LETTER

Therapeutic efficacy of the small molecule GS–5734 against Ebola virus in rhesus monkeys

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The most recent Ebola virus outbreak in West Africa, which was unprecedented in the number of cases and fatalities, geographic distribution, and number of nations affected, highlights the need for safe, effective, and readily available antiviral agents for treatment and prevention of acute Ebola virus (EBOV) disease (EVD) or sequelae1. No antiviral therapeutics have yet received regulatory approval or demonstrated clinical efficacy. Here we report the discovery of a novel small molecule GS–5734, a monophosphoramidate prodrug of an adenosine analogue, with antiviral activity against EBOV. GS–5734 exhibits antiviral activity against multiple variants of EBOV and other filoviruses in cell-based assays. The pharmacologically active nucleoside triphosphate (NTP) is efficiently formed in multiple human cell types incubated with GS–5734 in vitro, and the NTP acts as an alternative substrate and RNA-chain terminator in primer-extension assays using a surrogat e respiratory syncytial virus RNA polymerase. Intravenous administration of GS–5734 to nonhuman primates resulted in persistent NTP levels in peripheral blood mononuclear cells (half-life, 14 h) and distribution to sanctuary sites for viral replication including testes, eyes, and brain. In a rhesus monkey model of EVD, once-daily intravenous administration of 10 mg kg–1 GS–5734 for 12 days resulted in profound suppression of EBOV replication and protected 100% of EBOV-infected animals against lethal disease, ameliorating clinical disease signs and pathophysiological markers, even when treatments were initiated three days after virus exposure when systemic viral RNA was detected in two out of six treated animals. These results show the first substantive post-exposure protection by a small-molecule antiviral compound against EBOV in nonhuman primates. The broad-spectrum antiviral activity of GS–5734 in vitro against other pathogenic RNA viruses, including filoviruses, arenaviruses, and coronaviruses, suggests the potential for wider medical use. GS–5734 is amenable to large-scale manufacturing, and clinical studies investigating the drug safety and pharmacokinetics are ongoing.

The 2013–2016 outbreak of EVD in West Africa was the largest and most complex EBOV outbreak in the recorded history of the disease, with >28,000 EVD cases and >11,000 reported deaths1. Medical infrastructures in Guinea, Sierra Leone, and Liberia were seriously recrudescent in individuals who survived the acute disease have been documented2–5.

EBOV is a single-stranded negative-sense non-segmented RNA virus from the Filoviridae family. In addition to EBOV, other related viruses, namely Marburg, Sudan, and Bundibugyo viruses, have caused outbreaks with high fatality rates6. Although the efficacy of various experimental small molecules and biologics have been assessed in EVD animal models and in multiple clinical trials during the West African outbreak7–18, there are no therapeutics for which clinical efficacy and safety have been established for treatment of acute EVD or its sequelae. The availability of broadly effective antiviral(s) with a favourable benefit/risk profile would address a serious unmet medical need for the treatment of EBOV infection.

A 1′-cyano-substituted adenine C-nucleoside ribose analogue (Nuc) exhibits antiviral activity against a number of RNA viruses19. The mechanism of action of Nuc requires intracellular anabolism to the 1′-cyano-substituted adenine C-nucleoside ribose triphosphate (NTP) that persist with a half-life (t1/2) of 20–40 h in nonhuman primates. In contrast, the parent Nuc was less active, with EC50 values of 0.77 to 20 μM. As expected, treatment of EBOV-infected animals with GS–5734 caused rapid loading of cells with high levels of NTP that persist with a half-life (t1/2) of 24 h, followed by removal of GS–5734 (Extended Data Fig. 1a), resulting in up to 30-fold higher levels compared to incubation with Nuc (Fig. 1b). In cell-based assays, GS–5734 is active against a broad range of filoviruses including Marburg virus and several variants of EBOV (Fig. 1c). GS–5734 inhibits EBOV replication in multiple relevant human cell types including primary macrophages and human endothelial cells with half-maximum effective concentration (EC50) values of 0.06 to 0.14 μM (Table 1). As expected, the parent Nuc was less active, with EC50 values of 0.77 to >20 μM. Treatment with GS–5734 of liver Huh–7 cells infected with the EBOV Makona variant, isolated during the West African outbreak, resulted in profound dose-dependent reductions in viral RNA production and infectious virus yield (Extended Data Fig. 2). GS–5734 and Nuc inhibited replication of other human RNA viral pathogens including

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species, degrades the GS-5734 promoiety and adversely impacts its

...because high serum esterase activity, present in many rodent RNAsynthesis following efficient intracellular conversion to NTP. In contrast, NTP inhibited RSV RdRp-catalysed RNA synthesis (Fig. 1e) by inhibition of EBOV replication by targeting its RdRp and inhibiting viral RNA polymerases (Fig. 1e). These data suggest that GS-5734 selectively mature termination (Fig. 1f). In contrast, NTP did not inhibit human 

Extended Data Fig. 3). Consistent with the proposed mechanism of action, NTP inhibited RSV RdRp-catalysed RNA synthesis (Fig. 1e) by incorporating into the nascent viral RNA transcript and causing its premature termination (Fig. 1f). In contrast, NTP did not inhibit human RNA polymerases (Fig. 1e). These data suggest that GS-5734 selectively inhibits EBOV replication by targeting its RdRp and inhibiting viral RNA synthesis following efficient intracellular conversion to NTP.

Rodent models were not suitable for GS-5734 in vivo efficacy evaluations because high serum esterase activity, present in many rodent species, degrades the GS-5734 pro-moiety and adversely impacts its pharmacokinetic profile. Like humans, rhesus monkeys do not express high levels of serum esterase; rhesus lymphoid cells efficiently activated GS-5734 in vitro, although NTP levels were reduced relative to human cells (Extended Data Fig. 1b). In rhesus monkeys, intramuscular inoculation with clinically derived wild-type EBOV produces a fulminant lethal disease with pathophysiological responses that closely resemble human EVD cases, and nonhuman primates (NHP) are considered the most relevant EVD models well-suited for evaluating the efficacy of antiviral interventions when trials in infected humans are not feasible.

GS-5734 pharmacokinetics, metabolism, and distribution were examined in NHPs. Upon intravenous administration of a 10 mg kg⁻¹ dose in rhesus monkeys, GS-5734 exhibited a short plasma half-life (t₁/₂ = 0.39 h) with fast systemic elimination followed by the sequential appearance of transient systemic levels of the key intracellular intermediate alanine metabolite and more persistent levels of Nuc (Fig. 2a). GS-5734 rapidly distributed into peripheral blood mononuclear cells (PBMCs), and efficient conversion to NTP was apparent within 2 h of dose administration. In PBMCs, NTP represents the predominant biological replicates. For gel source data, see Supplementary Fig. 1.

...for 24 h (Fig. 2a, Extended Data Fig. 1c).
In cynomolgus monkeys, intravenous administration of a 10 mg kg⁻¹ dose of [¹⁴C]GS-5734 demonstrated that the drug-derived material distributed to testes, epididymis, eyes, and brain within 4 h of administration (Fig. 2b). Levels in the brain at 4 h were low relative to other tissues, but remained detectable above the drug plasma levels 168 h after dose administration. Taken together, the pharmacokinetic analysis indicates that once-daily dosing of GS-5734 provides sustained intracellular NTP levels and efficiently delivers drug metabolites to sanctuary sites where virus may persist.

