

Small molecule inhibitors reveal Niemann–Pick C1 is essential for Ebola virus infection

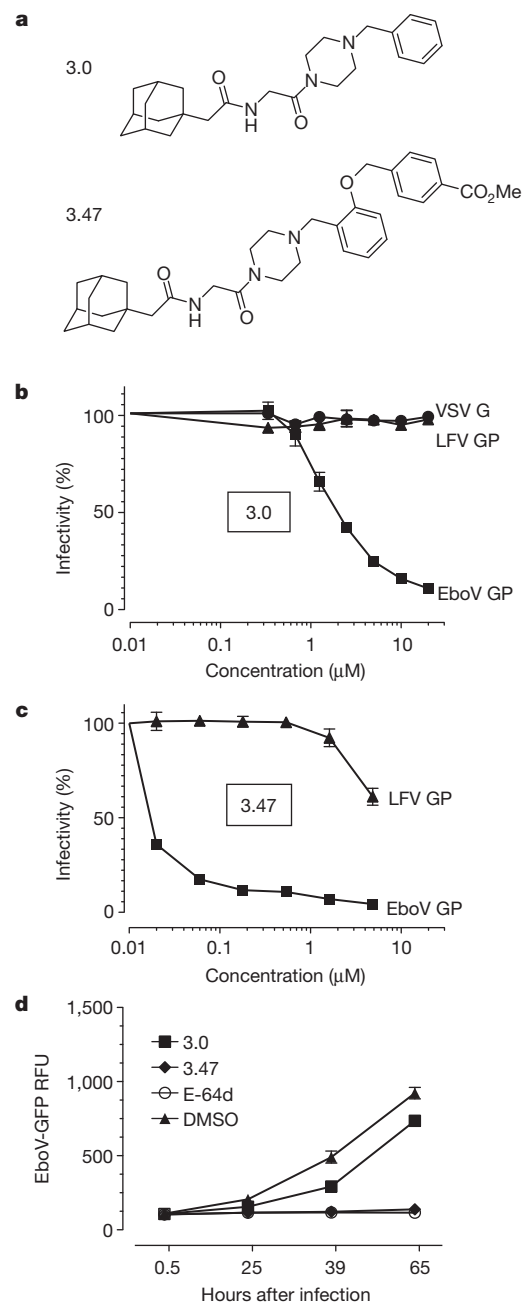
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Ebola virus (EboV) is a highly pathogenic enveloped virus that causes outbreaks of zoonotic infection in Africa. The clinical symptoms are manifestations of the massive production of pro-inflammatory cytokines in response to infection¹ and in many outbreaks, mortality exceeds 75%. The unpredictable onset, ease of transmission, rapid progression of disease, high mortality and lack of effective vaccine or therapy have created a high level of public concern about EboV². Here we report the identification of a novel benzylpiperazine adamantane diamide-derived compound that inhibits EboV infection. Using mutant cell lines and informative derivatives of the lead compound, we show that the target of the inhibitor is the endosomal membrane protein Niemann–Pick C1 (NPC1). We find that NPC1 is essential for infection, that it binds to the virus glycoprotein (GP), and that antiviral compounds interfere with GP binding to NPC1. Combined with the results of previous studies of GP structure and function, our findings support a model of EboV infection in which cleavage of the GP1 subunit by endosomal cathepsin proteases removes heavily glycosylated domains to expose the amino-terminal domain^{3–7}, which is a ligand for NPC1 and regulates membrane fusion by the GP2 subunit⁸. Thus, NPC1 is essential for EboV entry and a target for antiviral therapy.

To identify chemical probes that target EboV host factors, we screened a library of small molecules and identified a novel benzylpiperazine adamantane diamide, 3.0, that inhibits infection of Vero cells by vesicular stomatitis virus particles (VSV) pseudotyped with EboV Zaire GP, but not with VSV G or Lassa fever virus (LFV) GP (Fig. 1a, b). To verify that 3.0 is a bona fide inhibitor, we measured EboV growth on Vero cells for 96 h and found it was reduced by >99% in the presence of 3.0 (Supplementary Fig. 1a). We synthesized and tested more than 50 analogues of 3.0 and found that the addition of a (methoxycarbonyl) benzyl group at the ortho position of the benzene ring (compound 3.47) increased the potency, as measured by a single cycle of EboV GP-dependent infection, and efficacy, as measured by growth of EboV on Vero cells (Fig. 1a, c, d).

Figure 1 | Structure and function of Ebola virus entry inhibitors.

a, Compounds 3.0 and 3.47. **b**, **c**, Vero cells were grown in media containing increasing concentrations of 3.0 (**b**) or 3.47 (**c**) for 90 min before the addition of VSV particles encoding luciferase (**b**) or GFP (**c**) and pseudotyped with either EboV GP, VSV G or Lassa fever virus GP (LFV GP). Virus infection is reported as percent of luminescence units (RLU) or GFP-positive cells relative to cells exposed to DMSO vehicle alone. Data are mean \pm s.d. ($n = 4$) and is representative of three experiments. **d**, Vero cells were grown in media containing 3.0 (40 μ M), 3.47 (40 μ M), vehicle (1% DMSO) or the cysteine cathepsin protease inhibitor E-64d (150 μ M) 90 min before the addition of replication competent Ebola virus Zaire-Mayinga encoding GFP (multiplicity of infection (m.o.i.) = 0.1). Results are mean relative fluorescence units (RFU) \pm s.e.m. ($n = 3$).



