Discovery and Mechanism of Highly Efficient Cyclic Cell-Penetrating Peptides

Ziqing Qian, Agnieszka Martyna, Ryan L. Hard, Jiang Wang, George Appiah-Kubi, Christopher Coss, Mitch A. Phelps, Jeremy S. Rossman, and Dehua Pei

Supporting Information

Materials

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 (Louisville, KY). Cell culture media, fetal bovine serum, penicillin-streptomycin, 0.25% trypsin-EDTA, DPBS (2.7 mM potassium chloride, 1.5 mM monopotassium phosphate, 8.9 mM disodium hydrogen phosphate, and 137 mM sodium chloride) and Lucifer Yellow were purchased from Invitrogen (Carlsbad, CA). Fluorescein isothiocyanate, lissamine rhodamine B sulfonyl chloride, and 5-(and-6)-carboxynaphtho-fluorescein succinimidyl ester were purchased from Sigma Aldrich (St. Louis, MO), while cell proliferation kit (MTT) was purchased from Roche (Indianapolis, IN). 1-Palmitoyl-2-oleoyl-sn-glycero-3-phosphocholine (PC), 1-palmitoyl-2-oleoyl-sn-glycero-3-phosphoethanolamine (PE), L-α-phosphatidylinositol (Soy, PI), sphingomyelin (Brain, Porcine), bis(monooleoylglycero)phosphate (S,R isomer, BMP), and Top-Fluor-cholesterol were purchased from Avanti Polar Lipids (Alabaster, AL).

Cytotoxicity Assay

The cytotoxicity of CPPs was evaluated by the MTT assay with several mammalian cell lines. One hundred μL of A549, NIH 3T3, H1299, or HeLa (1×10^5 cells/mL) cells were placed in each well of a 96-well culture plate and allowed to grow overnight. Peptides were added to each well to a final concentration of 50 μM and the cells were incubated at 37 °C with 5% CO_2 for 24 h. Ten μL of the MTT stock solution was added into each well, using the growth medium (no cell) 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 Tecan Infinite M1000 PRO microplate reader. Each experiment was performed in triplicates and the cells without any peptide added were treated as control.

Preparation of Large Unilamellar Vesicles

LUVs with late endosomal membrane composition (50% PC, 20% PE, 10% PI, and 20% BMP) were prepared by extrusion methods. After mixing the designated lipids (chloroform solutions) in a test tube, the lipid mixture was dried gently, and the dried under vacuum overnight. The dried lipids were rehydrated in DPBS containing 70 mM calcein (pH 7.4) to bring the lipids to a concentration of 7 mM. The lipid film was hydrated in a 37 °C water bath for 1 h before 10 min vortex and 5 freeze/thaw cycles. Afterwards, the lipid solution was pushed through two polycarbonate filters (200 nm pore size) 20 times using Avanti manual extruder. The suspension became considerably translucent. Excessive free dye molecules were removed by passing through Sephadex-G25 twice. The resulting turbid and slightly orange LUV suspension (average diameter of ~110 nm) was stored at 4 °C and used for calcein release assay within 2-3 days.

Calcein Release Assay

The fluorescent calcein release from LUV was performed on 96-well plates and was monitored by Tecan M1000 Pro microplate reader using 490 nm laser excitation and a 520 emission filter. The initial fluorescence intensity (F_0) of properly diluted LUV solution was first measured before the adage of final 20 μM CPPs of interest. The release of calcein was monitored over time as incensement of fluorescence intensity (F_{time}). At the end of 30 min incubation, complete LUV lysis was induced by addition of 1% Trition X-100, leading to complete calcein release, which was measured as F_{comp}. The percent leakage was calculated by equation: %Calcein Release = $[(F_{time} - F_0)/(F_{time}-F_{comp})]\times 100$. The experiments were performed in triplicates and the %Leakage was plotted against time (min) as Mean ± Standard Deviation.

Preparation of Small Unilamellar Vesicles for FP Assay

SUVs mimicking the plasma membrane were prepared with 45% PC, 20% PE, 20% sphingomyelin, and 15% cholesterol (mol/mol). SUVs mimicking the late endosome membrane were prepared with 50% PC, 20% PE, 10% PI, and 20% BMP (mol/mol). After mixing the designated lipids (chloroform solutions) in a test tube, the lipid mixture was dried gently by blowing argon over the solution, and kept in a desiccator overnight. The dried lipids were rehydrated in DPBS to a final total lipid concentration of 7 mM. The suspension was rigorously mixed by vortexing and sonication using a 100-W Ultrasonic Processor on ice until it became clear. Metal debris was removed by centrifugation at 20,000 g at 4 °C for 5 min. A typical preparation yields a clear and homogeneous solution containing vesicles with average diameter of ~55 nm and a polydispersity (PdI) index smaller than 0.2 as determined by dynamic light scattering measurements using Zeta Sizer Nano Series (Malvern, Brookhaven, CT). The SUV solution was kept on ice and used for fluorescence polarization analysis on the same day.

Animals and CPP Administration

All animal studies were conducted according to the procedures our animal protocol approved by the Institutional Laboratory Animal Care and Use Committee at the Ohio State University. ICR male mice (6-week-old, ~25 g) were obtained from Harlan Laboratories (Madison, WI) and acclimated for 1 week before start of the study. The animals were housed in rooms maintained between 20-26 °C and 30-70% relative humidity and on a 12-h light/dark cycle with food and water available ad libitum. Mice were fasted prior to oral CPP administration. Mice were administered CPP dissolved in sterile PBS intravenously (IV) and orally (PO). The injection volume was 100 μl for IV and 250 μl for PO per mouse. Ten mice for each dosing route were used to perform the PK study. At each of the time points, 5, 10, 20, 30 minutes, and 1, 2, 4, 6, 8 and 24 hours post dosing, one mouse was sacrificed by CO_2 asphyxiation, and blood was immediately collected via cardiac puncture then transferred to heparinized tubes. After centrifugation, plasma was separated and collected for storage at -80 °C until processing and analysis.

