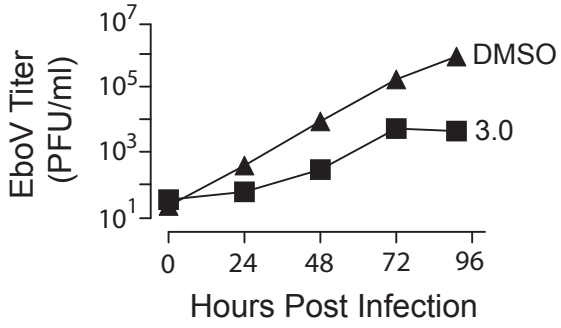
doi:10.1038/nature10380

a



b

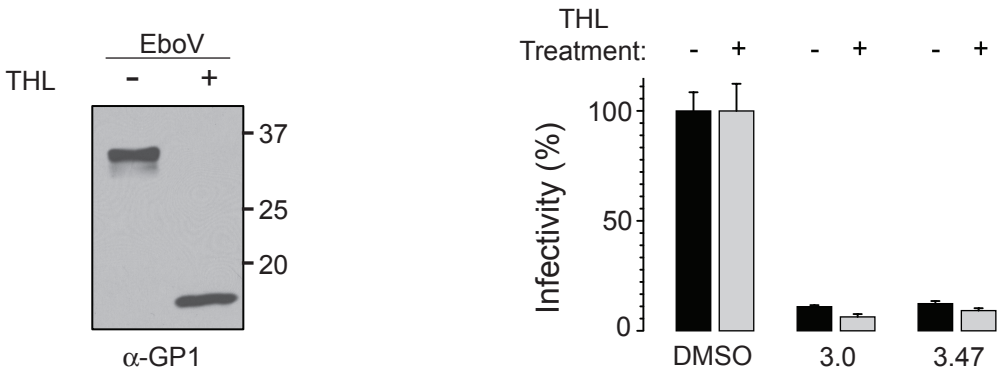


Figure S1.

a, EboV infection is inhibited by 3.0. Vero cells were cultured in media containing 3.0 (20 μM) or vehicle for one hour and then challenged with native ebolavirus Zaire-1995 (moi=0.1). Data for 3.0 is mean ± s.d. (n=4) and mean of duplicate DMSO-treated samples. (Note: Error bars are smaller than the bullets.)

b, (left) Compounds 3.0 and 3.47 inhibit infection by protease-cleaved virus particles. EboV pseudotyped particles were incubated with thermolysin and cleavage of GP1 was analyzed by immunoblot following deglycosylation with PNGaseF (left panel). Vero cells treated with 3.0, 3.47 (5 μM) or vehicle and exposed to native and thermolysin-cleaved VSV EboV GP particles(right panel). Infection was measured as in Fig 1b. Data is mean ± s.d. (n=4) and is representative of 3 experiments.

