Electronic Supplementary Material (ESI) for Chemical Communications.

Branched dimerization of Tat peptide improves permeability to HeLa and hippocampal neuronal cells

I. Abrrey Monreal,[1] Qian Liu,[2] Katherine Tyson,[3] Tyler Bland,[3] Doralyn S. Dalisay,[4] Erin V. Adams,[1] Gary A. Wayman,[3] Hector C. Aguilar,[2] and Jonel P. Saludes[1]*

[1]*Department of Chemistry,* [2]*Paul G. Allen School for Global Animal Health,* [3]*Department of Neuroscience, and* [4]*Institute of Biological Chemistry, Washington State University, Pullman, Washington 99164, U.S.A.*

Email: jonel.saludes@wsu.edu; Online: www.saludeslab.org; Tel: +1-509-335-1144

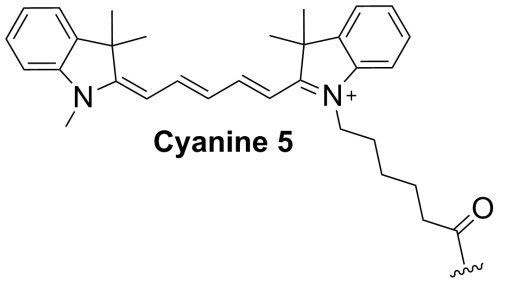
Table of Contents **Page**

A. Peptide Synthesis and Characterization

1. Peptide design

Compound	Name	Sequence
1	KLA	X-KLALKALKAALKLA-NH_2
2	R_8	X-RRRRRRRR-NH_2
3	Penetratin	X-RQIKIWFQNRRMKWKK-NH_2
4	TATp	X-GRKKRRQRRRPPQ-NH_2
5	TATp-D	Ac-GRKKRRQRRRPPQ(X)-K-K-NH_2

where X =



Cyanine 5

2. Solid Phase Peptide Synthesis

Peptides were synthesized using Biotage Initiator+ SP Wave microwave-assisted solid phase peptide synthesizer (Biotage, Charlotte, North Carolina) on Rink amide resin following standard Fmoc chemistry. Tatp-D (**5**) was also synthesized following the standard Fmoc chemistry on the Biotage Alstra fully automated microwave peptide synthesizer (Biotage, Charlotte, North Carolina) using ChemMatrix resin. Rink amide resin (4-(2',4'-Dimethoxyphenyl-Fmoc-aminomethyl)phenoxy resin 1% DVB), O-benzotriazole-N,N,N',N'-tetramethyl-uronium-hexafluoro-phosphate (HBTU), and Fmoc protected amino acids were purchased from Chem Impex (Wood Dale, IL) and used without further modification. All other reagents were purchased from Thermo Scientific or VWR and used without further purification. Typically, 0.10 mmol resin (Rink: 0.56 meq/g; ChemMatrix: 0.43 meq/g) was used, swollen in DMF for 30 minutes at 50 ºC with vortexing at 800 rpm. Fmoc deprotection was performed using 4 mL of 6% piperazine in DMF, irradiation to 75° C for 5 minutes, the liquid drained, the process repeated, then followed by thorough washing of the resin using DMF. Coupling steps were performed by adding 4 equiv. amino acid (0.4 mmol), 4 equiv. HBTU (0.4 mmol) and 8 equiv. DIPEA (0.8 mmol) in 4 mL total volume according to the peptide design in Section A.1, followed by irradiation to 75° C for 5 min. The efficiency of coupling and deprotection processes were monitored

using Kaiser Test (Reagent A: 0.4 mL of 0.001 M KCN in 20 mL pyridine; Reagent B: 0.5 g ninhydrin in 10 mL EtOH).

3. Cy5 labeling and Methyltrityl (Mtt) Deprotection

Peptides **1** - **4** were Fmoc deprotected and coupled to the carboxylic acid terminus of Cy5 (0.5 equiv.) using HATU (0.5 equiv.) and DIPEA (1.0 equiv.) in DMF at rt for 3h. The resin was washed with CH_2Cl_2 and MeOH wash cycles until the washings remained colorless, which was then dried overnight under reduced pressure. The peptides were cleaved using 5 mL of 95/2.5/2.5 TFA/TIS/H_2O (for compounds **1**, **2**, and **4**) or 90/2.5/2.5/2.5/2.5 TFA/EDT/TIS/Thioanisole/H_2O (for compound **3**) under Ar blanket for 2 h. The liquids were drained into a conical tube, the peptides precipitated using ice-cold Et_2O, the mixture centrifuged at 4 °C and 4,700 RCF, the supernatant decanted, the pellet resuspended in diethyl ether and centrifuged and the process repeated two more times. The resulting pellet was dissolved in deionized water, frozen and lyophilized. TATp-D (**5**) (0.025 mmol) was Fmoc deprotected, acetylated using Ac_2O (25% in DMF) and DIPEA (1.5 equiv.). Mtt protecting group at the ε-amino group of the *C*-terminal Lys was selectively removed using 1% TFA solution in CH_2Cl_2 (3.5 mL × 2 min × 17) at rt followed by thorough washing with CH_2Cl_2 and MeOH (5 mL x 3). The resin was then subjected to three 10 min stir-and-wash cycles in DMF containing 4 equiv. DIPEA followed by Cy5 labeling as described above. The peptide was cleaved using 2.5 mL of 95/2.5/2.5 TFA/TIS/H_2O. Cy5 labeling efficiency was monitored via HPLC compound **1** 55%, **2** 91%, **3** 18%, **4** 88%, **5** 26%.

4. Purification and Characterization

Peptides **1** - **5** were purified by reversed phase HPLC using Dionex UltiMate 3000 (Thermo Scientific) equipped with Chromeleon software version 6.80 SR14 and a C_{18} column (Waters X-Bridge BEH130, 10x250 mm, 5 μm). The mobile phase was composed of a linear gradient from 9:1 A/B (Solvent A: H_2O + 0.1% TFA; Solvent B: MeCN + 0.1% TFA) to 100% B in 40 min at a flow rate of 3.0 mL/min with detection set at 210, 254, 280, and 646 nm. Eluates were concentrated and lyophilized to yield fluffy blue powders of the TFA salt of the peptides. The peptides were prepared as chloride salts using 2 mM HCl solutions followed by lyophilization to remove residual TFA.[1] The purified peptides were characterized using Synapt G2 MALDI-TOF-MS (Waters, Milford, MA): Cy5-KLA (**1**), $[M]^+$ calculated for $C_{101}H_{168}N_{21}O_{15}^+$, 1915.3029; found, 1915.4335; Cy5-R_8 (**2**), $[M]^+$ calculated for $C_{80}H_{136}N_{35}O_9^+$, 1731.1255; found, 1731.1230; Cy5-Penetratin (**3**), $[M]^+$ calculated for $C_{136}H_{206}N_{37}O_{20}S^+$, 2709.5960; found 2709.0293; Cy5-TATp (**4**), $[M]^+$ calculated for $C_{102}H_{169}N_{38}O_{16}^+$, 2182.3579; found 2182.2307; TATp-D-Cy5 (**5**), $[M]^+$ calculated for $C_{188}H_{326}N_{77}O_{35}$, 4222.6097; found 4220.9980.