To evaluate the in vivo efficacy of GS-5734, we conducted a sequential two-part adaptive design study in EBOV-infected rhesus monkeys (Fig. 2c). In part 1, animals intramuscularly inoculated with EBOV were administered a 12-day treatment of vehicle (n = 3) or 3 mg kg⁻¹ GS-5734 beginning on day 0 (d0; 30–90 min following virus challenge) or day 2 (d2) (n = 6 per treatment group). Regardless of the time of initiation, GS-5734 treatment conferred an antiviral effect by reducing systemic viraemia relative to vehicle and an improved survival of 33% (2 out of 6) in the 3 mg kg⁻¹ d0 group and 66% (4 out of 6) in the 3 mg kg⁻¹ d2 group (Fig. 2d, e, Extended Data Fig. 4, Extended Data Tables 2, 3); however, mortalities observed in both treatment groups suggested that drug exposure at 3 mg kg⁻¹ was suboptimal. In part 2 of the efficacy study, GS-5734 was administered once at a loading dose of 10 mg kg⁻¹ followed by once-daily 3 mg kg⁻¹ doses beginning either 2 days (10/3 mg kg⁻¹ d2) or 3 days (10/3 mg kg⁻¹ d3) after virus exposure, or 10 mg kg⁻¹ doses were administered beginning 3 days after virus exposure (10 mg kg⁻¹ d3; n = 6 per group). All 12 animals in which GS-5734 treatments were initiated 3 days after virus exposure survived to the end of the in-life phase (Fig. 2d). However, the antiviral effects were consistently greater in animals administered repeated 10 mg kg⁻¹ GS-5734 doses (Fig. 2e, f, Extended Data Fig. 4, Extended Data Tables 2, 3). On day 4, plasma viral RNA was significantly decreased (P < 0.05), with geometric means reduced by ≥1.7 log₁₀ in all GS-5734-treated groups compared with combined vehicle-treated groups (Fig. 2e, f, Extended Data Table 3), and on days 5 and 7, when the geometric mean viral RNA concentration of the vehicle group exceeded 10⁹ copies ml⁻¹, viral RNA was detected at levels less than the lower limit of quantitation (8 × 10⁹ RNA copies ml⁻¹) in 4 of 6 animals in the 10 mg kg⁻¹ d3 group. Deep sequencing analysis of the EBOV RdRp (L) gene from all plasma samples positive for viral RNA showed no evidence of genotypic changes potentially associated with the emergence of GS-5734-resistant
or recrudescent cases of EVD, or in survivors with prolonged virus shedding and/or chronic clinical sequelae. The broad-spectrum antiviral activity of GS-5734 and its amenability to large-scale production warrants further assessment of its therapeutic potential against other human viral pathogens for which no treatment is available.

Online Content Methods, along with any additional Extended Data display items and Source Data, are available in the online version of the paper; references unique to these sections appear only in the online paper.

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1. World Health Organization. Ebola Situation Report - 3 February 2016. http://apps.who.int/ebola/current-situation/ebola-situation-report-3-february-2016 (2016).
2. Nanyonga, M., Saidu, J., Ramsay, A., Shindo, N. & Bausch, D. G. Sequence of Ebola virus disease, Kenema District, Sierra Leone. Clin. Infect. Dis. 62, 125–126 (2016).
3. Varkey, J. B. et al. Persistence of Ebola virus in ocular fluid during convalescence. N. Engl. J. Med. 372, 2423–2427 (2015).
4. Mate, S. E. et al. Molecular evidence of sexual transmission of Ebola virus. N. Engl. J. Med. 373, 2448–2454 (2015).
5. Deen, G. F. et al. Ebola RNA persistence in semen of Ebola virus disease survivors — preliminary report. N. Engl. J. Med. http://dx.doi.org/10.1056/NEJMoa1511410 (2015).
6. Kuhn, J. H. Filoviruses: A Compendium of 40 Years of Epidemiological, Clinical, and Laboratory Studies (SpringWien, 2008).
7. Kupferschmidt, K. & Cohen, J. Infectious diseases. Ebola drug trials lurch ahead. Science 347, 701–702 (2015).
8. Smither, S. J. et al. Post-exposure efficacy of oral T-705 (Favipiravir) against inhalational Ebola virus infection in a mouse model. Antiviral Res. 104, 153–155 (2014).
9. Oestereich, L. et al. Successful treatment of advanced Ebola virus infection with T-705 (favipiravir) in a small animal model. Antiviral Res. 105, 17–21 (2014).
10. McMullan, L. K. et al. The lipid moiety of brincidofovir is required for in vitro antiviral activity against Ebola virus. Antiviral Res. 125, 71–78 (2016).
11. Sissoko, O. et al. Favipiravir in patients with Ebola virus disease: early results of the JIKI trial in Guinea. Conference of Retroviruses and Opportunistic Infections abstr. 103-ALB (Seattle, 2015).
12. Chimerix. Brincidofovir Will Not Be Considered in Further Clinical Trials in Ebola Virus Disease. http://ir.chimerix.com/releasedetail.cfm?ReleaseID=893927 (2015).
13. Warren, T. K. et al. Protection against filovirus diseases by a novel broad-spectrum nucleoside analogue BCX4430. Nature 508, 402–405 (2014).
14. BioCryst Pharmaceuticals. BioCryst Announces Study Results for BCX4430 in a Non-Human Primate Model of Ebola Virus Infection. http://investor.shareholder.com/biocrost/releasesdetail.cfm?ReleaseID=888802 (2014).
15. Thi, E. P. et al. Lipid nanoparticle siRNA treatment of Ebola-virus-Makona-infected nonhuman primates. Nature 521, 362–365 (2015).
16. Qi, X. et al. Reversion of advanced Ebola virus disease in nonhuman primates with ZMapp. Nature 514, 47–53 (2014).
17. Olinger, G. G., Jr et al. Delayed treatment of Ebola virus infection with plant-derived monoclonal antibodies provides protection in rhesus macaques. Proc. Natl Acad. Sci. USA 109, 18030–18035 (2012).
18. Tekmira Pharmaceuticals Corporation. Tekmira Provides Update on TKM-Ebola. Guinea. http://www.sec.gov/Archives/edgar/data/1447028/000117184315003522/newsrelease.htm (2015).
19. Cho, A. et al. Synthesis and antiviral activity of a series of 1′-substituted 4-aza-7,9-diazadeganosenoic C-nucleosides. Bioorg. Med. Chem. Lett. 22, 2705–2707 (2012).
20. Murakami, E. et al. The mechanism of action of 3′-β-D-deoxy-2′-fluoro-2′-C-methylcytidine involves a 2′,3′-dideoxy-2′-D-fluoro-2′-C-methyluridine 5′-triphosphate, a potent inhibitor of the hepatitis C virus RNA-dependent RNA polymerase. Antimicrob. Agents Chemother. 52, 498–464 (2008).
21. Mackman, R. L., Parrish, J. P., Ray, A. S. & Theodore, D. A. Methods and compounds for treating respiratory syncytial virus infections. US Patent 2011045102. (2011).
22. Jäcomo, R., Becerra, A., Ponce de Leon, S. & Lazzcano, A. Structural analysis of monomeric RNA-dependent polynucleotides: Evolutionary and therapeutic implications. PLoS ONE 10, e0139001 (2015).
23. Bahar, F. G., Ohura, K., Oghara, T. & Imai, T. Species diversity of esterase C virus RNA-dependent RNA polymerase. Antimicrob. Agents Chemother. 52, 498–464 (2008).
24. Hunt, L. et al. Clinical presentation, biochemical, and haematological parameters and their association with outcome in patients with Ebola virus disease: an observational cohort study. Lancet Infect. Dis. 15, 1292–1299 (2015).
25. Martins, K. et al. Characterization of clinical and immunological parameters during Ebola virus infection of rhesus macaques. Viral Immunol. 28, 32–41 (2015).
Supplementary Information is available in the online version of the paper.

