Sorafenib impedes Rift Valley fever virus egress by inhibiting Valosin-containing protein function in the cellular secretory pathway

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Running Head: Sorafenib blocks RVFV egress by inhibiting VCP

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There is an urgent need for therapeutic development to combat Rift Valley fever virus (RVFV) infections, which causes devastating disease in both humans and animals. In an effort to repurpose drugs for RVFV treatment, our previous studies screened a library of FDA-approved drugs. The most promising candidate identified was the hepatocellular and renal cell carcinoma drug, sorafenib. Mechanism of action studies indicated sorafenib targeted a late stage in virus infection and caused a buildup of virions within cells. In addition, siRNA knockdown studies suggested that non-classical targets of sorafenib are important for the propagation of RVFV. Here we extend our previous findings to identify the mechanism by which sorafenib inhibits release of RVFV virions from the cell. Confocal microscopy imaging revealed that Gn colocalizes and accumulates within the endoplasmic reticulum (ER), and the transport of Gn from the Golgi to the host cell membrane is reduced. Transmission electron microscopy demonstrated that sorafenib caused virions to be present inside large vacuoles inside the cells. P97/Vasolin-containing protein (VCP), a protein involved in membrane remodeling in the secretory pathway and a known target of sorafenib, was found to be important for RVFV egress. Knockdown of VCP resulted in decreased RVFV replication, reduced Gn Golgi localization, and increased Gn ER accumulation. An intracellular accumulation of RVFV virions was also observed in VCP siRNA transfected cells. Collectively these data indicate that sorafenib causes a disruption in viral egress by targeting VCP and the secretory pathway, resulting in a buildup of virions within dilated ER vesicles.
In humans, symptoms of RVFV infection mainly include a self-limiting febrile illness. However in some cases, infected individuals can also experience hemorrhagic fever, neurological disorders, liver failure and blindness, which could collectively be lethal. The ability of RVFV to expand geographically outside sub-Saharan Africa is of concern, particularly to the Americas, where native mosquito species are capable of virus transmission. Currently, there are no FDA-approved therapeutics to treat RVFV infection, and thus there is an urgent need to understand the mechanisms by which the virus hijacks the host cell machinery to replicate. The significance of our research is in identifying the cellular target of sorafenib that inhibits RVFV propagation, so that this information can be used as a tool for further development of therapeutics used to treat RVFV infection.
INTRODUCTION

Rift Valley fever virus (RVFV) is a member of genus *Phlebovirus* in the family *Bunyaviridae*. RVFV is the causative agent of Rift Valley fever (RVF), an arboviral zoonosis that can cause severe disease in humans and livestock. In ruminants, the disease is characterized by spontaneous abortion rate 40-100%, as well as an increased incidence of mortality among neonates (1). Humans may contract the virus through a mosquito bite or through exposure to infected animal carcasses or body fluid. Human infections are generally asymptomatic or characterized by a mild febrile illness. However, a subset of infected people can progress to severe disease, with symptoms ranging from flu-like symptoms, hepatitis, maculo-retinitis, encephalitis, and fatal hemorrhagic fever that could result in death (2). RVFV has caused outbreaks of RVF throughout Africa and with recent expansion into Madagascar and parts of the Middle East (3). Studies with native mosquitoes in the Americas have demonstrated the potential of virus autochthonous transmission and thus the expansion to the Americas remains a concern. In addition, deliberate aerosol dissemination of the virus could enable widespread transmission of the disease and is thus considered a viable threat. Therefore, RVFV is classified as a Category A priority pathogen by the National Institute of Allergy and Infectious Diseases and ranked as the third most dangerous animal threat by the National Veterinary Stockpile (4). Currently, there are no licensed antiviral treatments or vaccines to prevent or treat RVFV infection, and the U.S. population remains vulnerable to natural disease outbreaks. Further research into the virulence mechanisms of the virus is necessary to develop safe and effective drugs against this disease.

The RVFV genome is comprised of a small (S), medium (M) and large (L) segment each of which is encapsidated by the nucleoprotein (N). Once encapsidated, the segments are referred to
as ribonucleoproteins (RNPs). The L-segment encodes for the viral RNA-dependent RNA polymerase (RdRp) or L protein (5). The genomic sense S-segment encodes for the N protein, while the antigenomic S-segment encodes for a non-structural protein NSs, a virulence factor known to modulate the host immune response (6). The M segment encodes for the major structural glycoproteins Gn and Gc as well as two non-structural proteins NSm1 and NSm2 which are involved in the suppression of cellular apoptosis (7, 8). A characteristic feature of bunyaviruses is that viral particle assembly occurs at the Golgi complex, from which virions have been shown to bud within Golgi vesicles and transport to the host cell plasma membrane for egress (9).