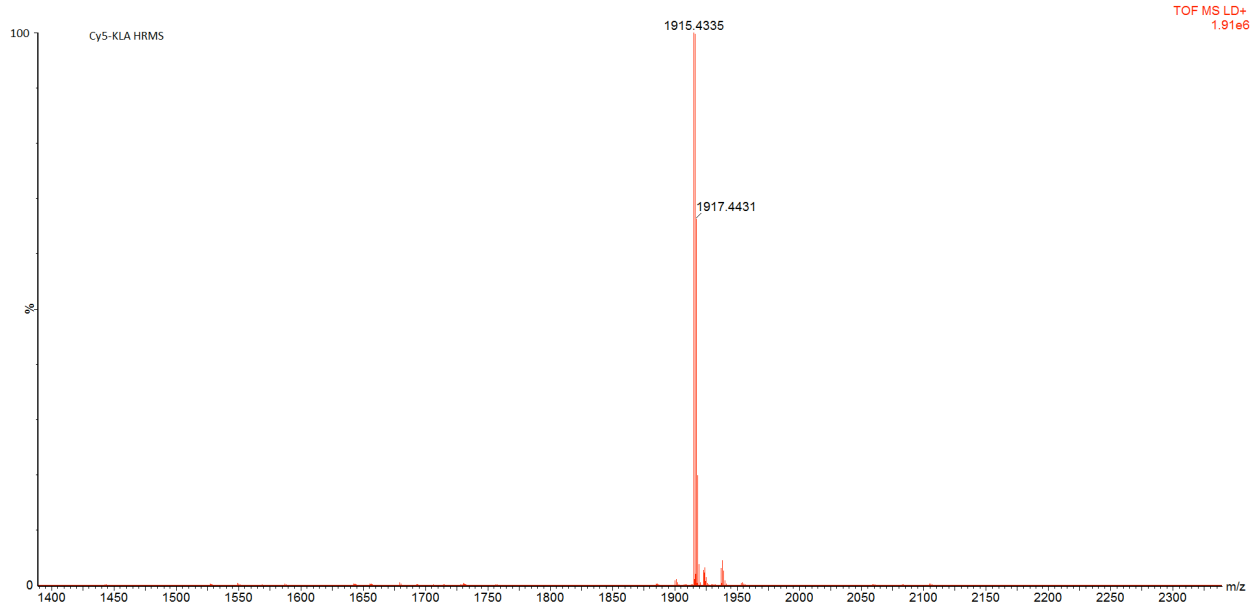


Figure S1. MALDI-TOF-MS of Cy5-KLA **1**.

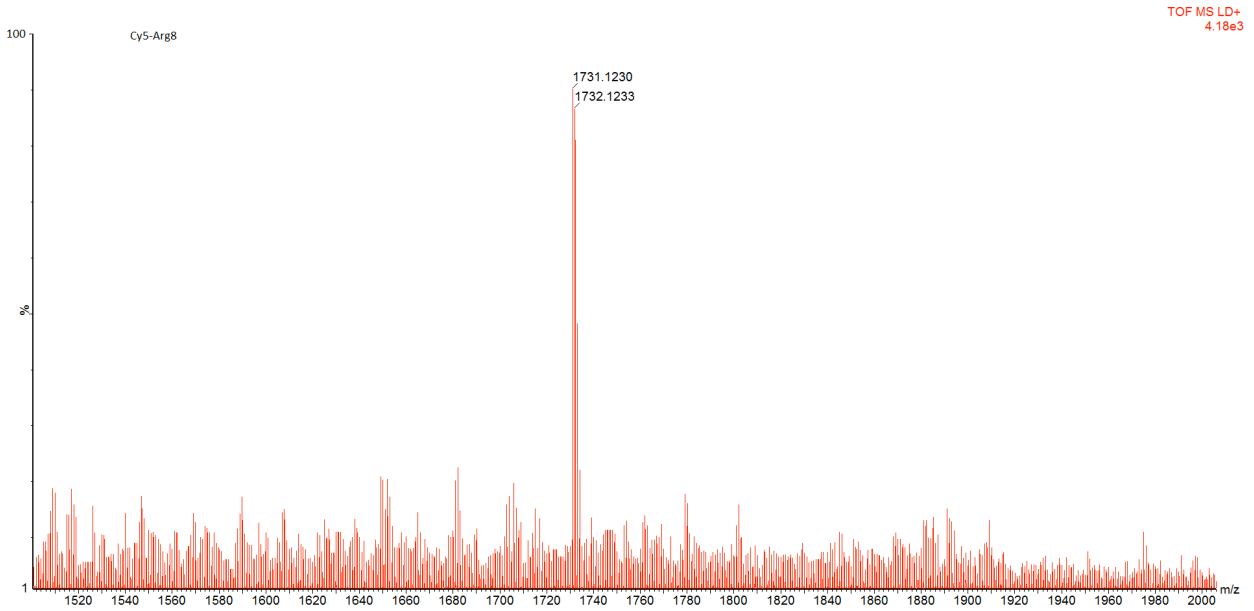


Figure S2. MALDI-TOF-MS of Cy5-R_8 **2**.

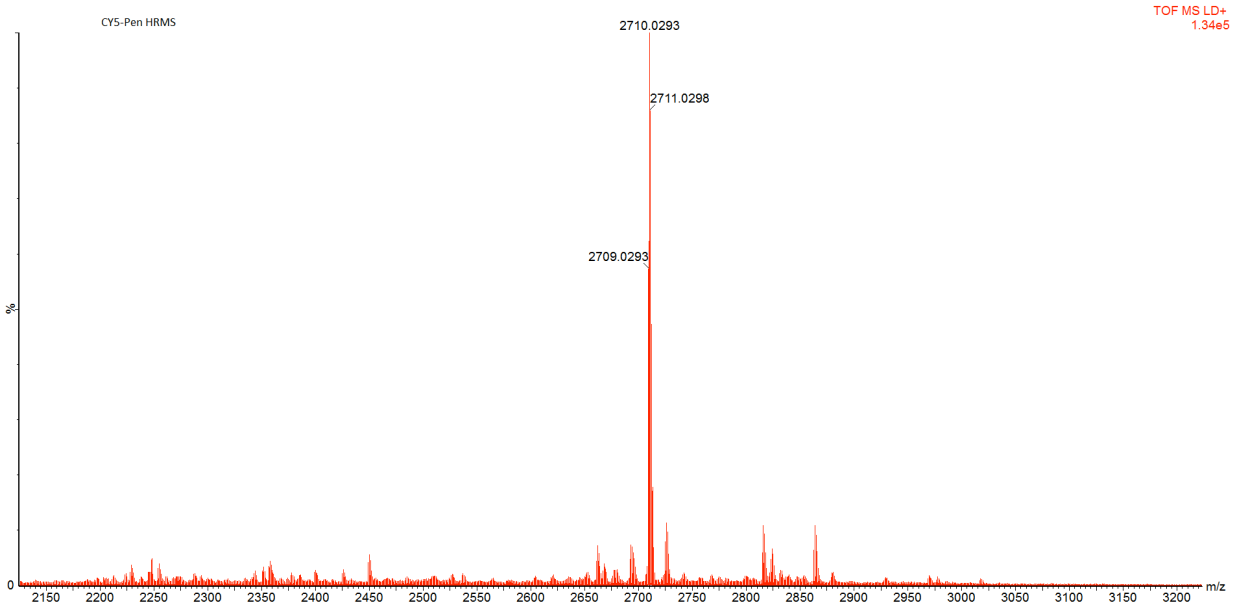


Figure S3. MALDI-TOF-MS of Cy5-Penetratin **3**.