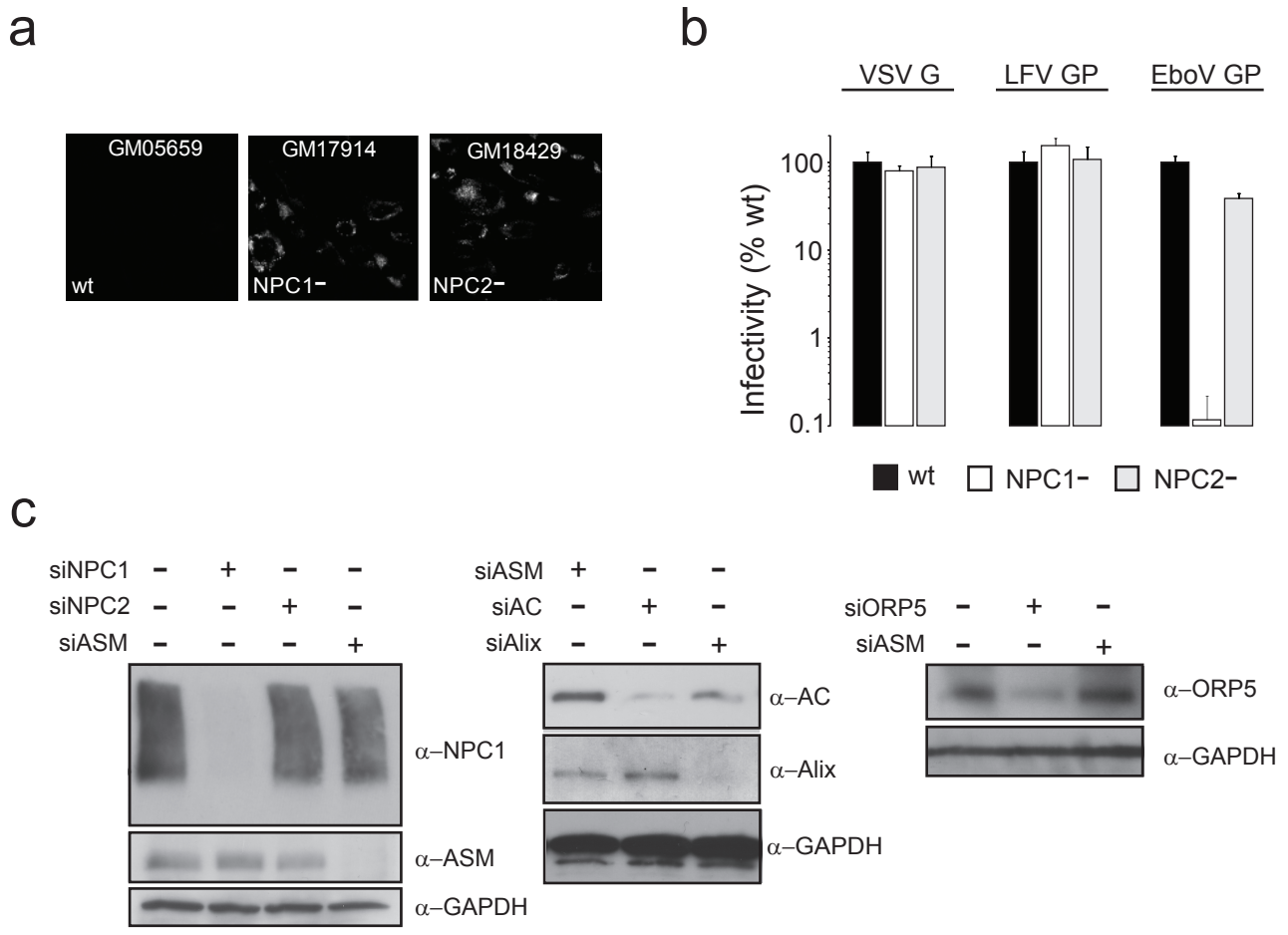


Figure S2. Analysis of proteins involved in cholesterol uptake in EboV GP infection

a, Niemann-Pick C1 and C2 cells contain cholesterol-rich vacuoles. Human fibroblast cell lines derived from patients with Niemann-Pick type C1 or C2 disease were analyzed for cytoplasmic cholesterol deposits using filipin staining. GM17914 (NPC1-) is a compound heterozygote with a frameshift and a missense mutation (I106T) that results in a misfolding[26]; GM18429 (NPC2-) is homozygous for a substitution that results in defective splicing of NPC2 RNA; and GM05659 (wt) fibroblasts are from a healthy human donor. Representative images are shown.

b, Ebolavirus infection of Niemann-Pick C1 and C2 cells. Wt, NPC1-, and NPC2- fibroblasts were exposed to VSV particles pseudotyped with VSV G, LFV GP or EboV GP. Infection was measured as in Fig 1c. Data is mean ± s.d. (n=3) and is representative of 3 experiments.

c, Protein expression in siRNA treated HeLa cells from Fig 2b. Expression of NPC1, NPC2, ASM, Alix, acid ceramidase (AC) and ORP5 was knocked-down in HeLa cells using SMARTpool siRNA (20 nM, Dharmacon). After 72 hours, cells were assessed for infection (Fig 2b) or protein expression. Protein expression was measured by immunoblot of cell lysates using anti-ASM 1H7 (Genzyme), anti-NPC1 (Abcam), anti-AC (BD Biosciences), anti-Alix (Biolegend), and anti-ORP5 (Abcam).

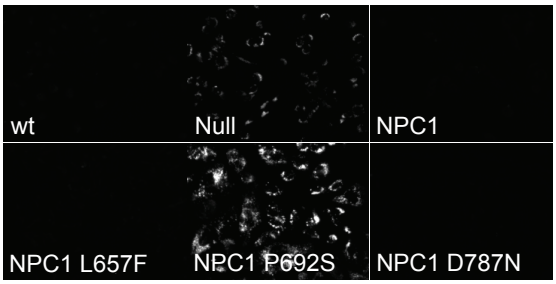


Figure S3. Phenotype of cells expressing NPC1 mutant proteins.

CHO_{wt}, CHO_{null}, and CHO_{null} cells stably expressing wild type mouse NPC1, NPC1 L657F, NPC1 P692S, and NPC1 D787N were fixed and stained with filipin as in Figure S2a. Representative images are shown.

