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Repurposed FDA-Approved drug sorafenib reduces replication of Venezuelan equine encephalitis virus and other alphaviruses

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ABSTRACT

The New World alphaviruses –Venezuelan, eastern, and western equine encephalitis viruses (VEEV, EEEV, and WEEV respectively) – cause a febrile disease that is often lethal in equines and children and leads to long-term neurological sequelae in survivors. Endemic to the Americas, epizootic outbreaks of the three viruses occur sporadically in the continental United States. All three viruses aerosolize readily, replicate to high titers in cell culture, and have low infectious doses. Additionally, there are no FDA-approved vaccines or therapeutics for human use. To address the therapeutic gap, a high throughput assay utilizing a luciferase reporter virus, TC83-luc, was performed to screen a library of commercially available, FDA-approved drugs for antiviral activity. From a group of twenty compounds found to significantly decrease luminescence, the carcinoma therapeutic sorafenib inhibited replication of VEEV-TC83 and TrD *in vitro*. Additionally, sorafenib inhibited replication of EEEV and two Old World alphaviruses, Sindbis virus and chikungunya virus, at 8 and 16 h post-infection. Sorafenib caused no toxicity in Vero cells, and coupled with a low EC_{50} value, yielded a selectivity index of > 19. Mechanism of actions studies suggest that sorafenib inhibited viral translation through dephosphorylation of several key proteins, including eIF4E and p70S6K, leading to a reduction in viral protein production and overall viral replication.

1. Introduction

The New World equine encephalitides, eastern, Venezuelan, and western equine encephalitis viruses (EEEV, VEEV, and WEEV, respectively), are endemic to North, Central, and South America and cause significant mortality and morbidity in equines and humans. There have been epizootic outbreaks of all three viruses in the United States (Strauss and Strauss, 1994). These viruses are primarily transmitted by *Culex, Culiseta*, and *Coquillettidia* mosquito species amongst enzootic bird or rodent hosts (Jupp, 2013). The viruses are asymptomatic in their enzootic hosts but can cause a range of neurological pathologies often resulting in death in equines and long-term sequelae in humans. VEEV, the model species, causes a biphasic febrile illness with lymphatic manifestations that leads to involvement of the central nervous system (CNS) in severe cases. Severe symptoms include seizures, ataxia, and coma, and occur more frequently in children (Steele and Twenhafel, 2010). The equine encephalitis viruses cause emerging infectious diseases with the potential for weaponization. The viruses readily aerosolize, replicate to high titers in culture, and have low infectious doses (Sidwell and Smee, 2003). The mosquito species that harbor the viruses are also found in parts of Europe, making accidental import a concern (Jupp, 2013).

Currently, there are no FDA-approved therapeutics or vaccines for human use against the New World alphaviruses, making them a priority for government funding agencies (Reichert et al., 2009). A live, attenuated strain of VEEV, TC83, is used to vaccinate horses and at-risk personnel, but its high reactogenicity and moderate seroconversion makes it unacceptable for general human use (Pittman et al., 1996). TC83 was generated from serial passaging the virulent strain Trinidad-Donkey (TrD) in guinea pig heart cells; TC83 and TrD genomes differ in 11 nucleotides, only 2 of which are attenuating, making the possibility of back-mutations high (Kinney et al., 1989, 1993). TC83 and formalin-inactivated vaccines for VEEV (C-84), EEEV (TSI-GSD 104), and WEEV (CM 4884) are the only vaccines with Investigational New Drug status, and all require boosters (reviewed in (Hoke, 2005)). There are no prophylactic or therapeutic treatment options for VEEV infection beyond symptom management.

