# Supporting Information

## Wolf et al. 10.1073/pnas.0909587107

### SI Pharmacokinetic Methodology

**Mass Spectrometric Analyses of Pharmacokinetic Serum Samples.** To the thawed serum samples, methanol (700 μL) was added with the internal standard ($^2H_5$-LJ001, 200 pmol in 20 μL chloroform), and the mixtures were vigorously mixed and centrifuged (20,000 × *g*) for 2 min. The supernatants were transferred to HPLC injector vials and dried in a vacuum centrifuge. The dried residues were redissolved in chloroform (20 μL) to which acetonitrile (120 μL) and acetonitrile/water (50/50, 120 μL) containing 0.1% formic acid were added sequentially. The samples were mixed, and 200-mL aliquots of the resulting solutions were injected onto a reverse-phase HPLC column (Waters Xterra, 4.6 × 100 mm, 3.5-μm particle size), equilibrated in 90% buffer A (0.1% TFA in water)/10% buffer B (0.1% TFA in acetonitrile), and eluted (500 μL/min) with an increasing concentration of acetonitrile (min/% acetonitrile: 0/10; 5/10; 30/100; 33/100; 35/10; and 45/10). The effluent from the column was directed into an atmospheric pressure chemical ionization source (probe: 450 °C particulate and hydrocarbon-depleted air for nebulizing gas) connected to a triple quadrupole mass spectrometer (PE Sciex API III+, oriface at 65 V) operating in the positive ion multiple reaction monitoring tandem mass spectrometric mode in which the collision chamber was flooded with argon gas (collision gas thickness instrumental setting at 100), and the intensities of the transitions of parent (protonated molecules) to fragment ions (*m/z* 328.1 → 200.1 and 333.1 → 205.1 for LJ001 and $^2H_5$-LJ001, respectively) were recorded. Under these conditions, LJ001 and $^2H_5$-LJ001 eluted virtually simultaneously at 29 min. Peak areas were computed using the MacSpec version 3.3 software provided by the instrument manufacturer, and the amount of drug in each sample was calculated using a standard calibration curve prepared from the signals collected from standard samples containing increasing amounts of LJ001 and the same amount of $^2H_5$-LJ001. Preliminary experiments had shown that LJ001 produced a negligible signal with electrospray ionization but a prominent signal corresponding to the protonated molecule at *m/z* 328.1 (calculated as 328.0468 Da for $C_{17}H_{14}O_2S_2N$) during atmospheric pressure chemical ionization and that the pentadeuterated internal standard yielded a corresponding signal at *m/z* 333.1. Furthermore, during collisionally activated dissociation, both compounds produced numerous fragment ions, the most intense of which (at *m/z* 200.1 and 205.1 for LJ001 and $^2H_5$-LJ001, respectively) was assigned $C_{12}{}^1H_8OS$ and $C_{12}{}^1H_3{}^2H_5OS$ elemental compositions, respectively.

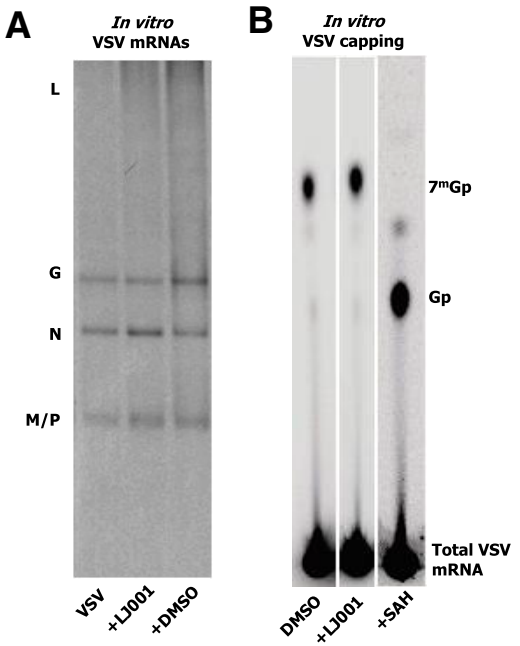
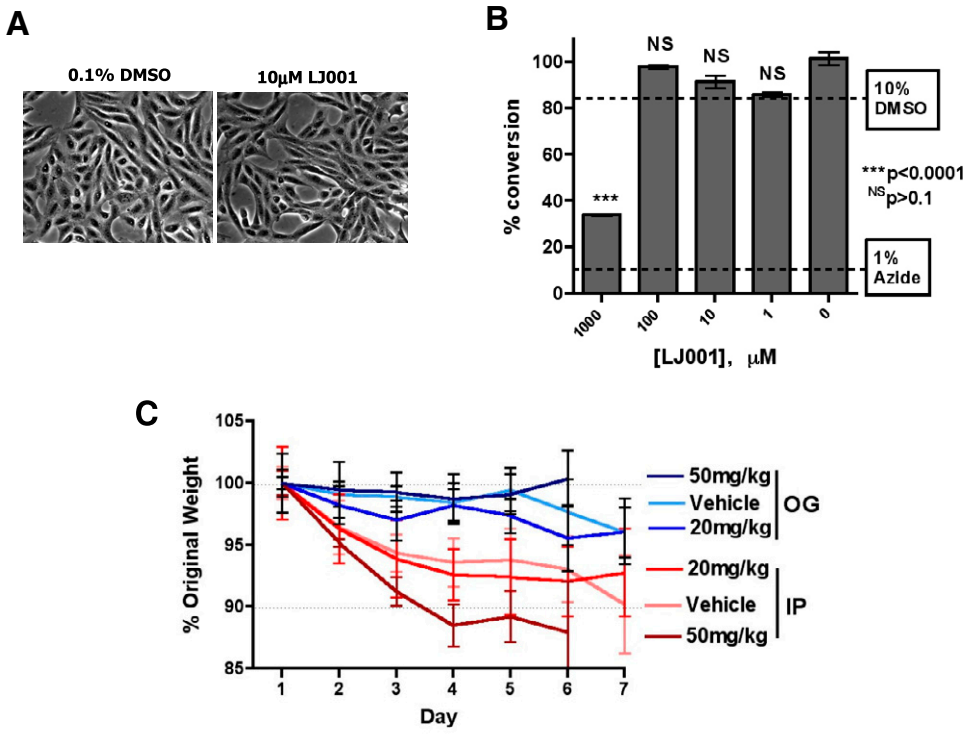