CHO Cell Line	Retroviral Titer (FFU/ml)							
	ZEboV GP	CIEboV GP	BEboV GP	SEboV GP	REboV GP	MARV GP	LFV GP	VSV G
wt	$(4.3 \pm 0.4) \times 10^6$	$(1.1 \pm 0.1) \times 10^6$	$(1.1 \pm 0.8) \times 10^7$	$(4.2 \pm 0.1) \times 10^6$	$(3.0 \pm 0.4) \times 10^5$	$(1.2 \pm 0.4) \times 10^6$	$(2.6 \pm 0.6) \times 10^5$	$(8.0 \pm 0.8) \times 10^6$
Null	< 4.0	< 4.0	4 ± 0	< 4.0	< 4.0	2.7 ± 2.3	$(2.2 \pm 0.9) \times 10^6$	$(2.7 \pm 1.5) \times 10^7$
NPC1	$(1.6 \pm 0.6) \times 10^6$	$(7.3 \pm 0.2) \times 10^5$	$(2.7 \pm 0.3) \times 10^6$	$(2.5 \pm 0.6) \times 10^6$	$(5.0 \pm 0.9) \times 10^4$	$(2.1 \pm 0.3) \times 10^5$	$(1.2 \pm 0.3) \times 10^6$	$(7.1 \pm 0.2) \times 10^6$

Figure S4. Infection of CHO cells by filovirus GP pseudotyped retrovirus particles.

CHOwt, CHO cells lacking NPC1 (CHO_{null}) and CHO_{null} cells stably expressing mouse NPC1 (CHO_{NPC1}) were exposed to MLV particles encoding LacZ and pseudotyped with GPs from ebolavirus Zaire (ZEboV), Côte d'Ivoire (CIEboV), Bundibungyo (BEboV), Sudan (SEboV), or Reston (REboV), marburgvirus (MARV), Lassa fever virus (LFV) or VSV G. Results are the mean ± s.d. (n=12).

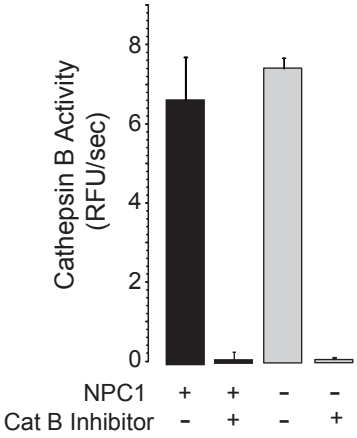


Figure S5. The relationship between NPC1 expression and cathepsin B activity in CHO cells.

CHO_{wt} and CHO_{null} cells were incubated in medium containing the Cat B inhibitor CA074 (80 μM) or vehicle (1% DMSO) for 4 hours, and Cat B protease activity was measured in cell lysates using a fluorogenic substrate[3]. Cat B activity (V0, relative fluorescence units (RFU)/sec) is plotted. Results are mean ± s.d. (n=9).

