Electronic Supplementary Material (ESI) for ChemComm.

Monitoring the cytosolic entry of cell-penetrating peptides using a pH-sensitive fluorophore

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Supporting Information

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Experimental Procedures

Material. Reagents for peptide synthesis were purchased from Chem-Impex (Wood Dale, IL), NovaBiochem (La Jolla, CA), or Anaspec (San Jose, CA). Rink Amide Resin LS (100-200 mesh, 0.2 mmol/g) was purchased from Advanced ChemTech. Cell culture media, fetal bovine serum, penicillin-streptomycin, 0.25% trypsin-EDTA, and DPBS (2.7 mM potassium chloride, 1.5 mM monopotassium phosphate, 8.9 mM disodium hydrogen phosphate, and 137 mM sodium chloride) were purchased from Invitrogen (Carlsbad, CA). Bis[tris(hydroxymethyl)methylamino] propane, fluorescein isothiocyanate, Lissamine Rhodamine B sulfonyl chloride, 5(6)-carboxyfluorescein, rhodamine B, and naphthofluorescein and 5-(and-6)-carboxynaphtho-fluorescein succinimidyl ester were purchased from Sigma Aldrich (St. Louis, MO).

Peptide Synthesis and Labelling. Peptides were synthesized on Rink Amide resin LS (0.2 mmol/g) using standard Fmoc-HATU chemistry. The typical coupling reaction contained 5 equiv. of Fmoc-amino acid, 5 equiv. of 2-(7-aza-1H-benzotriazole-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate (HATU) and 10 equiv. of diisopropylethylamine (DIPEA) and was allowed to proceed with mixing for 75 min. The peptides were deprotected and released from the resin by treatment with 92.5:2.5:2.5:2.5 (v/v) trifluoroacetic acid (TFA)/water/phenol/triisopropylsilane (TIPS) for 2 h. The peptides were triturated with cold ethyl ether (3x) and purified by reversed-phase HPLC equipped with a C_{18} column. To generate fluorescently labelled peptide, an N^{ε}-4-methoxytrityl-L-lysine was added to the C-terminus. After solid-phase peptide synthesis and before cleavage, the lysine side chain was selectively deprotected using 1% (v/v) TFA in DCM. The resin was incubated with 5 equiv. of a reactive fluorescent labelling reagent (fluorescein isothiocyanate, Lissamine rhodamine B sulfonyl chloride, or naphthofluorescein succinimidyl ester) and 5 equiv. of DIPEA in DMF overnight. The peptide was deprotected, triturated, and purified as described above. The authenticity of each peptide was confirmed by MALDI-TOF mass spectrometric analysis.

Determination of pH Profiles of Fluorophores. 5(6)-Carboxyfluorescein, rhodamine B, and naphthofluorescein were dissolved in DMSO to make 5 mM stock solutions, which were then diluted to 500 nM final concentration in buffers of the desired pH values. Solutions of $cF\Phi R_4^{FL}$, $cF\Phi R_4^{Rho}$, and $cF\Phi R_4^{NF}$ peptides (500 nM final concentration) were similarly prepared in buffers of the desired pH values. The buffers all contained 0.1 M 1,3-bis[tris(hydroxymethyl)methylamino]propane and the pH was adjusted to the desired values by adding hydrochloric acid. The fluorescence intensities of the above solutions were measured on a Molecular Devices Spectramax M5 spectrofluorimeter, with the excitation and emission wavelengths set at 485/525 nm for FL, 545/590 nm for Rho, and 595/660 nm for NF (with 5 nm bandwidth). The obtained fluorescence yields were converted into relative values using the fluorescence intensity at pH 9.5 as reference (100%). All data presented were the mean ± SD of three independent experiments.

Cell Culture. HeLa cells were maintained in growth medium consisting of DMEM, 10% fetal bovine serum (FBS) and 1% penicillin/streptomycin. Cells were cultured in a humidified incubator at 37 °C with 5% CO_2.