Mouse Plasma PK Sample Analysis

Plasma was thawed and extracted using methanol solution containing 1% (v/v) formic acid. Control samples of known concentrations (10 - 10,000 nM) were prepared in both methanol solution and clean plasma for standard curve calibration. The supernatants of centrifuged samples were analyzed by LC-MS/MS analysis using Thermo Dionex Ultimate 3000 RS LC systems with ZORBAX Extend C18 column (2.1 × 50 mm, 5 μm) and TSQ Quantum™ Triple Quadrupole Mass Spectrometer. The LC-MS/MS data was analyzed for plasma compound concentration with Thermo LCquan2.7. Plasma concentration-time data was analyzed by non-compartmental methods using default settings and the NCA analysis module in Phoenix WinNonlin v6.3 (Pharsight, Mountain View, CA). The resulting PK parameter estimates are presented in Table S2.

Table S1. Binding of FITC-Labeled CPPs to Late Endosomal Membrane (SUV2) at Different pH, Related to Table 2

CPP	EC_{50} at pH 7.4 (μM)[a]	EC_{50} at pH 5.5 (μM)[b]
1	224 ± 36	116 ± 20
2	755 ± 88	25 ± 6
4	542 ± 31	107 ± 31
7	109 ± 3	17 ± 2
9	33 ± 8	12 ± 3
11	93 ± 9	19 ± 6
12	26 ± 2	3.9 ± 0.6
R_9	470 ± 95	30 ± 9
Tat	2350 ± 440	403 ± 178
5.3	9.9 ± 1.3	3.2 ± 0.5

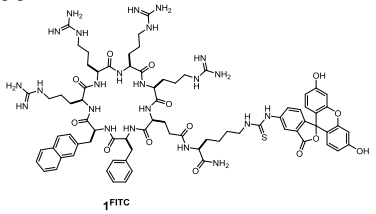
[a]Binding of FITC-labeled CPPs at pH 7.4 to SUV2 mimicking the late endosomal membrane of mammalian cells (50% PC, 20% PE, 10% PI, and 20% BMP) based on a FP assay. [b]Binding of FITC-labeled CPPs at pH 5.5 to the same SUV2 based on the FP assay.

Table S2. Pharmacokinetic Parameters for cFΦR_4 Administrated PO versus IV to Male ICR Mice, Related to Figure 5

	Dose (mg kg^{-1})	F (%)	C_{max} (nmol L^{-1})	$T_{1/2}$ (h)	AUC (nmol h L^{-1})	*CL_obs* (mL min^{-1})	*Vz_obs* (mL)
IV	1.5	-	12,174	1.02	6,711	0.08	7.51
PO	40	4	3,156	3.32	6,357	n.a.	n.a.

C_{max}, Peak plasma concentration; $T_{1/2}$, half-life; AUC, area under the curve; *CL_obs*, systemic plasma clearance; *Vz_obs*, terminal volume of distribution; F, bioavailability; n.a., not available.

A



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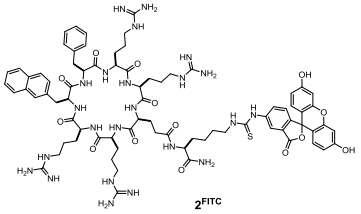

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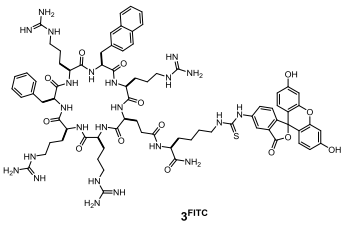

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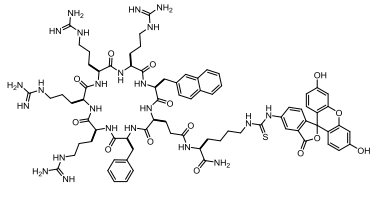

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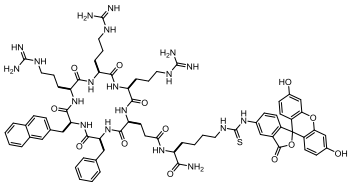

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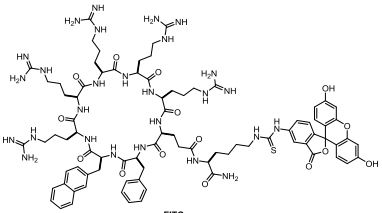

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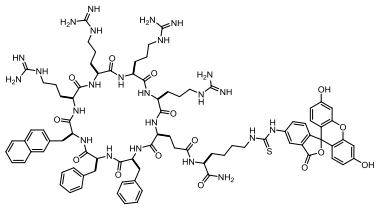

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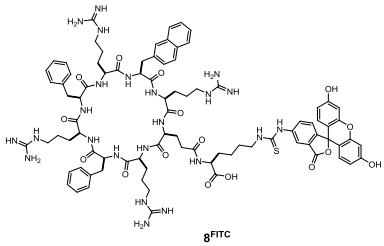

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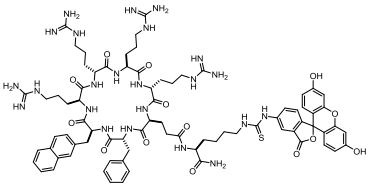

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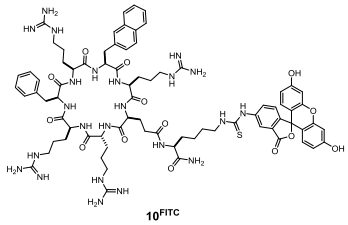

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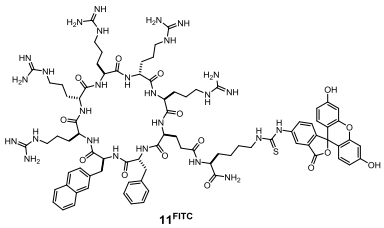