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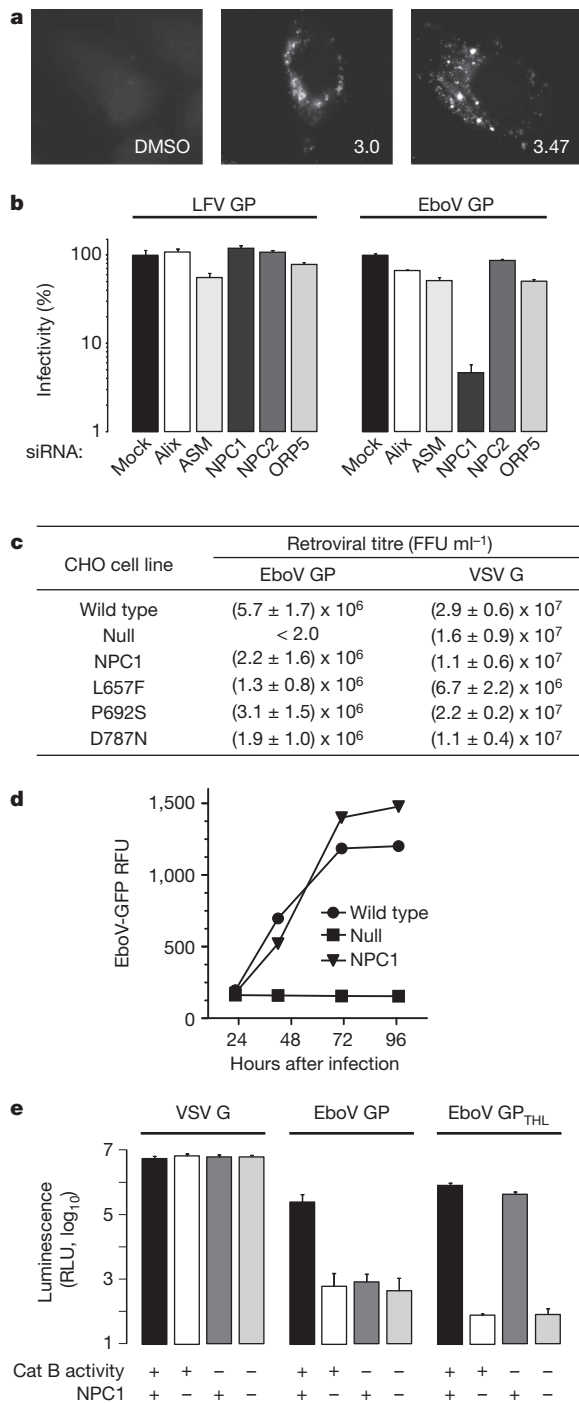


Figure 2 | NPC1 is essential for Ebola virus infection. **a**, HeLa cells were treated with 3.0 (20 μ M), 3.47 (1.25 μ M) or vehicle for 18 h, then fixed and incubated with the cholesterol-avid fluorophore filipin. **b**, HeLa cells were transfected with siRNAs targeting Alix, ASM, NPC1, NPC2 and ORP5. After 72 h, VSV EboV GP or LfV GP infection of these cells was measured as in Fig. 1c. Data are mean \pm s.d. ($n = 3$) and is representative of three experiments. **c**, CHO_{wt}, CHO_{null} and CHO_{null} cells stably expressing mouse NPC1 (CHO_{NPC1}) or NPC1 mutants L657F, P692S, D787N were exposed to MLV particles encoding LacZ and pseudotyped with either EboV GP or VSV G. Results are the mean \pm s.d. ($n = 4$) and is representative of three experiments. FFU, focus forming units. **d**, CHO_{wt}, CHO_{null} and CHO_{NPC1} cells were infected with replication competent Ebola virus Zaire-Mayinga encoding GFP (m.o.i. = 1). Results are mean relative fluorescence units \pm s.d. ($n = 3$). **e**, CHO_{wt} and CHO_{null} cells were treated with the cathepsin B inhibitor CA074 (80 μ M) or vehicle. These cells were challenged with VSV G particles or VSV EboV GP particles treated with thermolysin (EboV GP_{THL}) or untreated control (EboV GP). Infection was measured as in Fig. 1b. Data are mean \pm s.d. ($n = 9$).

Previous studies revealed that the endosomal protease cathepsin B is essential for EboV infection because it cleaves the GP1 subunit of GP^{3,4}. To address the possibility that 3.0 and 3.47 target this step, we measured cathepsin B activity in the presence of these compounds and found no effect *in vitro* or in cells (data not shown). Moreover, 3.0 and 3.47 inhibited infection by VSV EboV particles treated with thermolysin, a metalloprotease that faithfully mimics cathepsin cleavage of the GP1 subunit of GP (Supplementary Fig. 1b)^{4,9}. These findings demonstrate that cathepsin B is not the target of 3.0 and 3.47.