**Fig. S1.** LJ001 does not affect in vitro viral transcription, total mRNA production, and mRNA capping. (*A*) In vitro VSV mRNA was synthesized using 20 μg of purified VSV-Indiana and then treated with 10 μM LJ001 or 0.1% DMSO, followed by mRNA purification and radiodetection within an agarose-urea gel as described in (1). (*B*) Total in vitro VSV mRNA, prepared as described in *A*, was treated with tobacco acid pryophosphatase and subjected to TLC to assay for cap methylation. SAH, S-adenosylhomocysteine.

1. Li J, Fontaine-Rodriguez EC, Whelan SP (2005) Amino acid residues within conserved domain VI of the vesicular stomatitis virus large polymerase protein essential for mRNA cap methyltransferase activity. *J Virol* 79:13373–13384.




D

| | | Intraperitoneal | | | Oral Gavage | | |
|---|---|---|---|---|---|---|---|
| | | DMSO | Low | High | DMSO | Low | High |
| WBC | K/uL | 7.29 | 10.69 | 12.29 | 13.11 | 13.41 | 11.72 |
| NE | K/uL | 2.54 | 2.90 | 3.87 | 2.98 | 2.56 | 2.57 |
| LY | K/uL | 4.16 | 7.19 | 7.58 | 8.81 | 9.95 | 8.24 |
| MO | K/uL | 0.55 | 0.52 | 0.74 | 1.21 | 0.88 | 0.83 |
| EO | K/uL | 0.04 | 0.07 | 0.08 | 0.08 | 0.02 | 0.06 |
| BA | K/uL | 0.01 | 0.02 | 0.03 | 0.03 | 0.00 | 0.01 |
| NE | % | 33.96 | 26.97 | 31.58 | 23.24 | 19.15 | 22.69 |
| LY | % | 57.96 | 67.27 | 61.73 | 66.57 | 74.15 | 69.64 |
| MO | % | 7.54 | 5.02 | 5.91 | 9.19 | 6.53 | 7.06 |
| EO | % | 0.44 | 0.55 | 0.60 | 0.76 | 0.15 | 0.52 |
| BA | % | 0.10 | 0.19 | 0.19 | 0.25 | 0.03 | 0.10 |
| RBC | M/uL | 9.87 | 9.69 | 9.54 | 10.35 | 10.09 | 10.27 |
| Hb | g/dL | 15.1 | 15.0 | 14.5 | 15.9 | 15.6 | 15.6 |
| HCT | % | 54.6 | 53.1 | 51.3 | 58.3 | 57.0 | 56.8 |
| MCV | fL | 55.3 | 54.8 | 53.7 | 56.4 | 56.5 | 55.3 |
| MCH | pg | 15.3 | 15.5 | 15.2 | 15.4 | 15.5 | 15.2 |
| MCHC | g/dL | 27.6 | 28.2 | 28.4 | 27.3 | 27.4 | 27.5 |
| RDW | % | 18.0 | 17.6 | 17.8 | 18.3 | 17.3 | 18.8 |
| PLT | K/uL | 1258 | 897 | 1082 | 990 | 1021 | 962 |
| MPV | fL | 5.0 | 5.5 | 5.2 | 4.8 | 5.1 | 5.0 |
| CHOL | mg/dL | 113 | 136 | 134 | 117 | 126 | 143 |
| TRG | mg/dL | 130 | 116 | 301 | 171 | 191 | 199 |
| ALT | u/L | 37 | 45 | 67 | 49 | 47 | 64 |
| AST | u/L | 121 | 75 | 113 | 115 | 105 | 113 |
| ALK | u/L | 96 | 82 | 81 | 137 | 143 | 139 |
| TBIL | mg/dL | 0.2 | 0.2 | 0.3 | 0.3 | 0.3 | 0.3 |
| GLU | mg/dL | 227 | 315 | 239 | 236 | 247 | 298 |
| PHOS | mg/dL | 8.0 | 9.5 | 8.4 | 9.1 | 9.9 | 8.5 |
| TPR | g/dL | 6.1 | 6.3 | 5.8 | 5.9 | 6.1 | 5.8 |
| CAL | mg/dL | 10.9 | 12.1 | 11.4 | 11.1 | 11.1 | 11.4 |
| BUN | mg/dL | 17 | 19 | 25 | 20 | 21 | 30 |
| CRE | mg/dL | 0.4 | 0.4 | 0.4 | 0.4 | 0.3 | 0.4 |
| ALB | g/dL | 3.1 | 3.3 | 3.1 | 3.7 | 3.4 | 3.6 |

**Fig. S2.** LJ001 imparts no overt in vitro or in vivo toxicity. (*A*) Vero cells were washed, trypsinized, and split daily (i.e., fully passaged) in the continuous presence of 10 μM LJ001 or 0.1% DMSO (vehicle control) for 4 days and then were visualized at 25× magnification under brightfield (representative images).