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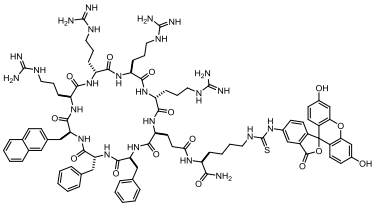

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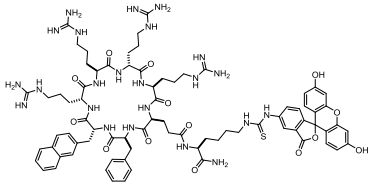

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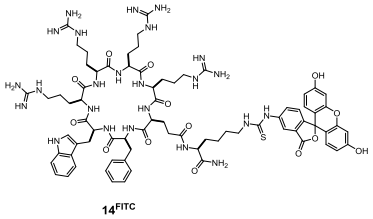

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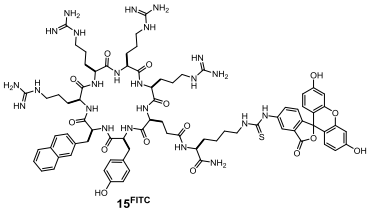

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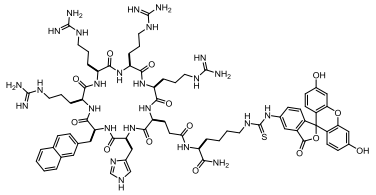

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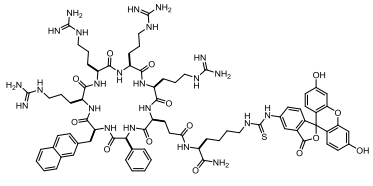

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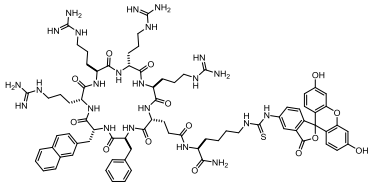

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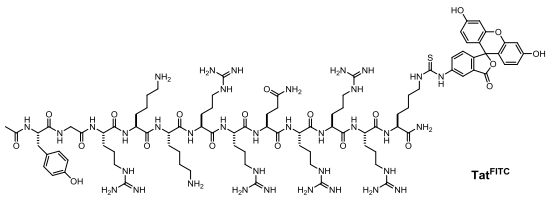

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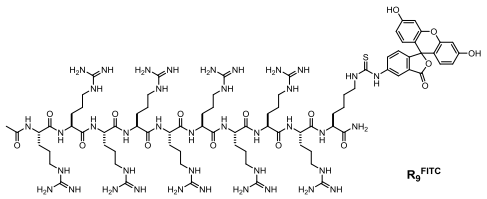
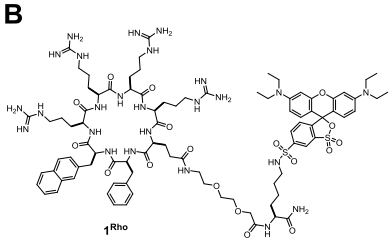


R$_9$^{FITC}

B



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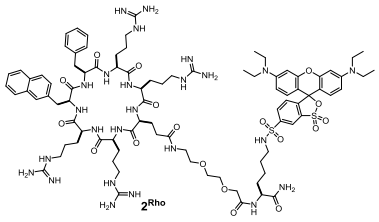

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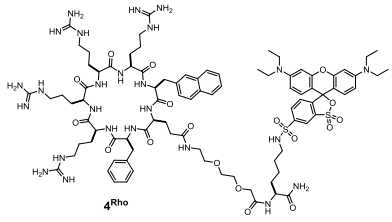

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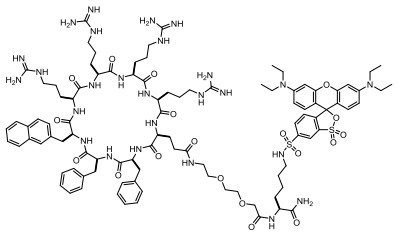

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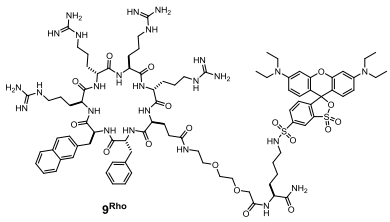

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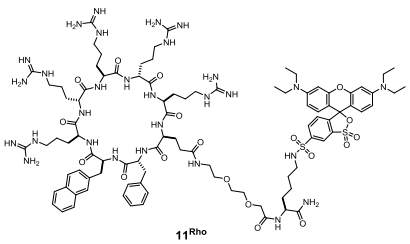

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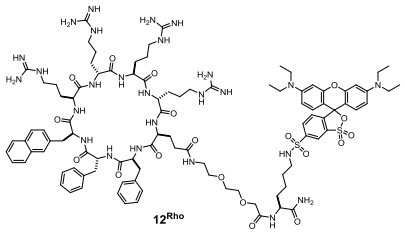

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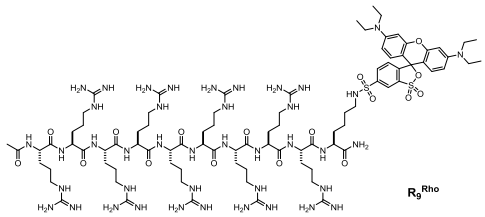

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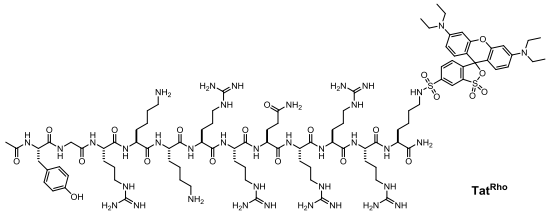