HeLa cells treated with 3.0 or 3.47 for more than 18 h developed cytoplasmic vacuoles that were labelled by cholesterol-avid filipin (Fig. 2a). The induction of filipin-stained vacuoles by the compounds

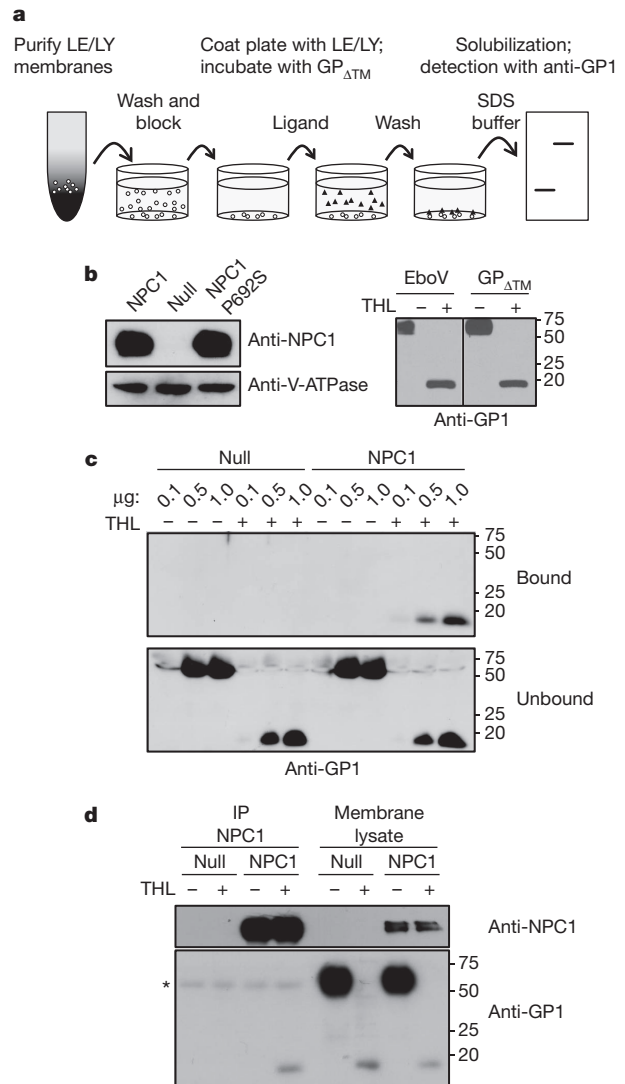


Figure 3 | Protease-cleaved EboV GP binds to NPC1. **a**, Schematic diagram of EboV GP1 binding assay used in panel c. **b**, left, LE/LY membranes from CHO_{NPC1}, CHO_{null} and CHO NPC1 P692S cells were analysed by immunoblot using antibodies to NPC1 or V-ATPase B1/2. Right, VSV-EboV GP particles and EboV GP_{ΔTM} protein were incubated in the presence or absence of thermolysin (THL) and analysed by immunoblot for GP1. **c**, EboV GP_{ΔTM} or thermolysin-cleaved EboV GP_{ΔTM} (0.1, 0.5, or 1.0 μ g) was added to LE/LY membranes purified from CHO_{null} or CHO_{NPC1} cells. Membrane bound and unbound GP1 were analysed by immunoblot. **d**, LE/LY membranes from CHO_{null} or CHO_{NPC1} cells were incubated with EboV GP_{ΔTM} or thermolysin-cleaved EboV GP_{ΔTM}. Following binding, membranes were dissolved in CHAPSO, NPC1 was precipitated using an NPC1-specific antibody, and the immunoprecipitate and the input membrane lysate were analysed by immunoblot for NPC1 (top) or GP1 (bottom). * IgG heavy chain.

indicated that they target one or more proteins involved in regulation of cholesterol uptake in cells. To test this hypothesis, we used mutant cell lines and cells treated with small interfering RNA (siRNA) to analyse proteins for which loss of activity had been previously associated with cholesterol accumulation in late endosomes^{10–12}. We found that EboV GP infection is dependent on the expression of Niemann–Pick C1 (NPC1), but not Niemann–Pick C2 (NPC2), acid sphingomyelinase (ASM), ALG-2-interacting protein X (Alix), or oxysterol binding protein 5 (ORP5) (Fig. 2b, Supplementary Fig. 2a–c). NPC1 is a polytopic protein that resides in the limiting membrane of late endosomes and lysosomes (LE/LY) and mediates distribution of lipoprotein-derived cholesterol in cells^{10,13}. To analyse the role of NPC1 in infection, we studied Chinese hamster ovary (CHO)-derived cell lines that differ in expression of NPC1. We found that the titre of a murine leukaemia virus (MLV) vector pseudotyped with EboV GP on wild-type CHO cells (CHO_{wt}) exceeded 10⁶ infectious units per ml (Fig. 2c). Importantly, CHO cells lacking NPC1 (CHO_{null}) were completely resistant to infection by this virus and infection of these cells was fully restored when NPC1 was expressed (CHO_{NPC1}). Thus, NPC1 expression is essential for EboV infection.

In CHO_{null} cells, LE/LY are enlarged and contain excess cholesterol (Supplementary Fig. 3)¹⁴. To determine if EboV infection is inhibited by endosome dysfunction secondary to the absence of NPC1, we studied a well-characterized NPC1 mutant P692S that is defective in cholesterol uptake and NPC1-dependent membrane trafficking^{13–15} and found that expression of NPC1 P692S fully supports infection of CHO_{null} cells (Fig. 2c). Conversely, gain-of-function mutants NPC1 L657F and NPC1 D787N (ref. 14) did not enhance EboV GP infection. Thus, EboV entry is strictly dependent on NPC1 expression, but not NPC1-dependent cholesterol transport activity. Consistent with the conclusion that NPC1 expression is essential for EboV

GP-dependent entry, we found that Ebola virus did not grow on CHO_{null} cells (Fig. 2d). In addition, we tested a single round of infection by MLV particles bearing GPs from the filoviruses EboV Sudan, EboV Côte d'Ivoire, EboV Bundibugyo, EboV Reston and Marburg virus and found that all are strictly NPC1-dependent (Supplementary Fig. 4). Because these viruses are not closely related¹⁶, these findings indicate that the requirement for NPC1 as an entry factor is conserved among viruses in the *Filoviridae* family.