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Author Information Viral genomic sequences have been deposited in GenBank (http://www.ncbi.nlm.nih.gov/genbank/) and accession numbers are supplied in Extended Data Table 5. Small molecule X-ray crystallographic coordinates and structure factor files have been deposited in the Cambridge Structural Database (http://www.ccdc.cam.ac.uk/) and accession numbers are supplied in the Supplementary Information. Reprints and permissions information is available at www.nature.com/reprints. The authors declare competing financial interests: details are available in the online version of the paper. Readers are welcome to comment on the online version of the paper. Correspondence and requests for materials should be addressed to S.B. (sina.bavari.civ@mail.mil) and T.C. (tomas.cihlar@gilead.com).
METHODS

Data reporting. No statistical methods were used to predetermine sample size for biochemical or cell-based assays, or for pharmacokinetic studies. Investigators were not blinded to outcome assessment during these investigations. For GS-5734 efficacy assessments in nonhuman primates, statistical power analysis was used to predetermine sample size, and subjects were randomly assigned to experimental group, stratified by sex and balanced by body weight. Study personnel responsible for assessing animal health (including euthanasia assessment) and administering treatments were experimentally blinded to group assignment of animals and outcomes.

Small molecules. GS-5734, Nuc, and NTP were synthesized at Gilead Sciences, Inc., and chemical identity and sample purity were established using NMR, HRMS, and HPLC analysis (Supplementary Information). The radio-labelled analogue $^{14}$C-GS-5734 (specific activity, 58.0 mCi mmol$^{-1}$) was obtained from Moravek Biochemicals (Brea, California) and was prepared in a similar manner described for GS-5734 using $^{14}$C[(trimethylsilyl)cyano (Supplementary Information). Small molecule X-ray crystallographic coordinates and structure factor files have been deposited in the Cambridge Structural Database (http://www.ccdc.cam.ac.uk/) and accession numbers are supplied in the Supplementary Information.

Viruses. RSV A2 was purchased from Advanced Biotechnologies, Inc. EBOV (Kikwit and Makona variants), Sudan virus (SUDV, Gulu), Marburg virus (MARV, Cb67), Junin virus (JUNV, Romero), Lassa virus (LASV, Josiah), Middle East respiratory syndrome virus (MERS, Jordan N3), Chikungunya virus (CHIV, AF 15561), and Venezuelan equine encephalitis virus (VEEV, SH3) were all prepared and characterized at the United States Army Medical Research Institute for infectious diseases (USAMRIID). EBOV containing a GFP reporter gene (EBOV–GFP), EBOV Makona (Liberia, 2014), and MARV containing a GFP reporter gene (MARV–GFP) were prepared and characterized at the Centers for Disease Control and Prevention.

Cells. HeP-2 (CCL-23), PC-3 (CCL-1435), HeLa (CCL-2), U2OS (HTB-96), Vero (CCL-81), HFF-1 (SCRC-1041), and HepG2 (HB-8065) cell lines were purchased from the American Type Culture Collection. Cell lines were not authenticated and were not tested for mycoplasma as part of routine use in assays. HeP-2 cells were cultured in Eagle’s Minimum Essential Media (MEM) with GlutaMAX supplemented with 10% fetal bovine serum (FBS) and 100 U ml$^{-1}$ penicillin and streptomycin. PC-3 cells were cultured in Kaighn’s F12 media supplemented with 10% FBS and 100 U ml$^{-1}$ penicillin and streptomycin. HeLa, U2OS, and Vero cells were cultured in MEM supplemented with 10% FBS, 1% l-glutamine, 10 mM HEPES, 1% non-essential amino acids, and 1% penicillin/streptomycin. HFF-1 cells were cultured in MEM supplemented with 10% FBS and 0.5 mM sodium pyruvate. HepG2 cells were cultured in Dulbecco’s Modified Eagle Medium (DMEM) with GlutaMAX supplemented with 10% FBS, 100 U ml$^{-1}$ penicillin and streptomycin, and 0.1 mM non-essential amino acids. The MT-4 cell line was obtained from the NIH AIDS Research and Reference Reagent Program and cultured in RPMI-1640 medium supplemented with 10% FBS, 100 U ml$^{-1}$ penicillin and streptomycin, and 2 mM l-glutamine. The Huh-7 cell line was obtained from C. M. Rice (Rockefeller University) and cultured in DMEM supplemented with 10% FBS and 0.5% non-essential amino acids.

Primary human hepatocytes were purchased from Invitrogen and cultured in William’s Medium E medium containing cell maintenance supplement. Donor profiles were limited to 18- to 65-year-old nonsmokers with limited risk factors. PBMCs were cultured in RPMI-1640 medium supplemented with 10% FBS, 5 to 50 ng ml$^{-1}$ granulocyte-macrophage colony-stimulating factor and 50 μM 3-mercaptopentoic acid to macrophage differentiation. The cryopreserved human primary renal proximal tubule epithelial cells were obtained from LifeLine Cell Technology and isolated from the tissue of human kidney. The cells were cultured at 90% confluency with RenalLife complete medium in a T-75 flask for 3 to 4 days before seeding into 96-well assay plates. Immortalized human microvascular endothelial cells (HMVEC-TERT) were obtained from R. Shao at the Pioneer Valley Life Sciences Institute. HMVEC-TERT cells were cultured in endothelial basal media supplemented with 10% FBS, 5 μg of endothelial growth factor, 0.5 mg hydrocortisone, and 1 ng ml$^{-1}$ phorbol 12-myristate 13-acetate (PMA) (Sigma-Aldrich). RNA POLII was purchased as part of the HeLaScribe Nuclear Extract in vitro Transcription System kit from Promega. The recombinant human POLRT and transcription factors mitochondrial transcription factors A (mtTFA or TFAM) and B2 (mtTFB2 or TFB2M) were purchased from Enzymax. RSV ribonucleoprotein (RNP) complexes were prepared according to a method modified from ref. 29.

Intracellular metabolism studies. The intracellular metabolism of GS-5734 was assessed in different cell types (HMVEC and HeLa cell lines, and primary human and rhesus PBMCs, monocytes and monocyte-derived macrophages) following 2-h pulse or 72-h continuous incubations with 10 μM GS-5734. For comparison, intracellular metabolism during a 72-h incubation with 10 μM of Nuc was completed in human monocyte-derived macrophages. For pulse incubations, monocyte-derived macrophages isolated from rhesus monkeys or humans were incubated for 2 h in compound-containing media following by removal, washing with 37°C drug-free media, and incubated for an additional 22 h in media which did not contain GS-5734. Human monocyte-derived macrophages, HeLa and HMVEC were grown to confluence (approximately 0.5, 0.2, and 1.2 × 10$^{6}$ cells per well, respectively) in 500 μl of media in 12-well tissue culture plates. Monocyte and PBMCs were incubated in suspension (approximately 1 × 10$^{6}$ cells ml$^{-1}$) in 1 ml of medium in micro centrifuge tubes.

LC-MS/MS. LC-MS/MS was performed using low-flow ion-pairing chromatography, similar to methods and descriptions previously. Briefly, analyses were separated using a 50 × 2 mm × 2.5 μm Luna C18(2) HST column (Phenomenex) connected to a LC-20ADXR (Shimadzu) ternary pump system and HTS PAL autosampler (LEAP Technologies). A multi-stage linear gradient from 10% to 50% acetonitrile in a mobile phase containing 3 mM ammonium formate (pH 5.0) with 10 mM dimethylhexylamine (DMH) in water for analysis by liquid chromatography coupled to triple quadrupole mass spectrometry (LC-MS/MS). Dried samples were then reconstituted in mobile phase A containing 3 mM ammonium formate (pH 5.0) with 10 mM dimethylhexylamine over 8 min at a flow rate of 150 μl min$^{-1}$ was used to separate analytes. Detection was performed on an API 4000 (Applied Biosystems) MS/MS operating in positive ion and multiple reaction monitoring modes. Intracellular metabolites malanamide, Nuc, N, N-dimethylphosphonate, nuceloside diphosphate, and nucleoside triphosphate were quantified using 7-point standard curves ranging from 0.274 to 200 pmol (approximately 0.5 to 400 nM) prepared in PBS in the assay medium. The total number of cells per sample was counted using a Countess automated cell counter (Invitrogen). EBOV Huh-7 and HMVEC antiviral assay. Antiviral assays were conducted in biosafety level 4 containment (BSL-4) at the Centers for Disease Control and Prevention. EBOV antiviral assays were conducted in primary HMVEC-TERT and in Huh-7 cells. Huh-7 cells were not authenticated and were not tested for mycoplasma or mycelia. Concentrations of compound were diluted in fourfold serial dilution increments in media, and 100 μl per well of each dilution was transferred in duplicate (Huh-7) or quadruplicate (HMVEC-TERT) on 96-well assay plates containing cell monolayers. The plates were transferred to BSL-4 containment, and the appropriate dilution of virus stock was added to test plates containing cells and serially diluted compounds. Each plate included four wells of infected cells.
untreated cells and four wells of uninfected cells that served as 0% and 100% virus inhibition controls, respectively. After the infection, assay plates were incubated for 3 days (Huh-7) or 5 days (HMVEC-TERT) in a tissue culture incubator. Virus replication was measured by direct fluorescence using a Biotek HTSynergy plate reader. For virus yield assays, Huh-7 cells were infected with wild-type EBOV for 1 h at 0.1 plaque-forming units (PFU) per cell. The virus inoculum was removed and replaced with 100 μl per well of media containing the appropriate dilution of compound. At 3 days post-infection, supernatants were collected, and the amount of virus was determined by endpoint dilution assay. Assays were conducted by preparing serial dilutions of the assay media and adding these dilutions to fresh Vero cell monolayers in 96-well plates to determine the tissue culture infectious dose that caused 50% cytopathic effects (TCID50). To measure levels of viral RNA from infected cells, total RNA was extracted using the MagMAX-96 Total RNA Isolation Kit and quantified using a quantitative reverse transcription polymerase chain reaction (qRT–PCR) assay with primers and probes specific for the EBOV nucleoprotein gene.