Previous studies conducted by our group demonstrated the utility of an FDA-approved hepatocellular and renal cell carcinoma drug, sorafenib, to inhibit RVFV replication. Studies suggested that sorafenib targets the RVFV in at least two stages in the viral life cycle; viral RNA synthesis (viral genome replication and/or viral transcription) and viral egress from the host cell, resulting in an intracellular buildup of virions. Sorafenib was first discovered as a Raf kinase inhibitor. However, our results showed that loss of Raf does not result in inhibition of RVFV (10), suggesting that an additional protein(s) is targeted by sorafenib in order to decrease viral replication/egress. Here, we investigate the mechanism by which sorafenib inhibits RVFV replication.
RESULTS

Sorafenib treatment causes the inhibition of viral egress. The process of RVFV virion egress consists of virion packaging and budding into the Golgi lumen followed by transport to the cell surface to exit the host cell (11). In order to provide further support for the finding that sorafenib inhibits viral egress, an assay that analyzes the ability of the viral glycoprotein Gn to reach the cell surface at increasing times post-infection was performed (Fig. 1A). This assay that has been optimized and used before in the study of RVFV egress (12, 13). All cells were fixed at 12hpi, and analyzed for the levels of Gn present on the cell surface. Infected cells treated with solvent control (DMSO) displayed 67% surface Gn staining, indicating normal viral egress (Fig. 1B, 1F). In contrast, the Gn staining were significantly reduced to 11% in cells treated with sorafenib immediately following infection (Fig. 1C, 1F). Similarly, when sorafenib was added to the cells at 8hpi (Fig. 1D), before the majority of viral egress is thought to occur, the levels of surface Gn were still significantly lower than the DMSO control at 37% surface Gn staining (Fig. 1F). However, when sorafenib was added at 10hpi (Fig. 1E), after the viral egress stage had already begun, the treatment was no longer able to inhibit viral Gn trafficking to the cell surface resulting in a surface staining of 70% (Fig. 1F) which is comparable to the DMSO control.

In order to corroborate these findings, intracellular distribution and levels of Gn were analyzed. To address this, a similar experiment to Fig. 1 was conducted (Fig. 2A) with only one condition altered. After cells were fixed at 12hpi, they were permeabilized before probing for Gn. This allowed for the detection of both intracellular and surface viral Gn, providing an indication of whether or not sorafenib affects Gn protein production and/or distribution. When treated with DMSO (Fig. 2B), total Gn staining appears punctate and perinuclear which could indicate its...
colocalization with the site of budding, the Golgi. In addition, quantitation of signal shows that
71% of total cells display Gn staining (Fig. 2F). When treated with sorafenib immediately post-
infection, a more diffuse phenotype is observed (Fig. 2C) with what appears to be a slight drop in
level of Gn staining at 55%, however, this decrease is not significant (p > 0.05) (Fig. 2F). Similar
results were obtained when cells were treated before the viral egress stage at 8 hpi (Fig. 2D),
with diffuse Gn staining in 75% of cells (Fig. 2F), comparable to the levels in the DMSO control.
When treated with sorafenib at 12 hpi after the majority of viral egress has begun (Fig. 2E), 75%
of cells were positive for total Gn which were again similar to the DMSO control (Fig. 2F). The
same experiment was conducted to determine total levels of N protein and no significant change
in total protein levels with sorafenib treatment was observed (Fig. 2G). Collectively, these results
reaffirm the hypothesis that sorafenib impedes the viral egress stage of the RVFV life cycle
while exhibiting no significant change in viral protein production at 12 hpi.