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Fresh LJ001 or DMSO was added daily. (*B*) Vero cells were plated at ~10% confluency and exposed to LJ001. Uptake of Alamar Blue substrate as cells proliferated (2 days) was measured by colorimetric measurements per the manufacturer's instructions (±SD). (*C*) We inoculated 18 BALB/cAnNCrl female mice at 6–8 weeks of age i.p.(IP) and by oral gavage (OG) daily for 7 days with 50 μL of 100% DMSO or LJ001 (in 100% DMSO) at 20 mg/kg (low) or 50 mg/kg (high) doses (*n* = three per group). Daily averaged weights of the animals in each group are shown (±SD). (*D*) On day 8, all animals were killed via $CO_2$ asphyxiation, and terminal blood samples were collected via cardiac puncture followed by complete blood cell count with differential and chemistry panel analyses. Results shown are averages of three individual animals. NS, not significant.

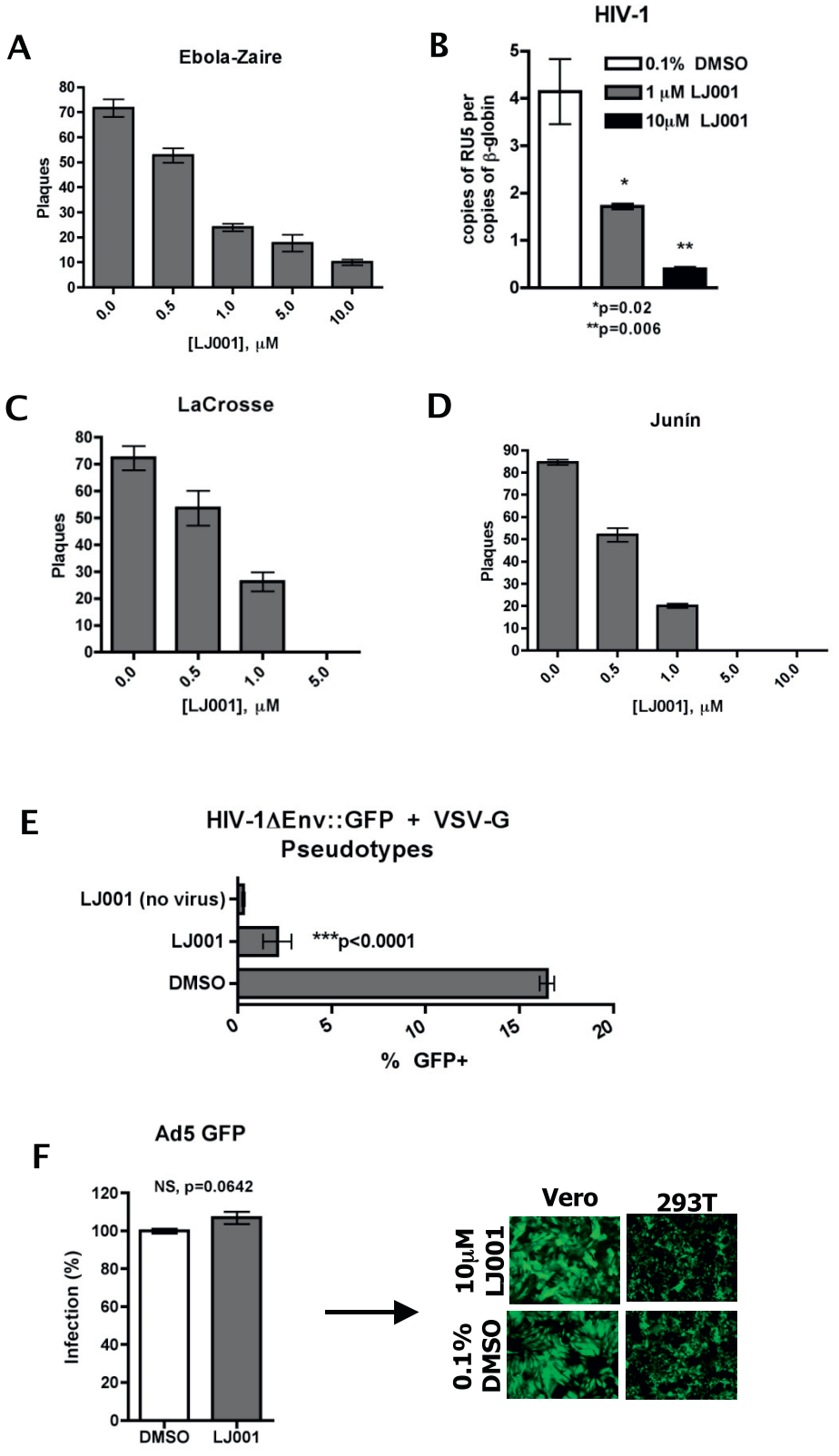



**Fig. S3.** (Continued)

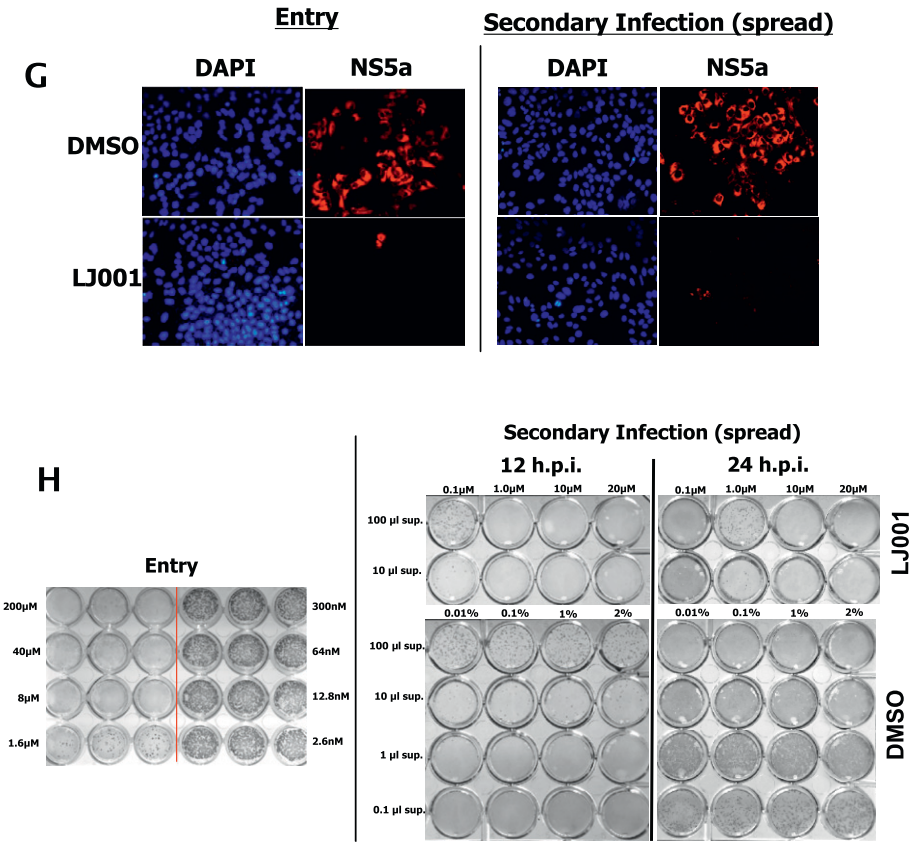



**Fig. S3.** (Continued)

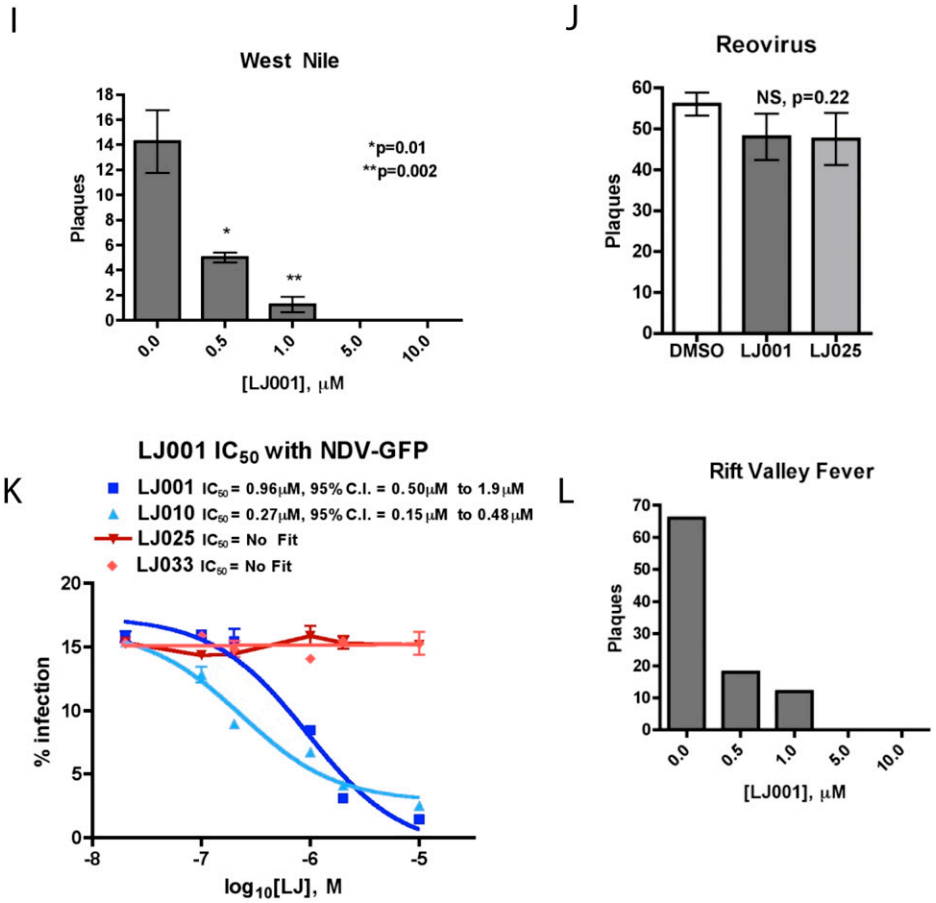



**Fig. S3.** Testing the broad-spectrum activity of LJ001. (*A*) One hundred plaque-forming units of Ebola-Zaire (Cat. A, Filovirus) were treated with LJ001 or DMSO for 20 min at 25 °C and then were used to infect VeroE6 cells for 1 h at 37 °C. Plaques were counted 10 d postinfection. Data shown are the average of triplicate experiments (± SD). (*B*) We treated 5.0 ng of an R5-HIV-1 (YU2) with LJ001 or DMSO for 20 min at 25 °C and used the treated R5-HIV-1 to infect 293-inducible cell lines, induced to express high levels of CD4/CCR5, at 37 °C. At 8 h postinfection, cells were harvested and assayed for early reverse transcription products via real-time qPCR. Data shown are the average of duplicate experiments (± SD). (*C*) Eighty plaque-forming units of La Crosse virus (Cat. B, Bunyavirus) were treated with LJ001 or DMSO for 20 min at 25 °C and then used to infect BHK-S cells for 1 h at 37 °C. Plaques were counted 4 d postinfection. Data shown are the average of