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Therapeutic Efficacy of the Small Molecule GS-5734 against Ebola Virus in Rhesus Monkeys

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Summary

The most recent Ebola virus outbreak in West Africa – 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 sequelae[1]. No antiviral therapeutics have yet received regulatory approval or demonstrated clinical efficacy. Here we describe the discovery of a novel anti-EBOV small molecule antiviral, GS-5734, a monophosphoramidate prodrug of an adenosine analog. GS-5734 exhibits antiviral activity against multiple variants of EBOV 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 alternate substrate and RNA-chain terminator in primer-extension assays utilizing a surrogate respiratory syncytial virus RNA polymerase. Intravenous administration of GS-5734 to nonhuman primates resulted in persistent

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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.

Disclaimers:

Opinions, interpretations, conclusions, and recommendations are those of the authors and are not necessarily endorsed by the U.S. Army or the Centers for Disease Control and Prevention, U.S. Department of Health and Human Services. Research was conducted under an IACUC 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 facility where this research was conducted is accredited by the Association for Assessment and Accreditation of Laboratory Animal Care, International and adheres to principles stated in the Guide for the Care and Use of Laboratory Animals, National Research Council, 2011.

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

Author Contributions: R.L.M., D.S., H.C.H., E.D., S.N., E.C., M.O.C., L.Z., W.L., B.S., Q.W., K.C., and L.W. were responsible for the synthesis, characterization, and scale-up of small molecules. T.K.W. designed and supervised activities associated with efficacy evaluations, and interpreted study results. J.W., K.S.S., N.L.G., G.D., S.A.V.T., and J.E.N. conducted in vivo efficacy studies and performed associated sample analyses. A.C.S., L.S.W., and L.G. coordinated efficacy study activities. M.K.L., M.F., L.K.M., designed and executed the initial in vitro antiviral testing against EBOV and analyzed data. V.S., C.J.R., D.N.G., and T.K. and B.P.E. designed and executed cell-based infection assays and analyzed these data. E.G. conducted quantitative PCR analysis. D.K.N. and C.L.W. performed anatomic pathology examinations and analyses of all nonhuman primate subjects. E.R.N., J.R.K., and G.P. conducted viral genomic sequence analyses. N.L., I.T., and R.S. developed and tested drug formulations. A.S.R., D.B., Y.P., and K.M.S. designed and executed the PK and metabolism studies and summarized results. M.P., O.B., M.R.B., and R.F. designed and conducted biochemical enzymatic assays. K.M.S., J.F., and Y.X. conducted cell based assays for cytotoxicity. P.W. conducted statistical analysis and S.S.C. oversaw the analysis. A.S.R., R.J., R.L.M., V.S., R.B., S.S., D.L.M., C.F.S., S.T.N., W.L., T.C., and S.B. designed experiments, evaluated results, and provided project oversight. T.K.W., A.S.R., R.J., D.S., M.P., and T.C. outlined and wrote the manuscript.

The authors from Gilead Sciences are employees of the company and may own company stock.

NTP levels in peripheral blood mononuclear cells (half-life = 14 h) and distribution to sanctuary sites for viral replication including testes, eye, and brain. In a rhesus monkey model of EVD, once daily intravenous administration of 10 mg/kg 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 of six treated animals. These results provide 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 expanded indications. GS-5734 is amenable to large-scale manufacturing, and clinical studies investigating the drug safety and pharmacokinetics are ongoing.

The most recent outbreak of Ebola virus disease (EVD) in West Africa, was the far largest and most complex Ebola virus (EBOV) outbreak in the recorded history of the disease with >28,000 EVD cases and >11,000 reported deaths[1]. Medical infrastructures in Guinea, Sierra Leone, and Liberia were seriously impacted by a loss of >500 healthcare workers[1]. Additionally, EVD-related sequelae (joint and muscle pain, as well as neurological, ophthalmic, and other symptoms) together with viral persistence and recrudescence in individuals who survived the acute disease have been documented[2–5].

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 – have caused outbreaks with high fatality rates[6]. Although the efficacy of various experimental small molecules and biologics have been assessed in multiple clinical trials during the West African outbreak[7–18], 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 favorable 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 viruses [19]. The mechanism of action of Nuc requires intracellular anabolism to the active triphosphate metabolite (NTP), which is expected to interfere with the activity of viral RNA-dependent RNA-polymerases (RdRp). Structurally, the 1′-cyano group provides potency and selectivity towards viral RNA polymerases, but because of slow first phosphorylation kinetics, modification of parent nucleosides with monophosphate promoieties have potential to greatly enhance intracellular NTP concentrations[20]. GS-5734, the single *Sp* isomer of the 2-ethylbutyl L-alaninate phosphosphoramidate prodrug (Supplementary Information), effectively bypasses the rate-limiting first phosphorylation step of the Nuc (Fig. 1a). In human monocyte-derived macrophages, incubation with GS-5734 rapidly loads cells with high levels of NTP that persist with $T_{1/2}$ = 24 h following 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 EC_{50} values of 0.06 to 0.14 μM (Table 1). As expected, the parent Nuc was less active with EC_{50} 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 the Nuc inhibited replication of other human RNA viral pathogens including respiratory syncytial virus, Junin virus, Lassa fever virus, and Middle East respiratory syndrome virus, but was inactive against alphaviruses or retroviruses (Table 1). Prior studies have reported activity of the Nuc against flaviviruses, parainfluenza type 3, and severe acute respiratory syndrome associated coronavirus but little or no activity against West Nile virus, influenza A, or Coxsackie A[19,21]. The antiviral activity of GS-5734 was selective as demonstrated by low cytotoxicity in a wide range of human primary cells and cell lines (Extended Data Table 1).

Isolation and expression of EBOV RdRp has been elusive, but the computational analysis of the catalytic palm subdomain demonstrated high sequence and structure homology with RSV RdRp[22] (Fig. 1d and Extended Data Fig. 3). Consistent with the proposed mechanism of action, NTP inhibited RSV RdRp-catalyzed 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[23]. 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 somewhat 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[24,25], 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 dose in rhesus monkeys, GS-5734 exhibits a short plasma half-life ($t_{1/2}$ = 0.39 h) with fast systemic elimination followed by the sequential appearance of transient systemic levels of the key intracellular intermediate alanine metabolite (Ala-Met) and more persistent levels of Nuc (Fig. 2a). GS-5734 is rapidly distributed into peripheral blood mononuclear cells (PBMCs), and efficient conversion to NTP is apparent within 2 h of dose administration. In PBMCs, NTP represents the predominant metabolite and is persistent with a $t_{1/2}$ = 14 h and levels required for >50% virus inhibition for 24 h (Fig. 2a; Extended Data Fig. 1c). In cynomolgus monkeys, intravenous administration of a 10 mg/kg dose of [^{14}C]GS-5734 demonstrated that the drug-derived material distributed to testes, epididymis, eyes, and brain within 4 h of administration (Fig. 2b). In brain, levels at 4 h were low relative to other tissues but

remained detectable above the drug plasma levels 168 h post dose. 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 regimen of vehicle (n = 3) or 3 mg/kg GS-5734 beginning on day 0 (D0; 30–90 min following virus challenge) or Day 2 (D2) (n = 6/treatment group). Regardless of the time of initiation, GS-5734 treatment conferred improved survival, 33% (2/6) in the 3 mg/kg D0 group and 66% (4/6) in the 3 mg/kg D2 group, and an antiviral effect by reducing systemic viremia relative to vehicle (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 was sub-optimal. In part 2 of the efficacy study, GS-5734 was administered once at a loading dose of 10 mg/kg followed by once-daily 3 mg/kg doses beginning either 2 days (10/3 mg/kg D2) or 3 days (10/3 mg/kg D3) after virus exposure, or 10 mg/kg doses were administered beginning 3 days after virus exposure (10 mg/kg D3) with n = 6/group. All animals (12/12) 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 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 ≥ 1.7 $\log_{10}$ 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 geometric mean viral RNA concentration of the vehicle group exceeded 10^9 copies/mL, viral RNA was detected at levels less than the lower limit of quantitation (8×10^4 RNA copies/mL) in 4 of 6 animals in the 10 mg/kg D3 group. Deep sequencing analysis of the EBOV RdRp (*L*) gene from all viral RNA-positive plasma samples showed no evidence of genotypic change(s) potentially associated with the emergence of GS-5734-resistant EBOV variants (Extended Data Table 5). The 10 mg/kg D3 GS-5734 regimen was associated with amelioration of EVD-related clinical disease signs (Fig. 2g; Extended Data Fig. 4) and markers of coagulopathy and end organ pathophysiology (Fig. 3a–f, Extended Data Table 4 and Extended Data Fig. 5). Although greater survival was observed in the 10/3 mg/kg D3 group than the 10/3 mg/kg D2 group, survival and viral RNA load were not statistically distinguishable (Fig. 2e) and likely represent natural endpoint variation associated with suboptimal therapeutic effect.

