Supporting Information

Ingallinella et al. 10.1073/pnas.0901007106

SI Materials and Methods

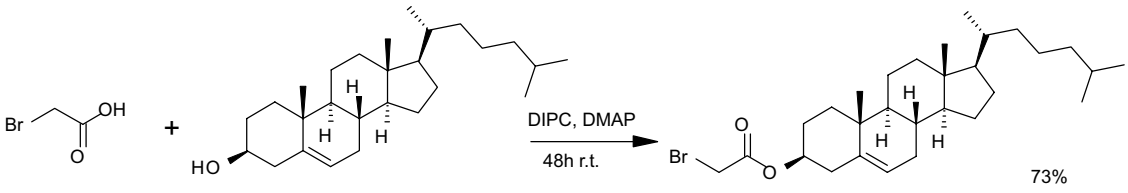
Peptide Synthesis. All peptides were synthesized by solid phase using Fmoc/tBu chemistry on a peptide synthesizer ABI433A (Applied Biosystems). For each peptide 0.75 g of resin Fmoc-Linker AM-Champion, 1% cross-linked (Biosearch Technologies) was used. The acylation reactions were performed for 60 min with 4-fold excess of activated amino acid over the resin-free amino groups. The amino acids were activated with equimolar amounts of 2-(1H-benzotriazole-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate (HBTU; Novabiochem) and a 2-fold molar excess of *N*,*N*-diisopropylethylamine (DIEA; Sigma–Aldrich) in dimethylformamide (DMF).

The side chain protecting groups were: tert-butyl for Asp, Glu, Ser, and Thr; trityl for Asn, Cys, His, and Gln; tert-butoxy-carbonyl for Lys, Tyr, and Trp; and 2,2,4,6,7-pentamethyldihydrobenzofuran-5-sulfonyl for Arg. Lys(N^{ε}-palmitoyl) (Bachem) was used as C-terminal residue for the synthesis of C34-Pam. N-terminal acetylation was performed at the end of the peptide assembly by reaction with a 10-fold excess of acetic anhydride in DMF.

At the end of the synthesis, the dry peptide–resins were treated individually with 20 mL of the cleavage mixture, 88% trifluoroacetica acid, 5% phenol, 2% triisopropylsilane, and 5% water for 1.5 h at room temperature. Each resin was filtered, and the solution was added to cold methyl-*t*-butyl ether to precipitate the peptide. After centrifugation, the peptide pellets were washed with fresh cold methyl-*t*-butyl ether to remove the organic scavengers. The process was repeated twice. Final pellets were dried, resuspended in H_2O and 20% acetonitrile, and lyophilized.

The crude peptides were purified by reverse-phase HPLC using semipreparative Waters RCM Delta-Pak C_4 cartridges (25 × 200 mm, 15 μm) and using as eluents (A) 0.1% trifluoroacetic acid in water and (B) 0.1% trifluoroacetic acid in acetonitrile, flow rate 30 mL/min. Analytical HPLC was performed on a Phenomenex Jupiter C_4 column (150 × 4.6 mm, 5 μm), flow rate 1 mL/min. The purified peptides were characterized by liquid chromatography–mass spectrometry using a Waters–Micromass LCZ platform.

Synthesis of Cholest-5-en-3-yl Bromoacetate (1). The synthetic scheme for the preparation of the precursor for chemoselective incorporation of cholesterol into cysteine-containing peptides is outlined below.



A mixture of 100 mg of cholesterol (Sigma–Aldrich) and 40 mg of bromoacetic acid (1.1 eq) (Sigma–Aldrich) was dissolved in 10 mL of anhydrous CH_2Cl_2. Then 44 μL (1.1 eq) of *N*,*N*-diisopropylcarbodiimide (DIPC; Sigma–Aldrich) and 1.5 mg (0.05 eq) of 4-dimethylaminopyridine (DMAP; Novabiochem) were added. The solution was left stirring at room temperature for 48 h and analyzed by TLC (30% CH_2Cl_2 in hexane). Afterward, the solvent was removed under vacuum, and the crude was purified by flash column chromatography on silica gel (BIOTAGE) with a gradient of 10–30% CH_2Cl_2 in petroleum ether to obtain 95 mg of the desired compound (1) as colorless oil (yield: 73%). The purified product was analyzed by NMR.

