GS-5734 and its parent nucleoside analog inhibit Filo-, Pneumo-, and Paramyxoviruses

Michael K. Lo1, Robert Jordan2, Aaron Arvey3, Jawahar Sudhamsu4, Punya Shrivastava-Ranjan1, Anne L. Hotard1, Mike Flint3, Laura K. McMullan1, Dustin Siegel2, Michael O. Clarke2, Richard L. Mackman2, Hon C. Hui2, Michel Perron2, Adrian S. Ray2, Tomas Cihlar2, Stuart T. Nichol1 & Christina F. Spiropoulou1

GS-5734 is a monophosphate prodrug of an adenosine nucleoside analog that showed therapeutic efficacy in a non-human primate model of Ebola virus infection. It has been administered under compassionate use to two Ebola patients, both of whom survived, and is currently in Phase 2 clinical development for treatment of Ebola virus disease. Here we report the antiviral activities of GS-5734 and the parent nucleoside analog across multiple virus families, providing evidence to support new indications for this compound against human viruses of significant public health concern.

Viruses belonging to the families Paramyxoviridae, Coronaviridae and Filoviridae include zoonotic and human pathogens that are of significant public health concern, ranging from vaccine-preventable diseases such as Measles (MV) and Mumps (MuV) viruses to highly pathogenic viruses such as Nipah (NiV), Middle East Respiratory Syndrome (MERS) and Ebola (EBOV) viruses, for which there are currently no approved therapeutics for human use. A broad spectrum therapeutic that can target multiple virus families would have a significant impact on disease burden in endemic areas. Nucleoside analogs are a class of small-molecule antivirals which can directly inhibit viral transcription and replication by targeting the viral RNA-dependent RNA polymerase, and have been used as backbone components of combination therapies against both Human immunodeficiency virus (HIV) and Hepatitis C virus (HCV)1-2. A 1′-cyano substituted adenine nucleoside analog GS-441524 (“Nuc”) was shown to have activity against HCV, dengue virus (DenV), parainfluenza type 3 virus (hPIV3), and severe acute respiratory syndrome coronavirus (SARS-CoV)3. GS-5734 is a monophosphate prodrug of Nuc that has shown activity against filoviruses and coronaviruses in cell-based antiviral assays4. More importantly, this compound protected non-human primates from lethal Ebola virus infection when given therapeutically beginning 3 days post-inoculation, and is currently in phase 2 clinical development for treatment of Ebola virus disease.

To determine the full spectrum of activity of Nuc and GS-5734 against a wide panel of human viral pathogens, the antiviral activity was determined using a variety of cell-based assays. Nuc was active against recombinant reporter-expressing NiV5, EBOV6,7, and Marburg viruses (MARV)8, with observed 50% effective inhibition concentration (EC50) values between 1–3 μM (Table 1; Supplementary Figure 1, Supplementary Table 1). We confirmed the activity of Nuc against wild-type EBOV (Makona strain), NiV (Malaysian and Bangladesh genotypes) and Hendra virus (HeV) using assays which measured inhibition of virus antigen expression, virus-induced cytopathic effect (CPE) (for NiV and HeV), and virus titer (Supplementary Figures 2–4, Supplementary Table 1). The EC50 values derived from the different assays using wild-type viruses closely paralleled the values observed from the reporter viruses (Supplementary Table 1) (For details regarding the different assays used, please see Methods section). Moreover, at concentrations between 5–16 μM, Nuc was able to reduce infectious virus production of NiV and EBOV by greater than 5 logs (Supplementary Figure 4). To explore the range of Nuc antiviral activity across the Paramyxoviridae and Pneumoviridae families, we tested Nuc against reporter viruses from representative genera including MV10, hPIV311, respiratory syncytial virus (RSV)12, human metapneumovirus13 (hMPV), as well as wild-type MV and MuV14 (Supplementary Figures 1 and 2). While EC50 values for Nuc against MV, hPIV3, hMPV, and RSV ranged between 0.5 to 2.1 μM, the EC50 value against MuV was consistently in the 7–12 μM range. However, when we tested Nuc against viruses from unrelated families such as