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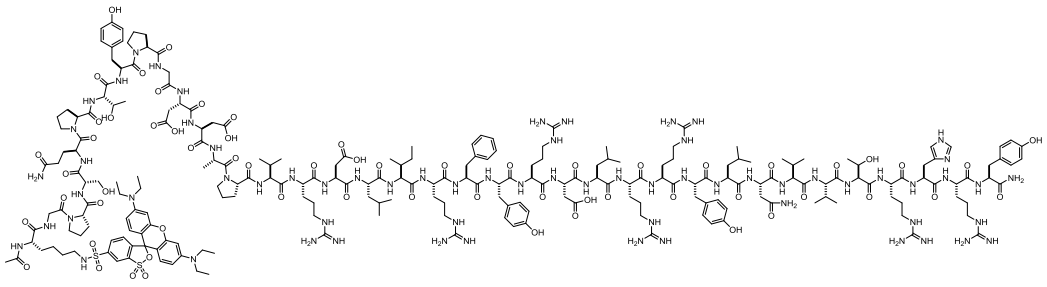

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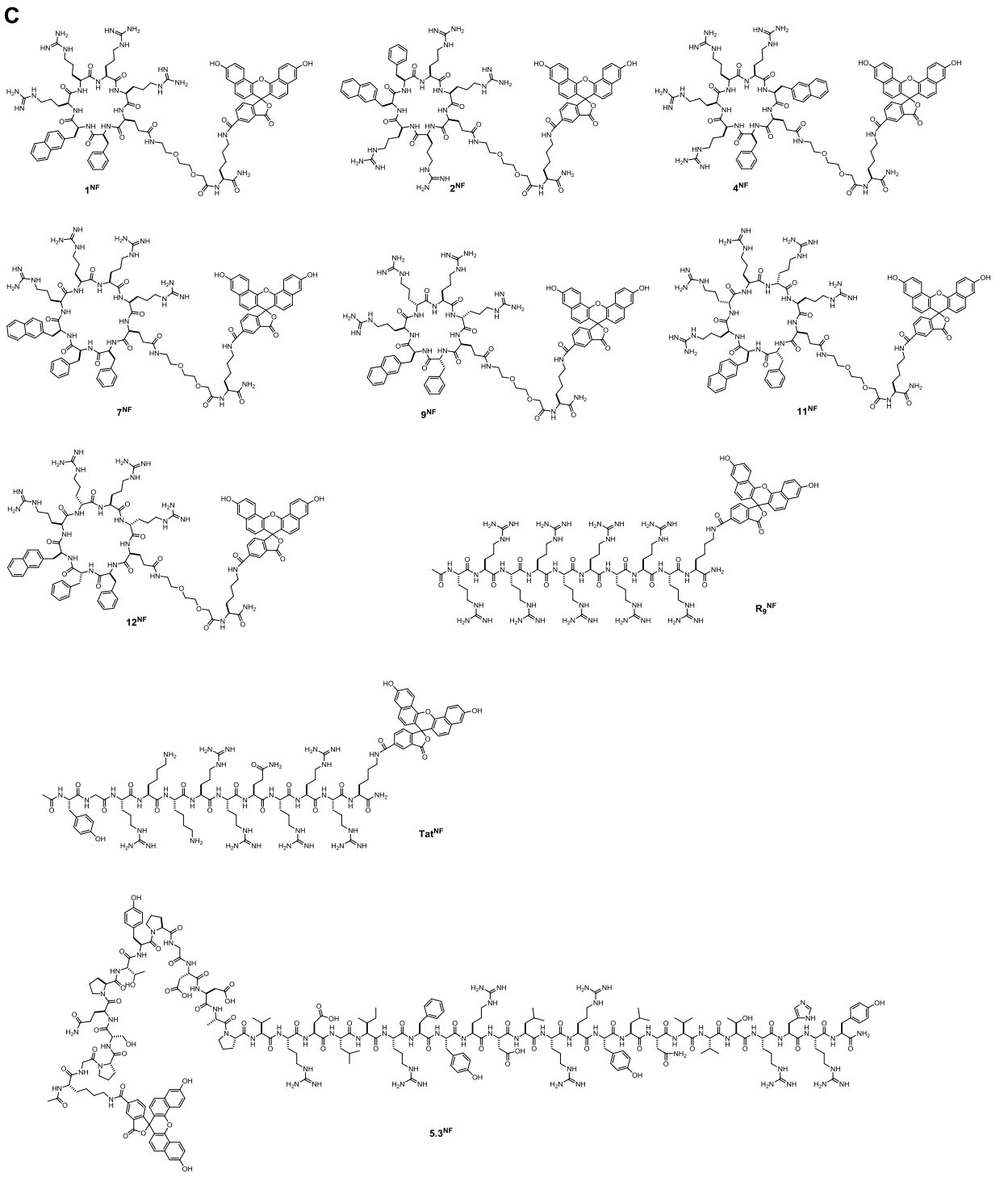


Figure S1. **Structures of Peptides Used in This Study, Related to Table 1 and Table 2**.
(A) FITC-labeled peptides in Table 1.
(B) Rho-labeled peptides in Table 2.
(C) NF-labeled peptides in Table 2.

