Supplementary Materials for "C-Peptide Inhibitors of Ebola virus Glycoprotein-Mediated Cell Entry: Effects of Conjugation to Cholesterol and Side Chain-Side Chain Crosslinking"

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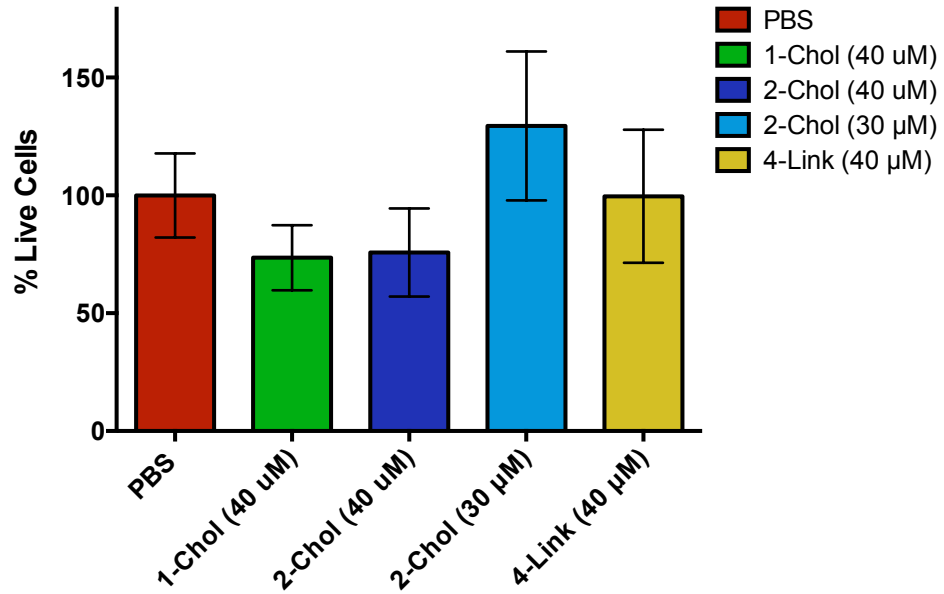


Figure S1. Cytotoxicity of C-peptides as determined by Cyto Tox Glo kit from Promega (Madison, WI)

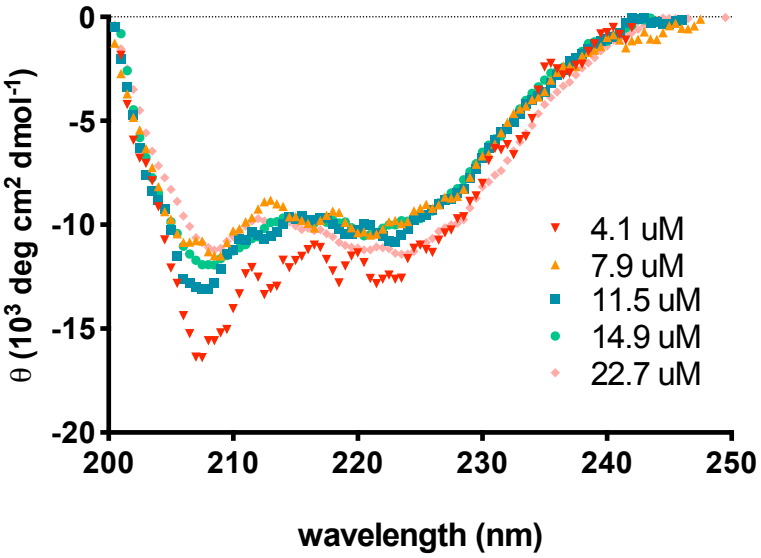
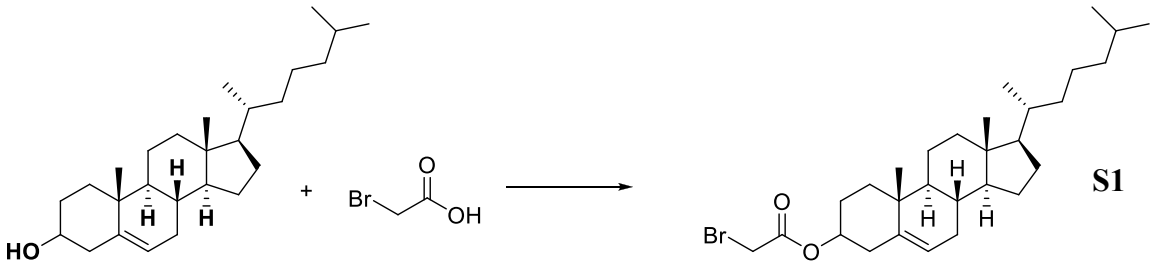