To this end, a library of FDA-approved compounds was screened *in vitro* for efficacy against VEEV infection, with the hopes that targeting a host pathway exploited by the virus would not only limit adaptive

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mutation of the virus, but may also have pan-alphavirus activity. Several groups of compounds affecting similar pathways were identified, such as inhibitors of growth factor receptors and DNA synthesis. One compound, sorafenib (known commercially as Nexavar), was found to significantly decrease viral replication with minimal toxicity to cells and has already been published as a potential inhibitor of Rift Valley fever virus (RVFV) (Benedict et al., 2015a) and hepatitis C virus (HCV) (Bürckstümmer et al., 2006). Sorafenib is a small molecule, broad tyrosine kinase (VEGFR) inhibitor, which blocks growth receptor autophosphorylation as well as downstream Raf kinases (C-Raf and to a lesser extent B-Raf) (Roberts and Der, 2007), and induces apoptosis through endoplasmic reticulum stress (Rahmani et al., 2007), autophagy (Park et al., 2010), and translation inhibition (Rahmani et al., 2005). It is currently used to treat hepatocellular, thyroid, and renal cell carcinomas (FDA, 2013). In addition to being well tolerated *in vitro*, sorafenib decreased viral titers significantly in VEEV TC83 and fully virulent VEEV TrD and EEEV, as well as the Old World alphaviruses, Sindbis virus (SINV) and the vaccine strain of chikungunya virus (CHIKV). Sorafenib's mechanism of action against VEEV was found to be unrelated to its inhibition of tyrosine and Raf kinases, but our data indicate that it interferes with the viral life cycle during translation through dephosphorylation of the eIF4E translation initiation factor (Rahmani et al., 2005).

2. Materials and methods

2.1. FDA-approved compound library screening

A FDA-approved compound library was purchased from Selleckchem (#L1300) and used for *in vitro* studies, as described previously (Benedict et al., 2015a). Briefly, compounds came suspended in DMSO at 10 mM. Compounds were further diluted to 10 μM in culture media. Vero cells were pre-treated with library compounds for 1 h, infected with TC83luc (MOI 0.1) for 1 h, washed with PBS, then media containing the compounds added back to the cells. At 16 h post-infection (hpi), Promega's BrightGlo was used to measure luminescence. DMSO in complete media, no greater than 0.1%, was included as the vehicle control.

2.2. Cell culture

Vero cells were maintained as described previously (Lundberg et al., 2013).

2.3. Viral infections and inhibitor treatments

Production of TC83 viral stocks has been described previously (Lundberg et al., 2016). The pTC83 plasmid was a kind gift from Dr. Ilya Frolov, University of Alabama (Kinney et al., 1993; Paessler et al., 2003). The VEEV-TC83luc virus was kindly provided by Dr. Slobodan Paessler of the University of Texas Medical Branch, Galveston (Patterson et al., 2011). SINV (EgAr 339) was obtained from BEI Resources. The vaccine strain of CHIKV (181/25) was kindly provided by Dr. Naomi Forrester, University of Texas Medical Branch, Galveston (Gorchakov et al., 2012). The VEEV ZPC738 TaV nLuc and EEEV FL93-939 TaV nLuc plasmid constructs (Sun et al., 2014) were provided by Dr. William Klimstra, University of Pittsburgh. VEEV-TC83, VEEV-TC83luc, SINV EgAr 339, and CHIKV 181/25 were utilized under BSL-2 conditions. VEEV-TrD and EEEV (GA97) were utilized under BSL-3 conditions. All work involving select agents is registered with the Centers for Disease Control and Prevention and conducted at George Mason University's Biomedical Research Laboratory, which is registered in accordance with federal select agent regulations.

When performing infections, virus was added to supplemented DMEM at a MOI of 1, unless otherwise stated. The viral media was added to cells and incubated at 37 °C for 1 h with rotation every 15 min. After infections, cell cultures were washed once with sterile 1X PBS. Crystal violet plaque assays determined viral titer as described previously (Lundberg et al., 2013).

Sorafenib tosylate and SC1 were purchased from Selleckchem (S1040 and S7752, respectively). Stock inhibitors were dissolved in sterile DMSO and diluted in complete culture media prior to viral infection unless otherwise noted. Cells were pre-treated for 2 h prior to infection then media containing inhibitors was removed, unless otherwise noted. After infection, cells were washed with sterile 1X PBS, and media containing compounds was added back to cells. Cells were cultured for an additional 16 h, unless otherwise noted. DMSO in complete media, no greater than 0.1%, was included as the vehicle control.

2.4. Cell viability assays

To determine inhibitor toxicity, Promega's CellTiter Luminescent Cell Viability Assay (G7571) was used to measure cellular viability 24 h after inhibitor treatment. The assay has been described previously (Lundberg et al., 2013).