Because NPC1 and cathepsin B are both essential host factors, we analysed their relationship during infection. In our initial experiment, we measured cathepsin B activity in CHO_{null} cells and found it was not significantly different from CHO_{wt} cells (Supplementary Fig. 5). To determine if NPC1 is required for virus processing by cathepsin B, we tested whether thermolysin-cleaved particles are dependent on NPC1. As expected, we found that thermolysin-cleaved particles are infectious and resistant to inactivation of cathepsin B when NPC1 is present (Fig. 2e). However, thermolysin cleavage did not bypass the barrier to virus infection of NPC1 deficient cells. Taken together, these findings indicate that cathepsin B and NPC1 mediate distinct steps in infection.

Previous studies suggest that the product of cathepsin B cleavage of the GP1 subunit of EboV GP is a ligand for a host factor^{6,17–20}. To test this hypothesis, we performed a series of experiments measuring binding of EboV GP to LE/LY membranes from CHO_{null}, CHO_{NPC1} and CHO_{P692S} cells (Fig. 3a, b, left panel). The source of EboV GP is a purified recombinant protein that is truncated just before the transmembrane domain (EboV GP_{ΔTM}). EboV GP_{ΔTM} is a trimer that is faithfully cleaved by thermolysin (Fig. 3b, right panel). We found that binding of EboV GP_{ΔTM} to LE/LY membranes is concentration-dependent, saturable, and strictly dependent on both thermolysin cleavage of GP1 and membrane expression of NPC1 or NPC1 P692S (Fig. 3c and Supplementary Fig. 6a, b). To determine if cleaved GP

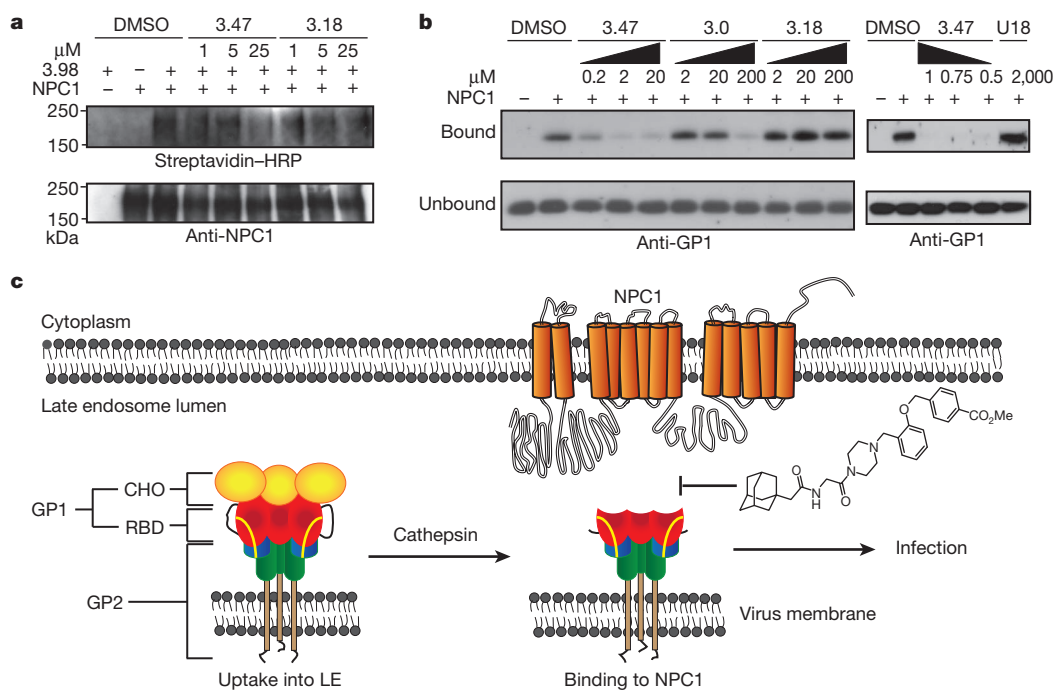


Figure 4 | NPC1 is a target of the small molecule inhibitors. **a**, LE/LY membranes from CHO_{null} or CHO_{NPC1} cells were incubated at the indicated concentrations of 3.47, 3.18 or DMSO (5%) before the addition of the photoactivatable 3.98 (25 μM). After incubation, 3.98 was activated by ultraviolet light and then conjugated to biotin. NPC1 was immunoprecipitated and analysed by immunoblot for conjugation of 3.98 to NPC1 using streptavidin–horseradish peroxidase (HRP) (top) and recovery of NPC1 (bottom). **b**, Thermolysin-cleaved EboV GP_{ΔTM} protein (1 μg) was added to LE/LY membranes from CHO_{null} or CHO_{NPC1} cells in the presence of DMSO (10%) or

the indicated concentrations of 3.47, 3.0, or 3.18 (left panel), and 3.47 or U18666A (U18, right panel). Membrane-bound and unbound GP1 were analysed by immunoblot. **c**, Proposed model of EboV entry. Following EboV uptake and trafficking to late endosomes^{24,25}, EboV GP is cleaved by cathepsin protease to remove heavily glycosylated domains (CHO) and expose the putative receptor binding domain (RBD) of GP1 (refs 6, 17–19). Binding of cleaved GP1 to NPC1 is necessary for infection and is blocked by the EboV inhibitor 3.47.

binds to NPC1, we performed a co-immunoprecipitation experiment. LE/LY membranes were incubated with EboV GP_{ΔTM} and then solubilized in detergent. NPC1 was recovered from the lysate by immunoprecipitation and the immune complexes were analysed for GP1. The findings indicate that cleaved EboV GP_{ΔTM} binds to NPC1 and that uncleaved EboV GP_{ΔTM} does not (Fig. 3d).