---

1Centers for Disease Control and Prevention, Atlanta, Georgia, USA. 2Gilead Sciences, Inc. Foster City, California, USA. Correspondence and requests for materials should be addressed to M.K.L. (email: mko2@cdc.gov) or C.S.F. (email: ccs8@cdc.gov)
Table 1. Mean in vitro antiviral activities of Nuc and GS-5734 across 7 virus families. VTR: virus titer reduction assay; AG: antigen reduction assay; REP: Reporter assay; CPE: cytopathic effect assay; SI: Selective Index = CC50/EC50; NT: Not tested; Rec.: recombinant. †>1 log10 reduction in virus yield at 24 hours post-infection; ‡>4 log10 reduction in virus yield at 48 (NiV) or 72 (EBOV) hours post-infection; §>5 log10 reduction in virus yield at 48 (NiV) or 72 (EBOV) hours post-infection.

| Virus Family | Virus | Strain | Assay Type | Nuc EC50/EC90 (μM)/[SI] | GS-5734 EC50/EC90 (μM)/[SI] |
|--------------|-------|--------|------------|------------------------|-----------------------------|
| Filo-        | EBOV  | Rec. Mayinga-GFP | REP | 1.6/6.7/31 | 0.066/0.203/151 |
|              |       | Rec. Mayinga-Glu | REP | 3.1/11/16 | 0.021/0.053/476 |
|              |       | Rec. Makona-ZSG | REP | 1.3/3.3/38 | 0.014/0.045/714 |
|              | MARV  | Makona | VTR | 1.0/2.5/50 | 0.003/0.019/666 |
|              |       | Rec. Bat371-Glu | REP | NT | 0.019/0.052/526 |
|              |       | Rec. Bat371-GFP | REP | 1.9/4.6/26 | 0.014/0.047/714 |
| Paramyxo-    | NiV   | Rec. M-Lac2AM | REP | 1.5/5.7/33 | 0.045/0.126/184 |
|              |       | Rec. M-GFP2AM | REP | 2.2/4.0/22 | 0.029/0.053/286 |
|              |       | M-1999 | VTR | 0.49/1.4/102 | 0.047/0.083/180 |
|              |       | B-2004 | VTR/CPE | 0.83/2.2/60 | 0.032/0.106/259 |
|              | HeV   | 1996 | VTR/CPE | 1.0/1.8/50 | 0.055/0.117/150 |
|              | hPIV3 | Rec. JS-GFP | REP | 0.51/1.0/98 | 0.018/0.35/461 |
|              | MV    | Rec. rMV40GFP(3) | REP | 1.0/2.6/50 | 0.037/0.073/224 |
|              |       | EZ vaccine | AG | 2.0/5.1/25 | NT |
|              | MuV   | IA 2006 | AG | 9.7/26.3/5 | 0.79/3.4/10 |
|              | RSV   | Rec. rgRSV224 (A2) | REP | 0.63/2.2/79 | 0.021/0.059/395 |
|              | ANDV  | Chole 9717669 | AG | NT | 7.0/10.1/1.4 |
| Pneumo-      | RVFV  | Rec. ZHV51-GFP | REP | No inhibition | No inhibition |
|              | CCHF  | Rec. IbAr 10200 | AG | No inhibition | No inhibition |
|              | ANDV  | Chole 9717669 | AG | NT | 7.0/10.1/1.4 |
| Bunya-       | LAVV  | Josiah | AG | No inhibition | 4.5/5.1/2.2 |
| Blabado-     | VSV   | New Jersey | CPE | No inhibition | No inhibition |
| Flavi-       | AHSV  | 200300001 | CPE | 49.9/ >150/[NT] | 4.2/17.6/[2.4] |
|              | KFDV  | P9605 | CPE | 46.3/ >350/[NT] | 1.8/3.4/[5.6] |
|              | TBEV  | Hypr | CPE | 51.2/ >150/[NT] | 2.1/3.5/[4.8] |
|              | OHFV  | Bogolovskova | CPE | 30.6/ <350/[NT] | 1.2/3.9/[8.3] |