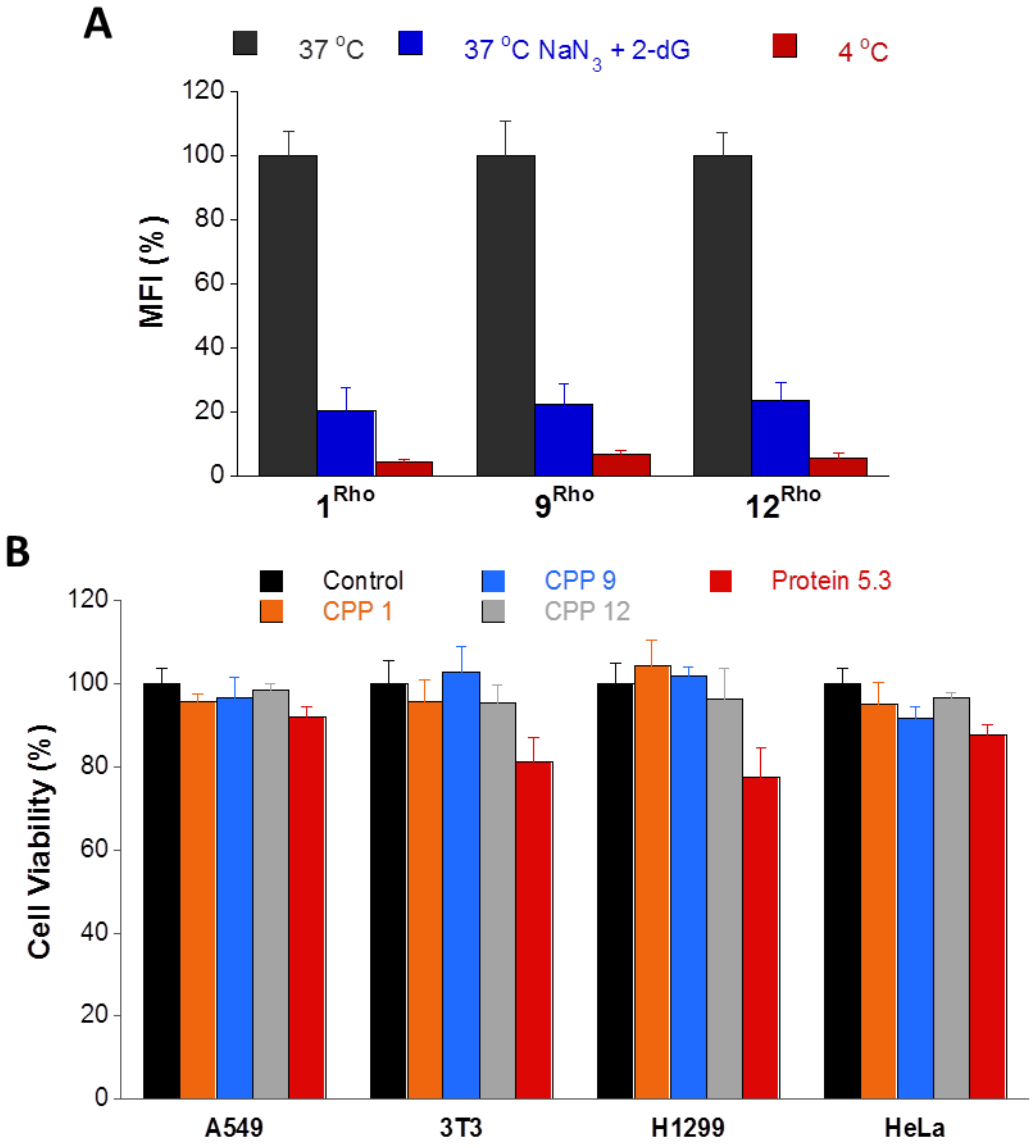


Figure S2. **Cellular Uptake Efficiency and Cytotoxicity of Selected CPPs, Related to Table 1 and Table 2.**

(A) Effect of energy depletion and low temperature on the Cellular Uptake of Cyclic CPPs. HeLa cells were treated with rhodamine B-labeled cyclic CPP (5 μM) for 2 h at 37 °C, 37 °C but in the presence of 10 mM NaN_3 and D-2-deoxyglucose, or 4 °C prior to flow cytometry analysis. Data reported are the mean ± SD from three independent experiments.

(B) Viability of four different mammalian cell lines (NIH 3T3, HeLa, and human lung cancer A549 and H1299 cells) after treatment without (control) or with 50 μM CPP **1**, **9**, **12**, or miniature protein **5.3** for 24 h (MTT assay).