Because the small molecules 3.0 and 3.47 inhibit infection of thermolysin-treated VSV EboV GP particles (Supplementary Fig. 1b) and inhibit cholesterol uptake from LE/LY into cells (Fig. 2a), both of which require NPC1, this suggests the possibility that these compounds directly target NPC1. To test this hypothesis, we synthesized the 3.47 derivative 3.98. This compound has anti-EboV activity and contains two additional functional moieties: an aryl-azide for photo-affinity labelling of target proteins and an alkyne for click conjugation with biotin²¹ (Supplementary Fig. 7). Compound 3.98 was incubated with LE/LY membranes, activated by ultraviolet light and coupled to biotin. NPC1 was then isolated by immunoprecipitation and analysed using streptavidin–horseradish peroxidase. The findings show that NPC1 is cross-linked to 3.98 and that cross-linking is inhibited by the presence of 3.47 but not by the closely related analogue 3.18, which has weak antiviral activity (Fig. 4a and Supplementary Fig. 7). In addition, we observed that overexpression of NPC1 conferred resistance to the antiviral activity of 3.0 and 3.47 (Supplementary Fig. 8), thus providing additional functional evidence supporting the conclusion based on the results of the cross-linking experiment using 3.98 that NPC1 is a direct target of the antiviral compounds.

The evidence that NPC1 is the target of the 3.0-derived small molecules selected for anti-EboV activity indicated that these compounds may interfere with binding of cleaved GP to NPC1. Consistent with this hypothesis, we found that 3.0 and 3.47 inhibited binding of cleaved EboV GP_{ΔTM} to NPC1 membranes in a concentration-dependent manner (Fig. 4b). Importantly, we observed a direct correlation between the potency of 3.47, 3.0 and 3.18 in inhibiting binding (Fig. 4b, left panel) and in inhibiting EboV infection (Supplementary Fig. 7). We also tested U18666A, a small molecule inhibitor of LE/LY cholesterol transport and membrane trafficking^{22,23}, and found that it does not inhibit binding of cleaved EboV GP to NPC1 membranes (Fig. 4b, right panel). These results support the conclusion that the 3.0-derived compounds inhibit EboV infection by interfering with binding of cleaved GP to NPC1.

Previous studies show that cleavage of GP by endosomal cathepsin proteases removes heavily glycosylated domains in the GP1 subunit and exposes the N-terminal domain^{3–7}. It has been proposed that binding of this domain to a host factor is essential for infection^{6,17–20}. The most straightforward interpretation of the findings in this report is that NPC1 is this host factor. This conclusion is based on the observations that NPC1 is strictly required for infection, that cleaved GP1 binds to NPC1, and that small molecules that target NPC1 are potent inhibitors of binding and infection.

Analysis of the EboV GP structure shows that the residues in the N-terminal domain of GP1 that mediate binding to NPC1 are interspersed with the residues that make stabilizing contacts with GP2 (ref. 5). This structural feature is consistent with the possibility that binding of cleaved GP1 to NPC1 relieves the GP1-imposed constraints on GP2 and promotes virus fusion to the limiting membrane (Fig. 4c). The role of cathepsin proteases in cleavage of GP1 to expose the NPC1 binding site during EboV infection is analogous to the role of CD4 in inducing a conformational change in gp120 to expose the co-receptor binding site during human immunodeficiency virus infection⁸. An alternative possibility is that binding of protease-cleaved GP1 to NPC1 is an essential step in infection, but virus membrane fusion is not completed until an additional signal is received, possibly including further cleavage of GP by cathepsin proteases, as has been proposed^{3,4,9}. These studies provide an example of how small molecules identified by screening and medicinal chemistry optimization can be used as molecular probes to analyse virus–host interactions.

METHODS SUMMARY

Screening of small molecules was performed at the New England Regional Centers of Excellence for Biodefense and Emerging Infectious Diseases at Harvard Medical School. Infection was assayed using VSV pseudotyped viruses encoding green fluorescent protein (GFP) or luciferase. Experiments with native Ebola virus were performed under BSL-4 conditions at the United States Army Medical Research Institute for Infectious Diseases. Cells were infected with EboV Zaire-Mayinga GFP and growth was measured by mean fluorescence. EboV GP_{ΔTM} is a derivative of EboV GP in which the transmembrane domain has been replaced by a GCN4-derived trimerization domain followed by a His₆ tag for purification. Late endosomes/lysosomes (LE/LY) were isolated by differential centrifugation and further purified by Percoll density gradient centrifugation. LE/LY were disrupted by incubation with methionine methyl ester and coated onto high binding ELISA plates. Following attachment, unbound LE/LY membranes were removed and plates were blocked. Bound membranes were incubated with the indicated amounts of native or thermolysin-cleaved EboV GP_{ΔTM} protein. Unbound EboV GP_{ΔTM} protein was removed, membranes were washed and bound EboV GP_{ΔTM} protein was recovered in SDS loading buffer and analysed by immunoblot using GP1 antiserum. Where applicable, membranes were pre-incubated with 3.0, 3.47, 3.18 or vehicle before the addition of EboV GP_{ΔTM}. To analyse EboV GP_{ΔTM} binding to NPC1, LE/LY membranes were dissolved in 10 mM CHAPSO, NPC1 was recovered by immunoprecipitation, and the immune complexes were analysed by immunoblot using GP1 antiserum.