In summary, GS-5734 is a potent and selective inhibitor of EBOV in multiple relevant permissive cell types. In an NHP model of clinical EVD, intravenous administration of GS-5734 results in rapid accumulation and persistence of intracellular NTP. Pronounced antiviral effects, amelioration of EVD signs, and significant survival benefit was achieved in EBOV-infected NHPs despite treatment initiation on day 3, a time when systemic viral RNA was detectable. These results represent the first case of substantive postexposure protection against EVD by a small-molecule antiviral compound in NHPs. Intravenous GS-5734 is currently being evaluated in multiple dose studies in healthy human volunteers to assess clinical safety and pharmacokinetics, and to help determine whether GS-5734 may provide therapeutic benefit in acute 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 significant human viral pathogens for which no treatment is available.

METHODS

Small Molecules

GS-5734, Nuc, and NTP were synthesized at Gilead Sciences, Inc. and chemical identity, sample purity were established using NMR, HRMS, and HPLC analysis (Supplementary Information). The radiolabeled analogue [^{14}C]GS-5734 (specific activity 58.0 mCi/mmol) was obtained from Moravek Biochemicals (Brea, CA) and was prepared in a similar manner described for GS-5734 using [^{14}C]trimethylsilylcyanide (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, Ci67), 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 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 (CDC)[26,27].

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 units/mL penicillin and streptomycin. PC-3 cells were cultured in Kaighn's F12 media supplemented with 10% FBS and 100 units/mL 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 units/mL 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 units/mL penicillin and streptomycin, and 2 mM L-glutamine. The Huh-7 cell line was obtained from Dr. Charles M. Rice at the Rockefeller

University and cultured in DMEM supplemented with 10% FBS, 100 units/mL penicillin and streptomycin, and 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 alcohol consumption. Upon delivery, the cells were allowed to recover for 24 h in complete medium with supplement provided by the vendor at 37 °C. Human peripheral blood mononuclear cells (PBMCs) were isolated from human buffy coats obtained from healthy volunteers (Stanford Medical School Blood Center, Palo Alto, CA) and maintained in RPMI-1640 with GlutaMAX™ supplemented with 10% FBS, 100 units/mL penicillin and streptomycin. Rhesus fresh whole blood was obtained from Valley Biosystems (Sacramento, CA). PBMCs were isolated from whole blood by Ficoll-Hypaque density gradient centrifugation. Briefly, blood was overlaid on 15 mL Ficoll-Paque™ (GE Healthcare Bio-Sciences AB, Piscataway, NJ), and centrifuged at 500 × g for 20 minutes. The top layer containing platelets and plasma was removed, and the middle layer containing PBMCs was transferred to a fresh tube, diluted with Tris Buffered Saline up to 50 mL, and centrifuged at 500 × g for 5 minutes. The supernatant was removed and the cell pellet was resuspended in 5 mL red blood cell lysis buffer (155 mM ammonium chloride, 10 mM potassium bicarbonate, 0.1 mM EDTA, pH 7.5). To generate stimulated PBMCs, freshly isolated quiescent PBMCs were seeded into a T-150 cm2 tissue culture flask containing fresh medium supplemented with 10 units/mL of recombinant human interleukin-2 (IL-2) and 1 μg/mL phytohemagglutinin-P at a density of 2×10^6 cells/mL and incubated for 72 h at 37°C. Human macrophage cultures were isolated from PBMCs that were purified by Ficoll gradient centrifugation from 50 mL of blood from healthy human volunteers. PBMCs were cultured for 7 to 8 days in in RPMI cell culture media supplemented with 10% FBS, 5 to 50 ng/mL granulocyte-macrophage colony-stimulating factor (GM-CSF) and 50 μM β-mercaptoethanol (BME) to induce 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 RenaLife complete medium in a T75 flask for 3 to 4 days before seeding into 96-well assay plates. Immortalized human microvascular endothelial cells (HMVEC-TERT) were obtained from Dr. Rong Shao at the Pioneer Valley Life Sciences Institute[28]. HMVEC-TERT cells were cultured in endothelial basal media supplemented with 10% FBS, 5 μg of epithelial growth factor, 0.5 mg hydrocortisone, and gentamycin/amphotericin-B.

Enzymes

RNA POLII was purchased as part of the "HeLaScribe® Nuclear Extract *in vitro* Transcription System" kit from Promega. The recombinant human POLRMT 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 Mason *et al.*[29].

Intracellular metabolism studies

The intracellular metabolism of GS-5734 was assessed in different cell types (HMVEC, HeLa, and primary human and rhesus PBMC, 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 or human were incubated for 2 h in compound containing media followed by removal, washing with 37°C drug-free media, and incubated for an additional 22 h in media not containing GS-5734. Human monocyte derived macrophages, HeLa and HMVEC were grown to confluence (approximately 0.5, 0.2, and 1.2×10^6 cells/well, respectively) in 500 μL of media in 12 well tissue culture plates. Monocyte and PBMC were incubated in suspension (approximately 1×10^6 cells/mL) in 1 mL of media in micro centrifuge tubes.

For adherent cells (HMVEC, HeLa, and monocyte derived macrophages), media was removed at select time points from duplicate wells, cells washed twice with 2 mL of ice cold 0.9% normal saline. For non-adherent cells (monocytes and PBMC), duplicate incubation were centrifuged at 5,000 rpm for 30 seconds to remove media. The cell pellets were re-suspended with 500 μL cell culture media (RPMI with 10% FBS) and layered on top of a 500 μL oil layer (Nyosil M25; Nye Lubricants, Fairhaven, MA) in a microcentrifuge tube. Samples were then centrifuged at room temperature at 13,000 rpm for 45 seconds. The media layer was removed and the oil layer was washed twice with 500 μL water. The oil layer was then carefully removed using a Pasteur pipet attached to vacuum. A volume of 0.5 mL of 70% methanol containing 100 nM of the analytical internal standard 2-chloro-adenosine-5′-triphosphate (Sigma-Aldrich, St. Louis, MO) was added to isolated cells. Samples were stored overnight at −20°C to facilitate extraction, centrifuged at 15,000 × *g* for 15 minutes and then supernatant was transferred to clean tubes for drying in a MiVac Duo concentrator (Genevac, Gardiner, NY). Dried samples were then reconstituted in mobile phase A containing 3 mM ammonium formate (pH5.0) with 10 mM dimethylhexylamine (DMH) in water for analysis by liquid chromatography coupled to triple quadrupole mass spectrometry (LC-MS/MS).

LC-MS/MS was done using low flow, ion pairing chromatography similar to methods described previously[30]. Briefly, analytes were separated using a 50 × 2 mm × 2.5 μm Luna C18(2) HST column (Phenomenex, Torrance, CA, USA) connected to a LC-20ADXR (Shimadzu, Columbia, MD) ternary pump system and HTS PAL autosampler (LEAP Technologies, Carrboro, NC). 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 over 8 minutes at a flow rate of 150 μL/min was used to separate analytes. Detection was performed on an API 4000 (Applied Biosystems, Foster City, CA) MS/MS operating in positive ion and multiple reaction monitoring modes. Intracellular metabolites Ala-Met, Nuc, NMP, NDP, and NTP were quantified using 7 point standard curves ranging from 0.274 to 200 pmol (approximately 0.5 to 400 μM) prepared in cell extract from untreated cells. Levels of adenosine nucleotides were also quantified to assure dephosphorylation had not taken place during sample collection and preparation. In order to

calculate intracellular concentration of metabolites, the total number of cells per sample were counted using a Countess automated cell counter (Invitrogen, Carlsbad, CA).

EBOV Huh-7 and HMVEC antiviral assay

Antiviral assays were conducted in biosafety level-4 containment (BSL-4) at the CDC. 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. Ten concentrations of compound were diluted in 4-fold serial dilution increments in media, and 100 μL per well of each dilution was transferred in duplicate (Huh-7) or quadruplicate (HMVEC-TERT) onto 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 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 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 quantified by endpoint dilution assay. The endpoint dilution assay was 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 ($TCID_{50}$). 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, Palo Alto, CA) in 2-fold dilution increments starting at 10 μM at 2 h prior to 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 prior to immune-staining, as described in Supplementary Table 1.