Sorafenib treatment of infected cells generates large intracellular vacuoles containing
virus. Previous literature has shown that sorafenib causes fragmentation of organelles within the
secretory pathway which include the ER and Golgi (14). Since we know that sorafenib causes an
intracellular buildup of virions (10), we wanted to obtain a general visualization of infected cells
that were treated with sorafenib compared to cells treated with DMSO. Images obtained by
Transmission Electron Microscopy (TEM) allow us to view the physical state of the organelles
and the localization of the virions in the respective conditions. As expected, when treated with
DMSO, we observe several small vesicles containing virus particles, which we can reasonably
deduce are Golgi vesicles containing virions being transported towards the plasma membrane to
be exocytosed (Fig. 3A). After sorafenib treatment, the appearance of large intracellular vacuoles
containing virions was observed (Fig. 3B). It has been reported in the literature that sorafenib
alters critical organelles of the secretory pathway as exemplified by the fragmentation of the
Golgi complex (14) and dilated ER lumens indicative of ER stress (15). Thus, we can
hypothesize that the vacuoles observed could be dilated ER or Golgi. Another possibility is that
they might be the result of membranes fusing together, unable to further vesiculate. These results
indicate that in the presence of sorafenib, RVFV is being trapped within these large intracellular
vacuoles, which correlates with the observed decrease in viral egress.

Sorafenib treatment prevents Gn from colocalizing with the Golgi and increases Gn
colocalization with the ER. Since a defect in egress was observed after sorafenib treatment,
confocal microscopy was used to determine whether sorafenib affects the colocalization of Gn
and potentially affects the virus budding into the Golgi. Vero (Fig. 4A) and Huh7 (Fig. 5A) cells
that were infected and treated with DMSO displayed a normal pattern of Gn distribution, where
the Gn signal colocalized with the signal from Golgi marker TGN46, indicated by the yellow
signal in the merged panels. In contrast, sorafenib treatment caused the cells to display a diffuse
Gn staining pattern, with Gn no longer colocalizing with the Golgi (Fig. 4B, 5B). Additionally,
the morphology of the Golgi is altered with sorafenib treatment, displaying a more fragmented
appearance as has been previously observed in the literature (14). Thus, the aberrance in viral
egress caused by sorafenib could be the result of the virions not being retained in the Golgi.

Given that sorafenib treatment prevented Gn from colocalizing with the Golgi, we hypothesized
that it was relocated to another location within the secretory pathway. To test this hypothesis, Gn
localization to the ER and/or autophagosomes was analyzed using calnexin and p62 as markers
of ER and autophagosomes, respectively. Infected Vero (Fig. 6A) and Huh7 (Fig. 7A) cells treated with DMSO displayed the characteristic perinuclear Gn staining of RVFV infected cells. There was a low level of colocalization with Gn and the ER marker calnexin (Fig. 6A, 7A), which is not surprising as Gn is translated on the rough ER. However, sorafenib treated cells exhibited a far greater level of colocalization with the ER as indicated by the prevalence of yellow staining compared to the DMSO control (Fig. 6B, 7B). Gn colocalization with p62 was not observed (data not shown), suggesting that Gn does not relocate to autophagosomes following sorafenib treatment. So far, our overall data supports the idea that in the presence of sorafenib, assembly of RVFV virions occurs in the Golgi, but prevents them from egressing by redirecting them to the ER after assembly where they remain.

siRNA knockdown of VCP causes a reduction in RVFV released into the extracellular medium. The classical target of sorafenib, Raf, was shown to not be important for RVFV replication in our previously published research (10). Therefore, we carried out an in-depth literature search to identify a candidate sorafenib target protein, which would induce the effects on the secretory pathway that are being observed, leading to the subsequent block in viral egress. One potential target of sorafenib is valosin-containing protein (VCP), a hexameric AAA+ ATPase (ATPases associated with diverse cellular activities), also known as p97. It has been shown that sorafenib prevents the tyrosine phosphorylation of VCP inducing ER stress, fragmentation of the Golgi, and disruption of the cellular secretory pathway (14). To determine whether VCP is important for viral egress, VCP expression was knocked down using siRNA and followed by RVFV infection. As a control, a very closely related AAA+ ATPase called NSF (N-ethylmaleimide sensitive fusion protein) (16) was also knocked down by
siRNA. NSF is also linked to the secretory pathway, but is instead involved in the heterotypic fusion between small transport vesicles and organelles (17). Twenty-four hours post-infection, extracellular supernatants were harvested and viral titers were analyzed by plaque assay to determine the levels of virus that were able to egress the cell. In conjunction with the supernatant harvest, protein lysates were collected from the cells and western blot analysis was performed in order to determine knockdown of the protein in question. Both VCP and NSF were successfully knocked down in comparison to the scrambled siNeg and the transfection reagent Dharmafect only treated controls (Fig. 8A). Viral titer analysis of the extracellular supernatants reveal that the knockdown of VCP was able to significantly reduce the level of infectious virus that was released from the cell, while knockdown of NSF showed no significant difference in virus titers from the controls (Fig. 8B). These results indicate that loss of VCP inhibits RVFV replication.