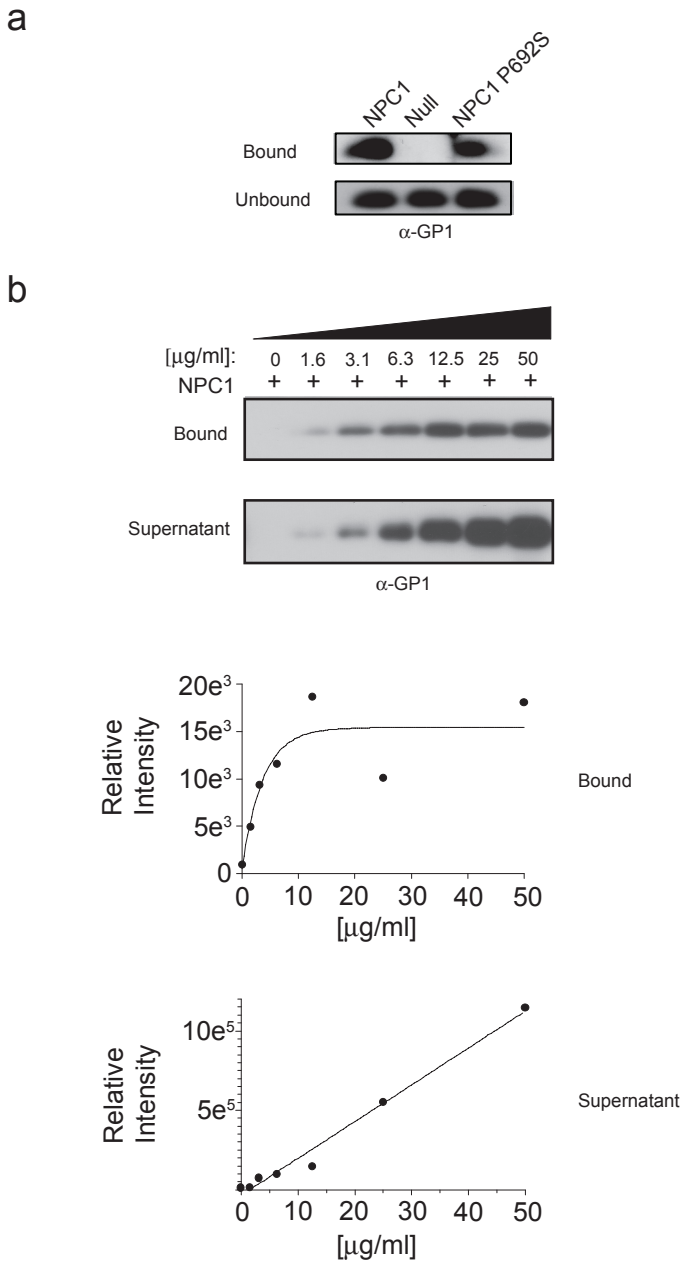


Figure S6.

a, EboV $GP_{\Delta TM}$ binds to membranes containing the NPC1 mutant P692S. Thermolysin-cleaved EboV $GP_{\Delta TM}$ protein (1 μg) was added to LE/LY membranes from CHO_{null}, CHO_{NPC1}, or CHO_{P692S} cells and analyzed as in Figure 3.

b, Saturable binding of thermolysin-cleaved EboV $GP_{\Delta TM}$ to NPC1 membranes. LE/LY membranes from CHO_{NPC1} were incubated with increasing concentrations of thermolysin-cleaved EboV $GP_{\Delta TM}$ as in Figure 3a. GP1 was analyzed in membrane bound and supernatant fractions using immunoblot (top). Densitometry was performed and the relative intensity of each GP1 band was measured using Quantity One Software (Bio-Rad). The data was used to plot the amount of GP1 in the supernatant and the amount bound to LE/LY membranes as a function of the input concentration of EboV $GP_{\Delta TM}$.

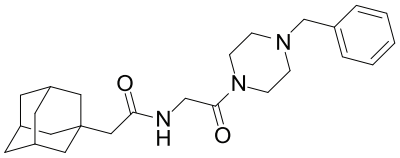
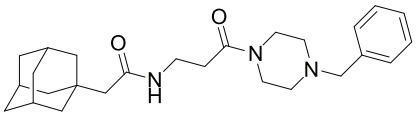
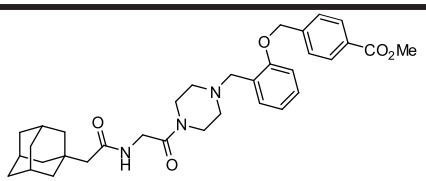
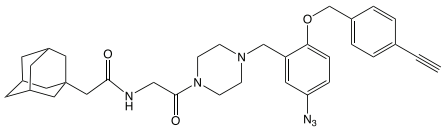
3.0		IC_{50}= 1.6 μM	IC_{90}= 5.8 μM
3.18		IC_{50}= 41 μM	IC_{90}= >50 μM
3.47		IC_{50}= 130 nM	IC_{90}= 400 nM
3.98		IC_{50}= 1 μM	IC_{90}= >40 μM

Figure S7. Structure and antiviral activity of 3.0 analogs 3.18, 3.47, and 3.98.

Inhibition of virus infection by 3.0 analogs 3.18, 3.47, and 3.98. Vero cells were incubated in the presence of 3.18, 3.47, 3.98 or vehicle for 90 minutes prior to the addition of VSV particles pseudotyped with EboV GP. Virus infection was calculated as in Fig. 1b. IC_{50} and IC_{90} values were determined from this data.

