulbecco’s PBS and then HEPES buffer as described above. Then, taking aliquots of each sample and treating them identically, UV absorbance spectra were recorded (Jasco V-630 Spectrometer, Spectra manager, all JASCO Corporation, Japan) as well as the fluorescence emission spectra (Jasco FP-6500 Spectrofluorometer, Spectra manager, all JASCO Corporation, Japan).
**Table S2.** Reaction setup for comparison of photophysical properties of GFP and cTAT-GFP.

<table>
<thead>
<tr>
<th>Reagents added in this order / µL (stock concentration)</th>
<th>cTAT-GFP</th>
<th>GFP mock 1</th>
<th>GFP mock 2</th>
<th>GFP (freezer)</th>
</tr>
</thead>
<tbody>
<tr>
<td>HPG-GFP (50µM)</td>
<td>200</td>
<td>200</td>
<td>200</td>
<td>200</td>
</tr>
<tr>
<td>PBS 1x without Ca,Mg</td>
<td>180</td>
<td>185</td>
<td>300</td>
<td>0</td>
</tr>
<tr>
<td>Azido-peptide (50 mM)</td>
<td>5</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Premixed CuSO₄ (20 mM) : THPTA (50 mM) = 1:2</td>
<td>15</td>
<td>15</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Aminoguanidine HCl (100 mM)</td>
<td>50</td>
<td>50</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Sodium Ascorbate (100 mM)</td>
<td>25+25</td>
<td>25+25</td>
<td>0</td>
<td>0</td>
</tr>
</tbody>
</table>

**Figure S12.** UV absorption spectra with focus on 280 nm to confirm similar protein concentration.
**Figure S13.** Full UV-Vis absorption spectra to confirm similar absorption maxima at 495 nm.

**Figure S14.** Fluorescence emission spectrum after irradiation at 495 nm confirm similar emission maxima at 509 nm.
5 Cellular Uptake

HeLa (human cervical carcinoma) cells were cultured under standard established conditions as indicated elsewhere. Cells were seeded at subconfluent concentration in optically proficient 0.17 mm glass bottom multiwell chambers (Evotec, Germany) the day before the live cell microscopy experiments were performed.

5.1 Cellular Uptake by Transduction Versus Endocytosis

![Figure S15. Cellular transduction and endocytic uptake in live cells. TAT-FITC and PTD4-TAMRA peptides were added to cultured media without serum and cells were incubated at 37°C. Left, differential interference contrast (DIC) images; middle, TAT (green) and PTD4 (red); right, overlay. (a) Representative cartoon, (b) Field captured with several cells, (c) Magnification of a single cell. Scale bar: 15 µm.]

![Figure S16. Live cell confocal images of the GFP, TAT-TAMRA and cTAT-GFP before and after washing. (a) GFP added to the extracellular medium, (b) TAT-TAMRA peptide added simultaneously with uncoupled GFP, (c) cTAT-GFP. Scale bar 15 µM.]

5.2 Live Cell Confocal Microscopy of Transduced Cells
5.3 Fluorescence Recovery After Photobleaching of Internalized GFP

Figure S17. The normalized fluorescence signal is plotted as a function of time (in seconds). Cells transfected with GFP (n=24) or transduced by 150 µM cTAT-GFP (n=11) were photobleached with 488 nm laser at full power for 100 ms in the nucleus or cytoplasm. Transfected GFP was employed as a control for maximal free diffusion (t_{1/2}=0.36±0.33 s). Fluorescence recovery of cTAT-GFP transduced cells (t_{1/2}=1.07±0.57 s) was comparable to that of transfected GFP, indicating that the transduced molecules are freely diffusing inside the cell. These observations are in agreement with our previous findings[5]. The slight different kinetics we observed can be attributed to the positively charged residues of the cTAT peptide influencing the interactions between the transduced protein and the crowded intracellular environment. Fluorescence recovery was measured at a speed of one frame per 192 or 374 ms for transfected GFP or cTAT-GFP transduced cells, respectively.
5.4 Cellular Uptake Does Not Require Macropinocytosis or Metabolic Energy

Currently, the two main proposed mechanisms of uptake for arginine-rich peptides, such as the TAT peptide, are macropinocytosis and either direct translocation across the plasma membrane at lower concentrations or transduction at higher concentrations.[6] Macropinocytosis requires metabolic energy while transduction is energy independent. In the present work we initially found that cTAT-GFP is able to get into cells in a HEPES buffer depleted from nutrients. This suggests that macropinocytosis or other energy dependent pathways might not be mandatory in the cellular uptake of cTAT-GFP, following in this way a similar mode of uptake as arginine-rich peptides.[6c] To directly investigate if macropinocytosis is involved in the mode of cellular entry of the cTAT-GFP complex we tested this uptake in the presence of amiloride, which is a specific macropinocytosis inhibitor. The formation of macropinosomes appears to be distinctively inhibited by amiloride and its analogues, and this property has been extensively used as an identifying feature of micropinocytosis.[7] As shown in Fig. S18 (a), in the presence of amiloride at 1mM we found uptake of cTAT-GFP in 76% of the cells.

