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Protein Phosphatase-1 Regulates Rift Valley Fever Virus Replication

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Abstract

Rift Valley fever virus (RVFV), genus *Phlebovirus* family *Bunyaviridae*, is an arthropod-borne virus endemic throughout sub-Saharan Africa. Recent outbreaks have resulted in cyclic epidemics with an increasing geographic footprint, devastating both livestock and human populations. Despite being recognized as an emerging threat, relatively little is known about the virulence mechanisms and host interactions of RVFV. To date there are no FDA approved therapeutics or vaccines for RVF and there is an urgent need for their development. The Ser/Thr protein phosphatase 1 (PP1) has previously been shown to play a significant role in the replication of several viruses. Here we demonstrate for the first time that PP1 plays a prominent role in RVFV replication early on during the viral life cycle. Both siRNA knockdown of PP1 α and a novel PP1-targeting small molecule compound 1E7-03, resulted in decreased viral titers across several cell lines. Deregulation of PP1 was found to inhibit viral RNA production, potentially through the disruption of viral RNA transcript/protein interactions, and indicates a potential link between PP1 α and the viral L polymerase and nucleoprotein. These results indicate that PP1 activity is important for RVFV replication early on during the viral life cycle and may prove an attractive therapeutic target.

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Keywords

L polymerase; phosphorylation; protein phosphatase 1; antiviral agent; Rift Valley fever virus; Bunyaviridae

1. Introduction

Members of the Bunyaviridae family are among the most widespread viruses in the world. Rift Valley fever virus (RVFV), genus *Phlebovirus* family *Bunyaviridae*, is an arthropod-borne-virus whose cyclic epidemics have had devastating economic effects on livestock populations throughout much of sub-Saharan Africa ¹. In humans, RVFV causes Rift Valley fever (RVF), which is characterized by a mild to moderate febrile illness. In a small percentage of patients retinitis with visual impairment, hemorrhagic liver necrosis, and permanent neurological damage can occur due to inflammation of the spinal cord and meninges ^{1,2}. Past outbreaks have been a source of concern as RVFV has proven adept in its ability to break past traditional geographic barriers, escaping continental Africa into the Arabian Peninsula and Madagascar ^{2,3}. The recent geographical expansion of West Nile virus and yellow fever virus are particularly troubling as RVFV is capable of being transmitted through similar arthropod vectors and has been isolated from up to 40 different species of mosquitoes in the field ⁴. Due to its increasing spread, host susceptibility, and in particular vector plasticity and ease of aerosolization, the CDC has listed RVFV as an emerging infectious disease and a Category A priority pathogen. Despite being recognized as a significant and emerging threat, there are currently no FDA licensed vaccines or therapeutics for RVF and there is an urgent need for their development.

RVFV contains a tripartite single stranded negative sense RNA genome composed of a large (L), medium (M), and small (S) segment. The viral RNA-dependent RNA polymerase or L protein (238 kDa) is encoded on the L segment, while the M segment encodes the precursors for the two glycoproteins Gc (56 kDa) and Gn (54 kDa), the nonstructural NSm protein, and a 78 kDa glycoprotein ¹. The S segment codes for the viral nucleoprotein N (26 kDa) and the nonstructural protein NSs (31 kDa) ¹. While the nonstructural proteins are replication dispensable; they do play a significant role in the pathogenesis of the disease *in vivo* ^{1,2}. The NSs protein in particular has been established as a major virulence factor due to its ability to suppress the host's innate immune response ^{1,2}.

Viruses are intraobligate cellular parasites reliant on manipulating host cellular signaling pathways in order to facilitate infection and viral replication. Host targeted therapeutics have become increasingly popular as they provide novel targets and insights to existing pathogens, along with a decreased likelihood of viral adaptation. Our previous studies have demonstrated that RVFV infection results in the phosphorylation of a large number of host signaling proteins such as ataxia telangiectasia mutated (ATM), p53, p38, extracellular signal regulated kinase (ERK), and other mitogen-activated protein kinases (MAPK) ⁵⁻⁸. The phosphorylation of viral and host proteins can play a critical role in regulating both viral replication and the host response to an invading pathogen.

Protein phosphatase 1 (PP1) is a well characterized and conserved Ser/Thr phosphatase holoenzyme comprised of a regulatory subunit, and one of three highly homologous catalytic subunits PP1 α , PP1 γ , or PP1 β/δ ⁹. The catalytic subunits interact with over 200 regulatory proteins including components of the MAPK signaling cascade such as ERK1/2, c-Jun N-terminal kinase (JNK), and p38⁹. PP1 catalytic subunits typically bind to their regulatory subunits through a combination of short binding motifs, such as through the well-established RVxF motif and the recently identified SILK, MyPhoNE, SpiDoC and iDoHA motifs¹⁰. Numerous studies have shown that PP1 plays a prominent role in viral replication affecting the antiviral response, signal transduction, cell cycle checkpoint control, RNA splicing, and protein synthesis^{11–15}. While the significance of cellular kinases for RVFV has been partially described^{16–19}, relatively little is known about the impact of cellular phosphatases on viral replication.