**siRNA knockdown of VCP causes Gn localization patterns similar to sorafenib treatment.**

In order to determine the importance of VCP for RVFV egress, the staining patterns of Gn, VCP and the organelle markers were characterized by confocal microscopy following siRNA knockdown of VCP. siNeg treated control cells displayed the typical morphology of Gn localization (Fig. 8C). VCP staining was present both in the nucleus and the cytoplasm as expected (18). siRNA knockdown of VCP resulted in a very diffuse staining of Gn throughout the cell (Fig. 8D). Also, the siRNA knockdown of VCP proved to be robust as demonstrated by the very faint staining of VCP (Fig. 8D).

Since knockdown of VCP resulted in Gn staining that was analogous to the alteration in Gn localization caused by sorafenib, we next wanted to determine whether VCP knockdown also affected Gn colocalization with the secretory pathway organelles in a similar manner to sorafenib...
siNeg transfected RVFV infected cells again resulted in the distinctive Gn staining which colocalized closely with the Golgi marker (TGN46), as shown by the yellow staining indicated by the white arrows (Fig. 9A). Conversely in RVFV infected cells transfected with siVCP, Gn colocalization with the Golgi (TGN46) is highly reduced as indicated by the near absence of yellow staining (Fig. 9B). Furthermore, a low level of colocalization between Gn and the ER (calnexin) was observed in RVFV infected cells transfected with siNeg control (Fig. 10A). However, when RVFV infected cells were transfected with siVCP, cells displayed an increase in colocalization between Gn and the ER (calnexin) (Fig. 10B). These results demonstrate that loss of VCP results in Gn being relocalized to the ER. They also support the hypothesis that VCP plays a key role in the inhibition of viral egress caused by sorafenib.

**siRNA knockdown of VCP causes intracellular buildup of infectious virions comparable to sorafenib treatment.** When infected Huh7 cells were treated with sorafenib, we observed a block in viral egress that led to a buildup in infectious viral particles within the cell (10). To ensure that this effect was not cell type dependent we performed the same experiment in Vero cells (Fig. 11C, D). Cells were infected and treated with sorafenib immediately following infection. At 24hpi, extracellular supernatants were harvested and the remaining cells were lysed using repeated cycles of freezing and thawing in order to release the intact virions from within the cells. These extracts were analyzed by plaque assay and results were plotted as the total number of pfu in each fraction (Fig. 11C). These results are also plotted in terms of the percentage of intracellular virions in the total pool of extracellular and intracellular virions (Fig. 11D). The results show that cells treated with DMSO have about 40% of the virus pool retained within the cell. When the cells are treated with sorafenib, there is close to 100% of the virion
pool retained within the cell. We hypothesized that loss of VCP would cause a similar intracellular accumulation of infectious virions through disruption of the secretory pathway. To test this hypothesis, Huh7 cells were subjected to transfection of either siVCP or control siNeg prior to infection. At 24hpi, extracellular and intracellular fractions were harvested, analyzed by plaque assay and plotted as previously mentioned (Fig. 11A, B). Similar to cells treated with sorafenib, siRNA knockdown of VCP caused a significant reduction in virus released by the cell (Fig. 11A). Furthermore, intracellular fraction data demonstrates a significant increase in infectious virus particles observed within the cell following knockdown of VCP compared to the siNeg control. The results show that siNeg transfected cells have about 50% of the virus pool retained within the cell. When the cells are transfected with siVCP, there is close to 100% of the virion pool retained within the cell (Fig. 11B). These data demonstrate that loss of VCP inhibits viral egress.
DISCUSSION

This study is a follow up to our group’s work on screening FDA-approved drugs to find a therapeutic to combat RVFV infection. We found our top candidate sorafenib and our goal was to ascertain the mechanism by which sorafenib inhibits RVFV replication and to determine one of the host’s cellular targets that RVFV hijacks to propagate itself. We discovered that sorafenib was capable of inhibiting RVFV replication by impeding viral egress, while still allowing for viral protein production and virion assembly to occur. Our data showed that sorafenib caused virions to get trapped in large vesicles which we can conclude are formed from dilated ER since viral Gn no longer colocalized with the site of budding, the Golgi, but rather colocalized with the ER.