Arenaviridae (Lassa virus (LASV)), Rhhabdoviridae (Vesicular Stomatitis virus (VSV)), Bunyaviridae (Rift Valley Fever virus (RVFV)), Flaviviridae (Alkhurma Hemorrhagic Fever virus (AHFV), Kyasanur Forest Disease virus (KFDV), Omsk Hemorrhagic Fever virus (OHFV)), Tick-borne encephalitis virus (TBEV)), we observed little to no antiviral activity (Table 1; Supplementary Figures 1–3; Supplementary Table 1).

GS-5734 is a prodrug that is designed to deliver the nucleoside monophosphate into the cell, thereby circumventing the rate-limiting first phosphorylation step and allowing for efficient formation of the active triphosphate (Table 1, Supplementary Table 2; Supplementary Figures 6–8). Moreover, GS-5734 was able to reduce infectious virus production of NiV and EBOV by greater than 4 logs but at much lower concentrations than Nuc, reflecting the more efficient metabolism of the prodrug to the active form (Table 1, Supplementary Figure 10, Supplementary Table 2). To confirm that GS-5734 specifically inhibits viral transcription and replication, we measured GS-5734 activity against EBOV and NiV minigenome replication, and observed dose-dependent inhibition with EC50 values in the sub-micromolar range (Supplementary Figure 9; Supplementary Table 2). Of particular note is that compared to immortalized cells, GS-5734 had similar or even enhanced antiviral potency relative to Nuc in primary microvascular endothelial cells and macrophages, which are major cellular targets in human infection for NiV and EBOV respectively (Supplementary Figure 10; Supplementary Table 2).
To determine whether structural and sequence similarities of viral RNA-dependent RNA polymerases (RdRPs) would provide additional insight into the observed antiviral spectrum, we took a hybrid approach that used both structural homology and primary protein sequence information to align the secondary structure of nucleotide binding domains (NBDs) from known crystal structures of various viral RdRPs. Distinct ssRNA virus families do not necessarily share a common ancestor in the traditional phylogenetic sense, thus the primary sequence and structure of RNA-dependent RNA polymerases (RdRPs) can be quite divergent. We therefore examined all available viral RdRP crystal structures and utilized secondary structure to align the nucleotide binding domains in iterative fashion by removing non-homologous regions. This resulted in a compelling structural alignment for the nucleotide binding domains, despite the overall structures of RdRPs being very different (Fig. 1B).

For example, structural alignments of HCV against DenV (Fig. 1C) and VSV (Fig. 1D) RdRP nucleotide binding domains both show good overlap in the nucleotide binding regions, but have significant differences globally. This structural alignment informed the extraction of two 10-residue regions within motifs A and B that interact directly with the nucleotide. Sequence similarity clustering analysis of these regions correlated with the observed spectrum of antiviral activity across virus families (Fig. 2). For instance we observe that while HCV and DenV are both inhibited by GS-5734 and have high sequence similarity among motifs A and B, VSV which is not inhibited by GS-5734 has low sequence similarity among motifs A and B when compared to HCV and other negative strand RNA viruses susceptible to GS-5734 inhibition. Taken together, our results expand the spectrum of viruses susceptible to Nuc and GS-5734, and further define their respective antiviral activities across 7 virus families. The unique potent broad-spectrum activity of GS-5734 against paramyxoviruses, coronaviruses, and filoviruses provides an opportunity to explore the compound for the treatment of highly pathogenic infections such as NiV and MERS-CoV in non-human primates, in addition to EBOV and other filoviruses. Depending on further careful assessment of its safety and efficacy profile, GS-5734 could also be considered for the treatment of prevalent respiratory infections (RSV, hPIV3, hMPV), as well as for controlling recurring outbreaks of MV globally among under-vaccinated populations.