EBOV assay in HeLa and HFF-1 cells. Antiviral assays were conducted in BSL-4 at USAMRIID. HeLa or HFF-1 cells were seeded at 2,000 cells per well in 384-well plates. Ten serial dilutions of compound in triplicate were added directly to the cell cultures using the HP D300 digital dispenser (Hewlett Packard) in twofold dilution increments starting at 10μM at 2 h before infection. The DMSO concentration in each well was normalized to 1% using an HP D300 digital dispenser. The assay plates were transferred to the BSL-4 suite and infected with EBOV Kikwit at a multiplicity of infection of 0.5 PFU per cell for HeLa cells and with EBOV Makona at a multiplicity of infection of 5 PFU per cell for HFF-1 cells. The assay plates were incubated in a tissue culture incubator for 48 h. Infection was terminated by fixing the samples in 10% formalin solution for an additional 48 h before immune-staining, as described in Supplementary Table 1.

EBOV human Thrombogenic infection assay. Antiviral assays were conducted in BSL-4 at USAMRIID. Primary human macrophage cells were seeded in a 96-well plate at 40,000 cells per well. Eight to ten serial dilutions of compound in triplicate were added directly to the cell cultures using an HP D300 digital dispenser in threefold dilution increments 2 h before infection. The concentration of DMSO was normalized to 1% in all wells. The plates were transferred into the BSL-4 suite, and the cells were infected with 1 PFU per cell of EBOV in 100μl of media and incubated for 1 h. The inoculum was removed, and the media was replaced with fresh media containing diluted compounds. At 48 h post-infection, virus replication was quantified by immuno-staining, as described in Supplementary Table 1.

RSV A2 antiviral assay. For antiviral tests, compounds were threefold serially diluted in source plates from which 100μl of diluted compound was transferred to a 384-well cell culture plate using an Echo acoustic transfer apparatus. HEp-2 cells were added at a density of 5 × 104 cells per well, and infected by adding RSV A2 at a titer of 1 × 104 tissue culture infectious doses (TCID50) per ml. Immediately following virus addition, 20μl of the virus and cells mixture was added to the 384-well cell culture plates using aμFlow liquid dispenser and cultured for 4 days at 37°C. After incubation, the cells were allowed to equilibrate to 25°C for 30 min. The RSV-induced cytopathic effect was determined by adding 20μl of CellTiter-Glo Viability Reagent. After a 10-min incubation at 25°C, cell viability was determined by measuring luminescence using an Envision plate reader.

High content imaging assay detecting viral infection. Antiviral assays were conducted in 384- or 96-well plates in BSL-4 at USAMRIID using a high-content imaging system to quantify virus antigen production as a measure of virus infection. ‘No virus’ control and a ‘1% DMSO’ control were included to determine the percentage of infection for each well on the assay plates. The DMSO concentration in each well was normalized to 1% using an HP D300 digital dispenser. The assay plates were transferred to the BSL-4 suite and infected with 1PFU per cell MARV, which resulted in 50% to 70% of the cells expressing virus antigen in a 48-h period.

Sudan virus assay. HeLa cells were seeded at 2,000 cells per well in a 384-well plate, and compounds were added to the assay plates. Assay plates were transferred to the BSL-4 suite and infected with 0.08 PFU SUDV per cell, which resulted in 50% to 70% of the cells expressing virus antigen in a 48-h period.

Junin virus assay. HeLa cells were seeded at 2,000 cells per well in a 384-well plate, and compounds were added to the assay plates. Assay plates were transferred to the BSL-4 suite and infected with 0.3 PFU per cell JUNV, which resulted in >50% of the cells expressing virus antigen in a 48-h period.

Lassa fever virus assay. HeLa cells were seeded at 2,000 cells per well in a 384-well plate, and compounds were added to the assay plates. Assay plates were transferred to the BSL-4 suite and infected with 0.1 PFU per cell LASV, which resulted in >60% of the cells expressing virus antigen in a 48-h period.

Middle East respiratory syndrome virus assay. African green monkey (Chlorocebus sp.) kidney epithelial cells (Vero E6) were seeded at 4,000 cells per well in a 384-well plate and compounds were added to the assay plates. Assay plates were transferred to the BSL-4 suite and infected with 0.5 PFU per cell of MERS virus, which resulted in >70% of the cells expressing virus antigen in a 48-h period.

Chikungunya virus assay. U2OS cells were seeded at 3,000 cells per well in a 384-well plate, and compounds were added to the assay plates. Assay plates were transferred to the BSL-4 suite and infected with 0.5 PFU per cell CHIK, which resulted in >80% of the cells expressing virus antigen in a 48-h period.

Venezuelan equine encephalitis virus assay. HeLa cells were seeded at 4,000 cells per well in a 384-well plate, and compounds were added to the assay plates. Assay plates were transferred to the BSL-4 suite and infected with 0.1 PFU per cell VEEV, which resulted in >60% of the cells expressing virus antigen in a 20-h period.

Cytotoxicity assays. HEp-2 (1.5 × 105 cells per well) and MT-4 (2 × 105 cells per well) cells were plated in 384-well plates and incubated with the appropriate medium containing threefold serially diluted compound ranging from 15 nM to 100,000 nM. PC-3 cells (2.5 × 105 cells per well), HepG2 cells (4 × 105 cells per well), hepatocytes (1 × 106 cells per well), quiescent PBMCs (1 × 106 cells per well), stimulated PBMCs (2 × 105 cells per well), and RPTP cells (1 × 105 cells per well) were plated in 96-well plates and incubated with the appropriate medium containing threefold serially diluted compound ranging from 15 nM to 100,000 nM. Cells were cultured for 4–5 days at 37°C. Following the incubation, the cells were allowed to equilibrate to 25°C, and cell viability was determined by adding CellTiter-Glo viability reagent. The mixture was incubated for 10 min, and the luminescence signal was measured using a μFlow liquid dispenser and was not tested for mycoplasma as part of routine use in cytotoxicity assays.