Cytotoxicity Assay. MTT assays were performed to evaluate cyclic peptide's cytotoxicity against several mammalian cell lines. One hundred μL of HeLa cells (5×10^4 cells/mL) cells were placed in each well of a 96-well culture plate and allowed to grow overnight. Varying concentrations of the peptide (0-25 μM) were added to the each well and the cells were incubated at 37 °C with 5% CO_2 for 24. Ten μL of MTT stock solution (Roche Life Science) was added into each well. Addition of 10 μL of the solution to the growth medium (no cell) was used as a negative control. The plate was incubated at 37 °C for 4 h. Then 100 μL of SDS-HCl solubilizing buffer was added into each well, and the resulting solution was mixed thoroughly. The plate was incubated at 37 °C for another 4 h. The absorbance of the formazan product was measured at 570 nm using a Molecular Devices Spectramax M5 plate reader. Each experiment was performed in triplicates and the cells without any peptide added were treated as control.

Confocal Microscopy. One mL of HeLa cell suspension (~5 x 10^4 cells) was seeded in a 35 mm glass-bottomed microwell dish (MatTek) and cultured overnight. Cells were gently washed with DPBS twice and treated with rhodamine- or naphthofluorescein-labelled peptides (5 μM) in phenol-red free, HEPES supplemented DMEM containing 1% FBS at 37 °C for 2 h in the presence of 5% CO_2. After removal of the medium, the cells were gently washed with DPBS twice and imaged on a Visitech Infinity 3 Hawk 2D-array live cell imaging confocal microscope equipped with 60 X oil objective. In Fig. 2A, rhodamine fluorescence images were captured using 7% power of a 561-nm laser and 100 ms of exposure time. In Fig. 2B, naphthofluorescein images were captured using 15% power of a 642-nm laser and an exposure time of 400 ms. In Fig. S3, naphthofluorescein images were captured using 5% power of a 642-nm laser and 50 ms exposure time. Data were analyzed using MetaMorph Premier (Molecular Devices).

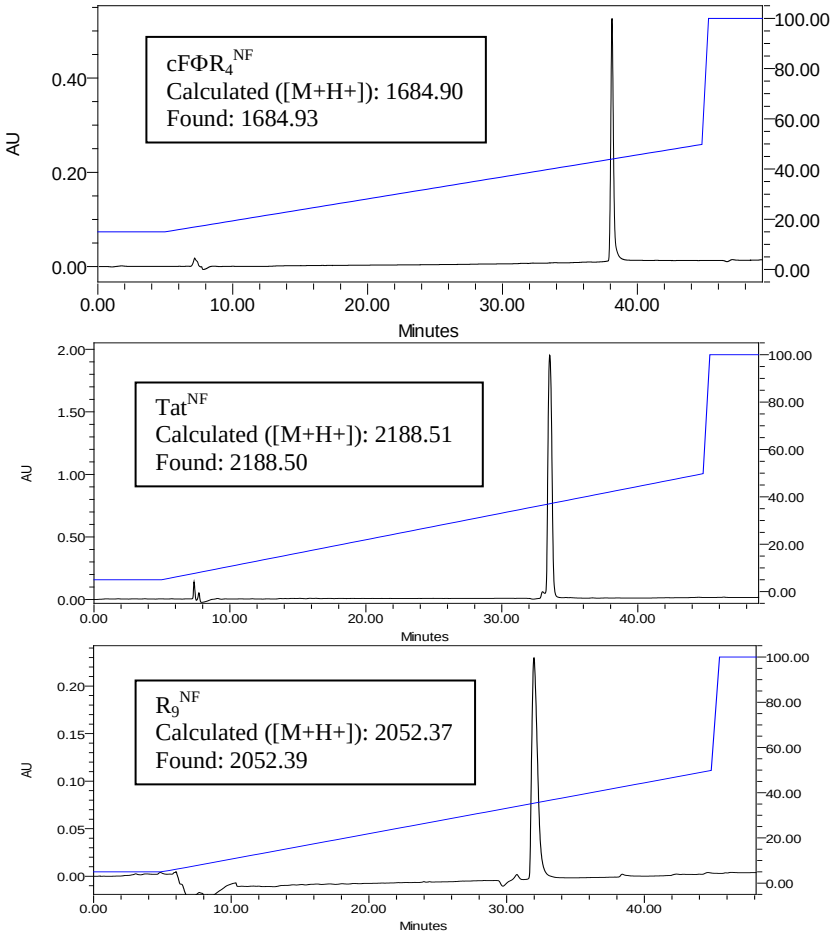
Flow Cytometry. HeLa cells were cultured in 12-well plates (1.5 x 10^5 cells per well) for 24 h. On the day of experiment, the cells were incubated for the desired periods of time (0-180 min) with 5 μM rhodamine- or naphthofluorescein-labelled peptide in phenol-red free, HEPES supplemented DMEM containing 1% FBS at 37 or 4 °C in the presence of 5% CO_2. At the end of incubation, the cells were washed with DPBS twice, detached from the plate with 0.25% trypsin, diluted into clear DMEM, pelleted at 250g for 5 min, washed twice with DPBS and resuspended in DPBS containing 1% bovine serum albumin, and analyzed on a BD FACS LSR II or Aria III flow cytometer. For the rhodamine-labelled peptides, a 561-nm laser was used for excitation and the fluorescence was analyzed under the PE channel. For naphthofluorescein-labelled peptides, a 633-nm laser was used for excitation and the fluorescence emission was analyzed under the APC channel. Data were analyzed using the Flowjo software (Tree Star).