**Methods**

**Small molecules.** GS-5734 and Nuc were synthesized at Gilead Sciences, Inc., and chemical identity and sample purity were established using NMR, HRMS, and HPLC analysis. To determine concentration response curves against each virus, we used either a 7-point or a 10-point 3-fold dilution series, with each compound concentration tested in quadruplicate.

**Cells.** HeLa (ATCC CCL-2) and Huh7 (Apath, LLC) cells were cultivated in Dulbecco’s Minimal Essential Medium (DMEM) supplemented with 7.5% fetal bovine serum (FBS), non-essential amino acids, and penicillin/streptomycin. HEK293T/17 (ATCC CRL-11286), Vero (ATCC CCL-81), NCI-H358 (ATCC CRL-5807), and
A549 (ATCC CCL-185) cells were cultivated in DMEM supplemented with 10% FBS. Primary human lung microvascular endothelial cells (HMVEC-L) cells (Lonza CC-2527) were cultivated using EGM-2 MV BulletKit (Lonza CC-3202). Human peripheral blood macrophages (Stemcell Technologies) were cultivated in Macrophage-SFM (Gibco). All cells were incubated at 37 °C in 5% CO2.

Viruses. Nipah virus (NiV-M, Malaysian genotype; NiV-B, Bangladesh genotype; NiV-Luc2AM recombinant Malaysia genotype expressing Renilla luciferase; NiV-GFP2AM, recombinant Malaysian genotype expressing ZsGreen fluorescent protein)5, Hendra virus (HeV), Measles virus (MV-EZ, Edmonston-Zagreb vaccine strain; rMV-EZGFP(3), recombinant Measles Edmonston-Zagreb strain expressing enhanced green fluorescent protein)10, Mumps virus (MuV, Iowa 2006), Ebola virus (EOBV, Makona variant; EBOV-GFP, recombinant Mayinga variant expressing green-fluorescent protein; EBOV-GLuc, recombinant Mayinga variant expressing Gaussia luciferase)6,21, Marburg virus (MARV-GFP, recombinant Bat 371 variant expressing enhanced green fluorescent protein; MARV-GLuc, recombinant Bat 371 variant expressing Gaussia luciferase)8,21, Lassa virus (LASV, Josiah), Andes virus (ANDV, Chile 9717869), Rift Valley Fever Virus (RVFV-GFP, ZH501)22, Alkhurma Hemorrhagic Fever virus (AHFV, 20030001), Omsk Hemorrhagic Fever virus (OHFV, Bogolovskaya), Kyasanur Forest Disease virus (KFDV, P9605), Tick-borne Encephalitis virus (TBEV, Hypr), and Vesicular Stomatitis virus (VSV, New Jersey) were propagated in either Vero E6 (ATCC CRL-1586) or Vero (ATCC CCL-81) cells, and were quantitated by 50% tissue culture infections dose (TCID₅₀) assay using the Reed and Muench method 23. EBOV-ZsG (recombinant Makona variant)7 was propagated and quantitated as above using Huh7 cells. The rgRSV was propagated and quantitated as above using HeLa cells12. Both hMPV-GFP (CAN97-83)13 and hPIV3-GFP (JS)11 were obtained from ViraTree.

Figure 2. GS-5734 potently inhibits multiple virus families. Clustering by sequence similarity of the nucleotide-interacting regions (motifs A and B) of viral RNA-dependent RNA polymerases correlates with activity of GS-5734 (indicated by color in legend). The tree branches do not necessarily reflect evolutionary relationships (or lack thereof). For details regarding methodology, please refer to methods. ⁴GS-5734 EC₅₀ against MuV is higher than 100 nM, with EC₅₀ of 0.79 μM. ⁵GS-5734 is potent (EC₅₀ < 4.5 μM) against AHFV, KFDV, TBEV, and OHFV. The parent nucleoside (Nuc) of GS-5734 is also active against DenV and HCV, but not WNV or YFV.³