Full Methods and any associated references are available in the online version of the paper at www.nature.com/nature.

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Supplementary Information is linked to the online version of the paper at www.nature.com/nature.

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Author Contributions M.C., J.M., T.R. and A.B. equally contributed to this work. K.C. and T.R. performed the inhibitor screen. K.L. synthesized and purified 3.0 analogues and T.R. tested them. T.R., A.B., J.M., Q.L. and M.C. carried out infection assays with pseudotyped viruses. A.B. performed microscopy. J.M. purified recombinant glycoprotein. M.C. and J.M. designed and performed binding assays. M.C. performed immunoprecipitation. D.O. provided NPC1 constructs, antibodies and CHO cell lines. Ebola virus infections were performed in the lab of L.H. by C.M.F.; J.C. supervised the project and wrote the manuscript. All authors reviewed the manuscript.

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METHODS

Cell lines. Vero, 293T, HeLa (ATCC) and human fibroblasts²⁶ (Coriell) were maintained in DMEM (Invitrogen) supplemented with 5% FetalPlex, 5% FBS (Gemini) or 10% FBS (HeLa, human fibroblasts). All CHO derived cell lines were grown as previously described^{14,27}. We have designated the CHO-K1 cell line as CHO_{wt}, CHO-M12 as CHO_{null}, CHO-wt8 as CHO_{NPC1}, and the CHO-derived cell lines expressing NPC1 mutants as CHO NPC1 P692S, CHO NPC1 L657F, and CHO NPC1 D787N. CHO/NPC1-1, designated here as CHO hNPC1, expresses high levels of human NPC1 (ref. 27).

Antibodies. Rabbit polyclonal anti-serum was raised against a peptide corresponding to residues 83 to 98 of Ebola virus Zaire Mayinga GP1 (TKRWGFRSGVPPKVVVC). Antibodies to NPC1 and V-ATPase B1/2 were obtained from Abcam and Santa Cruz, respectively.

Expression plasmids. Mucin domain-deleted EboV Zaire Mayinga GP (EboV GP) and VSV G were previously described³. Plasmids encoding Côte d'Ivoire-Ivory Coast GP, Sudan-Boniface GP, Reston-Penn. GP and Marburg-Musoke GP were obtained from Anthony Sanchez and the mucin domain-deleted (Δ Muc) derivatives were created: Zaire Δ Muc GP (amino acids 309–489 deleted), Côte d'Ivoire Δ Muc GP (amino acids 310–489 deleted), Sudan Δ Muc GP (Δ a.a. 309–490), and Reston Δ Muc GP amino acids 310–490 deleted). Bundibungyo-Uganda viral RNA was TRIzol-extracted and PCR was used to generate a construct that expresses a mucin-deleted GP (amino acids 309–489 deleted). A plasmid encoding Lassa fever virus GP1 was kindly provided by G. Nabel. A codon-optimized sequence encoding GP2 was generated and combined with the GP1 sequence in pCAGGS to complete a GP expression vector.

Production and purification of pseudotyped virions. VSV- Δ G pseudotyped viruses were created as described previously³. LacZ-encoding retroviral pseudotypes bearing the designated envelope glycoproteins were prepared as previously described²⁸.

Thermolysin digestion of EboV GP Virus and EboV GP_{ATM}. Purified EboV GP_{ATM} (50 μ g ml⁻¹) or VSV particles pseudotyped with EboV GP were incubated at 37 °C for 1 h with the metalloprotease thermolysin (Sigma, 0.2 mg ml⁻¹) in NT buffer (10 mM Tris-HCl pH 7.5, 135 mM NaCl). The reaction was stopped using 500 μ M phosphoramidon (Sigma) at 4 °C. Cleaved EboV GP_{ATM} was stored in phosphate buffered saline supplemented with 1 mM EDTA, 1 mM PMSF (Sigma) and 1 \times EDTA-Free Complete Protease Inhibitor Cocktail (Roche).

Infection assays with pseudotyped virus. VSV pseudotyped viruses expressing GFP were added to cells in serial tenfold dilutions and assayed using fluorescence microscopy. An infectious unit (i.u.) is defined as one GFP-expressing cell within a range where the change in GFP-positive cells is directly proportional to the virus dilution. For VSV expressing the luciferase reporter, pseudotyped virus was added to cells and luciferase activity was assayed 6–20 h post-infection using the firefly luciferase kit (Promega). Signal was measured in relative luminescence units (RLU) using an EnVision plate reader (Perkin Elmer). In experiments involving inhibitors, stock solutions of 3.0 (20 mM) and 3.47 (10 mM) in DMSO were diluted to a final concentration of 1% DMSO in media. Inhibitory activity was stable in the media of cultured cells for more than 72 h as assessed using a single cycle entry assay. Infection of target cells with LacZ-encoding retroviral pseudotypes was performed in the presence of 5 μ g ml⁻¹ polybrene (Sigma). Seventy-two hours post-infection, cells were stained for LacZ activity and titre was determined by counting positive foci and expressed as focus forming units (FFU) per ml of virus.