EBOV human macrophage 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 3-fold dilution increments 2 h prior to 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 100 μL 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 3-fold serially diluted in source plates from which 100 nL of diluted compound was transferred to a 384-well cell culture plate using an Echo acoustic transfer apparatus. HEp-2 cells at a density of 5×10^5 cells/mL were then infected by adding RSV A2 at a titer of $1 \times 10^{4.5}$ tissue culture infectious doses ($TCID_{50}$)/mL. Immediately following virus addition, 20 μL of the virus/cell mixture was added to the 384-well cell culture plates using a μFlow liquid dispenser and cultured for 4 days at 37°C. After incubation, the cells were allowed to equilibrate to 25°C for 30 minutes. The RSV-induced cytopathic effect was determined by adding 20 μL of CellTiter-Glo™ Viability Reagent. After a 10-minute 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. A “no virus” control and a “1% DMSO” control were included to determine the 0% and 100% virus infection, respectively. The primary and secondary antibodies and dyes used for nuclear and cytoplasmic staining are listed in Supplementary Table 1. The primary antibody specific for a particular viral protein was diluted 1,000-fold in blocking buffer (1× PBS with 3% BSA) and added to each well of the assay plate. The assay plates were incubated for 60 minutes at room temperature. The primary antibody was removed, and the cells were washed 3 times with 1× PBS. The secondary detection antibody was an anti-mouse (or rabbit) IgG conjugated with Dylight488 (Thermo Fisher Scientific, Waltham, MA, Cat. No. 405310). The secondary antibody was diluted 1,000-fold in blocking buffer and was added to each well in the assay plate. Assay plates were incubated for 60 minutes at room temperature. Nuclei were stained using Draq5 (Biostatus, Shepshed Leicestershire, UK) or 33342 Hoechst (ThermoFisher Scientific) for Vero and HFF-1 cell lines. Both dyes were diluted in 1× PBS. The cytoplasm of HFF-1 (EBOV assay) and Vero E6 (MERS assay) cells were counter-stained with CellMask™ Deep Red (Thermo Fisher Scientific, Waltham, MA). Cell images were acquired using a Perkin Elmer Opera confocal plate reader (Perkin Elmer, Waltham, MA) using 10× air objective to collect five images per well. Virus-specific antigen was quantified by measuring fluorescence emission at a 488 nm wavelength and the stained nuclei were quantified by measuring fluorescence emission at a 640 nm wavelength. Acquired images were analyzed using Harmony and Acapella PE software. The Draq5 signal was used to generate a nuclei mask to define each nuclei in the image for quantification of cell number. The CellMask Deep Red dye was used to demarcate the Vero and HFF-1 cell borders for cell-number quantitation. The viral-antigen signal was compartmentalized within the cell mask. Cells that exhibited antigen signal higher than the selected threshold were counted as positive for viral infection. The ratio of virus positive

cells to total number of analyzed cells was used to determine the percent infection for each well on the assay plates. Effect of compounds on the viral infection was assessed as percent inhibition of infection in comparison to control wells. The resultant cell number and percent infection were normalized for each assay plate. The Z′ values for all antiviral assays were >0.3. Analysis of dose response curve was performed using GeneData Screener software applying Levenberg-Marquardt algorithm (LMA) for curve fitting strategy. The curve-fitting process, including individual data point exclusion were pre-specified by default software settings. R^2-value quantified goodness of fit and fitting strategy was considered acceptable at $R^2 > 0.8$.

Marburg virus assay

HeLa cells were seeded at 2,000 cells/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 1 pfu per cell MARV, which resulted in 50% to 70% of the cells expressing virus antigen in a 48-h period. Virus infection was quantified by immuno-staining using antibodies that recognized the viral glycoproteins as described in Supplementary Table 1.

Sudan virus assay

HeLa cells were seeded at 2,000 cells/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. Virus infection was quantified by immuno-staining using antibodies that recognized the viral glycoproteins as described in Supplementary Table 1.

Junin virus assay

HeLa cells were seeded at 2,000 cells/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. Virus infection was quantified by immuno-staining using antibodies that recognized the viral glycoproteins as described in Supplementary Table 1.

Lassa fever virus assay

HeLa cells were seeded at 2,000 cells/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. Virus infection was quantified by immuno-staining using antibodies that recognized the viral glycoproteins as described in Supplementary Table 1.

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. Virus

infection was quantified by immuno-staining using antibodies that recognized the viral glycoproteins as described in Supplementary Table 1.

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 of CHIK, which resulted in >80% of the cells expressing virus antigen in a 48-h period. Virus infection was quantified by immuno-staining using antibodies that recognized the viral glycoproteins as described in Supplementary Table 1.

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. Virus infection was quantified by immuno-staining using antibodies that recognized the viral glycoproteins as described in Supplementary Table 1.

Cytotoxicity assays

HEp-2 (1.5×10^3 cells/well) and MT-4 (2×10^3 cells/well) cells were plated in 384-well plates and incubated with the appropriate medium containing 3-fold serially diluted compound ranging from 15 nM to 100,000 nM. PC-3 (2.5×10^3 cells/well), HepG2 (4×10^3 cells/well), hepatocytes (1×10^6 cells/well), quiescent PBMCs (1×10^6 cells/well), stimulated PBMCs (2×10^5 cells/well), and RPTEC (1×10^3 cells/well) cells were plated in 96-well plates and incubated with the appropriate medium containing 3-fold 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 Cell-Titer Glo viability reagent. The mixture was incubated for 10 minutes, and the luminescence signal was quantified using an Envision plate reader. Cell lines were not authenticated and were 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 promoter (3′-UGCGCUUUUUUACG-5′)[31–33]. RNA synthesis reactions were performed as described previously, except that the reaction mixture contained 250 μM rGTP, 10 μM rUTP, 10 μM rCTP, supplemented with 10 μCi [α^{32}P] CTP, and either included 10 μM rATP or no ATP. Under these conditions, the polymerase is able to initiate synthesis from the position 3 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 100 μM as specified in Fig 1f. RNA products were analyzed by electrophoresis on a 25% polyacrylamide gel, containing 7M 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 potassium acetate, 5% glycerol, 4.5 mM $MgCl_2$, 3 mM DTT, 2 mM EGTA, 50 μg/mL BSA, 2.5 U RNasin, 20 μM ATP, 100 μM GTP, 100 μM UTP, 100 μM CTP, and 1.5 μCi [α-^{32}P]ATP [3,000 Ci/mmol]). The radiolabeled nucleotide used in the transcription assay was selected to match the nucleotide analog being evaluated for inhibition of RSV RNP transcription.

To determine whether nucleotide analogs inhibited RSV RNP transcription, compounds were added using a 6-step serial dilution in 5-fold increments. After a 90-minute 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 minutes and run on a 1.2% agarose/MOPS gel containing 2M formaldehyde. The agarose gel was dried, exposed to a Storm phosphorimaging screen, and developed using a Storm phosphorimager.

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 $MgCl_2$, 100 ng CMV-(+) control DNA, and a natural mixture of NTPs were pre-incubated with various concentrations (0 μM to 500 μM) of the inhibitor at 30°C for 5 minutes. The natural mixture of NTPs contained 5–25 μM (equal to *K*m) of the competing ^{33}P-labeled NTP and 400 μM of the other three NTPs. The reaction was started by addition of 3.5 μL of HeLa+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/μL 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 minutes, and loaded onto a 6% polyacrylamide gel (8 M urea). The gel was dried for 45 minutes at 70°C and exposed to a phosphorimager screen. The full length product, 363 nucleotide runoff RNA, was quantified using a Typhoon Trio Imager and Image Quant TL Software.

Inhibition of human mitochondrial RNA polymerase

Twenty nanomolar POLRMT was incubated with 20 nM template plasmid (pUC18-LSP) containing POLRMT light-strand promoter region and transcription factors mtTFA (100 nM) and mtTFB2 (20 nM) in buffer containing 10 mM HEPES (pH 7.5), 20 mM NaCl, 10 mM DTT, 0.1 mg/mL BSA, and 10 mM $MgCl_2$[34]. The reaction mixture was pre-incubated to 32°C, and the reactions were initiated by addition of 2.5 μM of each of the natural NTPs and 1.5 μCi of ^{32}P-GTP. After incubation for 30 minutes at 32°C, reactions were spotted on DE81 paper and quantified.

Molecular modeling

A homology model of RSVA2 and EBOV polymerases were built using the HIV-RT X-ray crystal structure (PDB:1RTD). (Schrödinger Release 2015-1: Prime, version 3.9, Schrödinger, LLC, New York, NY, 2015, default settings with subsequent rigid body

minimization and side chain optimization. Loop insertions not in 1RTD of greater than 10 amino acids were not built).

Quantitative real-time PCR for in vivo studies

For quantitative assessment of viral RNA nonhuman primate plasma samples, whole blood was collected using a K3 EDTA Greiner Vacuette tube (or equivalent) and sample centrifuged at 2500 (± 200) RCF for 10 ± 2 min. To inactivate virus, plasma was treated with 3 parts (300 μL) TriReagent LS and samples were transferred to frozen storage (−60°C to −90°C), until removal for RNA extraction. Carrier RNA and QuantiFast High Concentration Internal Control (Qiagen) were spiked into the sample prior to extraction, conducted according to manufacturer's instructions. The viral RNA was eluted in AVE Buffer. Each extracted RNA sample was tested with the QuantiFast Internal Control RT-PCR RNA Assay (Qiagen) to evaluate the yield of the spiked-in QuantiFast High Concentration Internal Control. If the internal control amplified within manufacturer-designated ranges, further quantitative analysis of the viral target was performed. RT-PCR was conducted using an ABI 7500 Fast Dx using primers specific to EBOV glycoprotein. Samples were run in triplicate using a 5 μL template volume. For quantitative assessments, the average of the triplicate genomic equivalents (ge) per reaction were determined and multiplied by 800 to obtain ge/mL of plasma. Standard curves were generated using synthetic RNA. The limits of quantification for this assay are $8.0 \times 10^4 – 8.0 \times 10^{10}$ ge/mL of plasma. Acceptance criteria for positive template control (PTC), negative template control (NTC), negative extraction control (NEC), and positive extraction control (PEC) are SOP-specified. For qualitative assessments, the limit of detection (LOD) was defined as Ct 38.07, based on method validation testing. An animal was considered to have tested positive for detection of EBOV RNA when a minimum of 2 of 3 replicates were designated as "positive" and PTC, NTC, and NEC controls meet specified method-acceptance criteria. A sample was designated as "positive" when the Ct value was <LOD Ct.