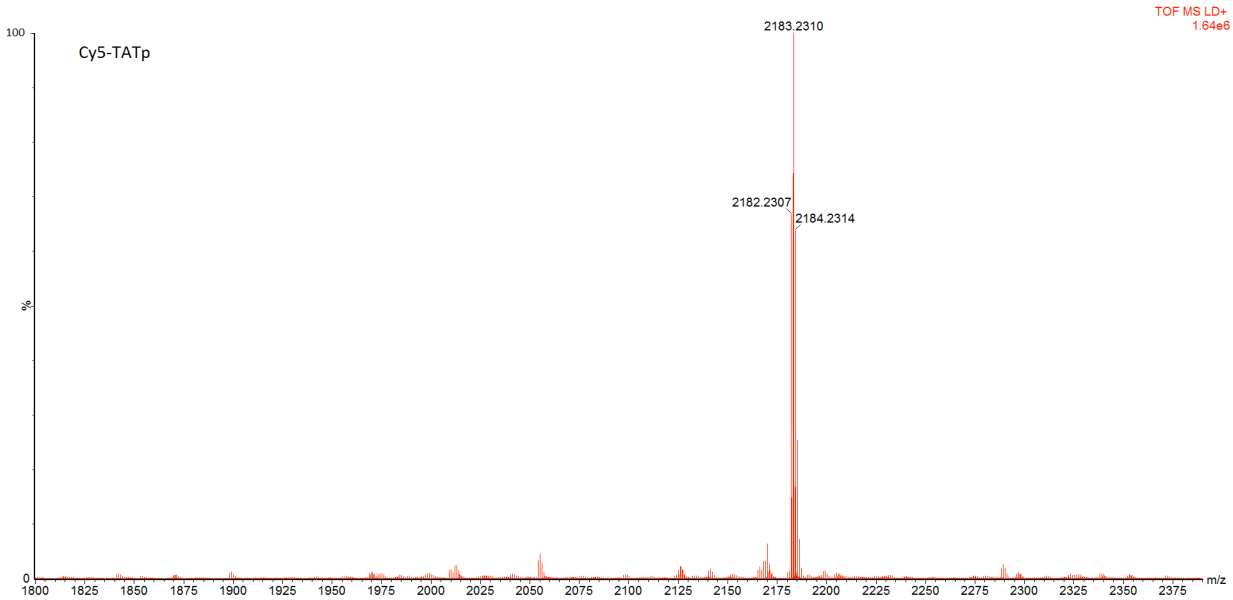


Figure S4. MALDI-TOF-MS of Cy5-TATp **4**.

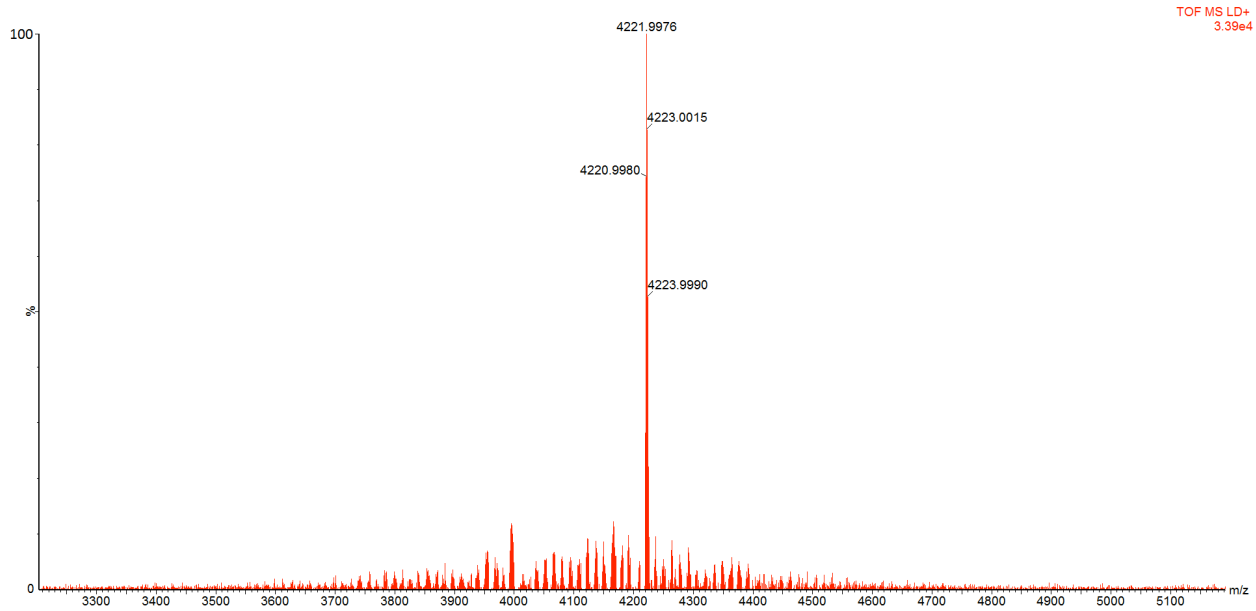


Figure S5. MALDI-TOF-MS of TATp-D-Cy5 **5**.

B. Fluorescence Activated Cell Sorting

HeLa cells ATCC (Manassas, VA) were incubated and passaged in 10 cm plates cultured in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% fetal bovine serum (FBS), 50 IU of penicillin/mL, 50 µg of streptomycin/mL, and 2 mM glutamine. For flow cytometry assays, HeLa cells were seeded in a 96-well plate to ~60% confluency, then incubated with 100 µL of Cy5 labeled peptides 0.25 to 5 µM peptide concentrations. While typical treatments are in the range of 2-16 µM for TATp and related CPPs,[2] we quickly determined that TATp-D (**5**) showed improved uptake at low concentrations. We sought to test the limits of this uptake at lower concentrations finding TATp-D (**5**) to be most efficient compared to TATp (**4**) at 0.25 µM concentrations. This follows our hypothesis that dimerization will increase the effective local concentration potentially leading to better translocation at lower doses. The cells were incubated at 37 °C for 1 h. Post incubation, the media was aspirated followed by washing with 1x DMEM phosphate buffered saline (DPBS, GIBCO, Life Technologies) before adding 100 µL of 1x Trypsin-EDTA (0.25% Trypsin-EDTA, GIBCO, Life Technologies) per well and incubating at 4 °C 30 min. The cells were then centrifuged at 500 RCF for 8 min followed by aspiration of the supernatant. The cells were then resuspended in DPBS followed by centrifugation, and this wash step was repeated a total of three times. The cells were resuspended in 200 µL of 0.5% paraformaldehyde solution in PBS and analyzed using the Guava EasyCyte 8HT flow cytometer (EMD Millipore, Germany) with detection set for fluorescence at 661 nm. All tests were done in triplicate. The data were processed using guavaSoft 2.6. In the low temperature experiments, two 96-well plates were prepared, one treated as described above and the other pre-incubated at 4 °C in a temperature regulated cooler for 30 minutes, followed by peptide treatment and incubation at 4 °C for 1 h while the first plate was incubated at 37 °C. Post incubation, both plates were treated and analyzed as described above.