Supplementary Table S1. Sequences of peptides used in this work

Abbreviation	Peptide Sequence[a]
$cF\Phi R_4^{Rho}$	cyclo(FΦRRRRQ)-K(Rho)-NH_2
$cF\Phi R_4^{FL}$	cyclo(FΦRRRRQ)-K(FL)-NH_2
$cF\Phi R_4^{NF}$	cyclo(FΦRRRRQ)-K(NF)-NH_2
R_9^{Rho}	Ac-RRRRRRRRR-K(Rho)-NH_2
R_9^{NF}	Ac-RRRRRRRRR-K(NF)-NH_2
Tat^{Rhod}	Ac-YGRKKRRQRRR-K(Rho)-NH_2
Tat^{NF}	Ac-YGRKKRRQRRR-K(NF)-NH_2

[a]Φ, L-2-naphthylalanine; Rho, rhodamine B; NF, naphthofluorescein; FL, fluorescein isothiocyanate.

Supplementary Figures



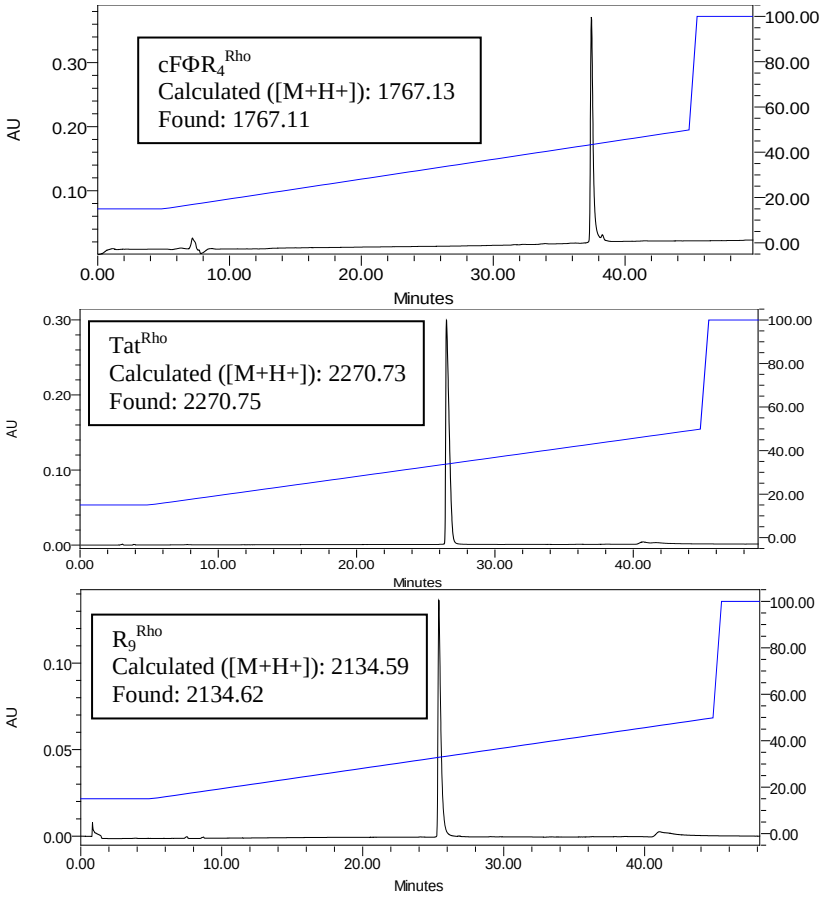


Fig. S1. RP-HPLC and MALDI-MS analysis of peptides used in the work. The purity of the product (>98%) was assessed by reversed-phase HPLC equipped with an analytical C_{18} column. The authenticity of the peptides was confirmed by MALDI-TOF MS analysis.