PP1 has previously been shown to play a significant role in the viral replication of papovavirus, adenovirus, human immunodeficiency virus 1 and 2 (HIV-1 & HIV-2), and Ebola virus (EBOV)^{11–15}. For EBOV, the switch between viral transcription and replication is dependent on the ability of PP1 to regulate the phosphorylation status of the viral polymerase cofactor VP30, which when phosphorylated at two N-terminal serine clusters promotes viral replication at the expense of viral transcription^{14,20,21}.

We recently developed small molecules that were efficient in inhibiting HIV-1^{22,23} as well as EBOV infection in a PP1 dependent manner²⁴. Initially, we developed a library of small molecules that were designed to specifically bind to the RVxF binding site of PP1 and identified the small molecule, 1H4, which inhibited HIV-1 transcription²². We further modified the 1H4 compound and identified compound 1E7-03, a tetrahydroquinoline derivative, which efficiently inhibited HIV-1²² and also efficiently inhibited transcription of the EBOV genome and replication of viral particles²⁴.

Our previous studies indicated that RVFV relies on a large number of cellular phosphosignaling events in order to regulate the cellular environment to facilitate viral replication^{5–8}. As PP1 is a critical regulator of numerous pathways utilized by RVFV, a compound screen of small molecule PP1 inhibitors was run against RVFV infected cells and found to inhibit viral replication (data not shown). Based on our previous studies and drug screenings, we hypothesize that PP1 activity may be important for RVFV replication through interaction with a viral substrate, and may potentially play a role in regulating the viral life cycle.

2. Materials and Methods

2.1 Viral Infections and Drug Treatments

The MP-12 strain of RVFV, a live attenuated strain derived from the ZH548 strain, was generated by 12 serial passages in MRC5 cells in the presence of 5-fluorouracil resulting in 25 nucleotide changes across the three viral genome segments²⁵. rMP-12-LV5 is a recombinant strain derived from MP-12 with a V5 tag inserted between amino acid position 1852 and 1853 of the viral L protein. The rMP-12-NSsdel (NSs) strain completely lacks the NSs ORF. For the rMP-12-NSsLuc (rMP-12-Luc) strain the NSs ORF was replaced with

a renilla luciferase reporter ²⁶. rMP-12-Flag (NSs-Flag) has a C-terminal Flag-tagged NSs ²⁶. Control experiments were performed with Influenza A/California/2009 (H1N1), obtained from BEI Resources (ATCC), in murine Darby canine kidney (MDCK) cells.

During viral inoculation, the growth media was removed, cells washed with phosphate buffered saline (PBS without calcium and magnesium), overlaid with a viral inoculum diluted in growth media, and incubated for 1 hour at 37°C at 5% CO₂. Following the 1 hour incubation, infectious supernatants were removed, cells washed with PBS, and growth media replaced. Unless otherwise noted, cells were pre- and post-treated during the course of an infection with either dimethyl sulfoxide (DMSO) or the PP1 targeting small molecule compound 1E7-03 ²². DMSO amounts were equalized and matched to corresponding drug treatment volumes and always less than 0.1% of final sample volume. Okadaic acid was used at a final concentration of 5 nM. Plaque assays were performed as previously described ^{27,28}.

2.2 Cell Culture

Human small airway lung epithelial cells (HSAECs) were isolated from an anonymous donor and grown in Ham's F12 medium according to the vendor's protocol (Cambrex Inc., Walkersville, MD). MDCK, 293T cells and Vero cells were cultured using Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% fetal bovine serum (FBS), 1% penicillin/streptomycin, and 1% glutamax [(American Type Culture Collection (ATCC))]. BSR-T7 cells, which stably express T7 RNA polymerase, were grown in minimal essential medium (MEM) supplemented with 10% FBS, 1% non-essential amino acids, 1% penicillin/streptomycin, 1% glutamax and 1 mg/ml G418²⁹. Cells were maintained at 37°C and 5% CO₂.

2.3 Western Blot Analysis

Cells were collected as previously described using a mixture of T-PER reagent (Pierce, IL), 2× Tris-glycine (sodium dodecyl sulfate) SDS sample buffer (Novex, Invitrogen), 33 mM dithiothreitol (DTT), and protease/phosphatase inhibitor cocktail tablets (1× Halt cocktail, Pierce) applied directly to cells prewashed in PBS ²⁷. Samples were then scraped, collected and boiled for 10 minutes. Cell lysates were separated in 4–12% Bis-Tris gels and transferred overnight using a wet transfer on PVDF. The membranes were blocked with either a 3% BSA or 3% boiled milk solution in PBS with Tween20 (PBS-T) for an hour at room temperature. Membranes were probed with anti-RVSV N, anti-RVSV Gn (#4519, ProSci), anti-V5 (Serotec), anti-Flag (Sigma Aldrich), anti-PP1α and β (Cell Signaling), anti-PP1β (Abcam), anti-peIF2α (S51) (Cell Signaling) and HRP conjugated actin, diluted in blocking buffer, then incubated overnight at 4°C. The blots were then washed four times with PBS-T and incubated with either secondary HRP-coupled anti-goat, anti-rabbit or an anti-mouse antibody diluted in blocking buffer. The blots were visualized by chemiluminescence using the SuperSignal West Femto Maximum Sensitivity substrate kit (Thermo Scientific) through the Molecular Imager ChemiDoc XRS system (Bio-Rad).