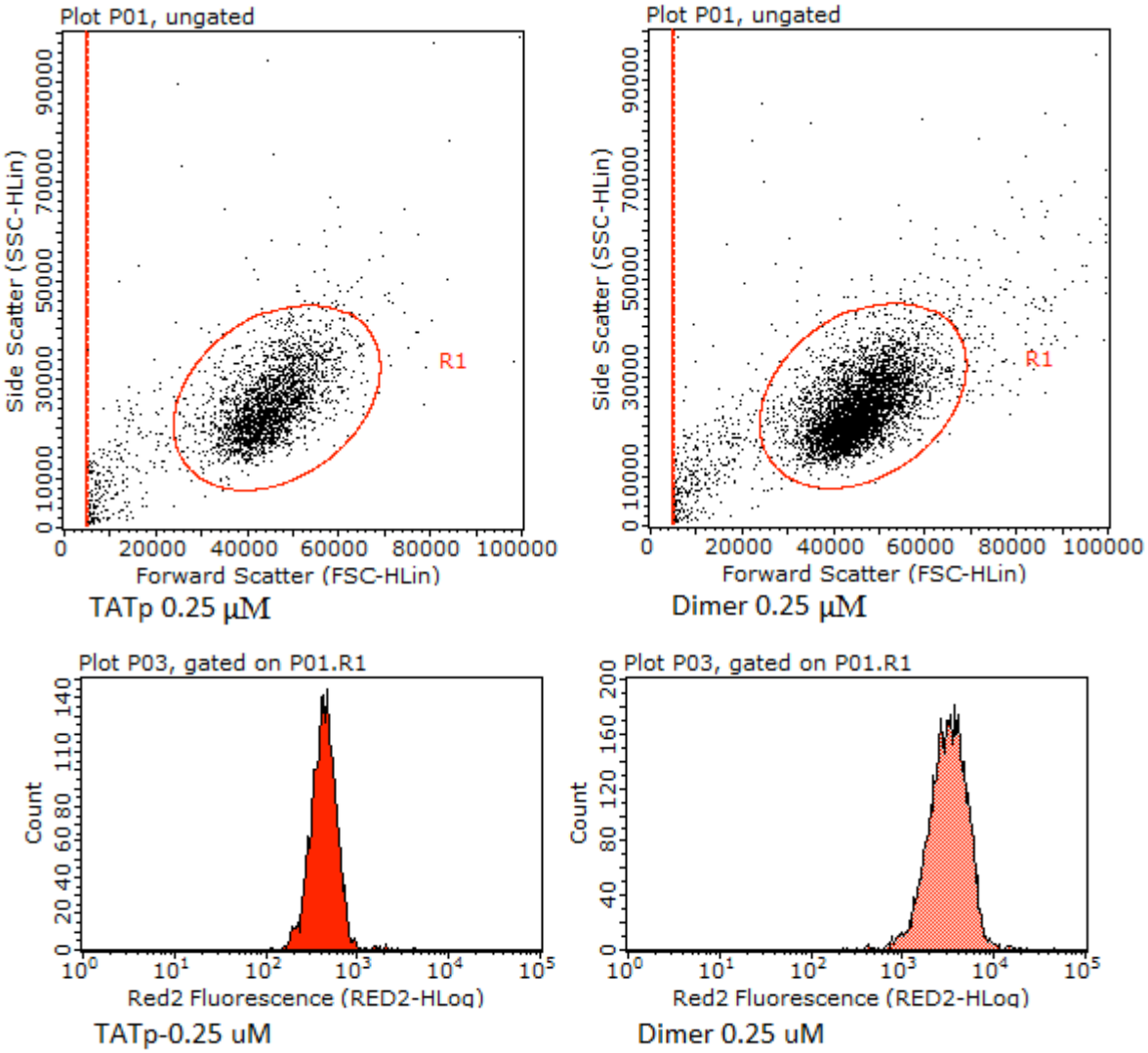


Figure S6. Fluorescence activated cell sorting data for Tatp (**4**) and Tatp-D (**5**). **Top**: Representative scatter plots for **4** and **5** at 0.25 µM peptide concentration. **Bottom**: Histograms showing fluorescence of HeLa cells from treatment with **4** and **5**.

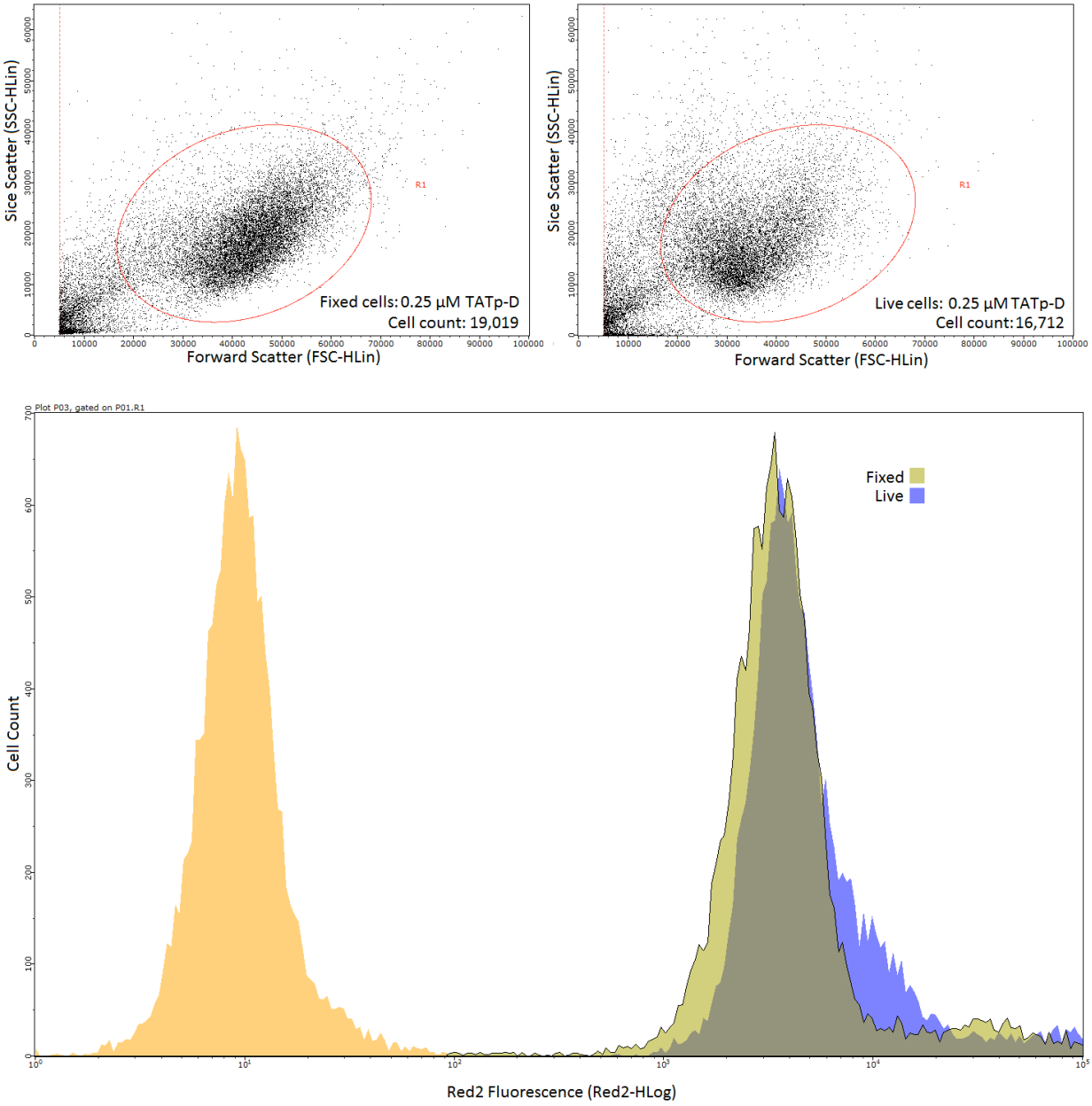


Figure S7. Comparison of flow cytometry histograms of fixed and unfixed cells. Scatter plots representing fixed (olive colored) and live (blue colored) HeLa cells treated with 0.25 µM TATp-D (**5**). Overlaid histograms show comparable profiles. Untreated cells are shown on the left (orange colored).