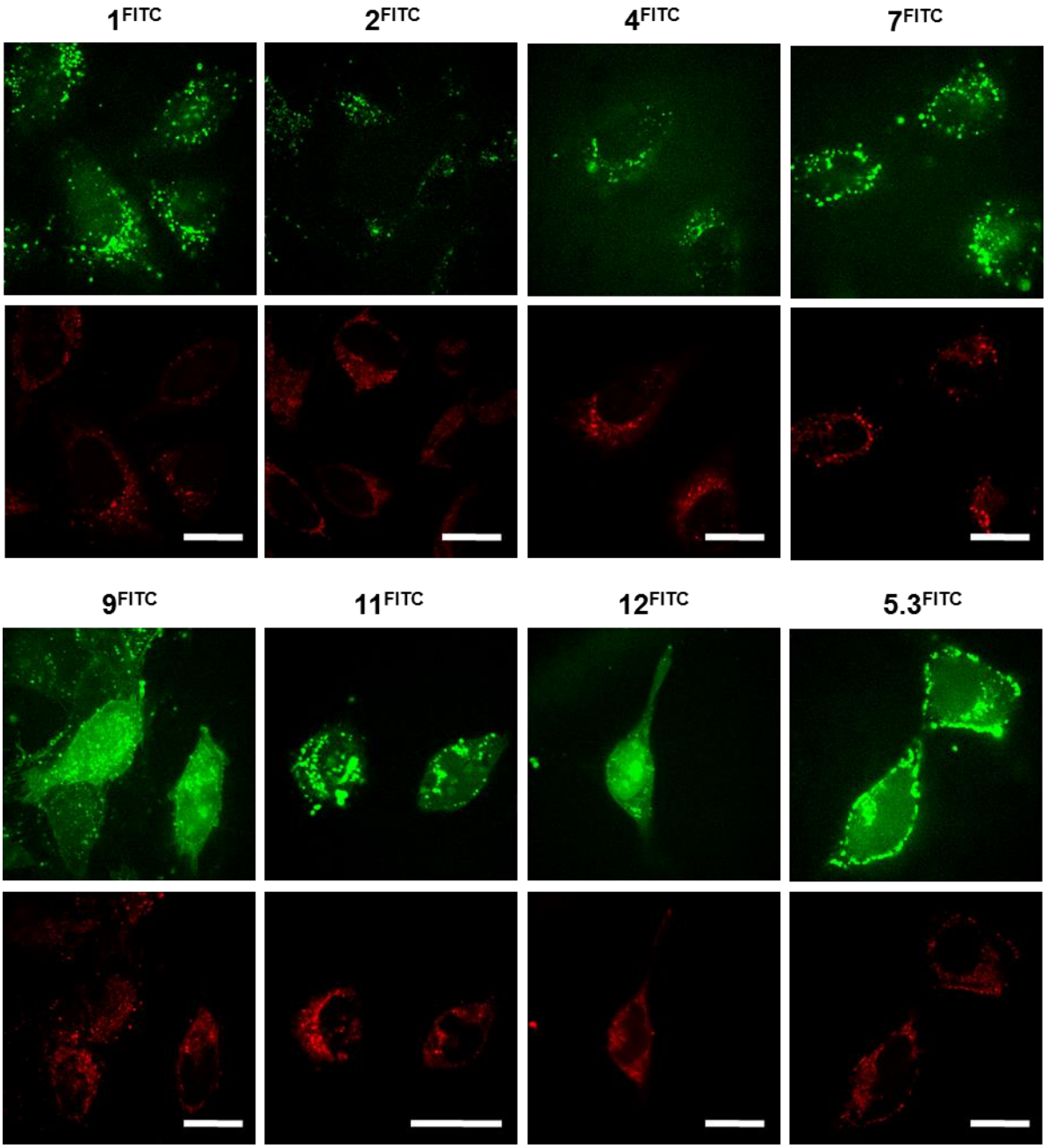


Figure S3. **Intracellular Distribution of FITC-Labeled CPPs, Related to Figure 2.**
Live-cell confocal microscopic images of HeLa cells after 2 h treatment with 5 μM FITC-labeled CPPs and 1.0 mg mL^{-1} $dextran^{Rho}$ and washing to remove extracellular peptides. Top panel, fluorescence of the CPP^{FITC} observed in the FITC channel; bottom panel, fluorescence of $dextran^{Rho}$ observed in the rhodamine channel. Scale bars indicate 20 μm.

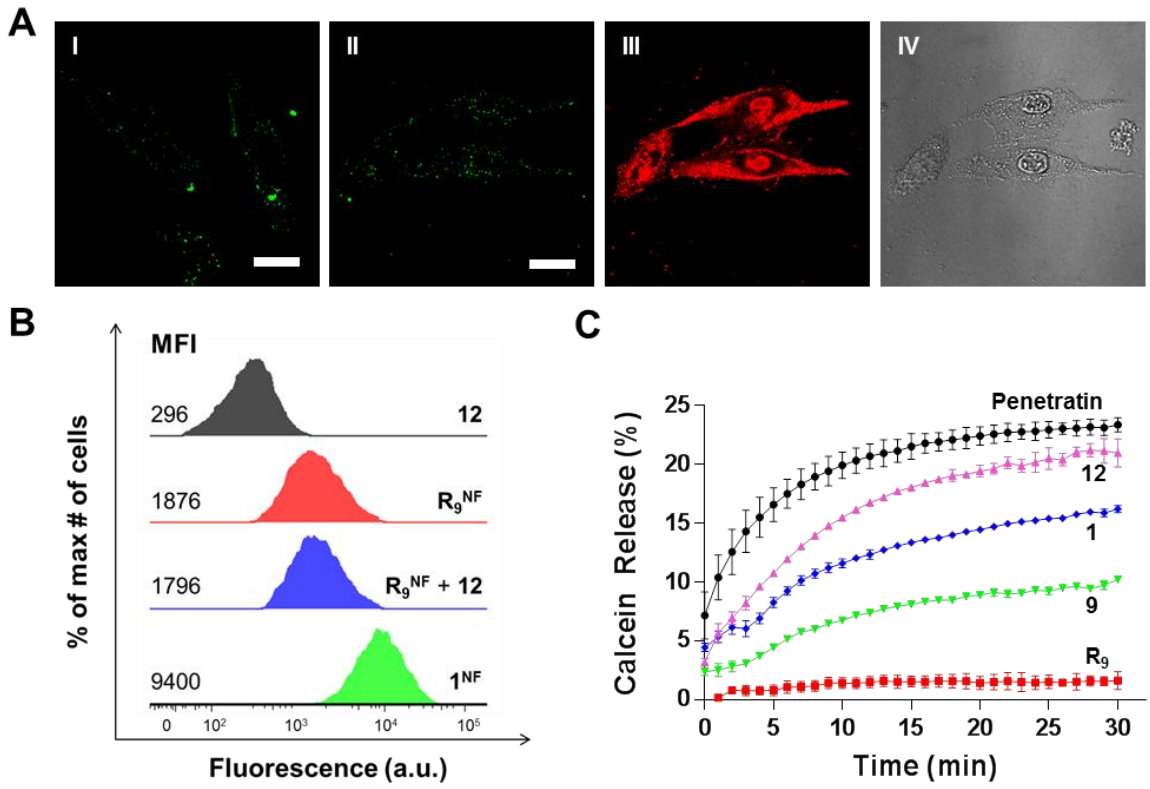


Figure S4. **Cyclic CPPs Do Not Disrupt Endosomal Membrane, Related to Figure 4.**
(A) Live-cell confocal microscopic images of HeLa cells after 2 h treatment with FITC-labeled dextran (1 mg ml^{-1}) in the absence (I) and presence of 5 μM $\mathbf{12}^{Rho}$ (II-IV) and washing to remove extracellular fluorescence. (I) and (II), FITC channel; (III), rhodamine channel; and (IV), DIC. The scale bars indicate 20 μm.
(B) Flow cytometry analysis of HeLa cells treated with 5 μM CPP **12** only (no label), 5 μM $\mathbf{R_9}^{NF}$ only, 5 μM $\mathbf{R_9}^{NF}$ and 5 μM CPP **12**, or 5 μM $\mathbf{1}^{NF}$ only.
(C) Calcein release from LUVs upon treatment with 20 μM CPPs **1**, **9**, **12**, $\mathbf{R_9}$, or penetratin. All values were relative to that of 1% Triton X-100 (100%).