Ebola virus infections under BSL-4 conditions. Vero cells or CHO cells were seeded to 96-well plates and exposed to EboV-GFP²⁹. Vero cells were incubated with 3.0 (40 μ M), 3.47 (40 μ M), E-64-d (150 μ M) or 1% DMSO 90 min before the addition of virus (m.o.i. = 0.1). Virus was added to CHO cells at m.o.i. of 1 as measured on Vero cells. Virus-encoded GFP fluorescence was determined using a SpectraMax M5 plate reader (Molecular Devices) at excitation 485 nm, emission 515 nm, cutoff 495 nm at 22.5, 42, 71 and 97 h post-infection. An additional inhibitor experiment was performed using 3.0. Vero cells were treated with 3.0 (20 μ M) or 1% DMSO alone for 4 h, and then infected with EBOV Zaire-1995 (m.o.i. = 0.1). After 1 h, the virus inoculum was removed, cells were washed, and fresh media containing 3.0 or DMSO was added. Cell supernatant was collected at 0, 24, 48, 72, or 91 h post-infection. RNA was isolated from the supernatant using Virus RNA Extraction kits (Qiagen) and EboV NP RNA was measured using a real-time RT-PCR assay³⁰. Virus titre was calculated using a standard curve obtained using a virus stock of known titre as determined by plaque assay.

Screen for Ebola virus entry inhibitors. Screening of small molecules was performed at the New England Regional Centers of Excellence for Biodefense and Emerging Infectious Diseases at Harvard Medical School. Vero cells were seeded in 384-well plates at a density of 5×10^3 cells per well using a Matrix WellMate (Thermo Scientific). The ChemBridge3, ChemDiv4, ChemDiv5 and Enamine2 compound libraries were transferred by robotics to the assay plates using stainless

steel pin arrays. The compounds were screened at a constant dilution to achieve a final concentration between 10 μ M and 60 μ M. After incubation for 2 h at 37 °C, viruses were dispensed into each well (m.o.i. = 1) and incubated for an additional 6 h to allow virus gene expression. Cells were lysed by addition of Steady-Glo (Promega) and after 10 min at room temperature luminescence was measured using an EnVision plate reader. Each compound was tested in duplicate. Candidate compounds that inhibited EboV GP infection by more than 80% were analysed for potency, selectivity and absence of cytotoxicity (using Cyto-Tox assay, Promega) and 3.0 (2-((3r,5r,7r)-adamantan-1-yl)-N-(2-(4-benzylpiperazin-1-yl)-2-oxoethyl)acetamide) was identified. The antiviral activity of the inhibitors was verified on human cells (HeLa, A549, 293T), mouse embryonic fibroblasts and Chinese hamster ovary cells.

Synthesis of 3.0 derivatives. Compound 3.47 (methyl 4-((2-((4-(2-((3r,5r,7r)-adamantan-1-yl)acetamido)acetyl)piperazin-1-yl)methyl)phenoxy)methyl)benzoate) was prepared via a multi-step synthesis starting from N-Cbz-piperazine. Thus, coupling of N-Cbz-piperazine with N-Boc-glycine followed by removal of the Boc group under acidic conditions yielded 4-Cbz-piperazine glycinamide. After acylation of the terminal amine with adamantane-1-acetyl chloride, the Cbz group was removed by hydrogenolysis to give (1-(adamantan-1-yl)acetamido)acetyl piperazine. The piperazine was then benzylated via reductive amination with 2-(4-methoxycarbonyl)benzylaldehyde using sodium triacetoxyborohydride to provide 3.47. Compound 3.18 was synthesized in a similar fashion. Compound 3.98 was prepared via a multi-step synthesis as follows. First, 2-hydroxy-5-nitrobenzaldehyde was alkylated by 4-ethynylbenzyl bromide in the presence of potassium carbonate in DMF. Resulting benzylaldehyde underwent reductive amination with 2-((3r,5r,7r)-adamantan-1-yl)-N-(2-oxo-2-(piperazin-1-yl)ethyl)acetamide using sodium triacetoxyborohydride. The nitro group was then reduced to aniline (SnCl₂), diazotized (NaNO₂), and the diazonium finally converted to azide to yield 3.98. See Supplementary Information for detailed experimental procedures and characterization data.

Protease inhibitors and protease activity assays. The measurement of cathepsin B activity and the use of the inhibitor CA074 (Sigma) have been previously described³.

Detection of intracellular cholesterol. Cells were stained with filipin (50 μ g ml⁻¹, Cayman Chemical) as previously described¹⁴. Images of stained cells were obtained using epifluorescence microscopy (Nikon Eclipse TE2000U). The images in the supplementary figures were processed using ImageJ software.

Production and purification of EboV GP_{ATM} soluble protein. EboV GP_{ATM} is a derivative of the mucin-deleted EboV Zaire-Mayinga GP in which the trans-membrane domain and carboxy terminus (amino acids 657–676) has been replaced by a GCN4-derived trimerization domain (MKQIEDKIEEILSKIYHIEN EIARIKKLIGEV) and a His₆ tag. The expression plasmid encoding EboV GP_{ATM} was transfected into 293T cells using lipofectamine2000. Eighteen to twenty-four hours later the culture medium was replaced with 293SFMII (Invitrogen) supplemented with 1 \times non-essential amino acids and 2 mM CaCl₂ and collected daily for 4 days. Media containing soluble EboV GP_{ATM} was filtered and PMSF (1 mM)/1 \times EDTA-Free Complete Protease Inhibitor Cocktail was added. EboV GP_{ATM} was purified by affinity chromatography using Ni-NTA agarose beads (Qiagen), dialysed against PBS using a 3 kDa dialysis cartridge (Pierce) and stored at –80 °C. Purity and integrity of EboV GP_{ATM} were analysed by SDS-PAGE.