In vitro RSV RNA synthesis assay. RNA synthesis by the RSV polymerase was reconstituted in vitro using purified RSV L/P complexes and an RNA oligonucleotide template (Dharmacon), representing nucleotides 1–14 of the RSV leader promoter3′-3′-1′-1′-3′ (5′-UCCGGCUUUUUACG-5′). RNA synthesis reactions were performed as described previously, except that the reaction mixture contained 250μM guanosine triphosphate (GTP), 10μM uridine triphosphate (UTP), 10μM cytidine triphosphate (CTP), supplemented with 10μCi [14C]GTP; and either included 10μM adenosine triphosphate (ATP) or no ATP. Under these conditions, 71% of the radioactivity was associated with the position 1 site of the promoter, but not the position 1 site. The NTP metabolite of GS-5734 was serially diluted in DMSO and included in each reaction mixture at concentrations of 10, 30, or 100μM as specified in Fig. 1f. RNA products were analysed by electrophoresis on a 25% polyacrylamide gel, containing 7 M urea, in Tris–taurine–EDTA buffer, and radiolabelled RNA products were detected by autoradiography.
RSV A2 polymerase inhibition assay. Transcription reactions contained 25 µg of crude RSV RNP complexes in 30 µL of reaction buffer (50 mM Tris-acetate (pH 8.0), 120 mM NaAcetate, 5% glyceral, 4.5 mM MgCl₂, 3 mM DTT, 2 mM EGTA, 50 µg ml⁻¹ BSA, 2.5 µR Na₂H₂¹⁴O, 20 µM ATP, 100 µM GTP, 100 µM UTP, 100 µM CTP, and 1.5 Gi (α³²P)ATP (3000 Ci mmol⁻¹)). The radiolabelled nucleotide used in the transcription assay was selected to match the nucleotide analogue being evaluated for inhibition of RSV RNP transcription.

To determine whether nucleotide analogues inhibited RSV RNP transcription, compounds were subjected to a five-step serial dilution. After a 90-min incubation at 30 °C, the RNP reactions were stopped with 350 µl of Qiagen RLT lysis buffer, and the RNA was purified using a Qiagen RNeasy 96 kit. Purified RNA was denatured in RNA sample loading buffer at 65 °C for 10 min and run on a 1.2% agarose/MOPS gel containing 2 mM formaldehyde. The agarose gel was dried, exposed to a Storm phosphorimaging screen, and developed using a Storm phosphorimagern.

Inhibition of human RNA polymerase II. For a 25 µl reaction mixture, 7.5 µl 1∶transcription buffer (20 mM HEPES (pH 7.2–7.5), 100 mM KCl, 0.2 mM EDTA, 0.5 mM DTT, 20% glycerol), 3 mM MgCl₂, 100 ng CMV positive or negative control DNA, and a mixture of ATP, GTP, CTP and UTP was pre-incubated with various concentrations (0–500 nM) of the inhibitor at 30 °C for 5 min. The mixture contained 5–25 µM (equal to Km) of the competing P-labelled ATP and 400 nM of GTP, UTP, and CTP. The reaction was started by addition of 3.5 µl of H2O and extract. After 1 h of incubation at 30 °C, the polymerase reaction was stopped by addition of 10.6 µl proteinase K mixture that contained final concentrations of 2.5 µg ml⁻¹ proteinase K, 5% SDS, and 25 mM EDTA. After incubation at 37 °C for 3–12 h, 10 µl of the reaction mixture was mixed with 10 µl of the loading dye (98% formamide, 0.1% xylene cyanol and 0.1% bromophenol blue), heated at 75 °C for 5 min, and loaded onto a 6% polyacrylamide gel (8 M urea) using a Waters Acquity ultra performance LC (Waters Corporation, Milford, MA, USA), a flow rate of 0.26 ml min⁻¹, and a gradient from Mobile phase A containing 0.2% formic acid in 99% water and 1% acetonitrile to mobile phase B containing 0.2% formic acid in 95% acetonitrile and 5% water over 4.5 min. For MS/MS analysis, we used a Waters Xevo TQ-S in positive multiple reaction monitoring mode using an electrospray probe. Plasma concentrations of GS-734, alanine metabolite and Nuc were determined using an 8-point calibration curve spanning a concentration range of over three orders of magnitude. Quality control samples were run at the beginning and end of the run to ensure accuracy and precision within 20%.

Intracellular metabolites in PBMCs were quantified by LC-MS/MS as described above for in vivo activation studies.
sequence of RNA-dependent RNA polymerase gene (L) of Ebola virus from all EBOV-RNA-positive rhesus monkey plasma samples obtained during the efficacy studies. Deep sequencing screening of the L gene was completed using previously described methods\textsuperscript{30,37}. Mutations and large subclonal events (≥10% of population) were reviewed for: non-synonymous substitutions in treated animals that succumbed to infection, any substitution enriched in treated populations regardless of survival status, and clusters of substitutions in any treated animal.

cDNA synthesis was performed using Superscript III First-Strand Synthesis System (Invitrogen). cDNA was amplified with Phusion Hot Start Flex DNA Polymerase (New England Biolabs) using overlapping 1,500-bp amplicons (primer information available upon request). After pooling and purification with AMPure XP Reagent (Beckman Coulter), PCR products were fragmented using the Covaris S2 instrument (Covaris). Libraries were prepared with the Illumina TruSeq DNA Sample Preparation kit (Illumina) on the Caliper SciclonG3 Liquid Handling Station (PerkinElmer). After measurement by real-time PCR with the KAPA qPCR Kit (Kapa Biosystems), libraries were diluted to 4 or 10 nM. Cluster amplification was performed on the Illumina cBot, and libraries were sequenced on the Illumina Nextseq or Illumina HiSeq 2500 using the 150 or 100 bp paired-end format. Viral assemblies were completed in DNAStar Lasergene nGen. Amplification primer removal, quality trimming, and trim-to-mer were performed on reads with a minimum similarity to the reference of 93% (four-base mismatch). A target depth of 1,200 is sought and single nucleotide polymorphisms at positions with fewer than 200 read depth were removed from the analysis. A consensus change was defined as a change relative to the deposited EBOV sequence (GenBank: AY354458) present in ≥50% of the population. Below that threshold, single nucleotide polymorphisms were considered subclonal substitutions and part of a minority subpopulation of the virus. Consensus sequence for the region covered in this screening is available in GenBank and the accession numbers are provided in Extended Data Table 5.

**Animal care.** Pharmacokinetic and radiolabelled tissue distribution studies in uninfected cynomolgus and rhesus macaques were conducted at Covance, Inc. Protocols were reviewed by an Institutional Animal Care and Use Committee (IACUC) at Covance. Efficacy experiments involving EBOV were performed in animal biosafety level 4 (ABSL-4) at USAMRIID. Research was conducted under an Institutional Animal Care and Use Committee approved protocol in compliance with the Animal Welfare Act, PHS Policy, and other federal statutes and regulations relating to animals and experiments involving animals. The facilities where this research was conducted are accredited by the Association for Assessment and Accreditation of Laboratory Animal Care, International and strictly adhere to principles stated in the Guide for the Care and Use of Laboratory Animals, National Research Council, 2011 (National Academies Press, Washington, DC).

**Statistics.** Combined vehicle group from part 1 and 2 (n = 6 animals total) was used as control group in all statistical comparisons of GS-5734 efficacy evaluations. The impact of GS-5734 treatment on the survival rates was estimated using Kaplan–Meier method and analysed by log-rank analysis using Dunnett–Hsu procedure to adjust for multiple comparisons. The effect on systemic viral RNA levels was assessed by analysis of variance (ANOVA), comparing each GS-5734 treatment group with vehicle group using Dunnett’s test to adjust for multiple comparisons. Wilcoxon rank-sum test without adjustment for multiple comparisons was used to compare the effects of GS-5734 treatment on haematology, coagulation, and clinical chemistry parameters. All data met the statistical assumptions of the test performed.