Figure S2. Concentration-dependent CD of **4-Link** in 10 mM NaOAc pH 4.6.

Experimental section.

Synthesis of bromoacetylated cholesterol (S1). All chemical reagents and solvents were obtained from Sigma-Aldrich and Acros and used without further purification unless otherwise noted. Cholesterol, *N,N*-diisopropylcarbodiimide and 4-(dimetylamino)pyridine were purchased from Sigma-Aldrich. Bromoacetic acid was purchased from MP Biomidicals LLC. Flash chromatography was performed using a Sorbent 60 Å 230- to 400-mesh silica gel. Analytical thin layer chromatography (TLC) was performed on glass-backed Analtech Uniplate silica gel plates, and compounds were visualized by staining with *p*-anisaldehyde or $KMnO_4$ stain. Organic extracts were dried over anhydrous $MgSO_4$ or Na_2SO_4, and the drying agent was removed by gravity filtration. Unless otherwise specified, all solvents were removed under reduced pressure, using a rotary evaporator. NMR spectra were obtained with Bruker DRX 600 spectrometers. ^1H chemical shifts (δ) are referenced to residual protic solvent ($CDCl_3$, 7.26 ppm) and coupling constants (*J*) are reported in hertz (Hz); ^{13}C chemical shifts (δ) are referenced to residual solvent ($CDCl_3$, 77.16 ppm) Electrospray

ionization mass spectra (ESI-MS) were obtained at the Albert Einstein Laboratory for Macromolecular Analysis and Proteomics.



S1

In an oven dried 250 mL RB flask with a solution of cholesterol (1.0 g, 2.59 mmol, 1.0 eq) and bromoacetic acid (0.4 g, 2.84 mmol, 1.1 eq) in 100 mL dry DCM was added *N,N*-diisopropylcarbodiimide acyl (0.359 g, 2.84 mmol, 0.44 mL, 1.1 eq), 4-(dimetylamino)pyridine (15 mg, 0.130 mmol, 0.05 eq). The reaction mixture was stirred at rt for 48 h. The reaction was stopped when TLC indicated the complete disappearance of starting material cholesterol (30% CH_2Cl_2 in hexanes). The solvent was evaporated *in vacuo*. Crude was further purified via flash chromatography (gradient eluent of hexanes to 10%-30% CH_2Cl_2 in hexanes) to provide 1.2 g product as white solid. ^{1}H NMR (300 MHz, CD_3OD) δ 5.39 (d, J = 4.2 Hz, 1H), 4.78-4.60 (m, 1H), 3.81 (s, 2H), 2.36 (d, J = 7.8 Hz, 1H), 2.05-1.80 (m, 5H), 1.72-0.84 (m, 33H), 0.68 (s, 3H); ^{13}C NMR (75 MHz, CD_3OD) δ 166.8, 139.3, 123.2, 76.3, 56.8, 56.3, 50.1, 42.5, 39.9, 39.7, 37.9, 37.0, 36.7, 36.3, 35.9, 32.1, 32.0, 28.4, 28.2, 27.7, 26.5, 24.4, 24.0, 23.0, 22.7, 21.2, 19.5, 18.9, 12.0.

HRMS *cacld* $[KC_{29}H_{47}BrO_2]^+$ 545.2397, observed 545.2368.