2.4 siRNA Transfections and Plasmids

293T cells were plated at 2×10^4 and then transfected the following day using Thermo Scientific DharmaFECT transfection reagent for 48 hours before RVFV infection. For siRNA either Thermo Scientific On-Targetplus SMARTpool for PP1CA siRNA (#L-008927-00) or Qiagen's All Stars siRNA negative control were used.

2.5 Cell Viability Assay

Cells were cultured, treated and infected as described above. At the indicated time points a cell viability assay using CellTiter-Glo Cell Luminescent Viability assay (Promega) was performed according to the manufacturer's protocol.

2.6 Quantitative RT-PCR

Cells were grown in 12-well plates, and collected in 350 μ l of Buffer RLT + β -mercaptoethanol. The RNA was extracted using RNeasy Mini Kit (Qiagen) in accordance with the manufacturer's protocol. The RNA was then DNase treated (DNase I-RNase-Free, Ambion) to remove any contaminating DNA. Four hundred nanograms (ng) of total RNA was used in the High Capacity RNA-to-cDNA kit by Applied Biosystems (#4387406) in accordance with the manufacturer's protocol. For qPCR, the template cDNA was added to a 20 μ l reaction with a SYBR® GREEN PCR master mix (Applied Biosystems) and 0.2 μ M of primer. Primer sequences: RVFV L segment (Forward GGTGGCATGTTCAATCCT and Reverse GCATTCTGGGAAGTTCTGGA), M segment (Forward AGTCCATAGCCCAGGTG TTG and Reverse CATTGAGCCAGGAAGGTTGT), S segment (Forward GAGAGGATCCGATTACTTTCTGTGATATCTGTTGATTTGC and Reverse GAGACTCGAGCTAATCAACCTCAACAAATCCATCATCATCA CTCTCC) and 18S (Forward TGAGAAACGGCTACCACATC and Reverse TTACAGGGCCTCGAAAGAGT). Fold changes were calculated relative to 18S using the Ct method and normalized to respective DMSO control samples.

2.7 Minigenome and RNA Immunoprecipitations

Minigenome transfections were performed as previously described³⁰, with the following modifications: BSR-T7 cells, which stably express T7 RNA polymerase, were utilized in place of 293T cells and the use of the pCT7pol plasmid. Cells were either transfected (12-well format) with a complete minigenome system (MG+) containing pT7-M-rLuc(-), pT7-IRES-fLuc, pT7-IRES-N, pT7-IRES-L, or a negative control (MG-) containing pT7-M-rLuc(-), pT7-IRES-fLuc, and pT7-IRES. pT7-IRES-fLuc which contains the T7 promoter, encephalomyocarditis virus (EMCV) internal ribosome entry site (IRES), and a firefly luciferase (fLuc) ORF was used as a transfection control, while pT7-IRES was used to equalize total plasmid levels between the MG+ and MG- systems³⁰. Following 16 hour post transfection, cells were then treated with either 1E7-03 or DMSO for 24 hours. Samples were then collected and read utilizing Promega's Dual-Glo Luciferase assay system. fLuc activities were reported as 1/100 of outputs.

RNA immunoprecipitations were performed in Vero cells infected with rMP-12 LV5 in cells that had been pre/post treated with the indicated compound. Cells were collected 16 hours post infection, and then fixed for 10 minutes at 37°C with 1% paraformaldehyde in complete

growth media. Samples were lysed using RIPA buffer (50 mM Tris-HCl, pH 7.5, 1% NP-40, 0.5% sodium deoxycholate, 0.05% SDS, 1 mM EDTA, 150 mM NaCl, protease cocktail inhibitor tablet) and sonicated. Immunoprecipitations were performed using Thermo Fisher Scientifics Protein G Dynabeads (#10004D), utilizing anti-RVFP N (mouse), anti-V5 (Serotec, mouse), anti-HA (Santa Cruz, mouse). Protein lysates and antibody/bead complexes were incubated at 4°C for 90 minutes, washed 5x in a high stringency RIPA buffer (50 mM Tris-HCl, 1%NP-40, 1% sodium deoxycholate, 0.1% sodium dodecyl sulfate, 1 mM EDTA, 1 M NaCl, 1 M urea, protease cocktail inhibitor tablets) and then treated with proteinase K. RNA was isolated utilizing TRIzol LS and Direct-zol RNA miniPrep kits from Zymo Research. QRT-PCR was performed using Thermo Fisher Scientifics RNA UltraSense One-Step Quantitative RT-PCR system, (#11732-927) with primers used for the viral M segment (Forward AGTCCATAGCCCAGGTGTTG and Reverse CATTGAGCCAGGAAGGTTGT). All viral M segment sample values were normalized to their respective input controls.

2.8 Statistical Analyses

All quantifications, unless otherwise noted, are based on data obtained from three independent samples calculated using unpaired Student's *t* test. Error bars in all figures indicate standard deviations (S.D.) with *p*-values less than 0.01. The test value of *p*<0.01 was considered highly significant.

EC₅₀ and CC₅₀ values were obtained using Graphpad's Prism 6 software, version 6.0g 2015. A CC₅₀ value was unattainable as the compound fell out of solution at >50 μM concentrations. For the EC₅₀ a bottom constraint of 183,000 pfu/ml was utilized. The low end of the selective index (CC₅₀/EC₅₀) was calculated using the highest soluble concentration of 1E7-03 (50 μM) as the CC₅₀ value.