2.5. Luciferase reporter assays

Luciferase reporter assays have been described previously (Lundberg et al., 2016). Briefly, Promega's BrightGlo Luciferase Assay System (E2610) was used to measure luminescence in white-walled, ninety-six well plates (Corning, 3610) seeded with 10,000 Vero cells/well at 16 h post-infection and read with Beckman Coulter's DTX880 Multimode Detector measuring luminescence after 100 ms integration per well.

2.6. RNA extraction and RT-quantitative PCR

RT-qPCR was performed as described previously (Lundberg et al., 2016). Briefly, Vero cells were pre-treated, infected with VEEV-TC83, then infectious media removed and drug media added back to cells after a sterile 1X PBS wash. Four or 8 h later, supernatants were collected to analyze extracellular viral RNA, and cells lysed and collected in Qiagen's RLT buffer to analyze intracellular viral RNA. Extracellular RNA was extracted using Ambion's MagMax Viral RNA Isolation Kit (AM 1836); intracellular RNA was isolated using Qiagen's RNeasy Mini Kit (74,104). RT-qPCR was performed using the Applied Biosystems StepOne Plus. Primers and probe against either the capsid region (Lundberg et al., 2016), amplifying both the genomic and subgenomic viral RNA, or viral RNA packaging signal (nt1057-1154), amplifying only genomic viral RNA (Carey et al., 2018; Kim et al., 2011) were used. The absolute quantification was calculated using StepOne Software v2.3 and based on the threshold cycle relative to the standard curve.

2.7. siRNA knockdown

siRNA knockdown of B-Raf and C-Raf was performed as described previously (Benedict et al., 2015a). eIF4E siRNA was obtained from Dharmacon (SMARTpool: ON-TARGETplus EIF4E siRNA, Catalogue # L-003884-00-0005). Vero cells were transfected according to the previously described parameters, infected with TC83 (MOI 1), then supernatants for plaque assays or protein lysates for western blot analysis were collected at 16 hpi (64 h post siRNA transfection).

2.8. Western blot analysis and antibodies

Cell lysates were collected and processed as previously described (Benedict et al., 2015a; Lundberg et al., 2016; Austin et al., 2012). Primary antibodies used were B-Raf at 1:2000 (Abcam, ab117860), C-Raf at 1:2000 (Abcam, ab124452), p-p70S6K (Thr389) at 1:1000 (Cell Signaling Technologies (CST), 9205S), p-eIF4E (Ser209) at 1:1000

(CST, 9741S), p-S6 Ribosomal protein (Ser 235/236) at 1:1000 (CST, 4856S), eIF4E (C46H6) at 1:1000 (CST, 2067), HRP-conjugated actin (Abcam, ab49900), VEEV capsid 1:3000 (BEI Resources, NR 9403), and VEEV Glycoprotein at 1:2000 (BEI Resources, NR 9404) diluted in blocking buffer (3% milk in PBS and 0.1% Tween-20 or 5% bovine serum albumin in TBS and 0.1% Tween-20 for phospho-antibodies). Secondary antibodies conjugated to HRP were goat anti-rabbit IgG at 1:2000 (Thermo Scientific, 32,460), goat anti-mouse IgG at 1: 1500 (Thermo Scientific, 2430), or donkey anti-goat at 1:5000 (Santa Cruz Biotechnologies, sc-2020) diluted in blocking buffer.

2.9. Statistical analysis

Unless noted otherwise, means were compared using the unpaired, two-tailed student T-test available on QuickCalcs, GraphPad's free online software.