Pharmacokinetic evaluations

Three uninfected male rhesus monkeys (*Macaca maniculata*) were used for the pharmacokinetic study. GS-5734 was formulated in solution at 5 mg/mL with 12% sulfobutylether-β-cyclodextrin in water, pH 3.5–4.0, and 2 mL/kg was administered by slow bolus (approximately 1 min) for a final dose of 10 mg/kg. Blood samples for plasma and PBMC were collected from a femoral vein/artery and were taken from each monkey over a 24-h period. Plasma samples were obtained at predose and at 0.083, 0.25, 0.5, 1, 2, 4, 8, and 24 h postdose. PBMC samples were obtained at 2, 4, 8, and 24 h. Blood samples for plasma were collected into chilled collection tubes containing sodium fluoride/potassium oxalate (NaF/K-Ox) as the anticoagulant and were immediately placed on wet ice, followed by centrifugation to obtain plasma. Blood samples for PBMC isolation were collected at room temperature into CPT vacutainer tubes containing sodium heparin for isolation. Plasma and isolated PBMC samples were frozen immediately and stored at ≤60°C until analyzed.

For plasma analysis, an aliquot of 25 μL of each plasma sample was treated with 100 μL of 90% methanol and acetonitrile mixture (1:1, v:v) and 10% water with 20 nM 5-(2-aminopropyl)indole as an internal standard. One hundred microliters of samples were

filtered through an Agilent Captiva 96 well 0.2 μm filter plate. Filtered samples were dried down completely for approximately 20 minutes and reconstituted with 1% acetonitrile and 99% water with 0.01% formic acid. An aliquot of 10 μL was injected for LC-MS/MS using a HTC Pal autosampler. Analytes were separated on a Phenomenex Synergi Hydro-RP 30A column (75 × 2.0 mm, 4.0 μm) using a Waters Acquity ultra performance LC (Waters Corporation, Milford, MA, USA), a flow rate of 0.26 mL/min, 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 minutes. MS/MS analysis used a Waters Xevo TQ-S in positive multiple reaction monitoring mode using an electrospray probe. Plasma concentrations of GS-734, Ala-Met and Nuc were determined using an 8-point calibration curve spanning a concentration range of over 3 orders of magnitude. Quality control samples run at the beginning and end of the run to ensure accuracy and precision within 20%. Intracellular metabolites in PBMC were quantified by LC-MS/MS as described above for in vitro activation studies.

Radiolabeled tissue distribution

Six cynomolgus monkeys were administered a single dose of [^{14}C]GS-5734 at 10 mg/kg (25 μCi/kg) by IV administration (slow bolus). Tissues were collected from 3 animals at 4 and 168 h post dose. The tissues were excised, rinsed with saline, blotted dry, weighed, and placed on wet ice. Tissues (testes, epididymis, eyes and brain; following homogenization) and plasma were analyzed by liquid scintillation counting. Concentrations were converted to ng equivalents of GS-5734 per gram of sample.

In vivo efficacy

Rhesus monkeys (*Macaca mulatta*) were challenged on day 0 by intramuscular injection with a target dose of 1000 pfu of EBOV-Kikwit (Ebola virus H.sapiens-tc/COD/1995/Kikwit), which was derived from a clinical specimen obtained during an outbreak occurring in The Democratic Republic of the Congo (formerly Zaire) in 1995. Challenge virus was propagated from the clinical specimen using cultured cells (Vero or Vero E6) for a total of four passages. Animals (3–6 years) were randomly assigned to experimental treatment groups, stratified by sex (with equal number of males and females per group) and balanced by body weight, using SAS® statistical software (Cary, North Carolina, USA). Study personnel responsible for assessing animal health (including euthanasia assessment) and administering treatments were experimentally blinded to group assignment of animals. The primary endpoint for efficacy studies was survival to day 28 following virus challenge. GS-5734 was formulated at Gilead Sciences in water with 12% sulfobutylether-β-cyclodextrin (SBE-β-CD), pH adjusted to 3.0 using HCl. Formulations were administered to anesthetized animals by bolus intravenous injection at a rate of approximately 1 min/dose in the right or left saphenous vein. The volume of all vehicle or GS-5734 injections was 2.0 mL/kg body weight. Animals were anesthetized using IM injection of a solution containing ketamine (100 mg/mL) and acepromazine (10 mg/mL) at 0.1 mL/kg body weight.

Animals were observed at least twice daily to monitor for disease signs, and animals that survived to day 28 were deemed to be protected. Study personnel alleviated unnecessary suffering of infected animals by euthanizing clinically moribund animals. The criteria used

as the basis for euthanasia of moribund animals were defined prior to study initiation and included magnitude of responsiveness, reduced body temperature, and/or specified alterations to serum chemistry parameters[35]. Serum chemistry was analyzed using a Vitros 350 Chemistry System (Ortho Clinical Diagnostics), and coagulation parameters were evaluated using a Sysmex CA-1500 coagulation analyzer (Siemens Healthcare Diagnostics). Hematology analysis was conducted using a Siemens Advia 120 Hematology System with multispecies software (Siemens Healthcare Diagnostics). On days in which GS-5734 or vehicle dosing were scheduled with blood sample collection for clinical pathology or viremia analysis, blood samples were collected immediately prior to dose administration.

Viral Genomic Sequence Analysis

Analysis of viral genomic sequence was conducted with the purpose of evaluating genomic sequence change patterns consistent with development of resistance against GS-5734. Attempts were made to obtain 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[36,37]. Mutations and large sub clonal 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 Invitrogen's Superscript III First-Strand Synthesis System. cDNA was amplified with Phusion Hot Start Flex DNA Polymerase (New England Biolabs) using overlapping 1,500-kb 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 SciCloneG3 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 1200 is sought and SNPs 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, SNPs were considered sub-clonal 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

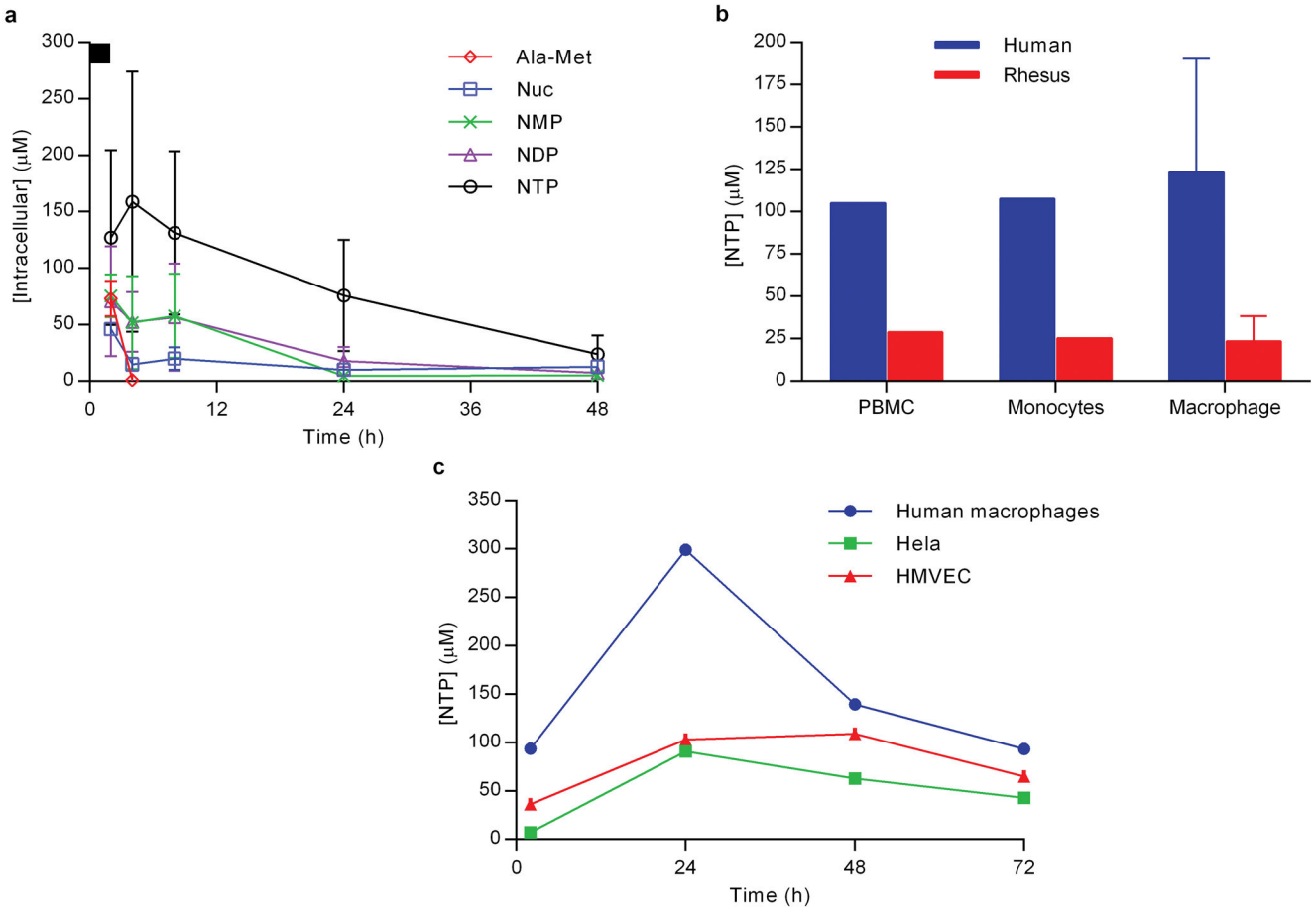
Pharmacokinetic and radiolabeled tissue distribution studies in uninfected cynomolgus and rhesus macaques were conducted at Covance, Inc. (Madison, WI). Protocols were reviewed by an Institutional Animal Care and Use Committee (IACUC) at Covance. Efficacy