Viral antigen reduction assays. Levels of viral replication was measured by either a fluorescence or chemiluminescence-based immuno-staining method using antibodies against the corresponding viral
antigen(s)\textsuperscript{24,25}. Fluorescence or luminescence was measured using an HD1 Synergy plate reader (Biotek). Untreated infected cell control values (after subtraction of reference values) were set at 100% viral antigen. EC\textsubscript{50} values were calculated using four-parameter variable slope non-linear regression fitting of mean values from assays performed in triplicate. NIV-M, NIV-B, HeV, MV-EZ, and MuV assays were performed in HeLa cells. EBOV, ANDV, LASV, and CCHFV assays were performed in HuH7 cells. Cells were seeded at 10,000 cells per well in either black opaque, clear-bottom (for fluorescence-based assay) or white (for chemiluminescence-based assay) opaque white plates, and compounds were added to the assay plates. Assay plates were transferred to the BSL-4 suite (where appropriate) and infected with 0.25 TCID\textsubscript{50} per cell with respective virus, and were fixed at 24–72 hpi with 10% formalin supplemented with 0.2% Triton-X 100 detergent before downstream staining with primary antibody specific for the respective virus, and secondary antibodies (conjugated with Dylight-488 or horseradish peroxidase). Primary antibodies used for each respective virus are indicated in the following: NIV-M, NIV-B, and HeV: [1A11C1]\textsuperscript{26}; EBOV: anti-EBOV rabbit polyclonal serum\textsuperscript{27}; MV-EZ: Abcam [2D7] ab9882; MuV: Abcam [7B10] ab9880; LASV: anti-LASV hyperimmune mouse ascites fluid\textsuperscript{28}; ANDV: anti-Pumaala virus nucleoprotein mouse monoclonal antibody\textsuperscript{29}; CCHFV: anti-CCHFV hyperimmune mouse ascites fluid\textsuperscript{30}.

Cytopathic effect (CPE) inhibition assays. Inhibition of virus-induced CPE was assayed using CellTiter-Glo 2.0 reagent (Promega) in a HD1 Synergy plate reader. Values were normalized to uninfected cell controls according to % viability as follows: % viability = [(specific value − reference value)/(DMSO control value − reference value)] × 100. Reference values were derived from control wells without cells. Uninfected cell control values (after subtraction of reference values) were set at 100% inhibition of CPE. EC\textsubscript{50} values were calculated using four-parameter variable slope non-linear regression fitting of values. NIV-M, NIV-B, HeV, and VSV assays were performed in HeLa cells and H358 cells (NIV-B), AHPV, KFDV, TBEV, and OHFV assays were performed in A549 cells. Cells were seeded at 10,000–20,000 cells per well in white opaque 96-well plates, and compounds were added to the assay plates. Assay plates were transferred to the BSL-4 suite and infected with 0.25–0.5 TCID\textsubscript{50} per cell, and were assayed with CellTiter-Glo 2.0 between 48–96 hpi.

Cell viability assay. The CellTiter-Glo 2.0 assay was used to determine viability of uninfected cells treated with 3-fold serial dilutions of the compounds for 72 h. Values were normalized to DMSO controls according to % viability as follows: % viability = [(specific value − reference value)/(DMSO control value − reference value)] × 100. Reference values were derived from control wells without cells. DMSO control values (after subtraction of reference values) were set at 100% viability. 50% viability/cytotoxicity (CC\textsubscript{50}) was calculated using four-parameter variable slope non-linear regression fitting of values.