3. Results

3.1. FDA-approved compound library screen

A reporter virus, TC83-luc, which encodes firefly luciferase downstream of a duplicated sub-genomic promoter (Patterson et al., 2011), was used to screen compounds for antiviral activity. A 420-FDA approved drug library was screened in a 96-well format, as described previously (Benedict et al., 2015a). Briefly, Vero cells were pre-treated with 10 μM compounds for 1 h, infected with TC83-luc (MOI 0.1) for 1 h, washed with PBS, then complete media containing each respective drug added back onto the cells and left for 16 h, when luciferase reporter activity was measured. Our overall testing strategy is displayed in Fig. 1. A total of 71 compounds demonstrated 50% inhibition or greater and were next tested for cytotoxity, to eliminate false positives. Compounds that reduced cell viability by more than 20% were discarded. Twenty-three compounds were then moved forward in the pipeline and tested in triplicate for their ability to inhibit VEEV-TC83-luc replication. Fig. 2A shows a selection of compounds that significantly inhibited luminescence normalized to the DMSO control. Groups or classes of compounds that emerged included tyrosine kinase inhibitors, growth factor receptor inhibitors, and inhibitors of DNA synthesis (Table 1).

From the screen, sorafenib tosylate (tradename Nexavar) was selected for further investigation due to its low cytotoxicity represented as a CC_{50} greater than 80 μM (Fig. 2B) after 24 h treatment, reduction of TC83-luc (MOI 1) replication in a dose-dependent manner which yielded an EC_{50} of 4.2 μM (Fig. 2C) at 16 h post-infection (hpi), and a selective index (SI – calculated by dividing the CC_{50} by the EC_{50}) of > 19.0 μM (Fig. 1). Sorafenib displayed an EC_{50} of 3.7 μM on VEEV TC83 infectious titers (Fig. 2D), which is in line with the EC_{50} determined using the TC83 luciferase virus. Sorafenib was also of particular interest because it was previously found to inhibit RVFV using a similar FDA-approved drug screen (Benedict et al., 2015a). By inhibiting two unrelated viruses, sorafenib could potentially be a pan-viral therapeutic, warranting further investigation.

3.2. Sorafenib reduces VEEV replication independently of its Raf-kinase inhibition activity

Sorafenib is a well-published inhibitor of Raf kinases, tyrosine kinases, and several growth factor receptors (Roberts and Der, 2007; Wilhelm et al., 2004a, 2006, 2008). Because sorafenib impacts multiple cellular pathways, we first wanted to know if its efficacy against VEEV was related to its ability to inhibit Raf kinases. A sorafenib derivative, known as SC1, retains the antifibrotic activities of sorafenib but lacks the ability to inhibit the Raf kinases (Chen et al., 2011; Su et al., 2015),