triplicate experiments (± SD). (*D*) One hundred twenty plaque-forming units of Junín virus (Cat. A, Arenavirus) were treated with LJ001 or DMSO for 20 min at 25 °C and then used to infect VeroE6 cells for 1 h at 37 °C. Plaques were counted 5 d post infection. Data shown are the average of triplicate experiments (± SD). (*E*) Fifty nanograms of HIV-1 (pNL-GFPΔ*env)* pseudotyped with VSV-G were treated with 10 μM LJ001 or DMSO for 10 min at 25 °C and used to infect 293T and Vero cells for 1 h at 37 °C. Cells were harvested 5 days after infection and assayed for GFP-positive cells via flow cytometry. Data shown are the average of triplicate experiments (± SD). This assay shows that cellular entry of the lentiviral genome was inhibited regardless of the envelope. (*F*) A 1:25 titration (resulting in ~90% infection) of unconcentrated supernatant produced from cells infected with recombinant Ad5-GFP was treated with 10 μM LJ001 or 0.1% DMSO for 10 min at 25 °C and used to infect 293T and Vero cells for 1 h at 37 °C (*Left*). At 18 h postinfection, cells were visualized under a fluorescent microscope and then were harvested and assayed for GFP-positive cells via flow cytometry (*Right*). Data shown are the average of triplicate experiments (± SD). No inhibition was seen with LJ001. Similar results were obtained for a recombinant Coxsackie-B-GFP virus. (*G*) JFH1 strain of Hepatitis C Virus was pretreated with 1 μM LJ001 or LJ025 for 10 min at 25 °C and used to infect Huh-7.5.1 cells. Entry: At 72 h postinfection, cells were scored for HCV NS5A protein by immunofluorescence as a measure of virus replication. Secondary infection: The supernatants (secreted virus) from the Entry experiment were used to infect naïve Huh-7.5.1 cells. At 72 h postinfection, the cells were scored for HCV NS5A protein by immunoflourescence as a measure of virus replication. (*H*) HPIV3 (MOI = 0.1) was pretreated with the indicated concentrations of LJ001 or DMSO for 20 min on ice and used to infect CV-1 cells. To assay for Entry, after 90 min at 37 °C, medium containing