Synthesis of C34-Chol. C34-Cholesterol was prepared by chemoselective thioether conjugation between the Cys-peptide precursor (Ac-WMEWDREINNYTSLIHSLIEESQNQQEKNEQELLGSGC) and the cholesterol derivative (1). In particular, 12 mg of purified Cys-peptide precursor (2.61 μmol) was dissolved in 600 μL of DMSO, and 1.59 mg of (1) (3.13 μmol, 1.2 eq), dissolved in 100 μL of THF, was added. Then 7 μL (1% by volume) of DIEA was added to the mixture, which was left stirring at room temperature. The reaction was monitored by liquid chromatography–mass spectrometry using a Waters–Micromass LCZ platform with a Phenomenex Jupiter C4 column (150 × 4.6 mm, 5 μm) using as eluents (A) 0.1% trifluoroacetic acid in water and (B) 0.1% trifluoroacetic acid in acetonitrile, and the following linear gradient: 30–70% (B) in 20 min to 80% (B) in 3 min, washing step at 80% (B) for 3 min, flow 1 mL/min.

After a 1-h incubation, the reaction was complete, and the resulting cholesteroylated peptide was purified by reverse-phase HPLC with semipreparative Waters RCM Delta-Pak C4 cartridges (25 × 200 mm, 15 μm), using as eluents (A) 0.1% trifluoroacetic acid in water and (B) 0.1% trifluoroacetic acid in acetonitrile, and an isocratic step at 50% (B) for 5 min followed by the linear gradient: 50–70% (B) in 20 min to 80% (B) in 3 min, washing step at 80% (B) for 3 min, flow 30 mL/min. The purified peptide was characterized by HPLC/MS on a Waters–Micromass LCZ platform (theoretical molecular mass, 5,020.7 Da; found, 5,020.0 Da).

Synthesis of Chol-C34. Similarly to the synthesis of C34-Chol, Chol-C34 was prepared by conjugation between the Cys-peptide precursor (Ac-CGSGWMEWDREINNYTSLIHSLIEESQNQQEKNEQELL) and the cholesterol derivative (1). In particular, 6.5 mg of purified Cys-peptide precursor (1.41 μmol) was dissolved in 400 μL of DMSO, and 0.9 mg of (1) (1.77 μmol, 1.2 eq), dissolved in 150 μL of THF, was added. Then 5 μL (1% by volume) of DIEA was added to the mixture, which was left stirring at room temperature.

After a 1-h incubation, the reaction was complete, and the resulting cholesteroylated peptide was purified as described above. The purified peptide was characterized by HPLC/MS (theoretical molecular mass, 5,020.7 Da; found, 5,020.3 Da).

Synthesis of T20-Chol. Similarly to the synthesis of C34-Chol, T20-Chol was prepared by conjugation between the Cys-peptide precursor (Ac-YTSLIHSLIEESQNQQEKNEQELLELDKWASLWNWFGSGC) and the cholesterol derivative (1). In

particular, 10.1 mg of purified Cys-peptide precursor (2.11 μmol) was dissolved in 500 μL of DMSO, and 1.18 mg of (1) (2.53 μmol, 1.1 eq), dissolved in 260 μL of THF, was added. Then 8 μL (1% by volume) of DIEA was added to the mixture, which was left stirring at room temperature.

After a 1-h incubation, the reaction was complete, and the resulting cholesteroylated peptide was purified as described above. The purified peptide was characterized by HPLC/MS (theoretical molecular mass, 5,223.0 Da; found, 5,221.6 Da).

Synthesis of C34-Acm. C34-Acm was prepared through reaction of the Cys-peptide precursor (Ac-WMEWDREINNYTSLIHSLIEESQNQQEKNEQELLGSGC) with iodoacetamide. In particular, 10.2 mg of purified Cys-peptide precursor was dissolved in 1 mL of 6 M guanidine chloride, 0.25 M Tris, 2 mM EDTA (pH 7.5). Then 6.0 mg of iodoacetamide (10 eq), dissolved in 200 μL of DMSO, was added.

After a 1-h incubation, the product was purified by reverse-phase HPLC. The purified peptide was characterized by HPLC/MS (theoretical molecular mass, 4,651.1 Da; found, 4,650.0 Da).