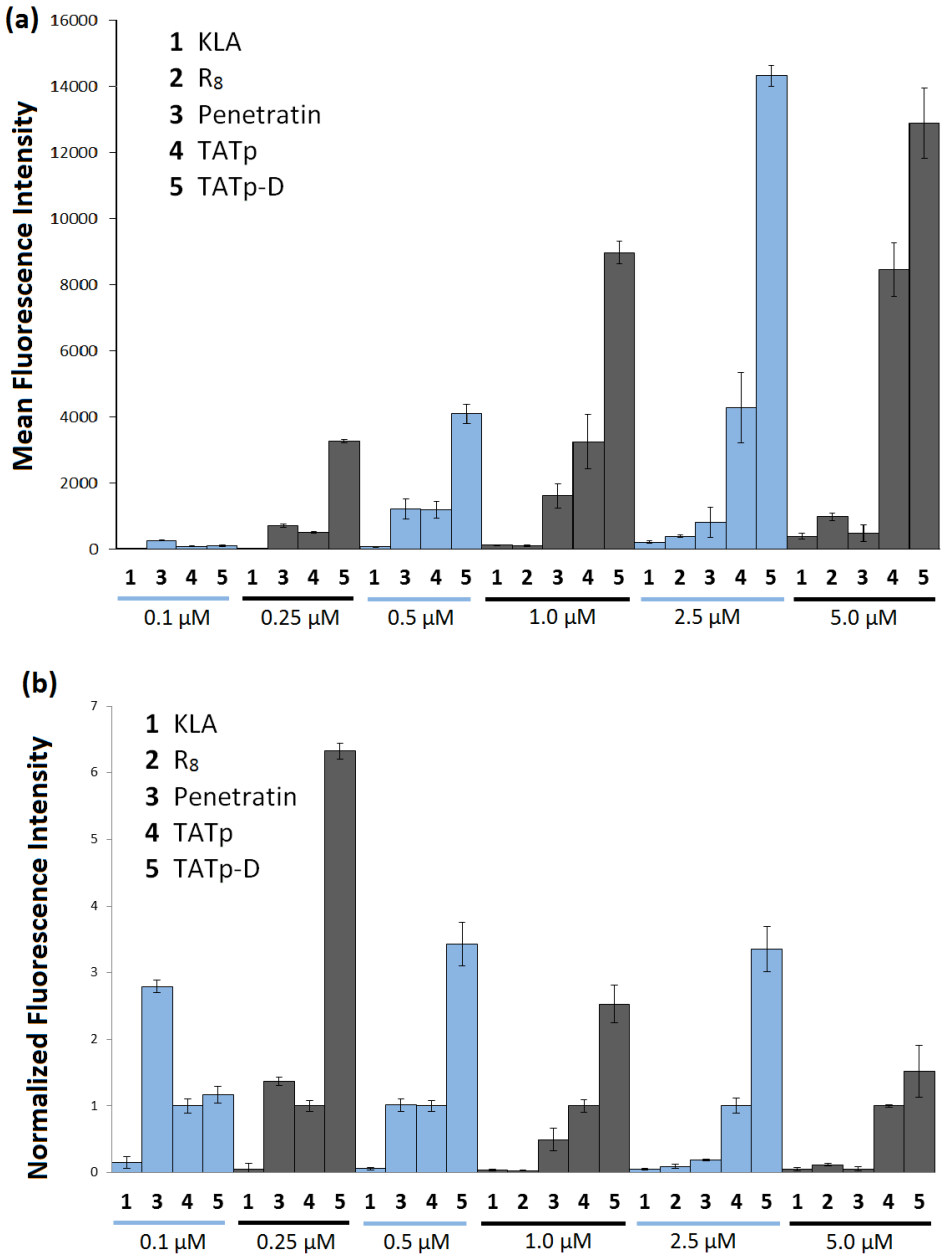


Figure S8. Membrane permeability to HeLa cells by flow cytometry. (a) Mean fluorescence intensity (MFI) of HeLa cells indicating that TATp-D (**5**) is very permeable vs other tested CPPs at 0.25-2.5 µM (b) MFI of HeLa cells normalized to TATp (**4**). Error represented as SD, n=3.

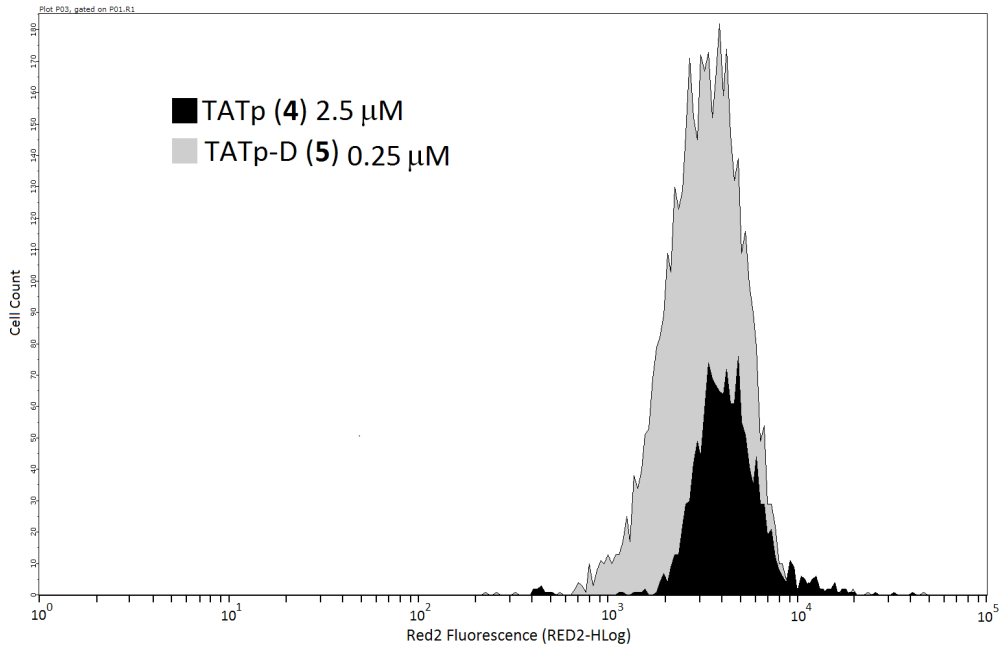


Figure S9. Overlaid histograms of HeLa cells treated with 2.5 µM Tatp (**4**) and 0.25 µM Tatp-D (**5**) showing comparable uptake of **5** at 10% the concentration of **4**.