experiments involving EBOV were performed in 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. The impact of GS-5734 treatment on the survival rates was estimated using Kaplan-Meier method and analyzed by log-rank analysis using Dunnett-Hsu procedure to adjust for multiple comparisons. The effect on systemic viral RNA levels was assessed by the 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 hematology, coagulation and clinical chemistry parameters. All data met the statistical assumptions of the test performed.

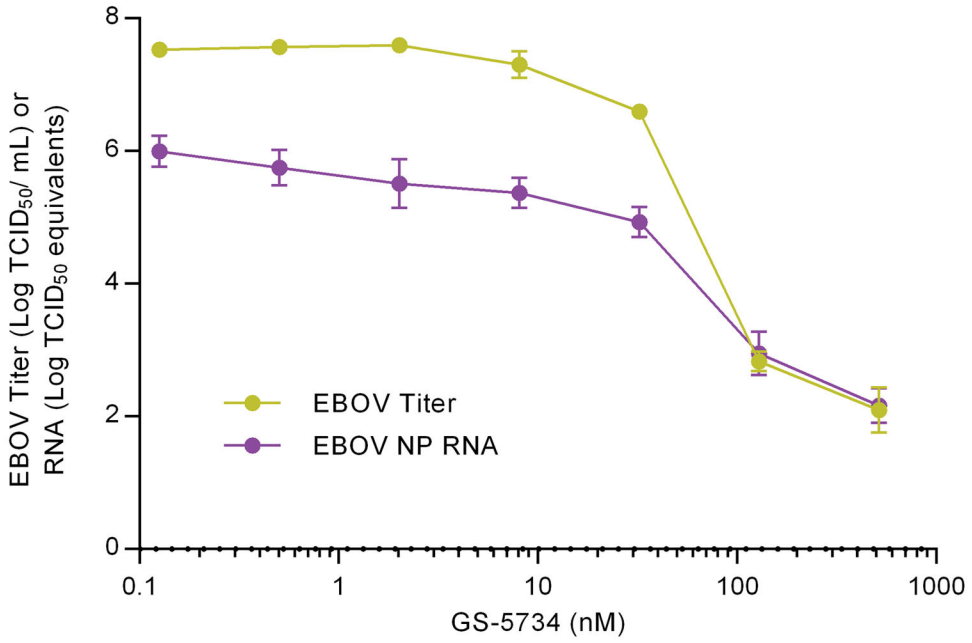
Extended Data



Extended Data Figure 1.

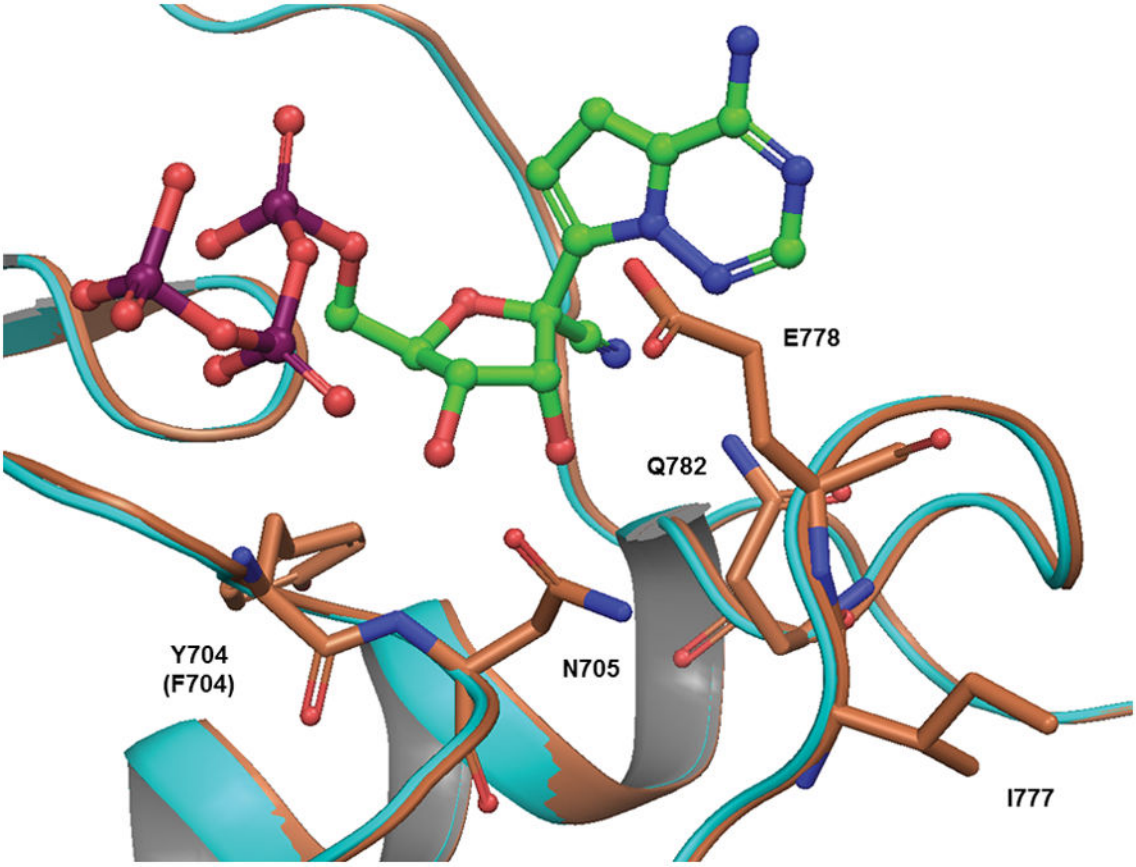
a, Intracellular metabolite profile in human macrophages. Following a 2-h pulse incubation (black bar at top of y-axis) of human monocyte-derived macrophages with 1 μM GS-5734 (mean ± s.d, from three donors.). GS-5734 is rapidly metabolized and not detected in cells. Transient exposure to the intermediate metabolite Ala-Met is observed followed by persistent Nuc and nucleotide analog exposure. The pharmacologically active NTP is formed quickly achieving a C_{max} at 4 h and persisted with a half-life of 16 ± 1 h in the three donors. Intracellular concentration estimated based on an intracellular volume of 1 pL/cell. **b,** Efficiency of GS-5734 activation in human and rhesus cells in vitro. Intracellular NTP concentrations formed in human and rhesus PBMC, monocytes, and monocyte-derived macrophages during a 2-h incubation with 1 μM GS-5734 (results are the mean ± SD of two (PBMC and monocyte) to six (macrophage) independent experiments done in cells from different donors). Intracellular concentrations estimated based on a cell volume of 0.2 pL/cell for PBMC and monocytes and 1 pL/cell for macrophage. **c,** Intracellular NTP levels required for inhibition of EBOV replication in cell culture. The diastereomeric mixture at phosphorous containing GS-5734 was incubated continuously for 72 h at 1 μM and levels of intracellular NTP were determined (results are the average of duplicate incubations done in

each cell type; two independent studies were done 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.