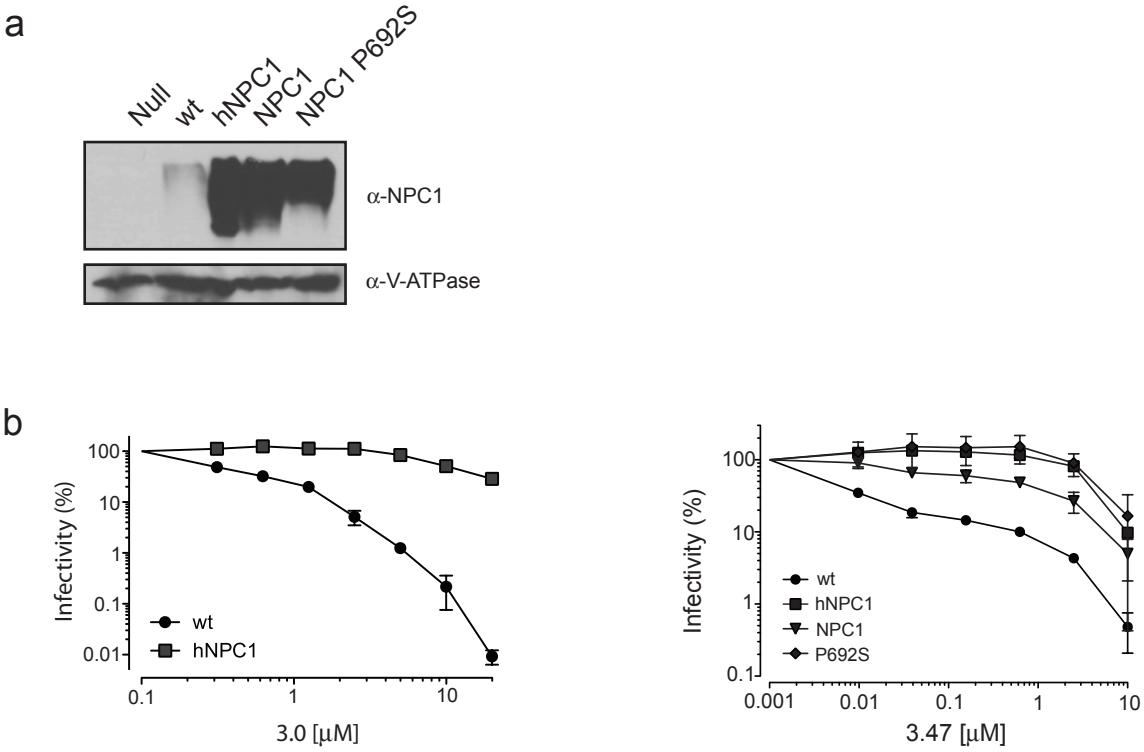


Figure S8. Effect of NPC1 expression on antiviral activity.

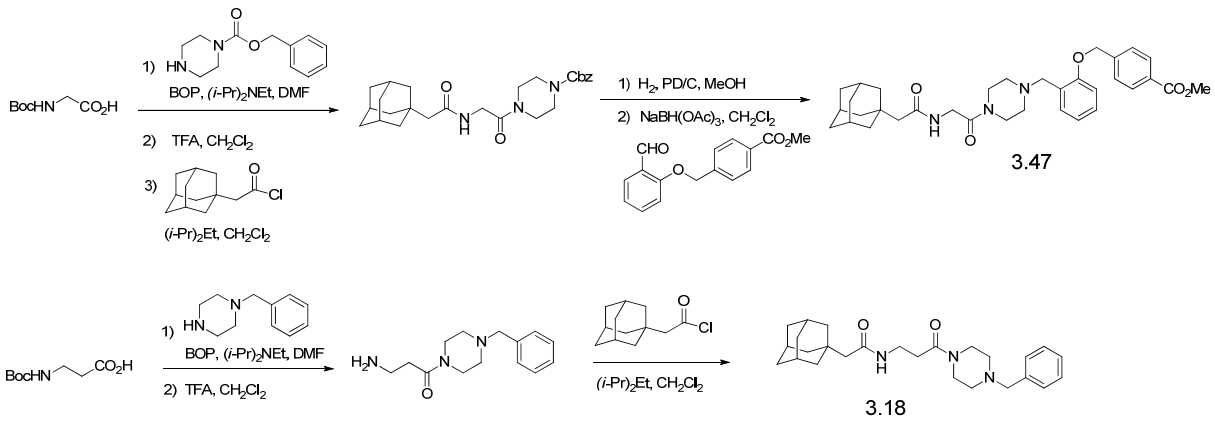
a, CHO_{null}, CHO_{wt}, CHO_{NPC1}, CHO_{hNPC1} and CHO_{P692S} cells were homogenized, and membranes in the post-nuclear supernatant were pelleted at 15000 x g. NPC1 and V-ATPase B1/2 in the pelleted membranes were detected by immunoblot as in Figure 3b.

b, CHO_{wt}, CHO_{hNPC1}, CHO_{NPC1}, CHO_{P692S} cells were incubated in the presence of increasing concentrations of 3.0 (left), 3.47 (right) or vehicle prior to the addition of VSV particles pseudotyped with EboV GP. Infection was calculated as in Fig 1b. Data is mean ± s.d. (n=4) and is representative of 3 experiments.

I. General information

^{1}H NMR spectra were recorded on a Varian Inova 600 MHz spectrometer with chemical shifts reported in parts per million (ppm) relative to an internal standard (trimethylsilane). Coupling constants (*J*) are reported in hertz (Hz). Standard resolution mass spectra were obtained on an Agilent 1200 Series HPLC (4.6 x 100 mm, 5 µm Phenomenex C18 reverse-phase column) and a 6130 Series mass spectrometer system; all mass spectra were obtained using electrospray ionization (EI) in positive ion mode. Standard reverse-phase HPLC conditions were as follows: mobile phase A = 0.1% formic acid in water; mobile phase B = 0.1% formic acid in acetonitrile. Solvents for synthesis were purchased as anhydrous grade and used without further purification. Reagents were purchased from commercial sources and used as received.