26. Uebelhoer, L. S. et al. High-throughput, luciferase-based reverse genetics systems for identifying inhibitors of Marburg and Ebola viruses. *Antiviral Res.* \textbf{106}, 86–94 (2014).
27. Towner, J. S. et al. Generation of eGFP expressing recombinant Zaire Ebolavirus for analysis of early pathogenesis events and high-throughput antiviral drug screening. *Virology* \textbf{332}, 20–27 (2005).
28. Shao, R. & Guo, X. Human microvascular endothelial cells immortalized with human telomerase catalytic protein: a model for the study of in vitro angiogenesis. *Biochem. Biophys. Res. Commun.* \textbf{321}, 788–794 (2004).
29. Mason, S. W. et al. Polyadenylation-dependent screening assay for respiratory syncytial virus RNA transcriptase activity and identification of an inhibitor. *Nucleic Acids Res.* \textbf{32}, 4758–4767 (2004).
30. Durand-Gasselin, L. et al. Nucleotide analogue prodrug tenofovir disoproxil enhances lymphoid cell loading following oral administration in monkeys. *Mol. Pharm.* \textbf{6}, 1145–1151 (2009).
31. Noton, S. L., Deflube, L. R., Tremaglio, C. Z. & Fears, R. The respiratory syncytial virus polymerase has multiple RNA synthesis activities at the promoter. *PloS Pathog.* \textbf{8}, e1002980 (2012).
32. Noton, S. L. et al. Respiratory syncytial virus inhibitor AZ-27 differentially inhibits different polymerase activities at the promoter. *J. Virol.* \textbf{89}, 7786–7798 (2015).
33. Tremaglio, C. Z., Noton, S. L., Deflube, L. R. & Fears, R. Respiratory syncytial virus polymerase can initiate transcription from position 3 of the leader promoter. *J. Virol.* \textbf{87}, 3196–3207 (2013).
34. Loderer, M. F. et al. Identification of multiple rate-limiting steps during the human mitochondrial transcription cycle in vitro. *J. Biol. Chem.* \textbf{285}, 16387–16402 (2010).
35. Warren, T. K. et al. Euthanasia assessment in Ebola virus infected nonhuman primates. *Viruses* \textbf{6}, 4666–4682 (2014).
36. Kugelman, J. R. et al. Emergence of Ebola Virus escape variants in infected nonhuman primates treated with the MB-003 antibody cocktail. *Cell Rep.* \textbf{12}, 2111–2120 (2015).
37. Kugelman, J. R. et al. Ebola virus genome plasticity as a marker of its passing history: a comparison of in vitro passing to non-human primate infection. *PLoS ONE* \textbf{7}, e50316 (2012).
Extended Data Figure 1 | Intracellular metabolism of GS-5734.

a, Intracellular metabolite profile in human macrophages. Following a 2-h pulse incubation (black bar at top of y axis) of human monocyte-derived macrophages with 1 μM GS-5734 (mean ± s.d., from three donors). GS-5734 is rapidly metabolized and not detected in cells. Transient exposure to the intermediate alanine metabolite (Ala-Met) is observed, followed by persistent Nuc exposure. The pharmacologically active NTP is formed quickly, achieving a maximum intracellular concentration at 4 h and persisted with a half-life of 16 ± 1 h in the three donors. Intracellular concentration was estimated on the basis of an intracellular volume of 1 pl per cell.

b, Efficiency of GS-5734 activation in human and rhesus cells in vitro. Intracellular NTP concentrations formed in human and rhesus PBMCs, monocytes, and monocyte-derived macrophages during a 2-h incubation with 1 μM GS-5734 (results are the mean ± s.d. of two (PBMC and monocyte) to six (macrophage) independent experiments performed with cells from different donors). Intracellular concentrations were estimated on the basis of a cell volume of 0.2 pl per cell for PBMCs and monocytes and 1 pl per cell for macrophages.

c, Intracellular NTP levels required for inhibition of EBOV replication in cell culture. The mixture of GS-5734 and its diastereomer on phosphorous was incubated continuously for 72 h at 1 μM and levels of intracellular NTP were determined (results are the average of duplicate incubations performed in each cell type; two independent studies were performed in HMVEC isolated from different donors). The corresponding EBOV EC_{50} values for the prodrug diastereomeric mixture were 100, 184, and 121 nM in human macrophages, HeLa, and HMVEC, respectively, suggesting that an average intracellular NTP concentration of approximately 5 μM is required for 50% inhibition of EBOV in vitro.
Extended Data Figure 2 | Inhibition of EBOV Makona replication by GS-5734. Huh-7 cells infected with wild-type EBOV (Makona) were incubated for 3 days in the presence of serial dilutions of GS-5734. The amount of infectious virus produced was quantified by endpoint dilution assay of culture media on fresh Vero cell monolayers and the tissue culture infectious dose that caused 50% infection (TCID$_{50}$) was determined. Independently, total RNA was extracted from infected cells and EBOV RNA levels were quantified using a nucleoprotein (NP) gene-specific qRT-PCR. Values represent mean ± s.d. of log$_{10}$-transformed values, $n$ = 4 replicates.
Extended Data Figure 3 | Homology model of RSV A2 (cyan) and EBOV (coral) polymerase based on HIV reverse transcriptase (PDB: 1RTD) with NTP (green and red representing the nucleoside and triphosphate portion, respectively).
Extended Data Figure 4 | Clinical signs of disease in individual rhesus monkeys exposed to Ebola virus. Animals were observed multiple times each day and were subjectively assigned a clinical disease score ranging from 0 to 5 based on responsiveness, posture, and activity. Maximum daily scores were converted to colour code, with darker colours indicative of more severe disease signs. The schematic was truncated to emphasize clinical scores during the acute disease phase, and none of the animals exhibited clinical disease signs outside of the times that are shown.
Extended Data Figure 5 | Amelioration of EVD clinical pathology by GS-5734 in rhesus monkeys. a–c, Group mean (n = 6 per group) values of d-dimer (a), activated partial thromboplastin time (b), and lipase (c). Black (open symbols), vehicle; red, 3 mg kg⁻¹ d0; green, 3 mg kg⁻¹ d2; blue, 10/3 mg kg⁻¹ d2; orange, 10/3 mg kg⁻¹ d3; black (closed symbols), 10 mg kg⁻¹ d3. Error bars omitted for clarity; x axes truncated at day 15. *P < 0.05 for comparison of mean change from d0 of vehicle and 10 mg kg⁻¹ d3 groups at day 7.
### Extended Data Table 1 | *In vitro* cytotoxicity of GS-5734 and Nuc in human cell lines and primary cells

|                | CC\(_{50}\) (µM)* |            |            |
|----------------|--------------------|------------|------------|
|                | GS-5734            | Nuc        | Puromycin  |
| **Human cell lines** |                    |            |            |
| HEp-2          | 6.0 ± 1.5          | > 100      | 0.53 ± 0.10|
| HepG2          | 3.7 ± 0.2          | > 100      | 0.73 ± 0.01|
| PC-3           | 8.9 ± 1.6          | > 100      | 0.52 ± 0.11|
| MT-4           | 1.7 ± 0.4          | 69.3 ± 25.7| 0.12 ± 0.03|
| **Human primary cells** |                |            |            |
| Hepatocytes    | 2.5 ± 0.6          | > 100      | 1.5 ± 0.6  |
| Renal proximal tubular epithelial cells (RPTEC) | 12.9 ± 6.2 | > 100 | 1.1 ± 0.3 |
| Quiescent PBMCs| > 20               | > 100      | 6.8 ± 1.4  |
| Stimulated PBMCs| 14.8 ± 5.8 | > 100      | 1.6 ± 0.2  |