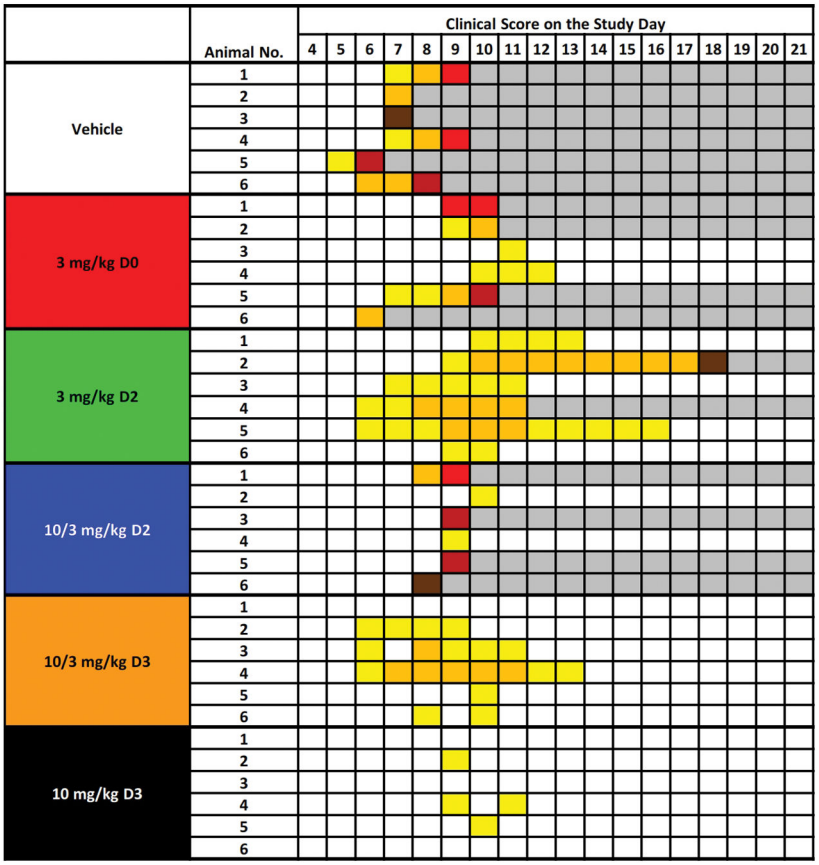


Extended Data Figure 2. Virus yield assay

Huh-7 cells seeded in 96-well plates were infected with wild-type EBOV (Makona) for 1 h at 0.1 plaque forming unit (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 quantified by endpoint dilution assay. The endpoint dilution assay was 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% infection (TCID50). To measure levels of viral RNA from infected cells, total RNA was extracted using MagMAX™-96 Total RNA Isolation Kit (Invitrogen, Carlsbad, CA) and quantified using a quantitative reverse transcription polymerase chain reaction (qRT-PCR) assay with primers and probes specific for the EBOV nucleoprotein gene. Values represent mean ± SD of log_{10}-transformed values, n=4 replicates.

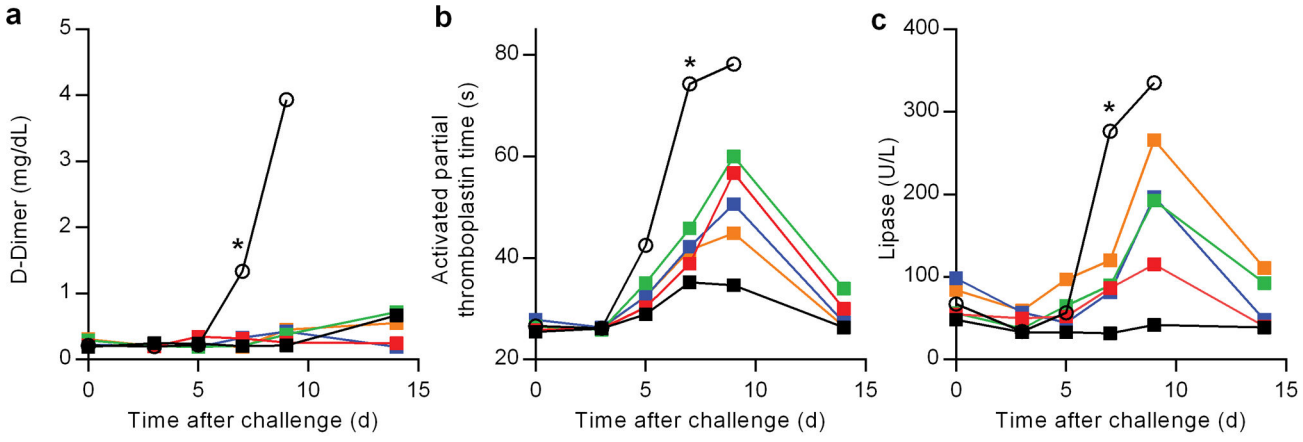


Extended Data Figure 3.
Homology model of respiratory syncytial virus (RSV) A2 (Cyan) and EBOV (coral) polymerase based on HIV-RT (PDB:1RTD) with NTP (green).



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 color code, with darker colors 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. Group mean (n=6/group) values of d-dimer (**a**), activated partial thromboplastin time (**b**), and lipase (**c**). Vehicle (black, open symbols), 3 mg/kg D0 (red), 3 mg/kg D2 (green), 10/3 mg/kg D2 (blue), 10/3 mg/kg D3(orange), 10 mg/kg D3 (black, closed symbols). Error bars omitted for clarity; x-axes truncated at day 15. *P<0.05 for comparison of mean change from Day 0 of vehicle and 10 mg/kg 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 ± SD of at least 2 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)]

		Plasma Viral RNA on Study Day														
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	6.6	9.0	–	10.0	–	9.5						
	2	ND	ND	ND	5.9	8.9	–	9.8								
	3	ND	ND	6.5	8.2	8.5	–	8.6								
	4	ND	ND	DET	6.6	8.4	–	8.4	–	8.1						
	5	ND	ND	5.8	8.8	10.0	10.3									
	6	ND	ND	5.4	7.4	9.4	–	9.2	8.7							
GS-5734 3 mg/kg D0																
	1	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	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
	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																

		Plasma Viral RNA on Study Day														
Treatment Description	**Animal #**	**0**	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**12**	**14**	**18**	**21/22**	**28/29**
	1	ND	ND	ND	DET	6.0	–	6.6	–	6.8	–	6.9	6.0	–	ND	ND
	2	ND	ND	ND	6.4	7.6	–	7.1	–	6.8	–	DET	ND	ND		
	3	ND	ND	ND	DET	6.6	–	7.5	–	8.2	–	5.2	ND	–	ND	ND
	4	ND	ND	ND	5.1	7.0	–	7.4	–	6.6	–					
	5	ND	ND	4.9	6.9	8.1	–	8.1	–	6.6	–	5.2	ND	–	ND	ND
	6	ND	ND	ND	DET	5.5	–	6.7	–	5.9	–	ND	ND	–	ND	ND
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
	3	ND	ND	ND	DET	6.1	–	7.8	–	7.5						
	4	ND	ND	ND	4.9	5.5	–	6.1	–	DET	–	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	5.3	6.2	7.1	–	6.8	–	6.0	–	ND	ND	–	ND	ND
	3	ND	ND	DET	5.1	6.9	–	7.0	–	7.0	–	6.7	ND	–	ND	ND
	4	ND	ND	ND	DET	7.0	–	8.1	–	8.3	–	6.2	DET	–	ND	ND
	5	ND	ND	ND	ND	DET	–	ND	–	5.5	–	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
	2	ND	ND	ND	DET	DET	–	DET	–	5.6	–	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); D, day; DET, detectable, but below the lower limit of quantitation (LLOQ, 8.0×10^4 copies/mL); ND, not detected, ie below limit of detection (LOD).

Extended Data Table 3

Summary and statistical analysis of plasma viral RNA

	Plasma Viral RNA, mean $\log_{10}$ copies/mL (P value*)					
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)

NA, not applicable due to no survivors in vehicle group.

*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/group, PCR sample assays performed in triplicate). EBOV RNA values reported as "<LOD" were substituted as 10^3 RNA copies/mL and values reported as ">LOD, <LLOQ" were substituted as LLOQ of 8.0×10^4 RNA copies/mL for computation purpose.

Statistically significant P-values (P < 0.05) are highlighted in bold.