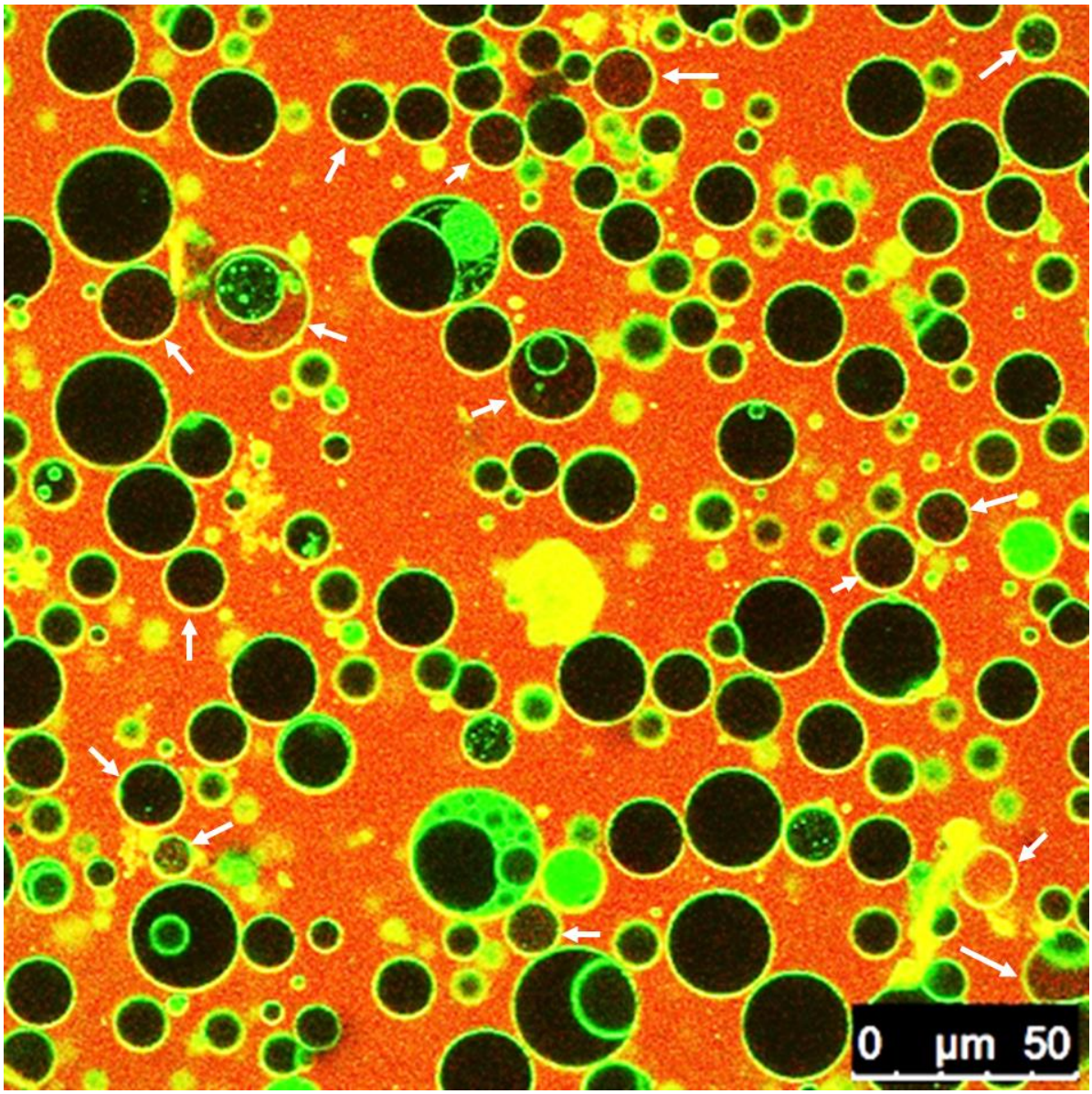


Figure S5. **Inward Budding and Vesicle Collapsing Result in Varying Levels of Intraluminal Lucifer Yellow Fluorescence, Related to Figure 4.**
Representative confocal microscopic image of GUVs after 1-h treatment with 5 μM CPP **12** (pH 7) and fluidic marker Lucifer yellow. GUVs containing visible intraluminal Lucifer yellow fluorescence are marked by white arrows. The brightness of the image was adjusted to show the intraluminal fluorescence.