Membrane binding assay. Indicated cells were washed with PBS twice, scraped in homogenization (HM) buffer (0.25 M sucrose, 1 mM EDTA, 10 mM HEPES pH 7.0), and disrupted with a Dounce homogenizer. Nuclei and debris were pelleted by centrifugation at 1,000g for 10 min. The post-nuclear supernatant was centrifuged at 15,000g for 30 min at 4 °C and the pellet, containing the LE/LY, was resuspended in a total volume of 0.9 ml composed of 20% Percoll (Sigma) and 0.4% BSA (Sigma) in HM and centrifuged at 36,000g for 30 min at 4 °C. Fractions (0.150 ml) were collected from the bottom to the top of the tube and those containing the highest β -N-acetylglucosamidase activity, as assessed by release of 4-methylumbelliferone from 4-methylumbelliferyl-N-acetyl- β -D-glucosaminide (Sigma), were pooled and incubated in 20 mM methionine methyl-ester (Sigma) for 1 h at room temperature. Following LE/LY disruption, 1 \times EDTA-Free Complete Protease Inhibitor Cocktail and 1 mM PMSF was added. The amount of purified LE/LY membranes used for the binding assay was normalized using the activity of the marker β -N-acetylglucosamidase and validated by immunoblot using V-ATPase B1/2 antibody (Supplementary Fig. 5).

Disrupted LE/LY membranes were coated on high-binding ELISA plates (Corning) overnight at 4 °C. Unbound membranes were removed and wells containing bound membranes were blocked for 2 h at room temperature with binding buffer (PBS, 5% FBS, 1 mM PMSF, 1 mM EDTA, 1 \times Complete Protease Inhibitor Cocktail). The indicated amount of purified EboV GP_{ATM}, pretreated or not with thermolysin, in binding buffer was added to each well and incubated for 1 h at room temperature. Unbound proteins were removed and wells were washed three

times with PBS. Membrane-bound EboV GP_{ATM} was solubilized in SDS-loading buffer. Bound and unbound EboV GP_{ATM} were detected by immunoblot using the EboV GP1 anti-serum. For binding assays in the presence of inhibitors, the immobilized membranes were pre-incubated at room temperature with the inhibitor or vehicle (10% DMSO) in binding buffer. After 30 min, thermolysin-cleaved EboV GP_{ATM} was added in the continuous presence of compound and bound and unbound GP was measured as described above.

Co-immunoprecipitation. CHO_{null} and CHO_{hNPC1} cells were homogenized as described above. The 15,000g membrane pellet was resuspended in HM buffer and protein content was measured using the BCA assay (Pierce). The LE/LY membranes contained in the 15,000g resuspended pellet were disrupted by incubation with 20 mM methionine methyl-ester for 1 h at room temperature. Membranes of equal protein content were incubated with indicated amounts of EboV GP_{ATM}, pre-treated or not with thermolysin, for 1 h at room temperature in the presence of Complete Protease Inhibitor Cocktail (Roche) and incubated for an additional hour on ice before the addition of membrane lysis buffer (12.5 mM CHAPSO, 150 mM NaCl, 1 mM EDTA, 10 mM Tris/HCl pH 7.4) for a final concentration of 10 mM CHAPSO. Proteins were solubilized on ice for 20 min and debris was removed by centrifugation at 12,000g for 10 min at 4 °C. The soluble membrane lysates were incubated with anti-NPC1 antibody for 1 h at 4 °C and then incubated with Protein A-agarose beads (Sigma) for an additional 4 h at 4 °C. Beads were then washed three times with 8 mM CHAPSO, 150 mM NaCl, 1 mM EDTA, 10 mM Tris/HCl pH 7.4 and immunoprecipitated product was eluted by incubation in 0.1 M glycine pH 3.5 for 5 min at room temperature. The eluted complex was then neutralized and analysed by immunoblot using the indicated antibody.

Photo-activation and click chemistry. Photo-activation and click chemistry were performed as described previously with some modifications²¹. Briefly, the 15,000g pellets from homogenized CHO_{hNPC1} or CHO_{null} cells were resuspended in PBS and incubated with the indicated concentrations of 3.47, 3.18 or DMSO for 10 min at room temperature. Membranes were then incubated with 25 µM of 3.98 for an additional 10 min and exposed to ultraviolet light (365 nm) for 1 min on ice. Proteins were solubilized in lysis buffer (1% Triton X-100, 0.1% NP-40, 20 mM HEPES pH 7.4) containing protease inhibitors and 150 µM of biotin-azide (Invitrogen) was added, followed by 5 mM L-ascorbic acid. The cycloaddition reaction (click chemistry) was initiated by the addition of 1 mM CuSO₄ and samples were incubated for 3 h at room temperature. NPC1 was immunoprecipitated and the product was resolved by SDS-PAGE, transferred to PVDF membrane, and analysed for conjugation of 3.98 to NPC1 using streptavidin-horseradish peroxidase (Sigma).

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