To strictly assess that the pathway of cell entry does not require metabolic energy, the delivery of cTAT-GFP was also carried out at 4 °C. Cells were incubated for 1 hour at 4 °C (or less) in a HEPES buffer lacking nutrients and glucose. Then the cells were placed on ice and the protein complex in solution (also kept in ice previously for 1 hour in a HEPES buffer) was added to the cells and incubated for an extra 40 min at 4 °C. The cells were washed three times with PBS and imaged immediately at the confocal microscope. The same protocol was simultaneously done for the TAT peptide (TAT-TAMRA) to compare the cTAT-GFP uptake side by side with the uncoupled TAT peptide. We detected uptake in all cases Fig S18 (b-d). In all cases cTAT-GFP and TAT-TAMRA were found in most cells freely distributed in the cytosol and labeling distinctively the nucleolus. The only difference was the intracellular intensity that was reduced at the lower concentration of the TAT peptide. This experiment rules out the requirement of any energy dependent cellular uptake pathway, including all endocytotic pathways, since at 4 °C all energy depending pathways are inhibited.

The DIC images show also that the cells remain morphologically healthy and we could see that after raising the temperature to 37 °C the cells continue undergoing cell division.
Figure S18. Cellular uptake of cTAT-GFP does not require macropinocytosis or any other energy-dependent pathway. (a) Cellular uptake of cTAT-GFP (75 µM) in the presence of a macropinocytosis inhibitor (amiloride at 1mM). We found uptake in 76 % of the cells. (b) Cellular uptake of cTAT-GFP at 4 °C in a HEPES buffer lacking nutrients and glucose. These conditions completely stop all cellular pathways that require metabolic energy. Under these conditions we still found uptake in 59 % of the cells (cells displaying nucleoli labeling by GFP in this case are explicitly marked with a star sign). (c) Uptake of 100 µM of TAT-TAMRA at 4 °C. (d) Uptake of 10 µM of TAT-TAMRA at 4 °C. Each experiment was repeated 3 times. The cellular uptake was quantified by counting as positive the cells that displayed clear nucleoli GFP or TAMRA labeling. Standard errors are shown between parentheses. Scale bar 15 µM.
5.5 Quantification of GFP Intracellular Concentration

Figure S19. Quantification of intracellular GFP. (a) Alkyne-GFP solution was prepared at different concentrations (40, 20, 10, 5, 2 µM) in an optically proficient micro-well dish. Fluorescence intensity (FI) of each micro-well was imaged by confocal microscopy over 10 z-planes (z = 0.59 µm). Two different ROIs per field per z-plane, and at least two fields were imaged. FI of each ROI was plotted as a function of z-planes. Each empty circle represents one ROI. Note how FI is homogenous over ROIs or z-planes, and proportional to GFP concentration (left graph). The ratio of two ROIs per field was
plotted as a function of z-planes in the right graph. The ratio is homogenous over z-planes as well as concentrations (overall: 0.99±0.03). Solid squares: negative control of fluorescence (HEPES buffer without alkyne-GFP).

(b) FI of ROIs inside or outside the cell contour from alkyne-GFP as well as cTAT-GFP or TAT-GFP was collected over z-planes. Importantly, cells are incubated as described in the methods and the imaging was performed before washing away the incubation solution. While alkyne-GFP was inert and its fluorescence remained unchanged in the medium, cTAT-GFP or TAT-GFP was partly adsorbed onto the plasma membrane. This is mainly due to the CPP moiety conjugated to GFP, which tends to interact with the plasma membrane. Hence, we observed that the FI of the medium is non-linearly reduced by a significant amount in samples treated with cTAT-GFP or TAT-GFP, compared to those which were treated with alkyne-GFP. The graph on the right shows the FI of ROIs from the extracellular space for alkyne-GFP (circles) and cTAT-GFP (squares) as a function of extracellular concentration. Note how FI of cTAT-GFP is lower than that of alkyne-GFP at the same concentration. Preliminary observations indicate a FI loss of ~15-35%. Such loss is expressed by the ratio between FI of ROIs in the extracellular space from samples treated with cTAT-GFP or TAT-GFP or alkyne-GFP (FI_{cTAT-GFP} / FI_{alkyne-GFP}), respectively. The loss of fluorescence was taken into account (as a correction factor) to further estimate GFP intracellular concentration.