A literature search revealed that sorafenib modulates the function of a protein called VCP which is involved in vesiculation and membrane remodeling in the secretory pathway. Interestingly, our results show that loss of VCP resulted in decreased Gn Golgi localization, increased Gn ER localization and increased accumulation of intracellular virions. VCP functions as a chaperone to control various cellular processes, primarily endoplasmic reticulum-associated protein degradation (ERAD), the ubiquitin–proteasome system (UPS) and ER and Golgi morphogenesis. VCP associates with a range of binding partners which determine its diverse activities. It is expected that the expression levels of the cofactors affect the function of VCP by competing for interaction and thus directing VCP to different protein machineries. When VCP interacts with a cofactor named p47, it regulates membrane fusion in ER and Golgi morphogenesis (19).
RVFV virions normally bud from the Golgi within small transport vesicles that travel towards the plasma membrane in order to get exocytosed. We observed that sorafenib treatment caused the virions to get trapped within large intracellular vacuoles unable to egress properly. Fusion of transport vesicles with their appropriate target membrane is caused by the binding of v-SNAREs (soluble N-ethylmaleimide-sensitive factor attachment protein proteins) embedded in the membrane of the transport vesicle to corresponding t-SNAREs in the target membrane. VCP utilizes ATP hydrolysis to break apart SNARE complexes within a restricted set of homotypic fusion pathways. This permits v-SNAREs and t-SNAREs to be recycled for additional rounds of transport. For both Golgi reassembly and yeast ER fusion, it is believed that the most likely ATPase target is a t-SNARE–t-SNARE pairing (syntaxin 5 and Ufe1p, respectively) (20). Thus, in the context of RVFV infection, loss of VCP could prevent SNARE complexes from breaking apart within homotypic fusion pathways, which would prevent Golgi vesicle formation.

NSF is a protein that is important in heterotypic vesicle formation, which includes ER-to-Golgi vesicle formation. Loss of NSF had no effect on RVFV replication/egress. VCP on the other hand, has been shown to play a role in enabling homotypic vesicle formation (20), which includes Golgi vesicle formation. Loss of VCP or sorafenib mediated inhibition of VCP resulted in virions not being able to bud from the Golgi properly in order to make their way to the plasma membrane to be exocytosed from the cell. Thus, we can deduce that VCP which is necessary for Golgi vesicle formation is utilized by the virus to egress from the Golgi complex. Gn colocalized primarily with the ER following sorafenib treatment and siRNA knockdown of VCP, suggesting that the intact heterotypic vesicle formation allows for the virions to be shuttled back to the ER.
Loss of VCP resulted in \(~1.5\) log drop in viral titers. In comparison, sorafenib treatment results in a 2-3 log decrease of virus, depending on the cell type analyzed \((10)\). These data suggest that sorafenib mediated inhibition of VCP is not the only factor contributing to RVFV inhibition. Our previous work revealed that sorafenib also interferes with an earlier step in the virus life cycle, most likely viral transcription and/or genome replication \((10)\). It is therefore likely that sorafenib inhibits a yet to be identified event/protein that suppresses viral RNA production. Recently published literature has revealed that sorafenib can associate with heat shock proteins through their N-termini in order to suppress their chaperone function \((21)\). Chaperones are necessary to maintain conformation of proteins so that they can preserve their function. If sorafenib-induced inhibition of heat shock proteins resulted in misfolded N protein or RdRp, this could inhibit the ribonucleoproteins from properly forming or could interfere with the functionality of the RdRp, thus causing a disruption in further steps of the virus life cycle. Interestingly, inhibition of heat shock proteins using the small molecular inhibitors 17-AAG and BAPTA-AM, reduces RVFV replication \((22)\). This inhibition was determined to be directed at a post-entry event, possibly influencing viral transcription or RNA replication. Hence, the interaction between chaperones and RVFV and the role that sorafenib plays in this interaction warrants further study.