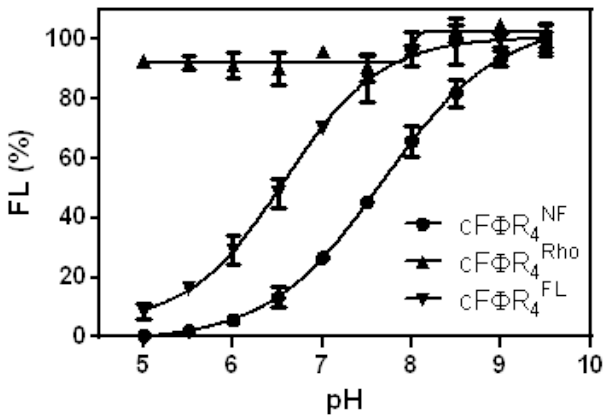


Fig. S2. Plot of the fluorescence intensity of FL- (Ex/Em = 485/525 nm), NF- (Ex/Em = 595/660 nm), and Rho-labelled $cF\Phi R_4$ (Ex/Em = 545/590 nm) as a function of pH. All values reported are relative to those at pH 9.5 (100%).

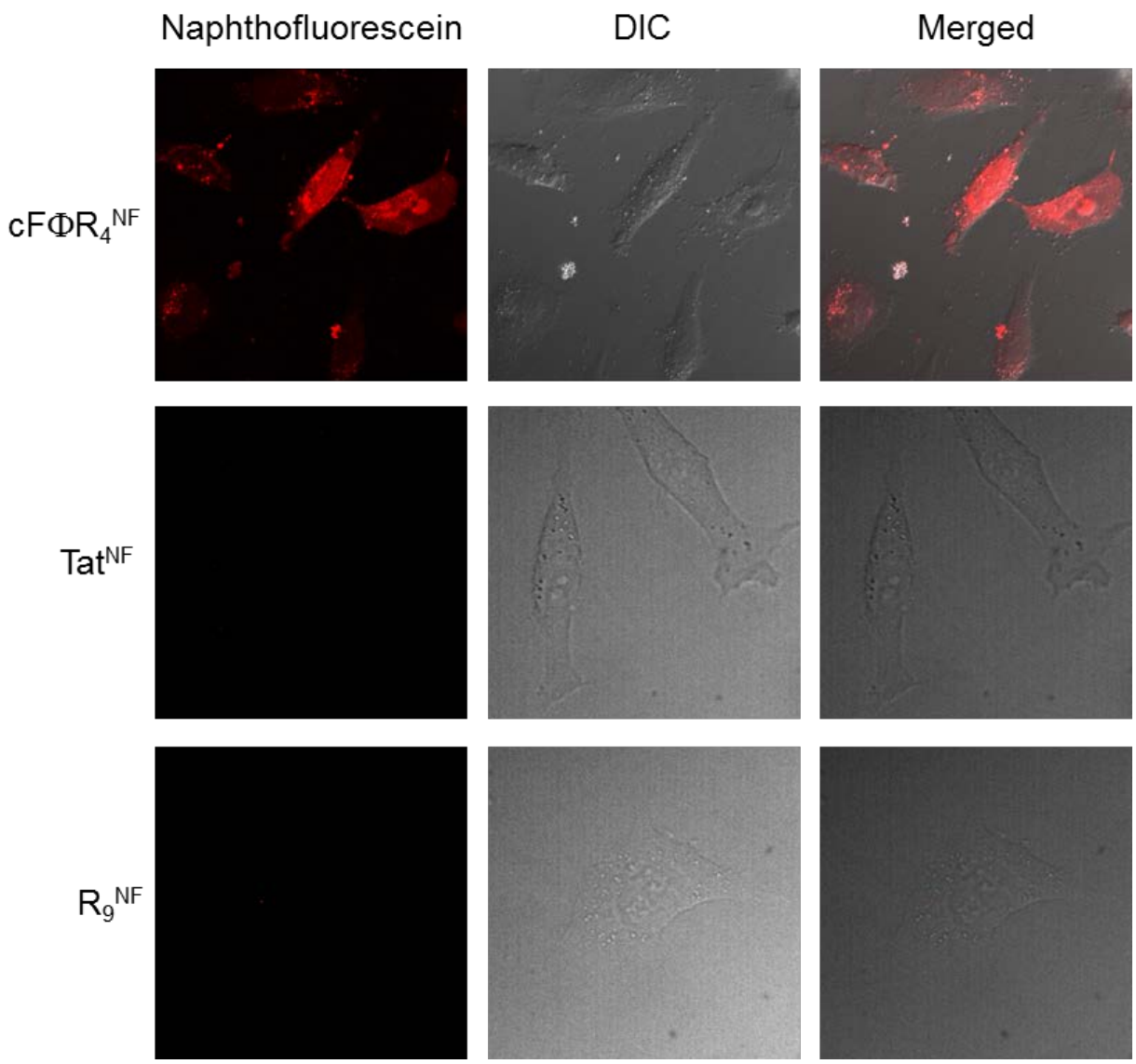


Fig. S3. Live-cell confocal microscopic images of HeLa cells treated for 2 h with 5 μM NF-labelled cFΦR$_4$, Tat, and R$_9$ peptides. All NF images were acquired using 5% power of a 642-nm laser and 50 ms exposure time.

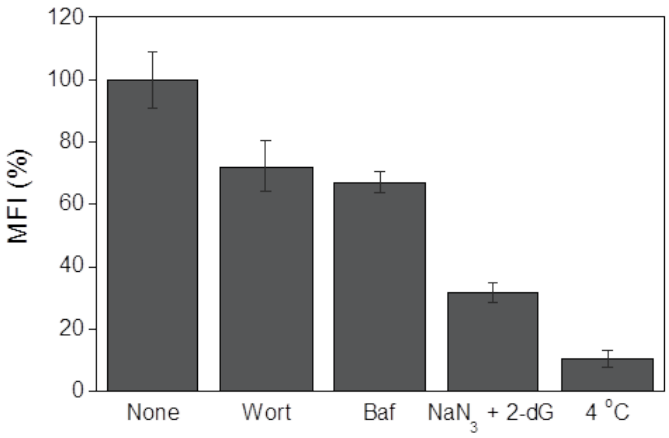


Fig. S4. Effect of wortmannin (Wort, 200 nM), bafilomycin (Baf, 200 nM), sodium azide/2-deoxy-D-glucose (NaN_3 + 2-dG, 10 mM each), or low temperature (4 °C) on the cytosolic entry of $cF\Phi R_4^{NF}$. HeLa cells were first incubated at 37 °C for 1 h in