II. Synthesis of 3.47 and 3.18



1. Synthesis of benzyl 4-(2-((tert-butoxycarbonyl)amino)acetyl)piperazine-1-carboxylate

   To a mixture of N-Cbz-piperazine (2.2 mL, 11.4 mmol) and Boc-Gly-OH (1.8 g, 10.4 mmol) in DMF (20 mL) was added BOP (6.0 g, 13.6 mmol) and diisopropylethylamine (5.4 mL, 31.2 mmol). After stirring for 16h at rt, the resulting mixture was treated with saturated brine and then extracted with EtOAc. The combined organic layers were washed with 5% aqueous $NaHCO_3$ and saturated brine, dried over sodium sulfate, filtered, and concentrated. The residue was purified by flash column chromatography eluting with Hexane:EtOAc (1:2) to obtain benzyl 4-(2-((tert-butoxycarbonyl)amino)acetyl)piperazine-1-carboxylate (3.5 g, 89 % yield) as a viscous oil. ESI-MS [M+Na]= 400.2 ;^{1}H NMR (600 MHz, $CDCl_3$) δ 7.39-7.2691 (m, 5H), 5.47 (br s, 1H), 5.15 (s, 2H), 3.96 (d, 2H, *J*=4.2), 3.62 (br s, 2H), 3.55-3.50 (m, 4H), 3.38 (br s, 2H), 1.45 (s, 9H).

2. Synthesis of benzyl 4-(2-(2-((3r,5r,7r)-adamantan-1-yl)acetamido)acetyl)piperazine-1-carboxylate

   To a cooled solution of the above compound (745 mg, 1.97 mmol) in CH_2Cl_2 (10 mL) at 0^oC TFA was added (1.5 mL, 19.7 mmol). After stirring for 3 h at ambient temperature, the reaction mixture was concentrated to give the crude 4-Cbz-piperazine glycinamide. To a solution of the crude 4-Cbz-piperazine glycinamide in CH_2Cl_2 (10 mL) at 0^oC diisopropylethylamine (1.7 mL, 10 mmol) and adamantan-1-acetyl chloride (419 mg, 1.97 mmol) were added and the reaction mixture was stirred overnight at RT. Water was added to the reaction mixture and the aqueous layer was extracted with CH_2Cl_2. The combined organic layers were washed with saturated brine, dried over sodium sulfate, filtered, and concentrated. The residue was purified by flash column chromatography eluting with CH_2Cl_2:MeOH (95: 5) to obtain benzyl 4-(2-(2-((3r,5r,7r)-adamantan-1-yl)acetamido)acetyl)piperazine-1-carboxylate (729 mg, 82 % yield for 2 steps). ESI-MS [M+1]=454.1; ^{1}H NMR (600 MHz, $CDCl_3$) δ 7.39-7.32 (m, 5H), 6.42 (br s, 1H), 5.15 (s, 2H), 4.07 (d, 2H, *J*=4.2), 3.64 (br s, 2H), 3.55-3.48 (m, 4H), 3.40 (br s, 2H), 2.01 (s, 2H), 1.97 (s, 3H), 1.70-1.62 (m, 12H).

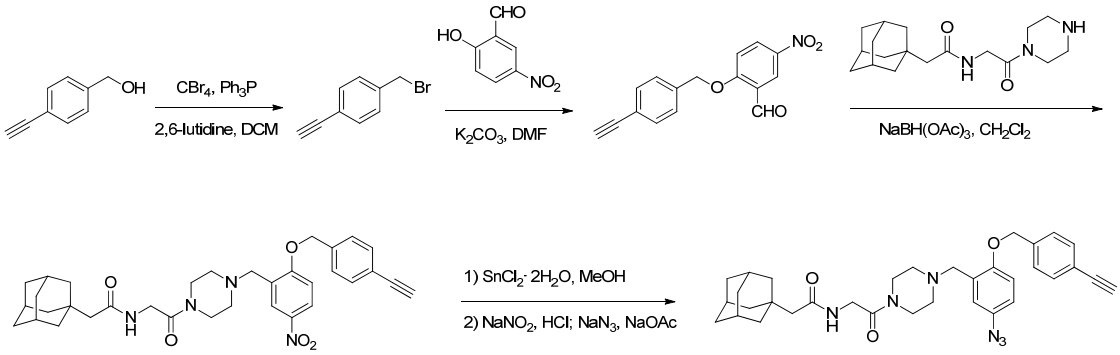
3. Synthesis of (methyl 4-((2-((4-(2-(2-((3r,5r,7r)-adamantan-1-yl)acetamido)acetyl)piperazin-1-yl)methyl)phenoxy)methyl)benzoate)