*Drug concentrations reducing the cell viability by 50% (CC\(_{50}\)) are presented. All CC\(_{50}\) values represent the mean ± s.d. of at least two independent experiments. Puromycin was included in experiments as a positive control for cytotoxicity.*
## Extended Data Table 2 | Individual plasma viral RNA (log_{10} (copies ml^{-1}))

| Treatment Description | Animal # | 0 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 | 12 | 14 | 18 | 21/22 | 28/29 |
|-----------------------|----------|---|---|---|---|---|---|---|---|---|----|----|----|----|-------|-------|
| **Vehicle**           |          |   |   |   |   |   |   |   |   |   |    |    |    |    |       |       |
| 1                     | ND       | ND | ND | ND | 6.6| 9.0| –  | 10.0| –  | 9.5|     |    |    |    |       |       |
| 2                     | ND       | ND | ND | ND | 5.9| 8.9| –  | 9.8 |   |    |     |    |    |    |       |       |
| 3                     | ND       | ND | ND | ND | 6.5| 8.2| 8.5| –  | 8.6|    |    |    |    |    |       |       |
| 4                     | ND       | ND | ND | DET| 6.6| 8.4| –  | 8.4 | –  | 8.1|    |    |    |    |       |       |
| 5                     | ND       | ND | ND | 5.8| 8.8| 10.0| 10.3|   |    |    |    |    |    |    |       |       |
| 6                     | ND       | ND | ND | 5.4| 7.4| 9.4| –  | 9.2 | 8.7|    |    |    |    |    |       |       |
| **GS-5734 3 mg/kg D0**|          |   |   |   |   |   |   |   |   |   |    |    |    |    |       |       |
| 1                     | ND       | ND | ND | ND | DET| 6.0| –  | 7.3 | 7.4 | 7.3|    |    |    |    |       |       |
| 2                     | ND       | ND | ND | ND | 4.9| –  | 6.8 | –  | 9.9|    |    |    |    |    |       |       |
| 3                     | ND       | ND | ND | ND | DET| 5.5| –  | 6.5 | 5.8 | –  | DET| ND | ND | ND |       |       |
| 4                     | ND       | ND | ND | ND | 4.9| –  | 5.8 | 5.6 | –  | DET| ND | ND | ND | ND |       |       |
| 5                     | ND       | ND | ND | DET| 5.7| –  | 9.1 | –  | 9.1 | 8.6|    |    |    |    |       |       |
| 6                     | ND       | ND | DET| 7.2| –  | 9.3 | –  |    |    |    |    |    |    |    |       |       |
| **GS-5734 3 mg/kg D2**|          |   |   |   |   |   |   |   |   |   |    |    |    |    |       |       |
| 1                     | ND       | ND | ND | ND | DET| 6.0| –  | 6.6 | 6.8 | 6.9 | 6.0 | –  | ND | ND |       |       |
| 2                     | ND       | ND | ND | ND | DET| 6.4| 7.6 | 7.1 | 6.8 | –  | DET| ND | ND | ND |       |       |
| 3                     | ND       | ND | ND | ND | DET| 6.6| –  | 7.5 | 8.2 | 5.2 | ND | –  | ND | ND |       |       |
| 4                     | ND       | ND | ND | ND | 5.1| 7.0 | 7.4 | 6.6 | –  |    |    |    |    |    |       |       |
| 5                     | ND       | ND | ND | DET| 5.5| –  | 6.7 | 5.9 | –  | ND | ND | –  | ND | ND |       |       |
| 6                     | ND       | ND | ND | DET| 5.5| –  | 6.9 | 8.1 | –  |    |    |    |    |    |    |       |       |
| **GS-5734 10/3 mg/kg D2**|        |   |   |   |   |   |   |   |   |   |    |    |    |    |       |       |
| 1                     | ND       | DET| 5.2| 5.8| 7.9 | –  | 8.2 | –  | 8.0|    |    |    |    |    |       |       |
| 2                     | ND       | ND | ND | ND | DET| –  | DET | ND | ND | ND | –  | ND | ND | ND |       |       |
| 3                     | ND       | ND | ND | ND | DET| 6.1| –  | 7.8 | 7.5|    |    |    |    |    |       |       |
| 4                     | ND       | ND | ND | ND | DET| 6.1| –  | DET | ND | ND | ND | –  | ND | ND |       |       |
| 5                     | ND       | ND | ND | ND | DET| 8.2 | –  | 8.2 | –  |    |    |    |    |    |    |       |       |
| 6                     | ND       | ND | ND | 5.3| 7.1 | –  | 6.9 | 8.1 | –  |    |    |    |    |    |    |       |       |
| **GS-5734 10/3 mg/kg D3**|        |   |   |   |   |   |   |   |   |   |    |    |    |    |       |       |
| 1                     | ND       | ND | DET| 5.4| 6.5 | –  | 6.5 | 5.0 | –  | ND | ND | –  | ND | ND |       |       |
| 2                     | ND       | ND | ND | 5.3| 6.2 | 7.1 | –  | 6.8 | 6.0 | –  | ND | ND | ND | ND |       |       |
| 3                     | ND       | ND | ND | DET| 5.1| 6.9 | 7.0 | 7.0 | 6.7 | ND | ND | –  | ND | ND |       |       |
| 4                     | ND       | ND | ND | DET| 7.0 | –  | 8.1 | 8.3 | 6.2 | DET | ND | ND | ND | ND |       |       |
| 5                     | ND       | ND | ND | DET| –  | ND | 5.5 | ND | ND | ND | ND | –  | ND | ND |       |       |
| 6                     | ND       | ND | DET| 5.8 | 6.6 | –  | 6.2 | 6.8 | –  | ND | ND | –  | ND | ND |       |       |
| **GS-5734 10 mg/kg D3**|        |   |   |   |   |   |   |   |   |   |    |    |    |    |       |       |
| 1                     | ND       | ND | DET| 5.7| 6.1 | –  | 6.0 | DET | ND | ND | ND | –  | ND | ND |       |       |
| 2                     | ND       | ND | ND | DET| DET | –  | 5.6 | –  | ND | ND | ND | –  | ND | ND |       |       |
| 3                     | ND       | ND | ND | 6.7 | DET | –  | ND | –  | ND | ND | ND | –  | ND | ND |       |       |
| 4                     | ND       | ND | ND | ND | DET| –  | ND | –  | ND | ND | ND | –  | ND | ND |       |       |
| 5                     | ND       | ND | ND | ND | ND | –  | ND | –  | ND | ND | ND | –  | ND | ND |       |       |
| 6                     | ND       | ND | DET| 5.6 | 6.9 | –  | 5.5 | 5.9 | –  | ND | ND | –  | ND | ND |       |       |

*Sample not collected (days 6, 8, 10, and 18 were unscheduled samplings of succumbed animals only). DET, detectable, but below the lower limit of quantitation \((8.0 \times 10^4 \text{ copies ml}^{-1})\); ND, not detected, that is, below limit of detection.*

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| Day | Vehicle | GS-5734 3 mg/kg D0 | GS-5734 3 mg/kg D2 | GS-5734 10/3 mg/kg D2 | GS-5734 10/3 mg/kg D3 | GS-5734 10 mg/kg D3 |
|-----|---------|----------------------|-------------------|-----------------------|-----------------------|---------------------|
| 3   | 4.77    | 3.32 (0.019)         | 3.32 (0.020)      | 3.36 (0.023)          | 4.33 (0.454)          | 3.63 (0.062)        |
| 4   | 7.24    | 4.66 (0.001)         | 5.52 (0.024)      | 4.49 (0.001)          | 5.06 (0.005)          | 4.81 (0.002)        |
| 5   | 9.05    | 6.04 (<0.001)        | 6.82 (0.002)      | 6.07 (<0.001)         | 6.52 (0.001)          | 5.12 (<0.001)       |
| 7   | 9.19    | 7.09 (0.013)         | 7.24 (0.015)      | 7.00 (0.007)          | 6.28 (0.001)          | 4.24 (<0.001)       |
| 9   | 8.76    | 7.55 (0.351)         | 6.82 (0.132)      | 6.30 (0.065)          | 6.42 (0.072)          | 4.22 (0.001)        |
| 12  | –       | 4.90 (NA)            | 5.05 (NA)         | 3.00 (NA)             | 4.14 (NA)             | 3.00 (NA)           |