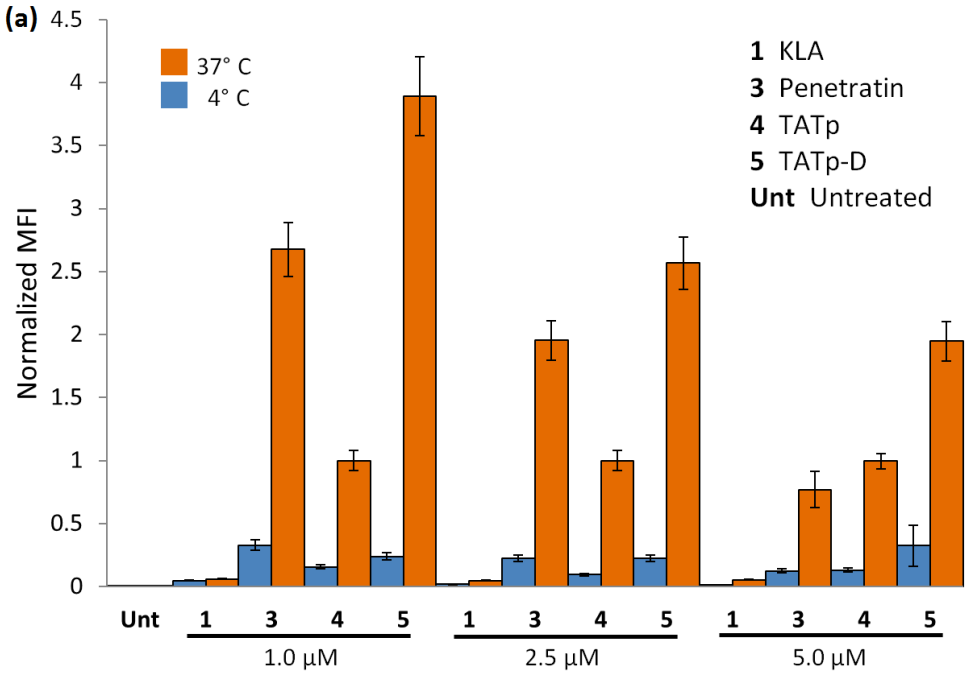


Figure S10. Flow cytometry profile of HeLa cells incubated at 4 and 37 °C. Mean fluorescence intensity chart of parallel experiments showing normalized fluorescence intensity of HeLa cells treated with Cy5 labeled peptides at various concentrations and incubated at 4 and 37 °C. Error bars represent ± standard deviation (n = 3).

C. Mechanism of Entry Studies

HeLa cells were prepared in 96-well plates as described in Section B. Following overnight incubation of HeLa cells, the media was replaced with 90 μL of fresh full media followed by addition of 10 μL of inhibitor stock solutions to a final concentration of 10 μM chlorpromazine, 50 μM wortmannin, 10 μM nocodazole, and 10 μM amiloride. HeLa cells were incubated with the inhibitors for 30 minutes at 37 °C prior to addition of TATp (**4**) and TATp-D (**5**) at 2.5 μM, followed by another 1 h incubation. The cells were then washed as described in Section B, resuspended in buffer and analyzed by flow cytometry as described in Section B, Studies were done in triplicate of 5,000 cells per well and percent uptake determined by normalizing the mean fluorescence intensity from cells treated with inhibitors to cells incubated with peptides only.

D. Cytotoxicity Assays

Cytotoxicity Profiling: MTT

Cellular toxicity of TATp (**4**) and TATp-D (**5**) against HeLa cells was evaluated colorimetrically using 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazoliumbromide (MTT) following an established protocol.[3] HeLa cells (1.0×10^4/well) were seeded into 96-well plates and incubated overnight. 10 μL of a 12 mM MTT stock solution was added to each well in phenol red free DMEM. The cells were incubated at 37 °C for 4 h. After incubation 100 μL of a 0.01M HCl 1% SDS solution (w/v) was added to each sample mixed thoroughly and incubated for 2 h. Following SDS incubation, the samples were thoroughly mixed and absorbance was measured at 570 nm using a Tecan Infinite M1000 PRO microplate reader (Männedorf, Switzerland). A solution of 0.1% Triton X-100 served as positive control. All tests were done in triplicate.

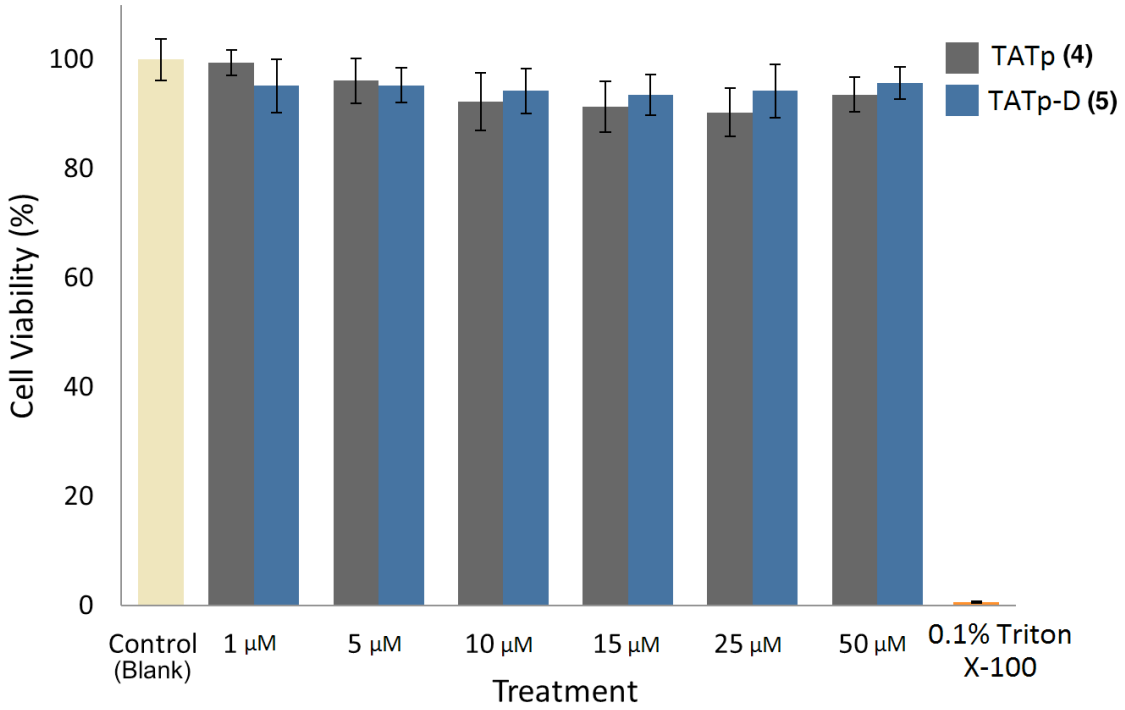


Figure S11. Cytotoxicity profile: MTT assay. Chart showing that Tatp (**4**) and Tatp-D (**5**) are not toxic to HeLa cells even at 10 times the highest tested concentration in flow cytometry assay. Data shown are the mean standard deviation of triplicate assays.

LDH Assay: Membrane Integrity

Membrane integrity was assayed using CytoTox-ONE™ Homogeneous Membrane Integrity Assay from Promega (part# G7891) following the supplier provided protocol.[6] HeLa cells were plated overnight as described above using phenol red free media. Cells were then treated in triplicate to TATp (**4**) and TATp-D (**5**) at varying concentrations for 4 hours at 37 °C. LDH release was then measured by incubating the media for 30 minutes with the provided reagents including blank cell free media for background correction, and media attained from lysed cells using the provided lysis buffer as the maximum LDH release. Fluorescence was then measured using a Tecan Infinite M1000 PRO microplate reader (Männedorf, Switzerland) with excitation set to 560 nm and emission wavelength measured at 590 nm. Percent cytotoxicity was measured by dividing measured fluorescence minus background by maximum LDH release minus background x100.