3. Results

3.1 Loss of PP1α reduces RVFV replication

PP1 has been implicated in the sensing and regulation of numerous host cellular process that affect the life cycle of RVFV^{31,32}. In order to determine if PP1 activity is linked to RVFV replication, the catalytic α-subunit of PP1 was knocked down using siRNA. For increased knockdown efficiency, 293T cells were used and transfected for 48hrs with siPP1α. Cells were then infected with the luciferase reporter virus rMP-12-Luc, and collected 24 hours post infection (hpi). PP1α knockdown resulted in a 50% reduction in viral luminescence in comparison to the siRNA control (Fig. 1A). PP1α siRNA knockdown was confirmed by western blot analysis at both 48 hours post transfection (time of initial infection) as well as 24 hpi (time of sample collection) (Fig. 1C).

As our viral reporter contains a luciferase substitution for the NSs ORF (a major virulence factor), confirmation experiments were performed using the fully intact MP-12 strain (Fig. 1B). Loss of PP1α in the context of full-length MP-12 supported our previous intracellular results and significantly reduced viral titers. Interestingly the loss of PP1α had a more dramatic influence on viral titers as compared to luciferase production, which suggests that PP1α may be inhibiting multiple stages of viral replication. As RVFV appears dependent on

PP1 α , overall levels of PP1 α were monitored over the course of an infection. While a slight increase was noted in PP1 α protein levels (Fig. 1D, compare lanes 1 and 2), there was no apparent change in PP1 levels over the course of the infection (compare lanes 2, 3, and 4). Collectively these results indicate that loss of PP1 α significantly decreases RVFV replication.

3.2 PP1-targeting small molecule compound, 1E7-03, decreases RVFV replication

Traditional enzymatic PP1 inhibitors, such as okadaic acid or microcystin, are highly toxic and demonstrate poor selectivity as they inhibit both PP2A and PP1^{33,34}. Okadaic acid has a strong preference for PP2A over PP1^{33,34}. We recently developed a PP1-targeting small molecule compound, 1E7-03 (Fig. 2A), that bound to PP1 *in vitro*²², 1E7-03 was shown to disrupt the interaction of HIV-1 Tat with PP1 and inhibited HIV-1 transcription²². In contrast to traditional inhibitors, 1E7-03 has no effect on the enzymatic activity of PP1 and may serve as a non-competitive inhibitor to prevent the binding of regulatory subunits to PP1. To confirm the ability of 1E7-03 to inhibit PP1, phosphorylation of eIF2 (an RVxF dependent interaction³⁵) was assessed in the presence and absence of 1E7-03. As expected, eIF2 phosphorylation was increased in cells treated with 1E7-03 (Fig. 2B), confirming the ability of 1E7-03 to inhibit PP1. When trialed against RVFV, PP1 inhibition by both okadaic acid and 1E7-03 reduced viral titers (Fig. 2C). 1E7-03 however demonstrated lower cellular toxicity (data not shown) and a significantly higher degree of viral inhibition than okadaic acid (Fig. 2C). In order to determine the ideal concentration for maximum efficacy when utilizing 1E7-03 against RVFV, the CC₅₀ (50% cytotoxicity concentration) and EC₅₀ (50% effective concentration) for 1E7-03 were identified (Fig. 2D and E). 1E7-03 was found to be self-limiting in its toxicity, as the compound became insoluble at >50 μ M when dissolved in DMSO, with a CC₅₀ >50 μ M (Fig. 2D). 1E7-03 was able to reduce viral titers by 2 logs at 10 μ M and an EC₅₀ of 3.5 μ M was determined (Fig. 2E). From these results the selective index (CC₅₀/EC₅₀) was estimated to be >14.3. This value was calculated using the highest soluble concentration of 1E7-03 (50 μ M) as the CC₅₀ value. These results indicate that 1E7-03 has a high degree of selectivity for RVFV (greater SI values indicate higher selectivity).

Both HepG2 liver cells and HSAECs were then assayed in order to look at relevant tissue models as RVFV is easily aerosolized and localizes to the liver during infection¹. Both cell types were infected (MOI 3) for 24 hrs while treated with 1E7-03^{36,37}. Treatment with 1E7-03 resulted in no significant cytotoxicity for either cell line (Fig. 3A), and reduced viral titers by nearly two-logs in both cell types (Fig. 3B). As the MP-12 strain is partially attenuated, we also utilized the virulent wild type ZH501 strain to confirm our results. Again, 1E7-03 demonstrated a similar 2 log drop in viral titers in comparison to DMSO (Fig. 3C). In order to further rule out general cellular toxicity, 1E7-03 was also tested against the influenza virus. No changes in either plaque morphology (data not shown) or viral titers were detected when comparing DMSO to 1E7-03 treated cells at either 24 or 48 hpi for influenza (Fig. 3D). Together these data indicate that 1E7-03 inhibits RVFV with minimal cellular toxicity and its effect is specific.