growth medium supplemented with the inhibitors or for 1 h at 4 °C (no inhibitor) and 5 μM $cF\Phi R_4^{NF}$ was added. The cells were incubated for an additional 2 h and the mean fluorescence intensity (MFI) of the cells was determined by FACS (633-nm laser, APC channel). All MFI values are relative to that of $cF\Phi R_4^{NF}$ alone ("None" = no inhibitor and at 37 °C) and represent the mean ± SD of three independent experiments.

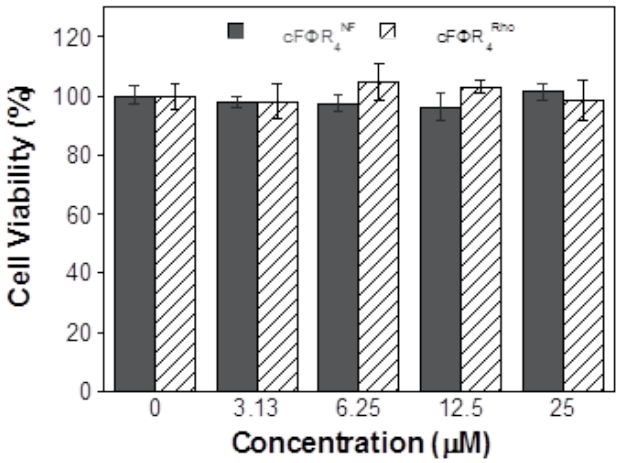


Fig. S5. Effect of $cF\Phi R_4^{NF}$ and $cF\Phi R_4^{Rho}$ (0-25 μM) on HeLa cell viability. HeLa cells were incubated with the indicated concentrations of the peptides for 24 h and the number of live cells was assessed by the MTT assay. All data are relative to that of control (no peptide, 100%) and represent the mean ± SD of three independent experiments.