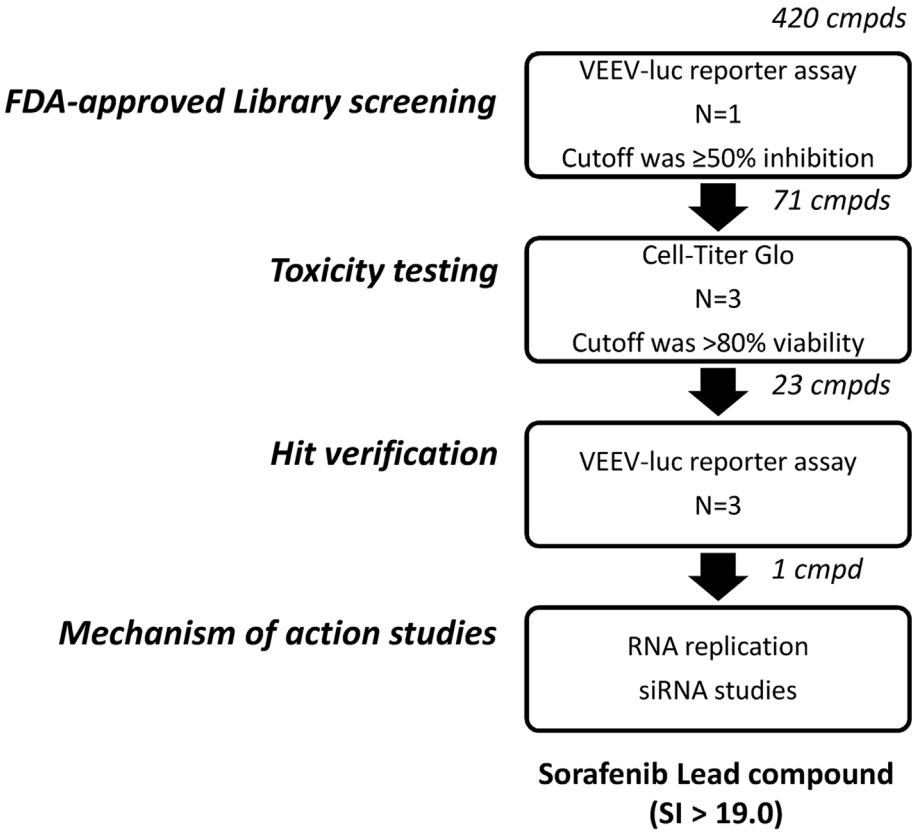


Fig. 1. Experimental process for screening FDA approved compounds for VEEV antiviral efficacy. Flow chart of the experimental setup used to identify effective VEEV inhibitors.