3.3 1E7-03 inhibits RVFV independently of NSs

NSs has been shown to down regulate host cellular transcription while potentially inhibiting the IFN response^{1,2}. Previous studies have also demonstrated dramatic activation of p53 phosphorylation and numerous phosphosignaling events linking the DNA damage response to NSs^{5,6}. Interestingly and unexpectedly, the results in Fig 1A indicated that loss of PP1 by siRNA resulted in the inhibition of RVFV even when NSs was absent (rMP-12-Luc). Based on this information, experiments were performed to determine if 1E7-03's viral inhibition was in anyway linked to NSs. To this end, the replication of the fully intact MP-12 strain was directly compared to that of the rMP-12- NSs strain (Fig. 4A). 1E7-03 treatment equally reduced viral titers (plaque assay) by two logs for both MP-12 and rMP-12- NSs. These results were further corroborated through the demonstration that 1E7-03 inhibits intracellular rMP12-Luc by over 50% in comparison to DMSO controls (Fig. 4B), which corresponded to our siRNA results in Fig 1A. Collectively these data indicate that NSs is not necessary for PP1 inhibition of RVFV replication.

3.4 1E7-03 treatment decreases RVFV viral protein levels

Viral protein levels in the context of 1E7-03 treatment were next analyzed to help determine if a specific protein was being impacted by PP1 deregulation. Due to limitations of commercial antibodies for RVFV, recombinant strains rMP-12-Flag (NSs-Flag) and rMP-12-LV5 were used alongside the unmodified MP-12 strain for viral protein detection. Cells were pre-treated with either DMSO or 1E7-03, infected (MOI 3), and collected at 8, 16, and 24 hpi. In the DMSO controls, the viral nucleoprotein (N) did not reach detectable levels until 16 hpi, and increased over time (Fig. 5A). Following 1E7-03 treatment, N protein was undetectable at 16 hpi, and dramatically reduced in comparison to our DMSO control at 24 hpi, suggesting translation was occurring, albeit at a much reduced capacity (Fig. 5A, compare lanes 6 and 10, with lanes 8 and 12). The viral NSs protein was detected as a faint band in the DMSO control at 8 hpi, with levels increased in comparison over subsequent time points (Fig. 5B). The addition of 1E7-03 completely abrogated expression of NSs, as well as the Gn protein (Fig. 5B). Earlier detection of NSs in comparison to the N protein may be due in part to the virus's ambisense coding strategy, or to differing antibody sensitivity. L polymerase detection in the DMSO controls occurred as early as 8 hpi, with an across the board decrease following 1E7-03 treatment (Fig. 5C). Overall PP1 inhibition appeared to globally decrease levels of viral protein accumulation, indicating a critical dependence on PP1 α .

3.5 1E7-03 treatment decreases RVFV RNA levels

Global inhibition of viral protein accumulation indicated that 1E7-03 treatment was affecting a critical and an early stage event during viral replication. In order to corroborate these findings and further narrow down the event that was being influenced by PP1 deregulation, RVFV viral RNA levels were examined in the context of 1E7-03 (Fig. 6A–C). Following 1E7-03 treatment, RNA levels for the L, M and S segments demonstrated an across the board decrease among viral RNA levels when compared to DMSO controls.

As 1E7-03 treatment of infected cells demonstrated reductions in viral RNA and protein levels; we wanted to ensure that 1E7-03 was not generally inhibiting cellular transcription or

translation. Previous studies utilizing the traditional PP1 inhibitor okadaic acid demonstrated a reduction in phosphorylation levels for cellular RNA polymerase II (pol II)³⁸. Over expression of a mutant nuclear inhibitor of PP1 (incapable of PP1 binding), has also been shown to significantly increase RNA pol II phosphorylation on C-terminal Serine 2 residues³⁹. Due to these concerns, the inclusion of a control experiment consisting of a CMV driven GFP reporter, which is transcribed by RNA pol II, was performed, where GFP reporter was transfected in the presence of either DMSO, 1E7-03 or actinomycin D (Fig. 6D). Following transfection and treatment, levels of the GFP reporter demonstrated no significant decrease for 1E7-03 treated samples in comparison to the DMSO control, while actinomycin D, a well characterized inhibitor of eukaryotic transcription⁴⁰, demonstrated a significant reduction in GFP levels.

Overall, decreases in both viral RNA and protein levels in 1E7-03 treated cells were observed across all of the time points tested, with no significant differences in cellular pol II driven GFP expression. These results indicate that 1E7-03 is specifically targeting an early stage event in the viral life cycle, potentially between viral entry and transcription/translation.

3.6 1E7-03 treatment results in early inhibition of viral replication post entry

In order to narrow down the point at which 1E7-03 was influencing the viral life cycle, a time of addition study was performed. A model using discrete timed drug pulses, in combination with continuously pre- and post-treated cells were utilized (Fig. 7A). For the pulse treatments, 1E7-03 was added solely at the indicated time points, removed, cells were then washed with PBS and drug free media added (Fig. 7B–C). Continuous treatments of DMSO or 1E7-03 were respectively used as high and low reference controls. The most dramatic inhibition was obtained when cells were continuously exposed to 1E7-03 (pre/post treatment), likely due to that multiple rounds of viral replication being influenced by the compound. The addition of 1E7-03 at 0–1 hpi was the second most effective treatment strategy, with a gradual tapering off in effectiveness at later time points. No significant changes in viral titers occurred by pre-treatment alone, indicating that viral entry was not affected. These results were confirmed when examining viral titers for the intact MP-12 strain (Fig. 7C). These data indicate that PP1 activity was most relevant at an early stage event during the viral life cycle, likely during the first few hours post entry, potentially acting on viral trafficking, transcription or replication.