virus and compounds was replaced with an overlay of agar-media and incubated overnight at 37 °C being assaying for the number of plaques formed. To assay for Secondary Infection, medium containing virus and compounds was replaced with regular media containing compounds at the same concentrations followed by overnight incubation at 37 °C. At 12 and 24 h postinfection, 10-μl and 100-μl aliquots from each condition were used for plaque assay as above. Similar results were obtained with SV5. (*I*) Fifty plaque-forming units of West Nile virus/well were pretreated with the indicated concentration of LJ001 for 20 min at 25 °C and used to infect Vero-E6 cells for 1 h at 37 °C. Then virus inoculum was removed, and cells were washed with PBS and overlaid with tragacanth. Data shown are the average of quadruplicate experiments (±SD). (*J*) Fifty plaque-forming units of Reovirus (T3D)/well were pretreated with LJ001, LJ025, or DMSO for 10 min at 25 °C and used to infect Madin-Darby canine kidney cells for 1 h at 37 °C. Then virus inoculum was removed, and cells were overlaid with 1% Bacto Agar (Fisher Chemicals) in DMEM. After 7 days, cells were stained with 0.03% neutral red and were assayed visually for plaque formation. Data shown are the average of duplicate experiments (±SD). (*K*) A 1:500 dilution of GFP replication-competent Newcastle disease virus harvested from the allantoic fluid of embryonated chicken eggs (100 pfu/egg) was pretreated with the indicated concentration of LJ-compound and then used to infect Vero cells for 1 h at 37 °C. The inoculum then was replaced with regular growth media, and after 18 h cells were harvested, fixed, and assayed for GFP expression via flow cytometry. Data shown are the average of duplicate experiments (±SD). (*L*) Fifty plaque-forming units of Rift Valley fever MP-12 virus (Cat. B, Bunyavirus) were treated with LJ001 for 20 min at 25 °C and then were used to infect BHK-S cells for 1 h at 37 °C. Plaques were counted 4 d postinfection. Data shown are the average of triplicate experiments (±SD).