NIV minigenome assay. A Nanoluciferase-based NiV minigenome assay was adapted from a previously developed NiV minigenome assay\textsuperscript{30}. Briefly, a bacteriophage T7 polymerase-based NiV minigenome was synthesized (Genscript) expressing a reporter fusion construct of Nanoluciferase (Promega) and mNeonGreen fluorescent protein\textsuperscript{31}. The open reading frame encoding the reporter fusion protein was flanked by a T7 promoter, hammerhead ribozyme, and NiV leader with N gene untranslated region at the 3′ end, and the L gene untranslated region, NiV trailer, and Hepatitis delta ribozyme at the 5′ end. HeLa cells (5,000 per well) were seeded in 96-well plates, and transfected with appropriate amounts of NiV support plasmids (N (50 ng/well), P (32 ng) and L (50 ng)), HeV minigenome (120 ng) and T7 polymerase (80 ng) prepared in RNase-free TE buffer, mixed with 0.8 μL/well LT-1 transfection reagent (Mirus Bio, Madison, WI) and 10 μL Opti-MEM/well. Complexes were incubated for 30 min at room temperature before adding to cells. Complexes were added directly to the cells 4 h post-transfection. For negative controls, the L plasmid was substituted with an equivalent amount of pcDNA 3.1 plasmid expressing the red fluorescent protein mCherry (Clontech). 48 h post-transfection of minigenome plasmids, 50 μL of Nanoluciferase assay buffer solution (Promega) was added directly to each well. Well contents were transferred to opaque white plates, and after three minutes, luminescence was read on a plate reader (HT-Synergy, Biotek). CellTiter-Glo 2.0 reagent (Promega) was added immediately following reporter minigenome luminescence reading to measure cell viability according to manufacturer's guidelines. The average raw luminescence value for untreated non-transfected cells was set as 100% cell viability. Percent (%) cell viability for individual wells were calculated by dividing their respective raw luminescence values by the average raw luminescence value for non-transfected cells. Reporter Nanoluciferase activity for each well was then normalized by dividing the nanoluciferase luminescence value by its respective % cell viability as determined above.

EBOV minigenome assay. A Gaussia luciferase-based EBOV minigenome assay was used\textsuperscript{32}. HuH7 cells (10,000 per well) were seeded and transfected with appropriate amounts of EBOV support plasmids (NP (50 ng/well), VP35 (5 ng/well), VP30 (5 ng/well)), EBOV minigenome (150 ng), and T7 polymerase (80 ng) prepared in RNase-free TE buffer mixed with 0.9 μL/well LT-1 transfection reagent (MirusBio) and 10 μL Opti-MEM/well. Complexes were incubated for 30 min at room temperature before adding to cells. Complexes were added directly to the cells 4 h post-transfection. For negative controls, the L plasmid was substituted with an equivalent inactive L plasmid. 48 h post-transfection, 25 μL of supernatant from each well was transferred to opaque white plates, and was combined with 50 μL of Renilla luciferase assay reagent (Promega) via injector and read for bioluminescence in a Biotek HD1 Synergy plate reader following a 0.2 s delay. CellTiter-Glo 2.0 reagent (Promega) was added to the transfected cells, and transferred to a white opaque 96-well plate to measure cell viability according to manufacturer's guidelines. The average raw luminescence value for untreated non-transfected cells was set as 100% cell viability. Percent (%) cell viability for individual wells were calculated by dividing their respective raw luminescence values by the average raw luminescence value for non-transfected cells. Reporter Gaussia luciferase activity for each well was then normalized by dividing the Gaussia luciferase luminescence value by its respective % cell viability as determined above.
Infectious virus yield reduction assays.  

**EBOV (Makona variant) assay.**  10,000 Huh7 cells or primary macrophages were infected with 0.25 TCID<sub>50</sub> per cell or 5 TCID<sub>50</sub> per cell, respectively for 1 h. Virus inoculum was then removed, cells were washed once with phosphate buffered saline, and replaced with culture medium containing compound in a 10-point 3-fold dilution series. At 24 hpi supernatants were harvested, serially diluted (10-fold) and mixed with 10,000 Vero cells per well in 96 well plates. At day 5 post-infection, plates were fixed with 0.2% Triton-X detergent, and stained with primary rabbit anti-EBOV serum, and after several washes the corresponding anti-rabbit Dylight 488 conjugated secondary antibody was added. Plates were assayed for focus forming units, and quantitated by TCID<sub>50</sub> assay using the Reed and Muench method. 