3.7 1E7-03 treatment decreases RVFV minigenome expression and viral protein binding to vRNA

Numerous viruses utilize PP1 in order to regulate viral transcription^{22–24,41}. Previous studies with 1E7-03 have demonstrated an inhibition of viral transcription for both HIV-1 (Tat-PP1 interaction) and EBOV (VP30-PP1 interaction)^{22–24,41}. In RVFV, 1E7-03 treatment appeared to generally inhibit viral RNA production among all three segments at an early stage in the viral life cycle. In order to determine if viral transcription and replication were directly being impacted by PP1 inhibition, a RVFV T7 RNA polymerase-driven minigenome (MG) reporter system was utilized³⁰. Cells were transfected with either a complete minigenome system (MG+), allowing expression and amplification of MG RNA

transcripts represented by renilla luciferase (rLuc), or a negative control (MG-) lacking the critical expression plasmids for the N and L proteins (incapable of viral amplification). In order to normalize differences in transfection efficiencies between sample sets, a fixed amount of pT7-IRES-fLuc plasmid was added to every MG system³⁰. Transfected cells were then treated with either DMSO or 1E7-03 and assayed to determine if the expression of the RVFV MG was being altered following treatment. Levels of firefly luciferase (fLuc) remained evenly and robustly expressed across all sample sets, indicating roughly equivalent levels of plasmid expression (data not shown). In the negative control system (MG-) expression of rLuc consistently remained at background levels across sample sets. The addition of the complete minigenome system (MG+) resulted in the expected rLuc expression in the presence of DMSO. In contrast, the addition of 1E7-03 reduced rLuc expression. After normalizing rLuc levels to respective fLuc transfection controls across sample sets (Fig. 8A), a significant drop in levels of rLuc demonstrated an inhibition of the expression of the RVFV MG in the presence of 1E7-03.

As MG RNA synthesis is reliant on interaction of the viral L and N proteins with viral RNA³⁰, an RNA immunoprecipitation (RNA-IP) was performed to determine if treatment with 1E7-03 had any influence on viral L and N protein binding to viral RNA. For the RNA-IP, cells were infected with RVFV in the presence of DMSO or 1E7-03, and collected at 16 hpi. RNA-IPs were then performed using antibodies against V5 (for L protein detection), N protein, or HA as a control. RNA from each of the immunoprecipitated samples was then extracted for qRT-PCR against the viral M segment. Genomic copies for the viral M segment were then normalized to their respective inputs to determine if changes in the level of viral RNA/protein binding were occurring. Infected cells treated with 1E7-03 resulted in a significant and dramatic decrease in the level of viral RNA bound to the N protein (Fig. 8B). A significant (50%) drop in viral RNA/L protein binding was also observed following 1E7-03 treatment (Fig. 8C). No significant changes between HA control samples (1E7-03 vs DMSO), were noted. Roughly equivalent levels of L protein was immunoprecipitated from DMSO or 1E7-03 treated samples (Fig. 8D, compare lanes 5 and 6). More N protein was immunoprecipitated from 1E7-03 treated cells (compare lanes 3 and 4), but this would not explain the decrease in viral RNA binding observed. Collectively, these results indicate that 1E7-03 treatment is affecting viral RNA production, potentially through the disruption of viral RNA/protein interactions.

4. Discussion

Viral interactions with the host cellular network are critical in the establishment of a successful infection. During an infection viruses must circumvent their host's natural defenses while hijacking cellular machinery in order to replicate. Host based therapeutics offer several advantages over viral targeted strategies in that they establish a much broader range of targets with decreased likelihood of viral adaptation.

The phosphorylation of both viral and cellular proteins have been shown to play a major role in the life cycle of numerous viruses, many of which exploit critical cellular signaling pathways through the selective activation of cellular phosphatases and kinases^{16,31,31,42}. Targeting key regulators of these signaling pathways, such as PP1, holds broad application

potential as PP1 regulates an enormous number of cellular functions and is highly conserved among eukaryotic cells. In order to overcome the inherent difficulties in nonspecifically disrupting all PP1 interactions, a library of small molecules were developed and modeled to specifically fit the RVxF-accommodating cavity of PP1⁴³. In the development of this library, a virtual screening of 300,000 compounds identified a tetrahydroquinoline derivative, 1E7-03²², that was devoid of toxicity and demonstrated increased *in vitro* efficacy in the inhibition of HIV-1 transcription by specifically disrupting the interaction of PP1 with HIV-1 Tat, which contains an RVxF binding motif^{36,37}. Additional confirmational studies with 1E7-03 treated samples have shown negligible changes among the classical regulatory subunits of PP1 α such as NIPP1 and PNUTS, or the expression of over 200 other host cellular proteins that were profiled using mass spectrometry (MS)³⁶. These studies suggest that targeting of PP1 can be done in a highly specific manner with few off target effects by limiting PP1 inhibition to a specific subset of PP1 interactors.