Extended Data Table 4

Statistical summary of selected clinical pathology parameters

	Mean Change from Baseline, Day 7 (P value compared with Group 1+4*)					
Parameter	**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**
Platelet count (10^3/μL)	−279	−118 (**0.012**)	−202 (**0.055**)	−155 (0.055)	−98 (**0.014**)	−65 (**0.008**)
PT (sec)	5.0	1.3 (**0.01**)	3.2 (**0.27**)	1.6 (0.06)	2.5 (**0.02**)	1.7 (**0.01**)
APTT (sec)	47.7	12.6 (**0.012**)	19.3 (**0.014**)	14.3 (**0.008**)	15.2 (**0.008**)	9.8 (**0.008**)
Fibrinogen (mg/dL)	2.5	−4.7 (**0.012**)	−5.5 (**0.008**)	−5.0 (**0.014**)	−5.0 (**0.014**)	−4.4 (**0.008**)
TT (sec)	50.2	−1.4 (**0.012**)	2.6 (**0.008**)	1.4 (**0.008**)	3.6 (**0.008**)	−1.5 (**0.008**)
Antithrombin (%)	−39.6	−6.1 (**0.012**)	−10.3 (**0.008**)	−7.9 (**0.008**)	5.6 (**0.008**)	3.1 (**0.008**)
D-dimer (mg/dL)	1.15	0.13 (**0.012**)	−0.09 (**0.008**)	0.11 (**0.008**)	−0.12 (**0.005**)	0.02 (**0.007**)
ALT (U/L)	340	14 (**0.012**)	24 (**0.008**)	116 (0.083)	28 (**0.008**)	32 (**0.008**)
AST (U/L)	1425	273 (**0.014**)	206 (**0.014**)	90 (**0.020**)	157 (**0.014**)	36 (**0.014**)
ALP (U/L)	1238	−69 (**0.012**)	−74 (**0.008**)	7 (**0.008**)	8 (**0.008**)	−66 (**0.008**)
CRK (U/L)	5420	1277 (**0.020**)	1002 (**0.014**)	841 (**0.014**)	682 (**0.014**)	96 (**0.014**)
GGT (U/L)	146	−12 (**0.012**)	−13 (**0.008**)	−2 (**0.008**)	1 (**0.008**)	−12 (**0.008**)
LDH (U/L)	8391	1006 (**0.020**)	2263 (**0.014**)	2358 (**0.014**)	2439 (**0.014**)	352 (**0.014**)
Bilirubin (mg/dL)	1.3	0 (0.071)	0 (**0.048**)	0 (**0.048**)	0 (**0.048**)	0 (**0.048**)
BUN (mg/dL)	60	0 (**0.021**)	1 (**0.028**)	2 (**0.036**)	5 (0.055)	1 (**0.021**)
Creatinine (mg/dL)	1.80	0.12 (**0.015**)	0.27 (**0.017**)	0.18 (**0.014**)	0.27 (**0.014**)	0.43 (0.066)
Lipase (U/L)	205	17 (0.14)	34 (0.12)	−17 (0.055)	36 (0.12)	−12 (**0.036**)
Triglycerides (mg/dL)	538	−7 (**0.012**)	52 (**0.008**)	63 (**0.008**)	420 (0.083)	−6 (**0.008**)
CRP (mg/dL)	48.6	48.8 (0.83)	43.8 (1.0)	41.2 (0.31)	35.3 (0.24)	13.2 (**0.008**)
Albumin (g/dL)	−1.5	−0.8 (**0.012**)	−1.2 (0.170)	−0.8 (**0.036**)	−0.8 (**0.022**)	−0.7 (**0.008**)
Total protein (mg/dL)	−1.1	−0.5 (**0.034**)	−0.9 (0.27)	−0.6 (0.17)	−0.4 (**0.035**)	−0.4 (**0.008**)
Chloride (mEq/dL)	−14	−3 (**0.011**)	−5 (**0.008**)	−6 (**0.013**)	−6 (0.067)	0 (**0.008**)
Phosphate (mEq/dL)	0.2	−2.1 (**0.036**)	−2.5 (**0.021**)	−1.5 (0.12)	−1.0 (0.65)	−0.3 (0.93)
Sodium (mg/dL)	−17	−8 (**0.019**)	−10 (**0.042**)	−9 (0.054)	−7 (**0.042**)	−5 (**0.014**)

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; D, day; GGT, gamma glutamyl transferase; LDH, lactate dehydrogenase; PT, prothrombin time; TT, thrombin time.

*Wilcoxon rank-sum test without adjustment for multiple comparisons using combined Group 1+4 as control group for the analysis (n=6 animals/group). Statistically significant P-values (P < 0.05) are highlighted in bold.

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 frameshifting 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 frameshifting 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 frameshifting 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 frameshifting 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 frameshifting 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 Description	Animal #	Day	Survival Outcome	Genbank Accession Number	% *L* Gene Coverage	Polymerase Amino Acid and Codon Changes	% of Population with Change	iSNV Description
								treatment groups and controls >2% of population. Causes a frameshifting 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 frameshifting deletion at the end of a large homopolymer region.
10 mg/kg D3	1	5	Survived	KU321154	86.5	K (AAG) @659 N (AAt)	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 frameshifting insertion at the end of a large homopolymer.

iSNV, intra-host Single Nucleotide Variant.

Genomic sequence analysis was conducted on all samples containing quantifiable concentrations of viral RNA (i.e. >LLOQ 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.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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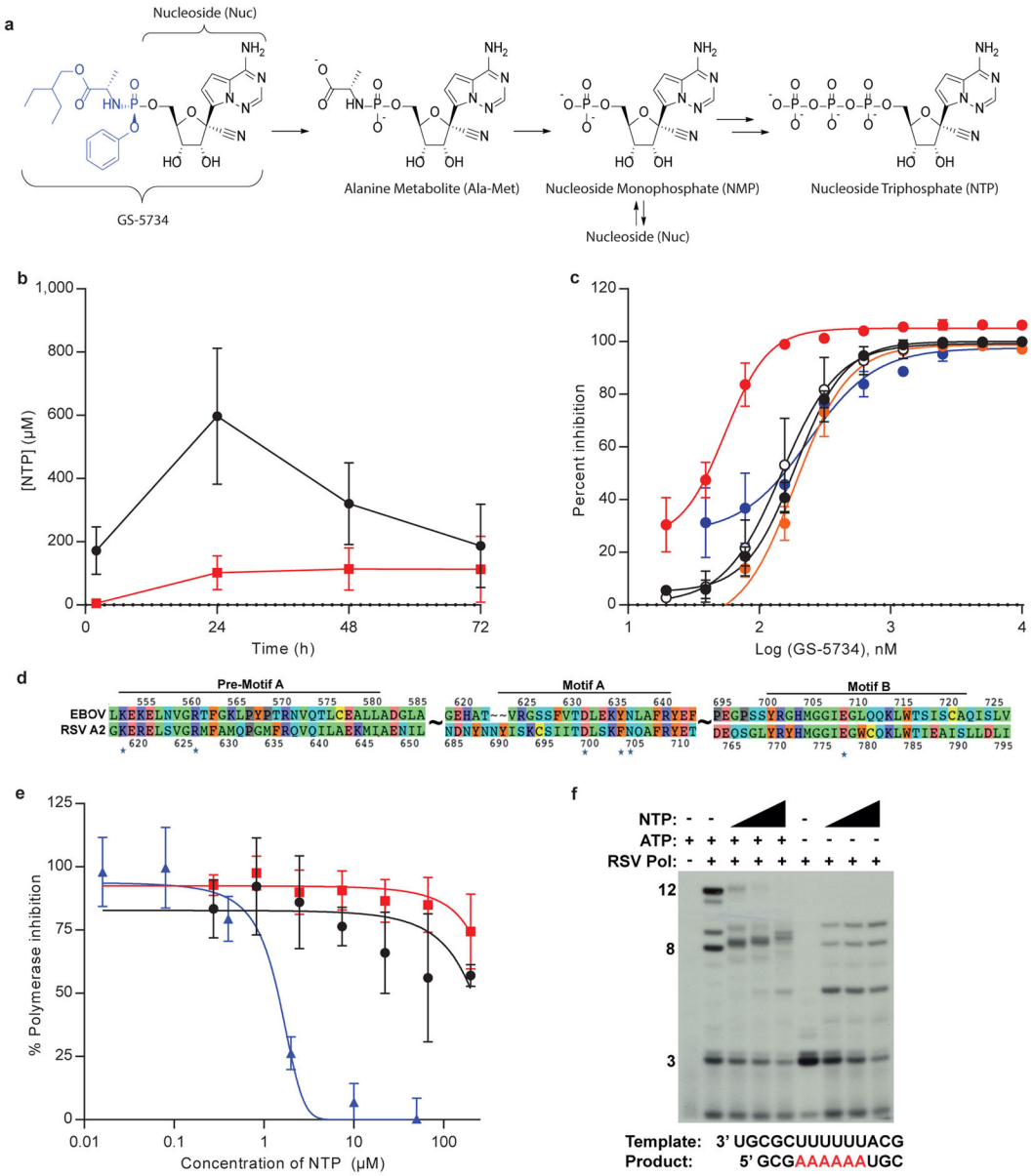

Figure 1.

Metabolism and mechanism of antiviral activity of GS-5734. **a**, Chemical structures of GS-5734 and metabolic conversion to NTP. **b**, NTP formation in human monocyte-derived macrophages following 72-h incubation with 1 μM GS-5734 (black) or Nuc (red); mean ± SD, from 3 donors. **c**, Antiviral activity of GS-5734 in HeLa cells against EBOV-Makona (black symbols), EBOV-Kikwit (open symbols), MARV (red), BDBV (orange), SUDV (blue); mean ± SD from triplicates. **d**, Amino acid sequence homology of EBOV and RSV RdRp active site. *, residues predicted to contact NTP. **e**, Inhibition of RSV RdRp (blue), but not human RNA Pol II (black) or mitochondrial (mt) RNA (red) polymerases by NTP ; mean ± SD, n=3 biological replicates. **f**, NTP-induced RNA chain termination in RSV RdRp primer-extension assay. For gel source data, see Supplementary Figure 1.