Collectively our data has resulted in our working model of sorafenib mediated viral egress inhibition (Fig. 12). In the absence of sorafenib treatment (Fig. 12A), viral proteins are translated by ribosomes that are either free-floating in the cytoplasm or are embedded in the ER. Gn and Gc are initially expressed as a precursor polypeptide which is then co-translationally cleaved, leading to the maturation of both glycoproteins. Once matured, Gn and Gc form oligomers which are transported from the ER to the Golgi apparatus due to the presence of a Golgi-targeting
signal located within the Gn C-terminal sequence (9). Once the proteins are ready to move on to the Golgi, ER-to-Golgi vesicles containing the viral proteins are able to form and are shuttled to the Golgi. Within the Golgi, the Gn and Gc oligomers organize into the virus envelope as cylindrical hollow spikes that cluster into an icosahedral lattice of 122 distinct capsomers. Published data have revealed that distinct regions of the Gn cytosolic tail are required for binding RdRp and N to package within the virion (5). Once the virions are formed, they are released into the lumen of the Golgi in virus-filled vesicles. These vesicles subsequently fuse with the plasma membrane, releasing mature virions into the extracellular medium (11).

In the presence of sorafenib treatment, the localization of Gn is dramatically altered (Fig. 12B). The majority of Gn now colocalizes with the ER. The large vacuoles containing virus that were observed in the TEM images are hypothesized to be dilated ER. We also note fragmentation of the Golgi complex as visualized in Fig. 4B. Since we observed fully assembled virions in the TEM images (Fig. 3B) and also detected a buildup of infectious virus particles within the cell (10), we can infer that the virions are being allowed to assemble properly with sorafenib treatment. This leads us to believe that the viral proteins are able to be transported from the ER to the Golgi within vesicles to be assembled into virions within the Golgi. However, since the majority of Gn was found to colocalize with the ER, we hypothesize that the assembled virions were prevented from egressing to the plasma membrane due to the inhibited function of VCP. Instead, the virions are then shuttled back to the ER facilitated by NSF, where they remain.
MATERIALS AND METHODS

Cells, Virus and Drug treatment. Vero (ATCC CCL-81) cells were grown in Dulbecco’s modified minimum essential medium (DMEM) supplemented with 10% heat-inactivated fetal bovine serum (FBS), 1% penicillin/streptomycin and 1% L-glutamine. Huh7 cells (a kind gift from Dr. Charles M. Rice, Rockefeller University, NY, NY) were grown in DMEM containing 1% L-glutamine, 1% NEAA, 10% FBS, 1% penicillin/streptomycin and 1% sodium pyruvate. Cells were maintained at 37°C in humidified 5% CO₂. Unless noted otherwise, all cells were plated at a density of 5.0 × 10⁵ cells in 6-well plates, 2.5 × 10⁵ cells in 12-well plates, and 1 × 10⁴ cells in 96-well plates.

Recombinant RVFV strain (r)MP12 was rescued and titered as previously described (10). Infections were performed under BSL-2 conditions. Cells were infected with virus at a multiplicity of infection (MOI) of 10 in drug treatment experiments and MOI of 0.1 in siRNA knockdown experiments. Inocula were removed after 1 h, washed one time with 1× phosphate-buffered saline (PBS), and replaced with fresh medium. Infection was allowed to proceed for a specific duration of time, as indicated for each experiment. Infectious virus was detected by plaque assay (23). The intracellular infectivity assay was performed as described in (10).

Sorafenib Tosylate (S1040) was purchased from Selleckchem and made into 10 mM stocks dissolved in DMSO and stored at -80°C. Drug treatments were made at a concentration of 10 µM in complete medium appropriate for the respective cells.
**Immunofluorescence and Antibodies.** At the end of the specified incubation time, virus-infected cells were fixed in 10% neutral buffered Formalin (Sigma, St. Louis, MO) for a minimum of 15 minutes. Briefly, formalin-fixed cells were either untreated or, when necessary, permeabilized (to stain for intracellular expression of RVFV Gn or N proteins) with 0.1% (v/v) Triton-X 100 in PBS for 15 minutes at room temperature. Cells were washed three times with PBS, blocked in 3% bovine serum albumin/PBS, and treated with the corresponding primary antibody at 37 °C for 1 h followed by three additional washes with 1× PBS and secondary antibody treatment. The mouse monoclonal antibodies 4D4 and R3-1D8-1-1a were used to detect RVFV glycoprotein (Gn) and RVFV nucleocapsid (N) respectively. All antibodies to viral antigens were purified from the hybridoma stocks at USAMRIID (available from BEI Resources). The rabbit monoclonal antibody ab109240 (Abcam) was used to detect VCP. The sheep polyclonal antibody AHP500GT (AbD Serotec) was used to detect TGN46. The rabbit polyclonal antibody sc11397 (Santa Cruz Biotechnology) was used to detect Calnexin. The rabbit monoclonal antibody 3924S (Cell Signaling Technologies) was used to detect NSF. Cell nuclei were labeled with Hoechst 33342 (Life Technologies) at a 1:10,000 dilution or DAPI at 1:1000 dilution. Alexa 488-conjugated goat anti-mouse secondary, Alexa 568-conjugated goat anti-rabbit or Alexa 568-conjugated donkey anti-sheep antibodies (1:500; Life Technologies) were used to visualize primary antibodies.