   A mixture of benzyl 4-(2-(2-((3r,5r,7r)-adamantan-1-yl)acetamido)acetyl)piperazine-1-carboxylate (729 mg, 1.61 mmol) and 10% palladium on carbon (146 mg) in MeOH (10 mL) was stirred for 18 hr under hydrogen atmosphere (1atm). After the palladium catalyst was filtered on celite, the filtrate was concentrated to give crude (1-(adamantan-1-yl)acetamido)acetyl)piperazine (400 mg) as a colorless viscous oil. To a solution of the crude material in CH_2Cl_2 (20 mL), 2-(4-methoxycarbonyl)benzyloxybenzaldehyde (406 mg, 1.50 mmol) and sodium triacetoxyborohydride (397 mg, 1.88 mmol) were added. After stirring for 16h at RT, the mixture was poured into water and extracted with EtOAc. The combined extracts were washed with saturated brine, dried over sodium sulfate, filtered, and concentrated. The residue was purified by flash column chromatography eluting with CH_2Cl_2:MeOH (95: 5) to give (methyl 4-((2-((4-(2-(2-((3r,5r,7r)-adamantan-1-yl)acetamido)acetyl)piperazin-1-yl)methyl)phenoxy)methyl)benzoate) (517 mg, 56 % yield for 2 steps) as a pale yellow oil. ESI-MS [M+1]=574.1; ^{1}H NMR (600 MHz, $CDCl_3$) δ 8.06 (d, 2H, *J*=8.4), 7.51 (d, 2H, *J*=7.8), 7.34 (d, 1H, *J*=7.8), 7.24 (t, 1H, *J*=7.8), 6.97 (t, 1H, *J*=7.8), 6.90 (d, 1H, *J*=8.4), 6.48 (br s, 1H), 5.15 (s, 2H), 4.03 (d, 2H, *J*=4.2), 3.93 (s, 3H), 3.65(br s, 4H), 3.40 (t, 2H, *J*=4.8), 2.50 (br s, 4H), 2.01 (s, 2H), 1.98 (br s, 3H), 1.70-1.62 (m, 12H).

   Compound 3.18 was prepared from Boc-β–Ala-OH and N-benzylpiparazine in a manner similar to that described for compound 3.47. ESI-MS [M+1]=424.1; ^{1}H NMR (600 MHz, $CDCl_3$) δ 7.34-7.26 (m, 5H), 6.27 (br s, 1H), 3.61 (t, 2H, *J*=4.2), 3.52 (m, 4H), 3.42 (t, 2H,

J=5.4), 2.51 (t, 2H, *J*=5.4), 2.41 (t, 4H, *J*=5.4), 1.96 (br s, 3H), 1.89 (s, 2H), 1.70-1.59 (m, 12H).

III. Synthesis of 3.98



1. Synthesis of 4-ethynyl benzyl bromide

   A solution of triphenylphosphine (3.9 g, 15 mmol) in CH_2Cl_2 (5 mL) was added dropwise to a cold mixture of 4-ethynylbenzylalcohol (1.0 g, 7.6 mmol), tetrabromomethane (4.5 g, 14 mmol), and 2,6-lutidine (4.4 mL, 38 mmol) in CH_2Cl_2 (20 mL) at 5^{o}C and the mixture was stirred for 16h at RT. After concentration in vacuo, the residue was treated with ether (40 mL) and the resulting solid was removed by filtration. The filtrate was washed with 1% HCl solution and then water, dried over sodium sulfate, filtered, and concentrated in vacuo. The residue was purified by flash column chromatography to give 4-ethynylbenzyl bromide (1.4 g, 95%) as a pale yellow oil. ^{1}H NMR (600 MHz, $CDCl_3$) δ 7.46 (d, 2H, *J*=7.8), 7.34 (d, 2H, *J*=7.8), 4.47 (s, 2H), 3.10 (s, 1H).

2. Synthesis of 2-((4-ethynylbenzyl)oxy)-5-nitrobenzaldehyde

   A mixture of (4-ethynylphenyl)methanol (1.4 g, 7.2 mmol), 2-hydroxy-5-nitrobenzaldehyde (1.0 g, 6.6 mmol), and potassium carbonate (2.7 g, 20 mmol) in DMF (12 mL) was stirred overnight at RT. Then water (50 mL) was added and the mixture was extracted with EtOAc. The combined extracts were washed with saturated brine, dried over sodium sulfate, filtered, and concentrated in vacuo. The residue was purified by flash column chromatography to give 2-((4-ethynylbenzyl)oxy)-5-nitrobenzaldehyde (1.1 g, 60%) as a yellow oil. ^{1}H NMR (600 MHz, $CDCl_3$) δ 10.5 (s, 1H), 8.73 (s, 1H), 8.41 (d, 1H, *J*=9.6), 7.56 (d, 2H, *J*=8.4), 7.40 (d, 2H, *J*=8.4), 7.14 (d, 1H, *J*=9), 5.34 (s, 2H), 3.11 (s, 1H).