**NiV-M assay.**  10,000 HeLa cells or 5,000 primary HMVEC-L cells were infected with 0.25 TCID<sub>50</sub> NiV per cell and 1 TCID<sub>50</sub> per cell, respectively for 1 h. Virus inoculum was then removed, cells were washed once with phosphate buffered saline, and replaced with culture medium containing compound in a 10-point 3-fold dilution series. At 48 hpi supernatants were harvested, serially diluted, and mixed with 10,000 Vero cells per well in 96-well plates. At day 5 post-infection, plates assayed for CPE, and then viral titers were quantitated by TCID<sub>50</sub> assay using the Reed and Muench method.

**NiV-B and HeV assay.**  10,000 HeLa cells were infected with 0.25 TCID<sub>50</sub> virus per cell. Virus inoculum was left in the growth medium containing compound in a 7-point 3-fold dilution series. At 24 hpi supernatants were harvested, serially diluted, and mixed with 10,000 Vero cells per well in 96-well plates. At day 5 post-infection, plates assayed for CPE, and then viral titers were quantitated by TCID<sub>50</sub> assay using the Reed and Muench method.

**EC<sub>50</sub>** values for all infectious virus yield reduction assays were calculated using four-parameter variable slope non-linear regression fitting of mean values derived from quadruplicate or triplicate samples.

Sequence similarity clustering analysis.  To estimate virus polymerase similarity, we took a hybrid approach that used both structural homology and primary protein sequence information. Distinct ssRNA virus families do not necessarily share a common ancestor in the traditional phylogenetic sense, thus the primary sequence and structure of RNA-dependent RNA polymerases (RdRPs) can be quite divergent. We therefore examined all available viral RdRP crystal structures and utilized secondary structure to align the nucleotide binding domains in iterative fashion by removing non-homologous regions. This resulted in a compelling structural alignment for the nucleotide binding domains (Supplementary Figure 12), although the overall structures of RdRPs were quite divergent. This allowed us to determine functionally homologous nucleotide binding residues (motifs A and B). We extracted the primary amino acid sequences (10 residues each) and aligned them by homology to viral RdRPs without known crystal structures using the global-local pairwise alignment in the Biostrings package in R. Grouping of these sequences was determined by calculating a distance matrix followed by hierarchical linkage clustering. The distance matrix was estimated by taking the negative values of Smith-Waterman alignments on the extracted regions using MUSCLE using default parameters. Clustering was performed using hclust in R. Results were comparable to those derived from multiple sequence alignments on the extracted regions using MUSCLE using default parameters.

Crystal structures examined included the following PDB entries:

- 4WTA (HCV)
- 5HMZ (Dengue)
- 2HCN (West Nile)
- 5A22 (VSV)
- 4QPX (Norovirus)
- 1XR5 (Rhinovirus 14)
- 1XR6 (Rhinovirus 1B)
- 1TP7 (Rhinovirus 16)
- 4K4Y (Coxsackie)
- 3N6M (EV71)
- 4K4S (Polio)
- 1RTD (HV).