High-content quantitative imaging data were acquired and analyzed on an Opera confocal reader (model 3842 [Quadruple Excitation High Sensitivity] or model 5025; PerkinElmer, Waltham, MA) at two exposures using a 10× air objective. Analyses of the images were accomplished within the Opera or Columbus environment using standard Acapella scripts. (24)
Transmission electron microscopy (TEM). Vero cells were infected with MP12 MOI 10 followed by treatment with sorafenib for 24h. Cells were then fixed and processed as described in (25).

siRNA knockdowns. Huh7 cells, plated at 120,000 cells per well in 12-well plates or 280,000 cells per well on coverslips in 6-well plates, were transfected with SMARTpool siRNAs targeting VCP (10nM; Dharmacon, # L-008727-00-0005), NSF (50nM; Dharmacon, # L-009401-00-0005), or negative control siRNA (50 nM; Dharmacon # D-001810-01-05). All transfections were performed using Dharmafect 2 reagent (Thermo scientific, # T-2002-02). An untreated control with Dharmafect 2 reagent alone was also performed. After 24h, transfection media was replaced with complete media and cultured for additional 48 hours before infection. Protein lysates and extracellular media supernatants were collected at 24hpi (hours post-infection). Protein expression was measured by western blot analysis and infectious titers determined by plaque assay. Western blots were performed according to (10). For confocal microscopy, coverslips were fixed at 24hpi, processed and imaged as mentioned earlier.

Statistics. All quantifications are based on data obtained from triplicate samples unless indicated otherwise. Error bars in all figures indicate standard deviations. P-values were calculated using unpaired Student's t test. P-values below 0.05 were considered significant.
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FIGURE LEGENDS

Figure 1. Sorafenib treatment causes the inhibition of viral egress. Vero cells were infected with MP12 MOI 10 and either treated with DMSO immediately following infection (B), treated with Sorafenib immediately following infection (C), treated with Sorafenib at 8h post-infection (D) or treated with Sorafenib at 10h post-infection (E). Cells were fixed, left unpermeabilized, and stained for virus glycoprotein Gn on the cell surface at 12h post-infection. The percentage of cells that stained positive for Gn expression was determined by high-content quantitative image-based analysis (See Materials and Methods). Means and standard deviations for three biological replicates are plotted (F). *p<0.05

Figure 2. Sorafenib treatment does not affect intracellular Gn protein levels. Vero cells were infected with MP12 MOI 10 and either treated with DMSO immediately following infection (B), treated with Sorafenib immediately following infection (C), treated with Sorafenib at 8h post-infection (D) or treated with Sorafenib at 10h post-infection (E). Cells were fixed, permeabilized, and stained for virus glycoprotein Gn both intracellularly and on the cell surface at 12h post-infection. The percentage of cells that stained positive for Gn expression was determined by high-content quantitative image-based analysis (See Materials and Methods). Means and standard deviations for three biological replicates are plotted (F). Cells were fixed, permeabilized, and stained for viral N protein both intracellularly and on the cell surface at 12h post-infection. The percentage of cells that stained positive for N expression was determined by high-content quantitative image-based analysis (See Materials and Methods). Means and standard deviations for three biological replicates are plotted (G).
Figure 3. Sorafenib treatment of infected cells generates large intracellular vacuoles containing virus. Vero cells were infected with MP12 MOI 10 and treated with DMSO (A) or treated with Sorafenib (B). Samples were prepared and analyzed by transmission electron microscopy.

Figure 4. Sorafenib treatment prevents Gn from colocalizing with the Golgi in Vero cells. Vero cells were infected with MP12 MOI 10 and either treated with DMSO immediately following infection (A) or treated with Sorafenib immediately following infection (B). Cells were fixed, permeabilized, and stained for Gn, trans-Golgi network protein 46 (TGN46) and Hoechst at 12h post infection.