(c) To estimate GFP intracellular concentration in subcellular compartments (e.g. cytoplasm or nuclei), the ratios of indicated ROIs were calculated. Such ratios were then corrected by the aforementioned correction factor. The corrected ratios were then multiplied by the original GFP extracellular concentration in the medium. On the right side, the distribution of labelled cells (cytoplasm and nuclei) is shown for 50, 100, 150 µM cTAT-GFP (green boxes). Corrected concentration for unlabeled cells is shown in grey and was used to calculate a transduction threshold. The latter are indicated by dashed red and grey lines set at one and two standard deviations above the mean corrected concentration of the unlabeled cells, respectively.

5.6 Movie of Transduced Cells – Motility and Mitosis

Movie 1. cTAT-GFP transduced cells continue moving and undergoing mitosis. First is shown a larger area capturing the motility of a cell followed by mitosis. Both are complex metabolic pathways that require the cell to be viable as well as having an active complex enzymatic network. This is followed by a number of cells undergoing mitosis at different times (indicated in the upper right corner of the DIC images) after being transduced by cTAT-GFP.
6 References


cTAT: HRMS. Measured on a LCT Premier Micromass Technologies system (a) Table of calculated m/z that correspond to found masses, (b) full ESI-MS spectrum and (c) zoom on the five times protonated mass.

(a) | Protonation | 2x  | 3x  | 4x  | 5x  |
---|---|---|---|---|---|
M  | 637.0572 | 478.0449 | 382.6375 |
M + 1 TFA | 675.0539 | 506.5424 | 405.4355 |
M + 2 TFA | 713.0506 | 535.0399 |
M + 3 TFA | 1126.0670 | 751.0472 |
M + 4 TFA | 1183.0620 |
M + 5 TFA | 1240.0570 |
**cTAT A: HRMS.** Measured on an Agilent 6210 ToF LC-MS system (a) Table of calculated m/z that correspond to found masses, (b) full ESI-MS spectrum and (c) zoom on the five times protonated mass.

(a)

<table>
<thead>
<tr>
<th>protonation</th>
<th>2x</th>
<th>3x</th>
<th>4x</th>
<th>5x</th>
</tr>
</thead>
<tbody>
<tr>
<td>M</td>
<td>1033.6005</td>
<td>689.4030</td>
<td>517.3042</td>
<td>414.0449</td>
</tr>
<tr>
<td>M + 1 TFA</td>
<td>1090.5955</td>
<td>727.3996</td>
<td></td>
<td></td>
</tr>
<tr>
<td>M + 2 TFA</td>
<td>1147.5905</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

(b) [Full ESI-MS spectrum]

(c) [Zoom on the five times protonated mass]
cTAT B: HRMS. Measured on an Agilent 6210 ToF LC-MS system (a) Table of calculated m/z that correspond to found masses, (b) full ESI-MS spectrum and (c) zoom on the five times protonated mass.

(a) Table of calculated m/z that correspond to found masses

<table>
<thead>
<tr>
<th>protonation</th>
<th>2x</th>
<th>3x</th>
<th>4x</th>
</tr>
</thead>
<tbody>
<tr>
<td>M</td>
<td>1026.6083</td>
<td>678.0748</td>
<td>508.8081</td>
</tr>
<tr>
<td>M + 1 TFA</td>
<td>1073.6033</td>
<td>716.0715</td>
<td></td>
</tr>
<tr>
<td>M + 2 TFA</td>
<td>1130.5983</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

(b) Full ESI-MS spectrum

(c) Zoom on the five times protonated mass
**TAT: HRMS.** Measured on a LCT Premier Micromass Technologies system (a) Table of calculated m/z that were correspond to found masses, (b) full ESI-MS spectrum and (c) zoom on the five times protonated mass.

(a) | protonation | 2x | 3x | 4x | 5x | 6x |
---|---|---|---|---|---|---|
M | 623.7129 | 468.0366 | 374.6309 | 312.3603 |
M+1 TFA | 661.7096 | 496.5341 |
M+2 TFA | 699.7062 | 525.0316 |
M+3 TFA | 1106.0504 | 737.7029 |
M+4 TFA | 1163.0454 |
M+5 TFA | 1220.0404 |