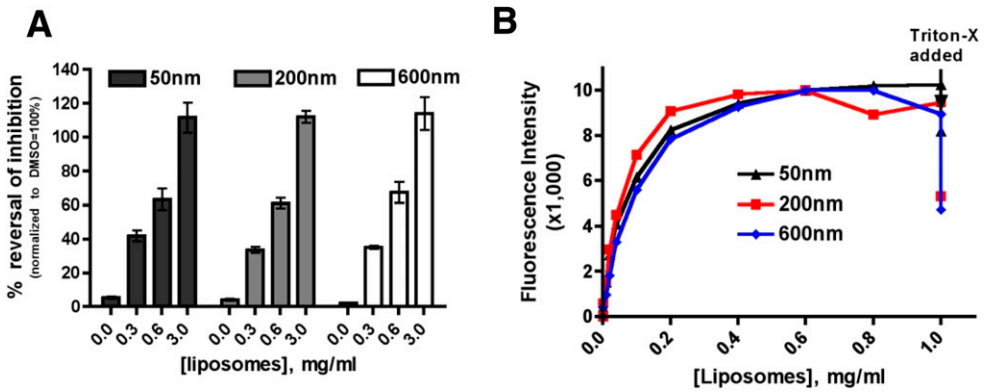



**Fig. S4.** LJ001 binds, perturbs, and irreversibly targets the viral lipid membrane. (*A*) Differentially sized liposomes were purified via size exclusion and are of the same composition as those used in Fig. 3. Vero cells were infected with pVSV, as previously described, after being pretreated simultaneously with 10 μM LJ001 and the indicated size and concentration of liposomes. Data are from a single representative experiment (±SD). (*B*) Fluorescence intensity signal (510 nm) after titration of liposomes of the indicated size into 10 μM LJ001 was determined as described in the main text (Fig. 3). Background scattering caused by the presence of liposomes alone was subtracted as previously described.

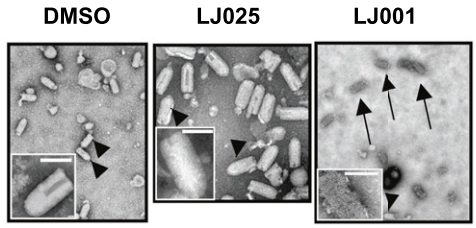



**Fig. S5.** Electron microscopy of LJ001-treated virions. Purified pVSV was treated with 300 μM LJ001, LJ025 (inactive analog), or DMSO (vehicle control), and incubated at 25 °C for 10 min, followed by staining with 2% phosphotungstic acid and visualization by electron microscopy. Shown are representative images. Arrows indicate disrupted particles; arrowheads indicate selected representative intact particles. *Insets* are at 75,000× magnification. (Scale bars, 100 nm.) Note that the membrane of the LJ001-treated virion (*Inset*) has a ruffled or moth-eaten appearance that is not apparent in the DMSO- or LJ025-treated virions (*Insets*).