In addition to HIV-1, 1E7-03 has also been trialed against EBOV²⁴. For EBOV, the ribonucleoprotein complex includes VP30, NP, VP35 and the L-polymerase^{44,45}. The polymerase complex mediates both the transcription of individual genes and replication of the genome. Previous studies have demonstrated that the phosphorylation of EBOV VP30 at serine clusters 29–31 and 42–46 blocks the ability of the viral polymerase to function during transcription, but not genome replication^{44–46}. In this process, VP30 functions as a switch between viral transcription and replication^{44–46}. 1E7-03 was found to increase EBOV VP30 phosphorylation in a PP1 dependent manner, and effectively suppressed replication of EBOV in cultured cells²⁴. Further analysis of the effect of 1E7-03 on EBOV transcription and replication using a mini-genome system showed reduction of EBOV transcription, but not replication²⁴.

1E7-03 regulation of the viral life cycle of RVFV, may be due to the inhibition of viral transcription or replication in a manner similar to VP30 for EBOV or Tat for HIV-1, where the viral L polymerase or N protein are substrates for PP1 and reliant on its regulation. For RVFV, the viral RNA-dependent polymerase is involved in both viral mRNA transcription and in the genomic replication of viral RNA. The synthesis of viral mRNA is accomplished through the uncoating and release of pseudohelicoidal ribonucleoproteins (RNPs) which are tightly associated with the L polymerase and support primary transcription^{1,47,48}. Subsequent viral protein production allows replication to initiate through an unknown switch, which copies the entire template and is not reliant on a primer for initiation^{1,47,49}. It may be that PP1 is regulating the switch between viral transcription and replication, or regulating the formation of RNP's, as our results indicate that PP1 inhibition through 1E7-03 treatment decreases viral RNA production, along with viral RNA binding to the L and N proteins. Alternatively, PP1 may be regulating the phosphorylation of an intermediate protein that is needed for RVFV RNA production. Interestingly, viral titers were more dramatically influenced by loss or inhibition of PP1 as compared to viral luciferase production, suggesting that multiple steps in replication are influenced. These data also support the model of genome replication being inhibited as luciferase production would not be as dramatically influenced in this mode of inhibition. These areas are of great interest and are being actively investigated.

PP1 has also been demonstrated to regulate the innate immune responses for numerous RNA viruses, such as: influenza virus, paramyxovirus, dengue virus, and picornavirus⁴². Recent studies have indicated two separate cytoplasmic RNA helicases: retinoic acid-inducible gene-I (RIG-I) and melanoma differentiation-associated gene 5 (MDA5), as a key line in the induction of interferon (IFN) and proinflammatory cytokines during an antiviral response^{32,50}. Studies have indicated that post translational modifications of RIG-I and MDA5, through phosphatases such as PP1 α and PP1 γ , play a critical role in their ability to regulate antiviral activity, as PP1 mediated dephosphorylation leads to their activation^{51,52}. While RIG-I has been identified as critical for IFN production and protection against infection in RVFV⁵⁰, it remains to be seen if deregulation of PP1 is able to disrupt RIG-I and MDA5 activity, particularly as no effect was noted on influenza replication in an 1E7-03/RVxF dependent fashion, which is a known deregulator of PP1-RIG-I activation.

Collectively this study indicates that inhibition of PP1 α demonstrates promise in controlling RVFV multiplication in a potentially therapeutic manner, along with the potential to provide novel and critical insight on the mechanisms governing viral-host interactions.

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- Protein Phosphatase-1 (PP1) is a critical host factor regulating RVFV replication.
- PP1 α deregulation by siRNA or 1E7-03 (small molecule inhibitor) decreases viral titers *in vitro* across multiple cell lines.
- 1E7-03 inhibits RVFV viral RNA production, potentially through modulation of viral N and/or L proteins.
- PP1 deregulation may prove an attractive host targeted therapeutic model, as it is a highly conserved target.

Figure 1. Loss of PP1α reduces RVFV replication

A) 293T cells were transfected for 48hrs with either siPP1α or a siRNA negative control. Cells were then infected with the MP-12-Luc viral reporter (MOI 3). Luminescence was measured at 24 hpi. **B)** 293T cells were treated as described in panel A using the rMP-12 strain. Viral supernatants were collected (24 hpi) and titers measured by plaque assay. **C)** Cells were transfected and infected as described in panel A. Protein lysates were collected at 0 and 24 hpi for western blot analysis with anti-PP1α and actin antibodies. **D)** Levels of PP1α were checked during the course of an infection in 293T cells (rMP-12, MOI 3) and compared to an uninfected sample. Each data set was performed in triplicate and repeated at least twice, *p values < 0.01.

Figure 2. PP1-targeting small molecule compound, 1E7-03, decreases RVFV replication
A) Chemical structure of compound 1E7-03. **B)** HSAECs treated with either DMSO or 1E7-03 (10 μ M) for 16 hrs were western blotted for RVxF dependent PP1 substrate p-eIF2 α . **C)** HSAECs were pre-treated with DMSO, okadaic acid, or 1E7-03, infected with rMP12 (MOI 3), and post-treated with compounds. Viral supernatants were collected at 16 hpi and viral titers determined by plaque assays. **D)** HSAECs were plated and tested against varying concentrations of 1E7-03 along with a solvent control. Triplicate samples for each time point were collected 24 hours post treatment and assayed using Promega's Celltiter-Glo ATP based viability assay. **E)** The EC₅₀ was identified in HSAEC's by pre/post treating the cells with 1E7-03 which were infected with the MP-12 strain (MOI 0.1). Samples were then collected at 24 hpi and analyzed by plaque assay. Sample sets were performed in triplicate, *p values < 0.01, **p values < 0.0001.