3. Synthesis of 2-((3r,5r,7r)-adamantan-1-yl)-*N*-(2-(4-(2-((4-ethynylbenzyl)oxy)-5-nitrobenzyl)piperazin-1-yl)-2-oxoethyl)acetamide

Sodium triacetoxyborohydride (210 mg, 0.99 mmol) was added to a mixture of 2-((4-ethynylbenzyl)oxy)-5-nitrobenzaldehyde (222 mg, 0.79 mmol) and 2-((3r,5r,7r)-adamantan-1-yl)-*N*-(2-oxo-2-(piperazin-1-yl)ethyl)acetamide (210 mg, 0.66 mmol) in CH_2Cl_2 (12 mL) at 5^oC. After stirring for 4 h at RT, the mixture was poured into water and extracted with EtOAc. The combined extracts were washed with saturated brine, dried over sodium sulfate, filtered, and concentrated in vacuo. The residue was purified by flash column chromatography to give 2-((3r,5r,7r)-adamantan-1-yl)-*N*-(2-(4-(2-((4-ethynylbenzyl)oxy)-5-nitrobenzyl)piperazin-1-yl)-2-oxoethyl)acetamide (178 mg, 46%). ^1H NMR (600 MHz, $CDCl_3$) δ 8.32 (s, 1H), 8.15 (d, 1H, *J*=8.4), 7.53 (d, 2H, *J*=8.4), 7.37 (d, 2H, *J*=9), 6.96 (d, 1H, *J*=9), 6.47 (br s, 1H), 5.13 (s, 2H), 4.06 (s, 2H), 3.68 (m, 2H), 3.63 (s, 2H), 3.44 (m, 2H), 3.08 (s, 1H), 2.50 (m, 4H), 2.01(s, 2H), 1.98 (br s, 3H), 1.71-1.60 (m, 12H).

4. Synthesis of 2-((3r,5r,7r)-adamantan-1-yl)-*N*-(2-(4-(5-azido-2-((4-ethynylbenzyl)oxy)benzyl)piperazin-1-yl)-2-oxoethyl)acetamide

A mixture of 2-((3r,5r,7r)-adamantan-1-yl)-*N*-(2-(4-(2-((4-ethynylbenzyl)oxy)-5-nitrobenzyl)piperazin-1-yl)-2-oxoethyl)acetamide (178 mg, 0.30 mmol) and tinchloride dehydrate (343 mg, 1.5 mmol) in methanol (12 mL) was heated at 90^oC for 6 h. The reaction mixture was cooled to RT and concentrated in vacuo. The residue was dissolved in CH_2Cl_2 and then treated with saturated $NaHCO_3$. The organic phase was separated and the aqueous phase was extracted with CH_2Cl_2. The combined extracts were washed with saturated brine, dried over sodium sulfate, filtered, and concentrated to give the corresponding amine (46 mg; ESI-MS [M+1]= 555.0). A solution of sodium nitrite (17 mg, 0.25 mmol) in water (1 mL) was added to a solution of the crude amine above (46 mg, 0.083 mmol) in 6N-HCl (1 mL) at 0^oC under N_2 atmosphere. After stirring for 15 min, the mixture was then added dropwise to a stirred solution of sodium azide (16 mg, 0.25 mmol) and sodium acetate (102 mg, 1.2 mmol) in water (5 mL) at 0 ^{o} C. After the addition was completed, the reaction mixture was warmed to RT, stirred for 4 h, and then extracted with CH_2Cl_2. The combined extracts were washed with saturated brine, dried over sodium sulfate, filtered, and concentrated in vacuo. The residue was purified by preparative LCMS, using the predicted molecular weights to trigger fraction collection, to give 2-((3r,5r,7r)-adamantan-1-yl)-*N*-(2-(4-(5-azido-2-((4-ethynylbenzyl)oxy)benzyl)piperazin-1-yl)-2-oxoethyl)acetamide (5 mg). ESI-MS [M+1]= 581.0; ^1H NMR (600 MHz, $CDCl_3$) δ 7.55 (d, 1H, *J*=7.8), 7.51 (d, 1H, *J*=7.8), 7.38 (t, 1H, *J*=7.8), 7.30 (t, 1H, *J*=7.2), 7.07 (s, 1H), 6.88-6.94 (m, 2H), 6.47 (br s, 1H), 5.23 (s, 2H), 4.04 (d, 2H, *J*=3.6), 3.65 (br s, 2H), 3.62 (s, 2H), 3.40 (br s, 2H), 3.33 (s, 1H), 2.49 (br s, 4H), 2.01 (s, 2H), 1.97 (br s, 3H), 1.70-1.62 (m, 12H).