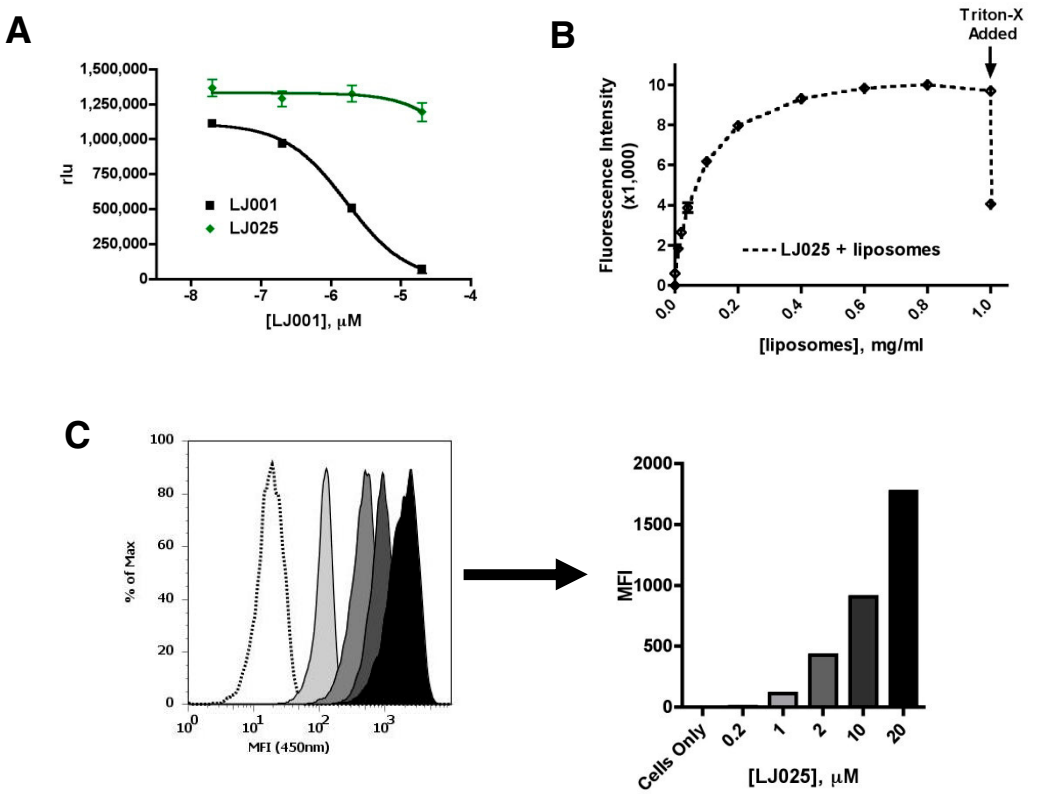



**Fig. S6.** Membrane binding of LJ is necessary but not sufficient for antiviral activity. (*A*) Pseudotyped VSV (pVSV+NiV-F/G) was pretreated with the indicated concentration of LJ001 or LJ025 for 10 min at 25 °C and then was used to infect Vero cells for 1 h at 37 °C. Data are from a representative experiment (± SD). (*B*) Fluorescence intensity signal after titration of liposomes into 10 μM LJ025 (LJ025 peak excitation: 410 nm; emission: 460 nm; laser: 405 nm; detector: 455 nm) was performed as described in the main text (Fig. 3). Again, background scattering caused by the presence of liposomes alone was subtracted as previously described (±SD). (*C*) (*Left*) Twenty-five thousand Vero cells were stained with the increasing concentrations of LJ025 for 30 min at 37 °C in normal growth medium and then were harvested by trypsinization or scraping and analyzed for mean fluorescence intensity by flow cytometry (excitation: 401 nm; emission: 450 nm). (*Right*) Bar graph showing mean fluorescence intensity values with increasing concentrations of LJ025. Data shown are from a single representative experiment. rlu, relative light units.

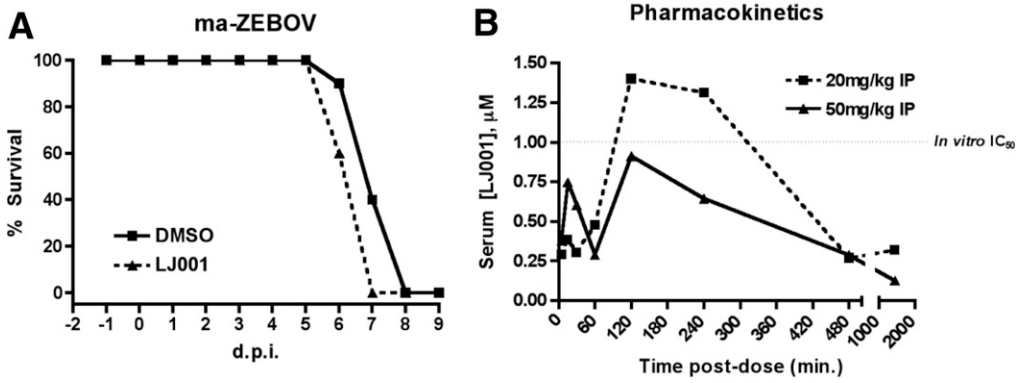



**Fig. S7.** Postchallenge efficacy and pharmacokinetics of LJ001. (*A*) One thousand plaque-forming units of mouse-adapted Ebola-Zaire virus (ma-ZEBOV) were used to infect female BALB/c mice ($n = 10$) via i.p. injection in a 0.2-mL volume. Immediately after challenge, the animals were dosed i.p. with 50 mg/kg LJ001 in 100% DMSO at a dose volume of 50 μL. Mice then were re-dosed every 24 h for 7 d. d.p.i., days postinfection. (*B*) Male Sprague-Dawley rats ($n = 3$) were implanted with jugular vein catheters (JVC) and dosed singly i.p. (IP) with LJ001 at 20 mg/kg or 50 mg/kg. Blood samples (300 mL) were taken by JVC at the time intervals indicated and placed in K2-EDTA tubes. LJ001 was detected via liquid chromatography/atmospheric pressure chemical ionization/tandem MS and quantified using pentadeuterated LJ001 as an internal standard. MS detection, analysis, and quantification methodology are described in *SI Text*. Surprisingly, the peak serum concentration was higher in the 20-mg/kg group than in the 50-mg/kg group, suggesting that the 100% DMSO delivery vehicle was not optimal.

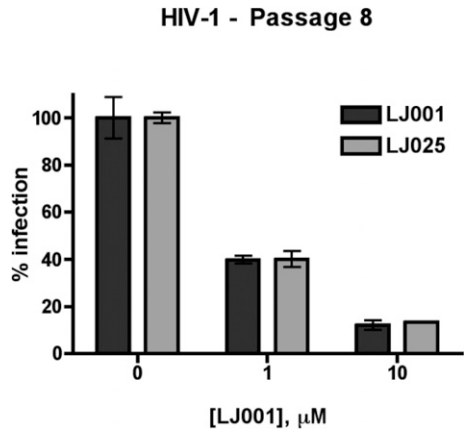



**Fig. S8.** Development of resistance to LJ001. HIV-1 (JRCSF) was passaged in the continuous presence of 6 μM LJ001 or LJ025 on GHOST-R5 cells. Viral replication cultures were split 1:4 twice weekly, and replication was monitored by the LTR-GFP reporter as well as by the p24 antigen level in the supernatant. After eight consecutive passages (~4 weeks), LJ001-passaged virus or LJ025-passaged virus was adjusted to the 25-ng equivalent of p24 and tested for sensitivity to LJ001 inhibition on fresh Ghost-R5 cells. Cells were assayed for GFP production via flow cytometry. Data shown are the average of duplicate experiments (±SD).