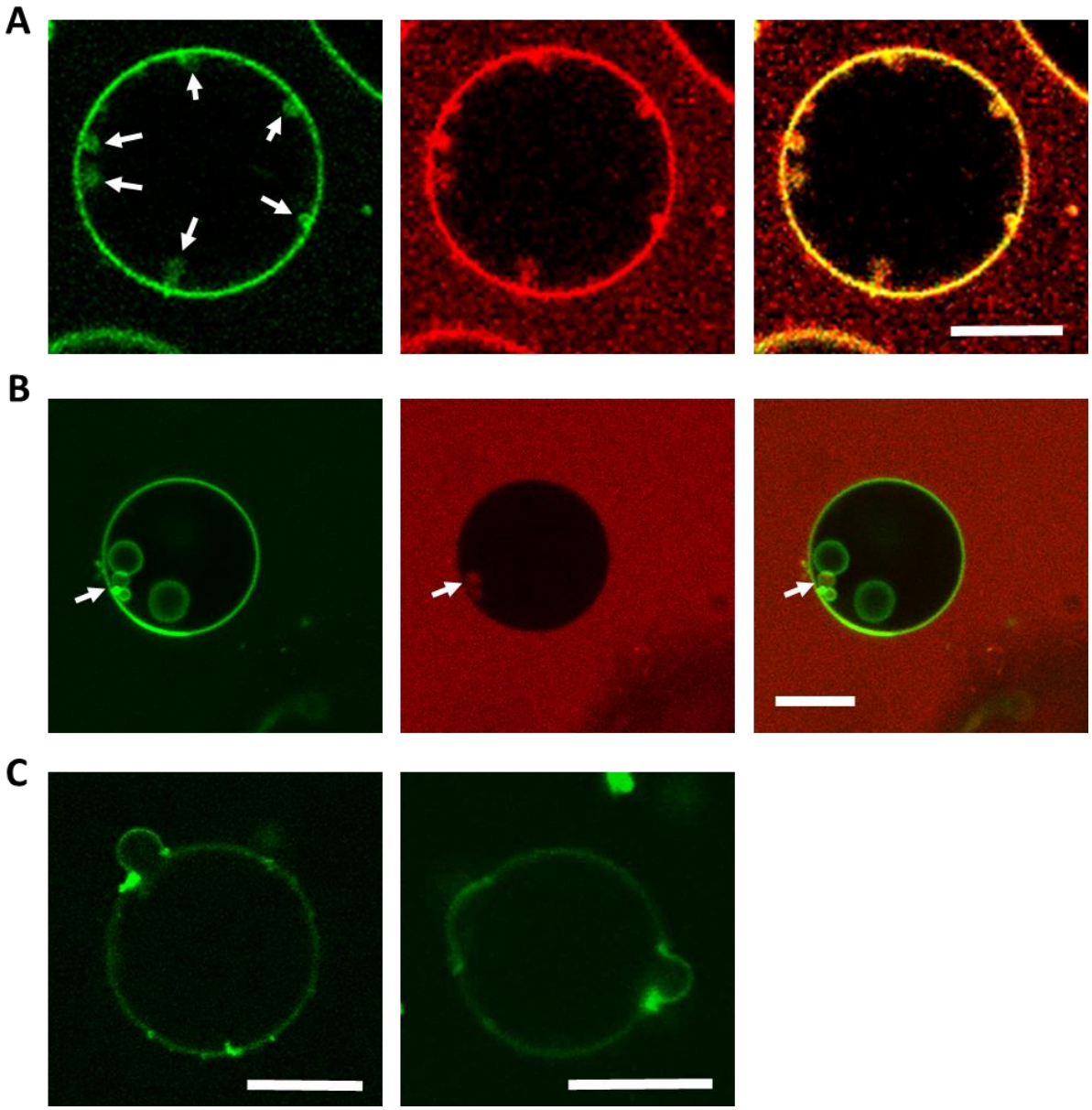
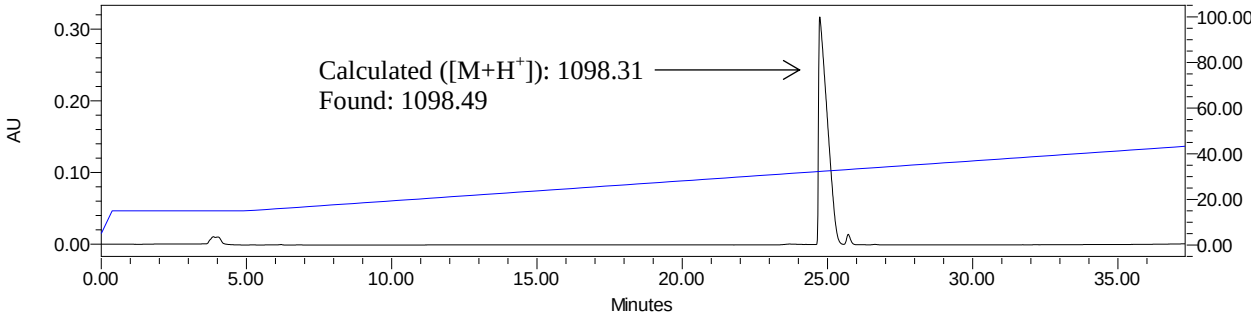


Figure S6. **Confocal Microscopic Images of Various Budding and Aggregation Intermediates from GUVs Treated with CPP 12, Related to Figure 4.**

(A and B) Endosomal membrane GUVs were prepared with 0.5% BODIPY-labeled cholesterol and treated with 5 μM CPP **12** (pH 7) and fluidic marker Lucifer yellow (shown in red). Nascent vesicles and/or lipid-peptide aggregates are marked by arrows. The scale bar indicates 10 μm.

(C) Budding intermediates derived from GUVs treated with 20 μM FITC-labeled CPP **12** at pH 5. Note the concentration of CPPs at the budding neck. The scale bar indicates 10 μm.

cyclo(FΦRRRRQ) (CPP **1**):



cyclo(fΦRrRrQ) (CPP **9**):

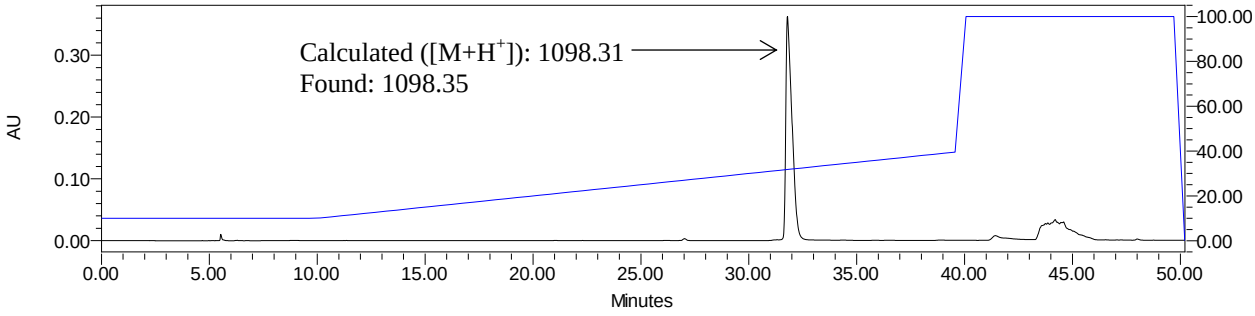


Figure S7. HPLC and MS Analysis of Representative Peptides Used in This Work.
The purity of the product (>98%) was assessed by reverse-phase HPLC equipped with an analytical C_{18} column. The authenticity of the peptides was confirmed by MALDI-TOF MS analysis.