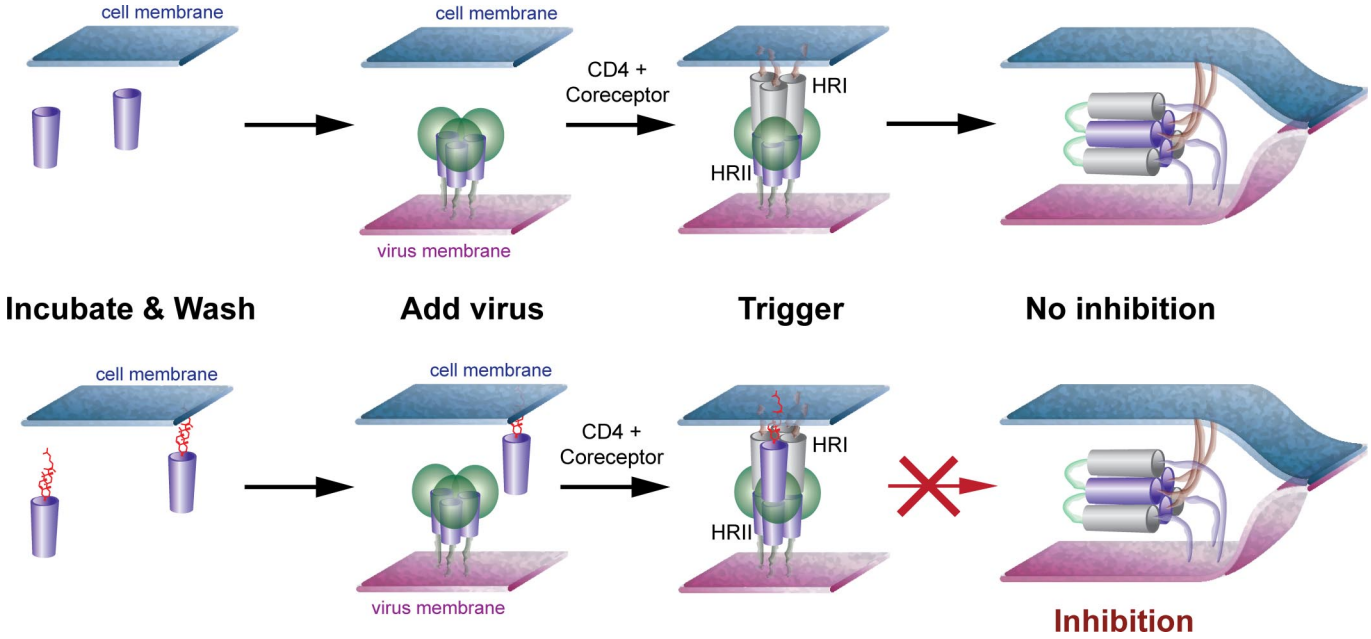


Fig. S1. Mechanism of action of C34-Chol. (*Upper*) Cells are incubated with C34 or C34-Acm and then washed thoroughly before addition of the virus. In this condition, most of the inhibitor is removed before initiation of the infection and cannot interfere with transition of the hairpin intermediate to the 6-helix bundle postfusion structure. (*Lower*) C34-Chol binds to the raft comparments of the membrane during the incubation, and because of its high affinity for this compartment, it is not removed during the washing step, remaining available for inhibition.

Table S1. Pharmacokinetic parameters of C34-Acm and C34-Chol in mice

	C34-Acm		C34-Chol	
Parameter	s.c.	i.v.	s.c.	i.v.
C_{max}, μM	1.0		2.1	
T_{max}, h	0.8		3.0	
$AUC_{(0\rightarrow\infty)}$, mM·h	1.4	0.7	13.9	18.9
$t_{1/2}$, h	0.8	0.6	6.5	3.0
V_d, L/kg		0.21		0.03
Cl, mL/min/kg		5.7		0.2

In addition to the subcutaneous (s.c., 3.5 mg/kg) administration shown in Fig. 2, intravenous (i.v., 1 mg/kg) administration was also performed. The vehicle was 10 mM glycine buffer (pH 10.2), 10% ethanol.