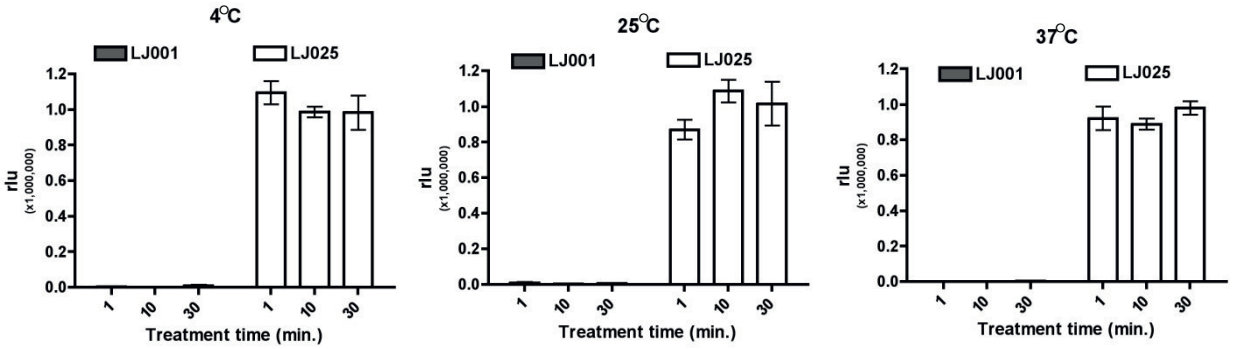
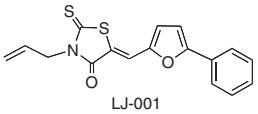
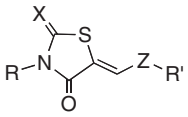



**Fig. S9.** Temperature/time independence of LJ inactivation. pVSV was pretreated with 10 μM LJ001 or LJ025 for the time and temperature indicated in 100-μL volume. The mixture then was immediately added to 6 mL of ice-cold PBS followed by ultracentrifugation through a 20% sucrose cushion. The resulting pellet was resuspended and was tested for infection as previously described. rlu, relative light units.

**Table S1. Structure–activity relationship analyses of the LJ-series compounds**




LJ-001




| Compound | R, Z, R′, X | $IC_{50}$, μM | 95% confidence interval | Compound | R, Z, R′, X | $IC_{50}$, μM | 95% confidence interval |
|---|---|---|---|---|---|---|---|
| LJ-001 | Allyl, 2,5-furyl, Ph, S | 1.0 | 0.74–1.90 | LJ-014 | Et, 2,5-furyl, H, S | Inactive** | N/A |
| LJ-002 | Et, 2,5-furyl, Ph, S | 0.7 | 0.27–1.76 | LJ-015 | Et, 2,5-furyl, 2-$NO_2C_6H_4$, S | 1.3 | 0.90–1.98 |
| LJ-003 | Pr, 2,5-furyl, Ph, S | 0.9 | 0.48–1.98 | LJ-016 | Et, 2,5-furyl, 2-$HOC_6H_4$, S | 2.2 | 1.05–4.40 |
| LJ-004 | Bn, 2,5-furyl, Ph, S | 1.1 | 0.42–2.90 | LJ-017 | Et, 2,5-furyl, 2-$NH_2C_6H_4$, S | 3.4 | 1.15–9.84 |
| LJ-005 | Me, 2,5-furyl, Ph, S | 1.5 | 0.80–2.85 | LJ-018 | Et, 2,5-thiophenyl, Ph, S | 4.1 | 0.95–17.80 |
| LJ-006 | Et, 2,5-furyl, 3-$ClC_6H_4$, S | 3.6 | 1.56–8.15 | LJ-019 | Et, 1,3-phenyl, Ph, S | Inactive** | N/A |
| LJ-007 | Et, 2,5-furyl, 3-$FC_6H_4$, S | 2.5 | 1.22–5.38 | LJ-020 | Et, 2,5-furyl, $C_6H_4$-2-$N_2^+BF_4^-$, S | 11.1*** | 0.43–286.9 |
| LJ-008 | Et, 2,5-furyl, 2-$FC_6H_4$, S | 2.4 | 1.70–3.60 | LJ-021 | $AcO(CH_2)_3$, 2,5-furyl, Ph, S | 0.9 | 0.50–1.62 |
| LJ-009 | Et, 2,5-furyl, 2-$ClC_6H_4$, S | 2.9 | 2.05–4.27 | LJ-022 | $HO(CH_2)_3$, 2,5-furyl, Ph, S | 0.8 | 0.53–1.16 |
| LJ-010 | Et, 2,5-furyl, 2-$MeOC_6H_4$, S | 0.8 | 0.57–1.28 | LJ-023 | Et, 5,2-oxazolyl, Ph, S | 2.5 | 0.68–9.07 |
| LJ-011 | Et, 2,5-furyl, 3-$MeOC_6H_4$, S | 1.5 | 1.14–1.86 | LJ-024 | Biotin-$CO_2(CH_2)_3$, 2,5-furyl, Ph, S | 1.9 | 0.86–3.99 |
| LJ-012 | Et, 2,5-furyl, 2-$CF_3C_6H_4$, S | 2.3 | 1.50–3.85 | LJ-025 | Allyl, 2,5-furyl, Ph, O | Inactive** | N/A |
| LJ-013 | Et, 2,5-furyl, Me, S | Inactive** | N/A | LJ-033 | Allyl, 2,5-furyl, Ph, S, dihydro | Inactive** | N/A |

The compound codes, chemical structures, and their respective $IC_{50}$ values (on NiV-pVSV infections) are shown.

**Inactive compounds have an estimated $IC_{50} > 100$ μM.

***All $IC_{50}$ values have been retested; upon retesting LJ020 gave 1.62 μM (95% confidence Interval, 0.74–3.56 μM).