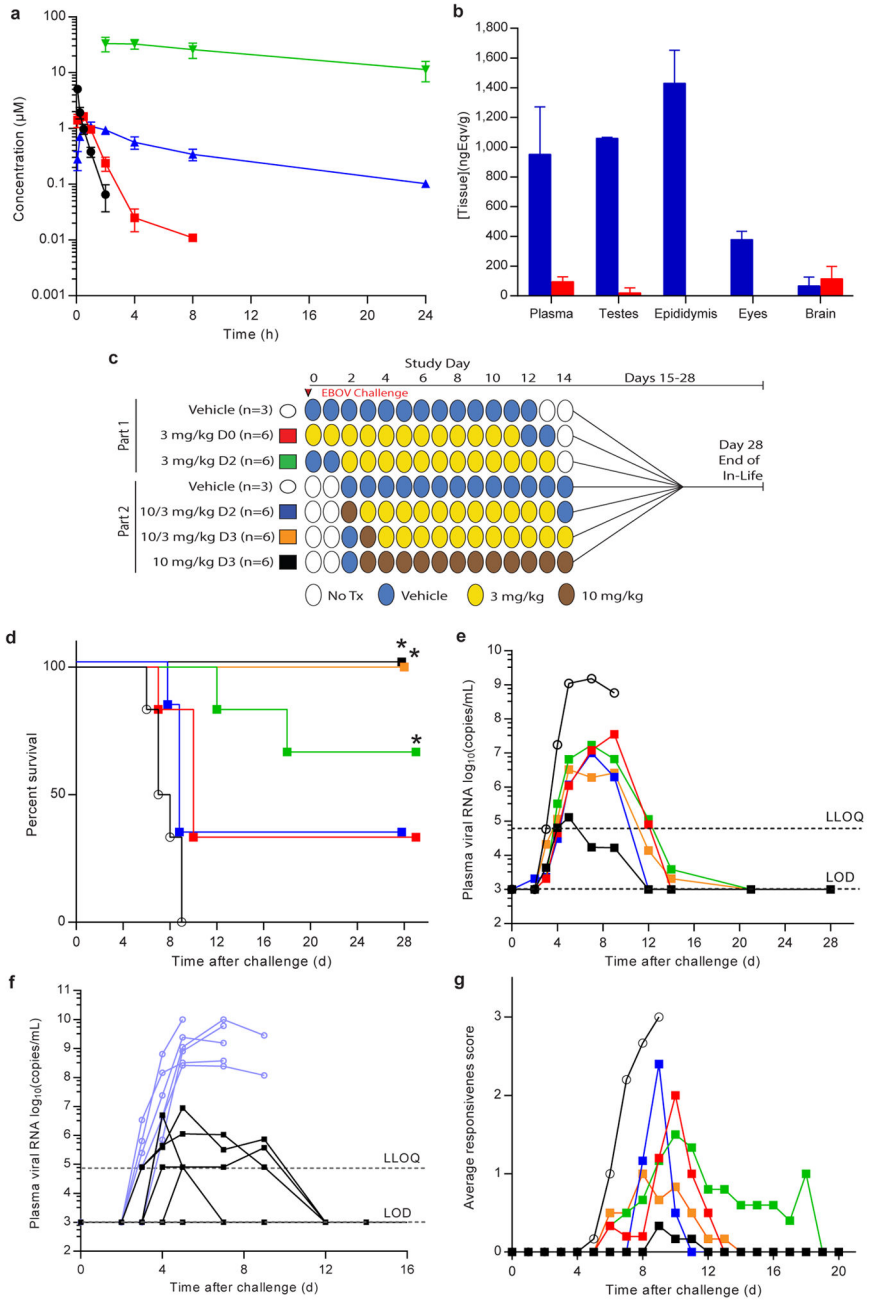

Figure 2.

GS-5734 pharmacokinetics and post-exposure protection against EBOV in rhesus monkeys. **a,** pharmacokinetics following intravenous administration of 10 mg/kg GS-5734 dose in healthy rhesus macaques (mean ± SD, n=3). Plasma GS-5734 (black), Ala-Met (red), and Nuc (blue); NTP in PBMCs (green). **b,** Tissue distribution of [^{14}C]GS-5734 and metabolites at 4 h (blue) and 168 h (red) following intravenous 10 mg/kg GS-5734 dose in healthy cynomolgus macaques (mean ± SD, n=3). **c,** Experimental design for GS-5734 efficacy evaluations in rhesus monkeys. **d**, Kaplan-Meier survival curves. *P<0.05 for treatment versus vehicle groups. **e,** Group geometric mean of plasma viral RNA concentrations;

LLOQ, lower limit of quantitation; LOD, limit of detection. **f,** Individual plasma viral RNA in vehicle (blue) or 10 mg/kg GS-5734 (black) groups. **g**, Group average clinical disease score. **d,e,g**, vehicle (black, open symbols), 3 mg/kg D0 (red), 3 mg/kg D2 (green), 10/3 mg/kg D2 (blue), 10/3 mg/kg D3(orange), 10 mg/kg D4 (black, closed symbols). Error bars omitted for clarity (**e,g**); x-axes truncated to emphasize acute disease phase (**f,g**).

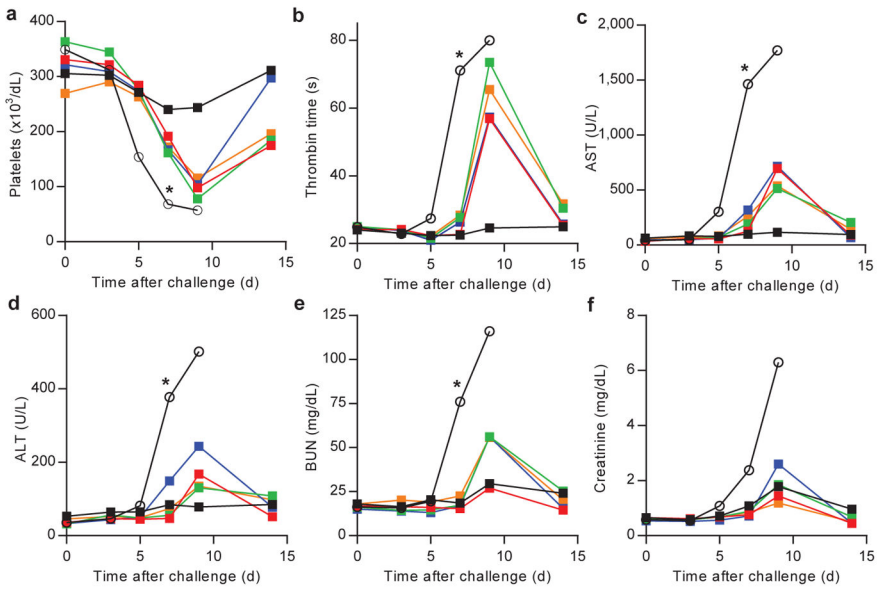

Figure 3.

Amelioration of EVD clinical pathology by GS-5734 in rhesus monkeys. Group mean (n=6/group) values of platelets (**a**), thrombin time (**b**), aspartate aminotransferase (AST, **c**), alanine aminotransferase (ALT, **d**), blood urea nitrogen (BUN, **e**), creatinine (**f**). Vehicle (black, open symbols), 3 mg/kg D0 (red), 3 mg/kg D2 (green), 10/3 mg/kg D2 (blue), 10/3 mg/kg D3(orange), 10 mg/kg D3 (black, closed symbols). Error bars omitted for clarity; x-axes truncated at day 15. *P<0.05 for comparison of mean change from Day 0 of vehicle and 10 mg/kg D3 groups at Day 7.

Table 1

Antiviral Activity of GS-5734 and Nuc

	Antiviral activity; EC_{50} / EC_{90} [μM]	
	GS-5734	**Nuc**
EBOV		
Primary macrophages *	0.086 / 0.18	>20 / >20
HeLa cells †	0.14 / 0.41	>20 / >20
HFF-1 *	0.13 / 0.26	>20 / >20
HMVEC-TERT cells †	0.06 / 0.22	0.77 / 3.12
Huh-7 cells †	0.07 / 0.22	1.49 / 6.04
Other human RNA viruses		
RSV ‡	0.019 / 0.051	0.75 / 2.84
JUNV §	0.47 / 1.33	
LASV §	1.48 / 2.80	
MERS §	0.34 / 4.24	
CHIV §	>20 / >20	
VEEV §	>20 / >20	
HIV-1 §	>20 / >20	

CC_{50} values for all compounds in primary human cells and human cell lines were greater than the highest concentration tested (> 20 μM).

* Mean values from duplicated titrations conducted in differentiated macrophages or HFF-1 cells in a single experiment (n = 1). Cells were infected with EBOV (Makona) for antiviral activity determination.

† Mean values from quadruplicate (HMVEC-TERT) or duplicate (Huh-7) titrations generated from two experiments (n = 2) or from multiple experiments (n = 6) for HeLa cells. Cells were infected with a replication competent reporter virus (EBOV-GFP) or wild type EBOV strain Zaire (HeLa) for antiviral activity determination.

‡ Mean values from two (GS-5734) or four (Nuc) independent experiments with each drug dilution tested in triplicate against the respiratory syncytial virus (RSV).

§ Mean values from duplicate titrations with each drug concentration tested in quadruplicate from a single experiment (n = 1). Junin virus (JUNV), Lassa fever virus (LASV), Middle East respiratory syndrome coronavirus (MERS), Chikungunya virus (CHIK), Venezuelan equine encephalitis virus (VEEV) and human immunodeficiency virus type 1 (HIV-1).