Figure 5. Sorafenib treatment prevents Gn from colocalizing with the Golgi in Huh7 cells. Huh7 cells were infected with MP12 MOI 10 and either treated with DMSO immediately following infection (A) or treated with Sorafenib immediately following infection (B). Cells were fixed, permeabilized, and stained for Gn, trans-Golgi network protein 46 (TGN46) and Hoechst at 12h post infection.

Figure 6. Sorafenib treatment increases Gn colocalization with the ER in Vero cells. Vero cells were infected with MP12 MOI 10 and either treated with DMSO immediately following infection (A) or treated with Sorafenib immediately following infection (B). Cells were fixed, permeabilized, and stained for Gn, ER marker Calnexin and DAPI at 12h post infection.
**Figure 7. Sorafenib treatment increases Gn colocalization with the ER in Huh7 cells.** Huh7 cells were infected with MP12 MOI 10 and either treated with DMSO immediately following infection (A) or treated with Sorafenib immediately following infection (B). Cells were fixed, permeabilized, and stained for Gn, ER marker Calnexin and DAPI at 12h post infection.

**Figure 8. Loss of VCP decreases RVFV viral release.** Huh7 cells were treated with either siRNA negative control or siRNA targeting VCP, followed by infection with MP12 MOI 0.1 for 24h. Protein levels were detected by Western blot (A). Level of virus in the cell supernatants was determined by plaque assay (B). Means and standard deviations for three biological replicates are plotted. *p<0.05. Huh7 cells were treated with either siRNA negative control (C) or siRNA targeting VCP (D), followed by infection with MP12 MOI 0.1. Cells were fixed, permeabilized, and stained for Gn, VCP and DAPI at 24h post infection.

**Figure 9. Loss of VCP prevents Gn from colocalizing with the Golgi.** Huh7 cells were treated with either siRNA negative control (A) or siRNA targeting VCP (B), followed by infection with MP12 MOI 0.1. Cells were fixed, permeabilized, and stained for Gn, trans-Golgi network protein 46 (TGN46) and DAPI at 24h post infection.

**Figure 10. Loss of VCP increases Gn colocalization with the ER.** Huh7 cells were treated with either siRNA negative control (A) or siRNA targeting VCP (B), followed by infection with MP12 MOI 0.1. Cells were fixed, permeabilized, and stained for Gn, ER marker Calnexin and DAPI at 24h post infection.
Figure 11. Loss of VCP inhibits RVFV viral egress comparable to sorafenib treatment.
Huh7 cells were treated with either siRNA negative control or siRNA targeting VCP, followed by infection with MP12 MOI 0.1 for 24h. Intra- and extracellular infectivity per infection as determined by plaque assay is plotted (A). Total infectivity (i.e. sum of intra- and extracellular infectivity) per infection was determined. The percentage of intracellular infectivity relative to the total was plotted for siNeg and siVCP transfected cells (B). Vero cells were treated with either DMSO or sorafenib, followed by infection with MP12 MOI 0.1 for 24h. Intracellular versus extracellular infectivity (C) and percentage of intracellular infectivity (D) were plotted as mentioned above. Means and standard deviations for three biological replicates are plotted. *p<0.05

Figure 12. Working model of sorafenib effect on viral egress. Normal RVFV egress (A). Disruption of egress following Sorafenib treatment (B).
Figure 1

A) Infection

0h 1h 2h 3h 4h 5h 6h 7h 8h 9h 10h 11h 12h

(B) (C) (D) (E)

DMSO Sorafenib

B) C) D) E)

F) % Gn-positive cells

0 20 40 60 80 100

1 8 10

* DMSO Sorafenib

Hours post-infection
Figure 2

Distribution Statement A: Approved for public release; distribution is unlimited.

A) Infection

B) DMSO

C) Sorafenib

D) Hours post-infection

E) DMSO

F) Sorafenib

G) % N-Positive cells

Hours post-infection
Figure 3

Distribution Statement A: Approved for public release; distribution is unlimited.
Figure 7

A) Gn Calnexin

DAPI Merge

20 µm

B) Gn Calnexin

DAPI Merge

20 µm
Figure 8

A) Western blot analysis showing VCP, NSF, N, and Actin proteins under different conditions (siNeg, siVCP, siNSF, Dhharmafect).

B) Bar graph depicting pfu/ml concentration for siNeg, siVCP, siNSF, and Dhharmafect, with * indicating a significant difference.

C) Confocal microscopy images of Gn and VCP stains with DAPI merge showing cellular distribution.

D) Additional confocal microscopy images with Gn and VCP stains in DAPI and Merge views.