Peptide synthesis. Peptides were synthesized on an Applied Biosystems 433A synthesizer using Nα-Fmoc protecting goup strategy. Rink amide resin (0.1 mmol) was loaded into the reaction vessel and each Fmoc-protected amino acid was loaded into reaction cartridges at 3-fold molar excess. N-terminal acetylation was accomplished using 50% acetic anhydride/DCM (see Table 1). Simultaneous side chain deprotection and cleavage was carried out by treatment with 95% TFA, 2.5% thioanisole, and 2.5% 1,2-ethanedithiol for 3 h. After the resin was filtered, the crude peptide was precipitated, washed twice with cold diethyl ether, and pelleted. The pellet was suspended in a water/acetonitrile mixture and then lyophilized. Crude lyophilized peptide was redissolved in water/acetonitrile and purified by reverse-phase HPLC on a Vydac C18 column

(10 μm, 25 x 21.2 mm) with water/acetonitrile mobile phases containing 0.1% TFA. Purified peptide was lyophilized and redissolved in 10mM phosphate buffer, pH 7.1. For viral entry inhibition studies, the trifluoroacetate counterions were exchanged for chloride ions by dissolving lyophilized peptide in 50 mM hydrochloric acid and lyophilizing. This process was repeated twice. Peptide purity was determined using analytical reverse-phase HPLC and estimated to be > 90% in all cases peptides. Peptide mass was confirmed using MALDI-TOF. Concentrations were determined by absorbance at 280nm using an extinction coefficient of 22,000 M^{-1} cm^{-1}.

For conjugation of peptides to cholesterol to produce **1-Chol**, **2-Chol**, and **3-Chol**, the appropriate sulfhydryl-containing precursor peptide was incubated with 1.2-fold molar excess **S1** (above) in DMSO/DIEA for 3 hrs. Reaction progress was monitored by HPLC; upon completion, the cholesterol-conjugated peptides were then purified by HPLC as above.

For side chain-side chain crosslinking of **4-Link** and **5-Link**, the appropriate precursors were synthesized as above using Nα-FMOC strategy. All side chains contained standard acid-labile protecting groups except Orn, which contained an Nδ-ALLOC side chain protecting group. Following production of the linear precursor, the resin was treated with 6 x 10^{-5} mols of triphenylphosphine Pd(0) in dry dichloromethane containing 5% acetic acid and 5% *N*-methylmorpholine for 3 hrs 1-2 times. Deprotection of the Orn amine was confirmed by Kaiser test. The resin was then treated with a solution of 5 mM iodoacetic anhydride in 5 mL dichloromethane. Iodoacetylation of the amine was confirmed by Kaiser test, if necessary this process was repeated. Simultaneous side chain deprotection and cleavage, and purification by HPLC were as described as above.

Viral entry assays. Viral entry inhibition assays were performed essentially as described in reference 12. Vero cell monolayers (7.5 x 10^4) cells were plated in a 48-well plate in the presence of 2% FBS and 1% Penicillin-Streptomycin (PenStrep). Vero cells were exposed to peptides dissolved to the appropriate concentration in PBS supplemented with calcium and magnesium for 1 hour at 37°C. VSV-GP pseudotyped virus or VSV-G recombinant virus was added to the wells and incubation at 37°C continued for an additional

1 hour. The peptide/virus combination was then removed via aspiration, and DMEM media supplemented with 2% FBS and 1% PenStrep containing 20 mM ammonium chloride to neutralize endosomal pH was added to all wells. The plates were incubated at 37°C overnight and infection was scored the following day by manually counting eGFP-positive cells under a fluorescence microscope.

Circular dichroism. All spectra were acquired on a Jasco J-815 spectrometer with a 1 mm quartz cuvette. Peptides were dissolved in the appropriate buffer (10 mM phosphate buffer, pH 7.1 or 10 mM sodium acetate, pH 4.6). Wavelength scans were obtained with 0.1 nm step size and a speed of 1 nm/sec from 200 - 250nm.