Figure 3. PP1-targeting small molecule compound decreases RVFV replication

A) Cellular toxicity for 1E7-03 (10 μ M) was determined using Promegas Celltiter-Glo ATP viability assay for both HepG2 cells and HSAECs by treating cells for 24 hrs. **B)** HepG2 and HSAECs were infected with MP-12 (MOI 3) and treated with 1E7-03 (10 μ M). Viral supernatants collected at 24 hpi were analyzed using plaque assays. **C)** HSAECs cells were treated as described in panel B, and infected with the fully virulent ZH501 strain. **D)** MDCK cells were infected with Influenza A (MOI 1) and treated as in panel B. Each data set was performed in triplicate and repeated at least twice, *p values < 0.01.

Figure 4. 1E7-03 inhibited viral replication independent of NSs

A) HSAECs were infected with either MP-12 or rMP12- NSs (MOI 3) and treated with DMSO or 1E7-03 (10 μ M). Viral titers were checked assayed at 16hpi. **B)** HSAECs were infected with the r MP-12-Luc virus and treated with 1E7-03 (10 μ M). Luminescence was analyzed at 16hpi. Each data set was performed in triplicate, *p values < 0.01.

Figure 5. 1E7-03 treatment decreases RVFV viral protein levels

A) HSAECs were treated with either 1E7-03 (10 μ M) or DMSO, and infected with MP-12 (MOI 3). Protein lysates were collected at various time points post-infection and probed by western blotting with anti-N protein and anti- β -actin antibodies. **B)** Cells were treated as described in panel A and infected with rMP-12-NSs-Flag (MOI 3). Western blotting was performed with anti-Flag, anti-Gn, and anti- β -actin antibodies. **C)** Cells were treated as described in panel A and infected with rMP-12-V5 (MOI 3). Western blotting was performed with anti-LV5 and anti- β -actin antibodies.

Figure 6. 1E7-03 globally decreases RVFV RNA levels

A–C) HSAECs were infected with MP-12 (MOI 3) and treated with either 1E7-03 (10 μ M) or DMSO. Cells were collected and intracellular RNA extracted at 4, 8, 16, and 24 hpi. QRT-PCR was performed using viral primers targeting the L, M or S segments of RVFV. Viral RNA levels for 1E-03 treated samples for each segment were normalized to DMSO samples which were set at 1. **D)** A CMV-GFP reporter (encoded by a eukaryotic class II-driven polymerase) was transfected in the presence of either DMSO, 1E7-03 (10 μ M) or actinomycin D (0.1 μ g/ml). Cells were collected 24 hpi with levels of GFP expression, shown as arbitrary fluorescent units (AU), by flow cytometry. Each data set was performed in triplicate and repeated at least twice, *p values < 0.01.

Figure 7. 1E7-03 treatment displays early inhibition of viral replication

A) Discrete pulses were used for drug additions (grey bars) at the times indicated, then washed with PBS and replaced with complete media alone. **B)** HSAECs were infected with rMP-12-Luc (MOI 3) and treated with 1E7-03 (10 μ M). 1E7-03 was added at the indicated times (panel A), and intracellular viral luminescence was analyzed for all sample sets at 16 hpi. **C)** HSAECs were infected as in panel B, utilizing the full length MP-12 strain, viral supernatants were collected at 16 hpi for plaque assay. Each data set represents biological triplicates, *p values < 0.01.

Figure 8. 1E7-03 treatment inhibits viral RNA production and viral protein binding to vRNA
A) BSR/T7 cells were transfected with either a complete (MG+) system containing pT7-M-rLuc(-), pT7-IRES-fLuc, pT7-IRES-N, pT7-IRES-L, or a negative control (MG-) containing only pT7-M-rLuc(-), pT7-IRES-fLuc, and pT7-IRES. Sixteen hours post transfection cells were treated with either 1E7-03 (10 μ M) or DMSO for 24 hours. RLuc values were normalized to fLuc levels, in order to account for differences in transfection efficiency and plasmid expression. **B–C)** Vero cells were treated with either 1E7-03 (10 μ M) or DMSO, infected with rMP12-LV5 (MOI 3) and collected at 16 hpi. Cells were fixed and immunoprecipitations (IPs) for the viral N and the L-V5 tagged proteins were performed, alongside an HA control for all sample sets. Stringent washes were performed following the IP for all sample sets, and bound RNA was extracted from the immunoprecipitated samples as well as the input controls. Viral RNA for the M segment was probed by qRT-PCR with genomic values normalized to input controls for each respective sample. Each data set was performed in triplicate and repeated at least twice, *p values < 0.01. **D)** Representative western blot showing equal amounts of viral N and L-V5 tagged proteins were immunoprecipitated between DMSO and 1E7-03 treated samples. *non-specific band