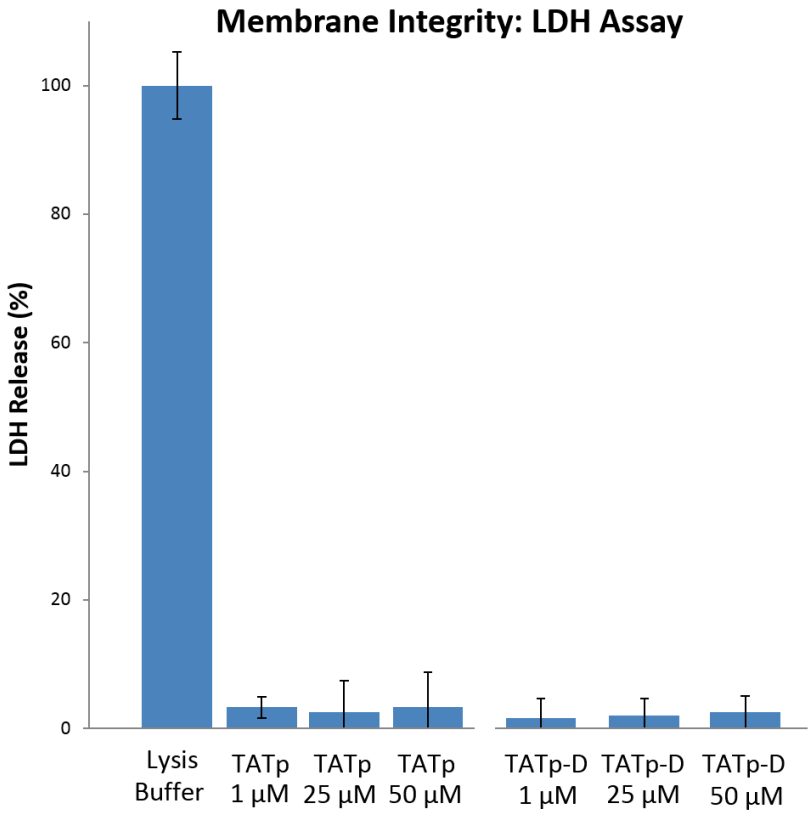


Figure S12. Membrane integrity: LDH Assay. Chart showing that TATp (**4**) and TATp-D (**5**) have insignificant effect on membrane integrity of HeLa cells up to 50 µM peptide concentration. Error represented as ± SD (n = 3)

Propidium iodide (PI) Co-Incubation: Effects of trypsinization

HeLa cells were plated in 96-well plates as stated above for flow cytometry assay. Cells were subjected to propidium iodide staining by treatment in triplicate using a minimum of 5,000 cells per well using 0.1 % PI solution in 200 µL flow cytometry buffer for 3 minutes prior to analysis at RT by gentle mixing. Cells analysed were subjected to 1 h treatment to **4** and **5** as stated above for 1 h at 5 µM followed by the flow cytometry washing protocol stated above before incubation with PI. Concurrently cells were subjected to either a 30 minute trypsinization or a 5 minute trypsinization of untreated cells in full media. A separate set of cells were subjected to 10% EtOH treatment for 90 minutes at 37 °C. PI fluorescence was measured using a guava easyCyte 8-HT flow cytometer excitation at 488 nm fluorescence measured using the yellow channel (583 nm) plotted with forward scatter to investigate the population of dead cells (Figure **S13**).

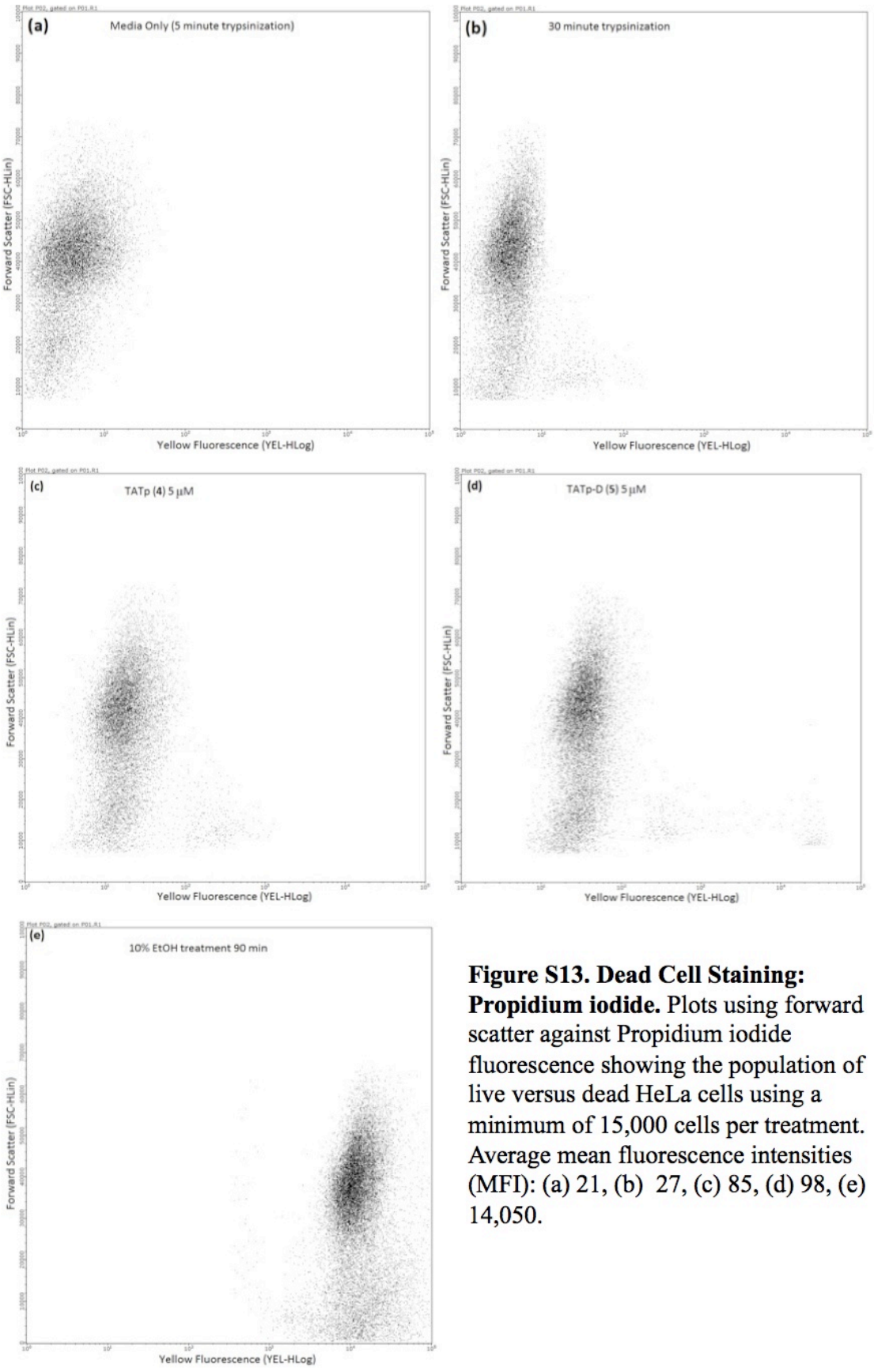


Figure S13. Dead Cell Staining: Propidium iodide. Plots using forward scatter against Propidium iodide fluorescence showing the population of live versus dead HeLa cells using a minimum of 15,000 cells per treatment. Average mean fluorescence intensities (MFI): (a) 21, (b) 27, (c) 85, (d) 98, (e) 14,050.