References

1. Cihlar, T. & Ray, A. S. Nucleoside and nucleotide HIV reverse transcriptase inhibitors: 25 years after zidovudine. *Antiviral Res* **85**, 39–58 (2010).
2. Eltahlal, A. A., Luciani, F., White, P. A., Lloyd, A. R. & Bull, R. A. Inhibitors of the Hepatitis C Virus Polymerase: Mode of Action and Resistance. *Viruses* **7**, 5206–5224 (2015).
3. Cho, A. et al. Synthesis and antiviral activity of a series of 1′-substituted 4-aza-7,9-dideazaadenosine C-nucleosides. *Bioorg Med Chem Lett* **22**, 2705–2707 (2012).
4. Warren, T. K. et al. Therapeutic efficacy of the small molecule GS-5734 against Ebola virus in rhesus monkeys. *Nature* **531**, 381–385 (2016).
5. Lo, M. K., Nichol, S. T. & Spiropoulou, C. F. Evaluation of luciferase and GFP-expressing Nipah viruses for rapid quantitative antiviral screening. *Antiviral Res* **106**, 53–60 (2014).
6. 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* **332**, 20–27 (2005).
7. Albarino, C. G., Wigginton Guerrero, L., Lo, M. K., Nichol, S. T. & Towner, J. S. Development of a reverse genetics system to generate a recombinant Ebola virus Makona expressing a green fluorescent protein. *Virology* **484**, 259–264 (2015).
8. Albarino, C. G. et al. Development of a reverse genetics system to generate recombinant Marburg virus derived from a bat isolate. *Virology* **446**, 230–237 (2013).
9. Afonso, C. L. et al. Taxonomy of the order Mononegavirales: update 2016. *Arch Virol* **161**, 2351–2360 (2016).
10. Rennick, L. J. et al. Live-attenuated measles virus vaccine targets dendritic cells and macrophages in muscle of nonhuman primates. *J Virol* **89**, 2192–2200 (2015).
Paramyxoviruses.

How to cite this article: Lo, M. K. GS-5734 and its parent nucleoside analog inhibit Filo-, Pneumo-, and

Acknowledgements

We would like to thank Linda J. Rennick and W. Paul Duprex for the rMV\textsuperscript{GFP}(3), Paul A. Rota for the MV-EZ, Donald R. Latner for the MuV, Eric Bergeron for the recombinant CCHFV, and Mark E. Peeples and Peter L. Collins for the rgRSV224. Anne L. Hotard is supported by an American Society for Microbiology Postdoctoral Fellowship. This work was funded by CDC core funding. The findings and conclusions in this report are those of the authors and do not necessarily represent those of the Centers for Disease Control and Prevention.

Author Contributions

M.K.L. executed cell-based infection, minigenome, and cytotoxicity assays, and analyzed the results. M.K.L., R.J., M.P., T.C., S.T.N., and C.F.S. designed experiments, executing cell-based infection assays. R.L.M., D.S., H.C.H., A.S.R., and M.O.C. were responsible for the synthesis, structural polymerase alignments and similarity clustering analyses. P.R., A.L.H., M.F., and L.K.M. assisted in characterizing, and scale-up of small molecules. M.K.L. designed experiments, and outlined and wrote the manuscript. M.K.L. executed cell-based infection, minigenome, and cytotoxicity assays, and analyzed the results. M.K.L., R.J., M.P., T.C., S.T.N., and C.F.S. designed experiments, executing cell-based infection assays. R.L.M., D.S., H.C.H., A.S.R., and M.O.C. were responsible for the synthesis, structural polymerase alignments and similarity clustering analyses. P.R., A.L.H., M.F., and L.K.M. assisted in characterizing, and scale-up of small molecules. M.K.L., R.J., M.P., T.C., S.T.N., and C.F.S. designed experiments, provided project oversight, evaluated results, and outlined and wrote the manuscript.

Additional Information

Supplementary information accompanies this paper at http://www.nature.com/srep

Competing financial interests: The authors affiliated with Gilead Sciences are employees of the company and may own company stock.

How to cite this article: Lo, M. K. et al. GS-5734 and its parent nucleoside analog inhibit Filo-, Pneumo-, and Paramyxoviruses. Sci. Rep. 7, 43395; doi: 10.1038/srep43395 (2017).

Publisher's note: Springer Nature remains neutral with regard to jurisdictional claims in published maps and institutional affiliations.

This work is licensed under a Creative Commons Attribution 4.0 International License. The images or other third party material in this article are included in the article’s Creative Commons license, unless indicated otherwise in the credit line; if the material is not included under the Creative Commons license, users will need to obtain permission from the license holder to reproduce the material. To view a copy of this license, visit http://creativecommons.org/licenses/by/4.0/

© The Author(s) 2017