*P values are from ANOVA comparing each GS-5734 treatment group with vehicle group using Dunnett’s test to adjust for multiple comparisons (n = 6 animals per group, PCR sample assays performed in triplicate). EBOV RNA values reported as ‘<LOD’ were substituted as 10^3 RNA copies ml^-1, and values reported as ‘>LOD, <LLOQ’ were substituted as LLOQ of 8.0 × 10^4 RNA copies ml^-1 for computation purpose. Statistically significant P values (P < 0.05) are highlighted in bold. NA, not applicable, owing to no survivors in vehicle group.
Extended Data Table 4 | Statistical summary of selected clinical pathology parameters

| Parameter                  | Vehicle 3 mg/kg | GS-5734 3 mg/kg | GS-5734 10 mg/kg |
|----------------------------|-----------------|-----------------|-----------------|
| Platelet count (10^3/μL)   | -279            | -118 (0.012)    | -202 (0.055)    |
| PT (sec)                   | 5.0             | 1.3 (0.01)      | 3.2 (0.27)      |
| APTT (sec)                 | 47.7            | 12.6 (0.012)    | 19.3 (0.014)    |
| Fibrinogen (mg/dL)         | 2.5             | -4.7 (0.012)    | -5.5 (0.008)    |
| TT (sec)                   | 50.2            | -1.4 (0.012)    | 2.6 (0.008)     |
| Anthrobin (%)              | -39.6           | -6.1 (0.012)    | -10.3 (0.008)   |
| D-dimer (mg/dL)            | 1.15            | 0.13 (0.012)    | -0.09 (0.008)   |
| ALT (U/L)                  | 340             | 14 (0.012)      | 24 (0.008)      |
| AST (U/L)                  | 1425            | 273 (0.014)     | 206 (0.014)     |
| ALP (U/L)                  | 1238            | -69 (0.012)     | -74 (0.008)     |
| CRK (U/L)                  | 5420            | 1277 (0.020)    | 1002 (0.014)    |
| GGT (U/L)                  | 146             | -12 (0.012)     | -13 (0.008)     |
| LDH (U/L)                  | 8391            | 1006 (0.020)    | 2263 (0.014)    |
| Bilirubin (mg/dL)          | 1.3             | 0 (0.071)       | 0 (0.048)       |
| BUN (mg/dL)                | 60              | 0 (0.021)       | 1 (0.028)       |
| Creatinine (mg/dL)         | 1.80            | 0.12 (0.015)    | 0.27 (0.017)    |
| Lipase (U/L)               | 205             | 17 (0.14)       | 34 (0.12)       |
| Triglycerides (mg/dL)      | 538             | -7 (0.012)      | 52 (0.008)      |
| CRP (mg/dL)                | 48.6            | 48.8 (0.83)     | 43.8 (1.0)      |
| Albumin (g/dL)             | -1.5            | -0.8 (0.012)    | -1.2 (0.170)    |
| Total protein (mg/dL)      | -1.1            | -0.5 (0.034)    | -0.9 (0.27)     |
| Chloride (mEq/dL)          | -14             | -3 (0.011)      | -5 (0.008)      |
| Phosphate (mEq/dL)         | 0.2             | -2.1 (0.036)    | -2.6 (0.021)    |
| Sodium (mg/dL)             | -17             | -8 (0.019)      | -10 (0.042)     |

*Wilcoxon rank-sum test without adjustment for multiple comparisons using a combined vehicle group as a control group for the analysis (n = 6 animals per group). Statistically significant P values (P < 0.05) are highlighted in bold.

ALP, alkaline phosphatase; ALT, alanine aminotransferase; APTT, activated partial thromboplastin time; AST, aspartate aminotransferase; BUN, blood urea nitrogen; CRK, creatine kinase; CRP, C-reactive protein; GGT, gamma glutamyl transferase; LDH, lactate dehydrogenase; PT, prothrombin time; TT, thrombin time.
Extended Data Table 5 | L gene deep sequencing screening sample description and metrics

| Treatment Description | Animal # | Day | Survival Outcome | Genbank Accession Number | % L Gene Coverage | Polymerase Amino Acid and Codon Changes | % of Population with Change | ISNV Description |
|-----------------------|----------|-----|------------------|--------------------------|-------------------|----------------------------------------|----------------------------|------------------|
| Vehicle               | 6        | 7   | Deceased         | KU321182                 | 100.0             | W (TGG) @191 (T+G)                     | 21.9                       | Volatile sub clonal substitution observed in most samples in all treatment groups and controls >2% of population. Causes a frameshift insertion at the end of a large homopolymer. |
| 3 mg/kg D0            | 2        | 7   | Deceased         | KU321189                 | 100.0             | E (GAA) @2173 E (GAg)                  | 99.5                       | Synonymous substitution unlikely selection pressure. |
| 3 mg/kg D0            | 6        | 5   | Deceased         | KU321084                 | 100.0             | G (GGT) @1160 G (GGc)                  | 44.5                       | Synonymous substitution unlikely selection pressure. |
| 3 mg/kg D2            | 1        | 7   | Survived         | KU321152                 | 100.0             | W (TGG) @191 (T+G)                     | 21.8                       | Volatile sub clonal substitution observed in most samples in all treatment groups and controls >2% of population. Causes a frameshift insertion at the end of a large homopolymer. |
|                       |          |     |                  |                          |                   | Q (CAA) @805 (+AA)                     | 26.6                       | Volatile sub clonal substitution observed in most samples in all treatment groups and controls >2% of population. Causes a frameshift insertion at the end of a large homopolymer. |
| 3 mg/kg D2            | 1        | 9   | Survived         | KU321162                 | 100.0             | W (TGG) @191 (T+G)                     | 13.8                       | Volatile sub clonal substitution observed in most samples in all treatment groups and controls >2% of population. Causes a frameshift insertion at the end of a large homopolymer. |
| 3 mg/kg D2            | 3        | 5   | Survived         | KU321165                 | 100.0             | W (TGG) @191 (T+G)                     | 23.4                       | Volatile sub clonal substitution observed in most samples in all treatment groups and controls >2% of population. Causes a frameshift insertion at the end of a large homopolymer. |
|                       |          |     |                  |                          |                   | Q (CAA) @1755 Q (CAG)                  | 99.0                       | Synonymous mutation unlikely selection pressure. |
| 3 mg/kg D2            | 4        | 9   | Deceased         | KU321088                 | 100.0             | W (TGG) @191 (T+G)                     | 13.9                       | Volatile sub clonal substitution observed in most samples in all treatment groups and controls >2% of population. Causes a frameshift insertion at the end of a large homopolymer. |
| 10/3 mg/kg D2         | 3        | 5   | Deceased         | KU321098                 | 81.2              | —                                      | 24.2                       | Non-coding substitution, unlikely selection pressure. |
| 10/3 mg/kg D3         | 2        | 9   | Survived         | KU321149                 | 100.0             | K (AAG) @341 K (Aaa)                   | 26.7                       | Synonymous substitution unlikely selection pressure. |
|                       |          |     |                  |                          |                   | I (ATC) @348 S (AgC)                   | 22.3                       | Non-synonymous substitution tolerated by survivor. |
| 10/3 mg/kg D3         | 3        | 7   | Survived         | KU321172                 | 99.3              | K (AAA) @1387 K (Aag)                  | 28.3                       | Synonymous substitution unlikely selection pressure. |
|                       |          |     |                  |                          |                   | F (TTT) @1827 (TT-)                   | 20.6                       | Indel causes a frameshift deletion at the end of a large homopolymer region. |
| 10 mg/kg D3           | 1        | 5   | Survived         | KU321154                 | 86.5              | K (AAG) @659 N (AAI)                   | 28.0                       | Non-synonymous substitution tolerated by survivor. |
|                       |          |     |                  |                          |                   | Q (CAA) @805 (+AA)                     | 40.2                       | Volatile sub clonal substitution observed in most samples in all treatment groups and controls >2% of population. Causes a frameshift insertion at the end of a large homopolymer. |

Genomic sequence analysis was conducted on all samples containing quantifiable concentrations of viral RNA (that is, >LOD as assessed by quantitative real-time PCR).
Sequences for which no change (defined as >2% of the population) was noted from the reference sequence are not shown.
ISNV, intra-host Single Nucleotide Variant.