E. Confocal Laser Scanning Microscopy

HeLa cells were maintained as described in Section B. Primary neuronal hippocampal cells were prepared as described previously.[4] Briefly, primary neuronal cells harvested from rat brains were grown in Neurobasal A media supplemented with 2% B27 and 1% 100x Glutamax (Invitrogen, Grand Island, NY) and transfected with Clover-β actin (green fluorescent protein) at DIV 6 using Lipofectamine 2000 (Invitrogen, Grand Island, NY) according to the manufacturer's protocol,[5] yielding 3–5% transfection efficiency that allowed visualization of select neurons via observed green fluorescence. Both cell types were plated on poly-lysine coated coverslips in 24-well plates. Coverslips were prepared in an incubation chamber at 37 °C and using 150 μL live imaging buffer (145mM NaCl, 2.5 mM KCl, 10 mM glucose, 10 mM HEPES, 2 mM $CaCl_2$, 1 mM $MgCl_2$, pH 7.4) with set concentrations of peptides. Microscopy studies were done using a Leica DMI6000 SD confocal microscope (Buffalo Grove, IL) equipped with a Yokogawa CSU-X1 spinning disk and the images were captured using a Hamatsu-R2 CCD camera. The samples were excited using ILE/4 integrated laser engine (Spectral Applied Research, Ontario, Canada) with excitation set to 488 nm, 150 mW max (green channel) and 640 nm, 100 mW max (far red channel) and laser power set to 10-25% power and exposure times of 3-500 ms. The cells were treated with various concentrations of TATp (**4**) and TATp-D (**5**), and peptide uptake was monitored by continuously imaging the live cells for 30 min at 30 s intervals. The cells were then fixed by treatment with 4% PFA in 1x Phems, pH 7.4, for twenty minutes followed by washing with PBS and mounting the coverslips to glass slides to generate fixed cell images. To measure the fluorescence intensity of the intracellular domain, the cytoplasm of HeLa cells and soma of neuronal cells were analyzed after 30 min incubation as described above. Measurements were done at 30 ms exposure with gain set to 100 and 640 nm laser excitation set to15 mW. Images were taken from 5-7 HeLa cells per treatment and 2-3 Clover-β actin-transfected neuronal cells per treatment. Intracellular fluorescence intensity was measured in triplicates and averaged using MetaMorph software from Molecular Devices (Sunnyvale, CA).

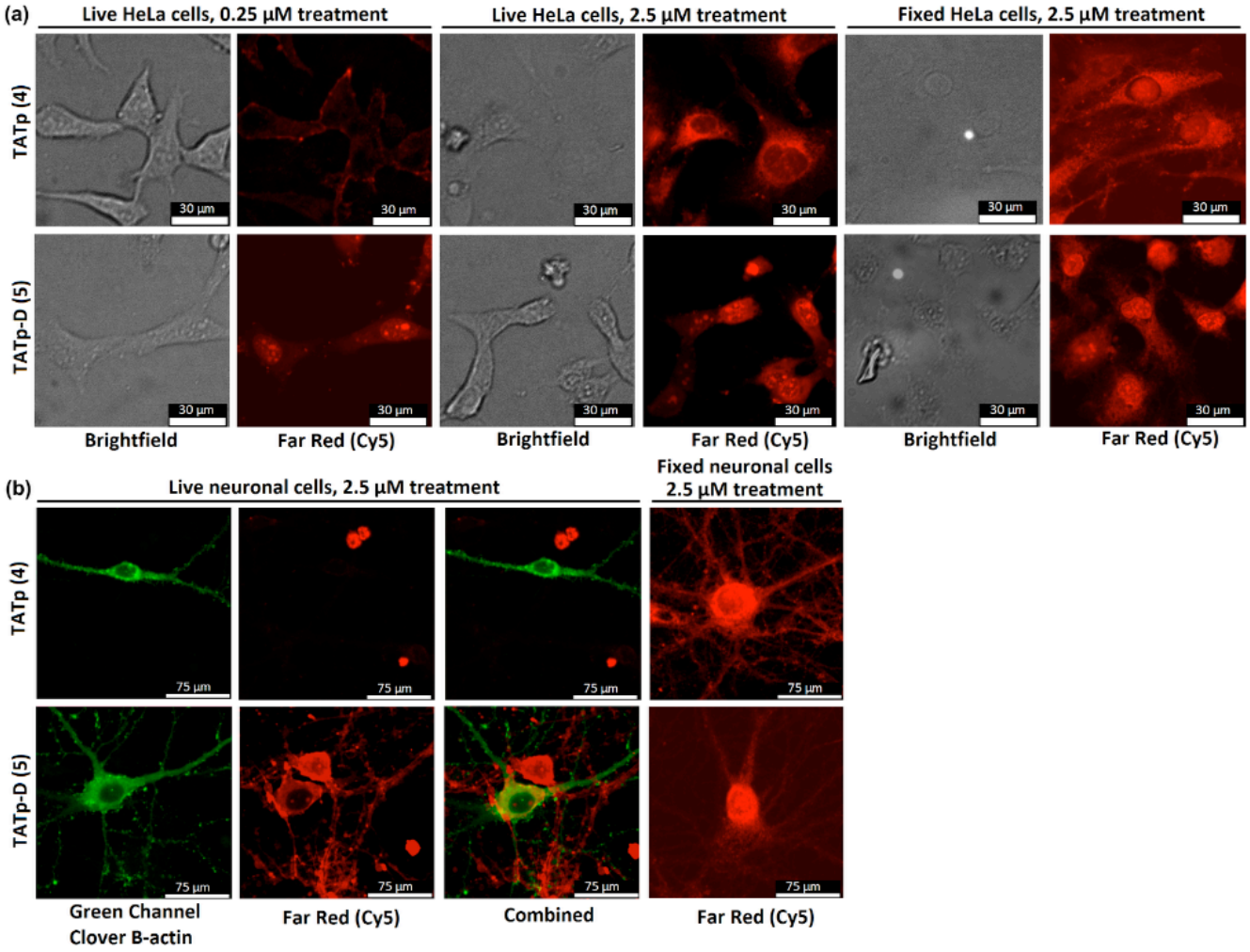


Figure S14. Confocal microscopy images taken at 20x magnification. (a) HeLa cells; (b) Primary hippocampal neuronal cells. Live cell images taken following 30 min incubation at 37 ^{o}C in imaging buffer. Fixed images taken after fixation in 4% paraformaldehyde for 20 min followed by washing in 1x PBS and mounting.

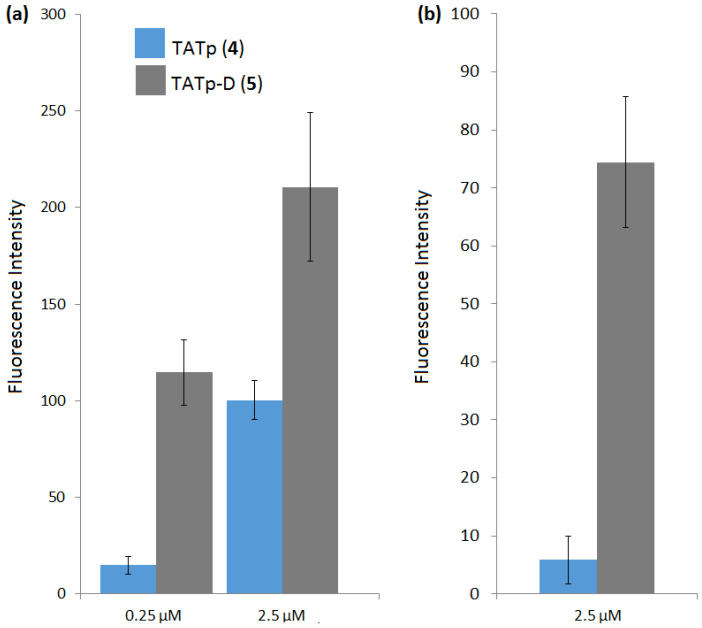


Figure S15. Fluorescence intensity of intracellular domain. (a) Fluorescence intensity of HeLa cells cytoplasm treated with 0.25 μM and 2.5 μM of TATp (**4**) and TATp-D (**5**), confirming our observation by flow cytometry that **5** at 0.25 μM has comparable translocation efficiency as **4** at 2.5 μM. (b) Fluorescence intensity of the soma of primary rat neurons treated with 2.5 μM of TATp (**4**) and TATp-D (**5**), demonstrating that **5** possesses about one-order of magnitude translocation efficiency than **4**.

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