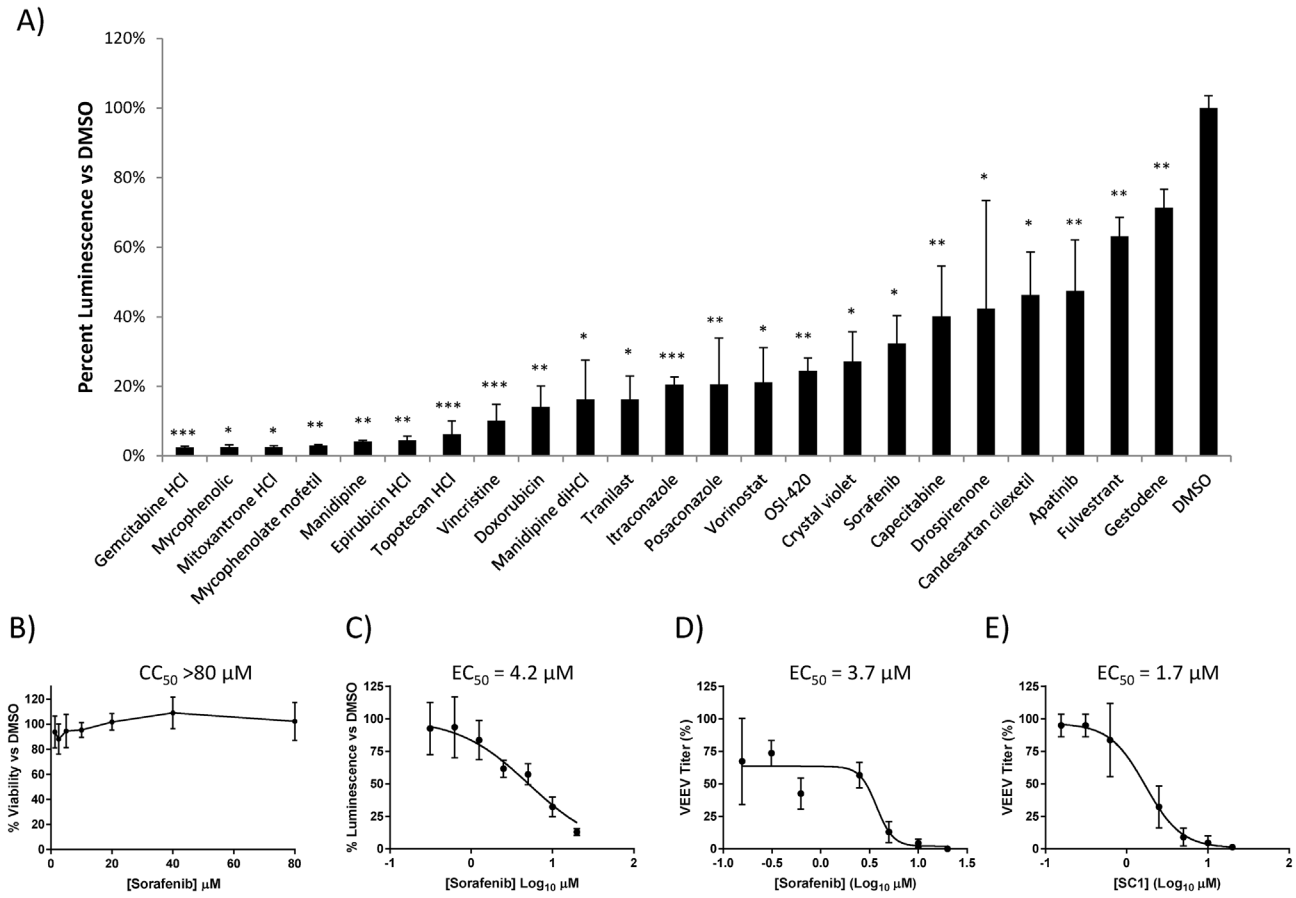


Fig. 2. Sorafenib is non-toxic in Vero cells and inhibits VEEV in a dose-dependent manner. (A) Vero cells in triplicate wells were pretreated with 10 µM of selected compounds for 1 h, infected with TC83-luc (MOI 0.1) for an hour, then compound-containing media added back to cells. After 16 hpi, luminescence was measured using Promega's BrightGlo and normalized to DMSO-treated infected controls. Data represents a selection of compounds that reduced luminescence by a statistically significant amount without reducing cell viability below 80% compared to DMSO treated controls. *p < 0.05, **p < 0.01, ***p < 0.001. (B) Vero cells were treated with decreasing concentrations of sorafenib to determine toxicity. After 24 h, Promega's CellTiter-Glo was performed to determine cell viability compared to DMSO treated controls. Data represent biological triplicates, and the experiment was performed twice. (C) Vero cells were pretreated with varying concentrations of sorafenib for 2 h, infected with TC83-luc (MOI 1) for 1 h, then drug-treated media added back to cells. After 16 hpi, luminescence was measured using Promega's BrightGlo and normalized to DMSO controls. Data represents biological triplicates, and the experiment was performed twice. (D and E) Cells were treated as described in panel C with either sorafenib (panel D) or SC1 (panel E). Supernatants were collected at 16 hpi for plaque assay analysis.

so it was chosen as a control. SC1 (Fig. 3A) was more toxic to Vero cells at 24 h than sorafenib (Fig. 2B), so a concentration of 10 µM was chosen for each compound for subsequent experiments.

To confirm that sorafenib could reduce VEEV TC83 replication and determine if this was dependent on inhibiting the Raf kinases, Vero cells were pre-treated with sorafenib, SC1, or DMSO (vehicle control) for 2 h, infected with TC83 for 1 h, washed, then complete media containing the respective compound was added back to cells (pre- and post-treatment regimen). Supernatants were collected at 8, 16, and 24 hpi and plaque assays performed to calculate viral titer. Both sorafenib and SC1 significantly reduced TC83 titer compared to the DMSO-treated control at all time points and displayed an EC_{50} of 1.7 µM on VEEV TC83 infectious titers (Fig. 2E), suggesting that sorafenib's Raf kinase inhibiting activity is not necessary for suppressing VEEV replication. To further confirm this supposition, triplicate Vero cell populations were transfected with siRNA against B-Raf, C-Raf (lanes 4–6), or both (lanes 7–9) for 48 h (Fig. 3C) then infected with TC83. Supernatants were collected 16 hpi and plaque assays performed. Compared to the siNeg (scrambled siRNA sequence) control (lanes 1–3, Fig. 3C), loss of B-Raf, C-Raf, or both in conjunction had no effect on viral titers (Fig. 3D). This, along with the SC1 results, confirmed that Raf kinases are not necessary for VEEV replication in Vero cells, and that sorafenib's ability to inhibit viral replication is independent of its activities as a Raf-kinase inhibitor.

3.3. Sorafenib inhibits VEEV replication post-entry, but prior to egress

To better define where in the viral life cycle sorafenib was having an effect, viral genomic RNA was measured at early time points (4 and 8 hpi) in the presence or absence of sorafenib treatment. Sorafenib treatment resulted in a decrease in extracellular viral RNA by 4 hpi (Fig. 4A), and an even greater decrease compared to DMSO by 8 hpi, indicating a reduction in released virions that impacts subsequent rounds of infection. RT-qPCR was next performed on intracellular viral RNA with primer and probe sets recognizing either the capsid region (genomic and subgenomic RNAs) or the viral packaging sequence (genomic viral RNA only). There was a decrease in intracellular genomic and subgenomic RNAs at 4 hpi (Fig. 4B) and a similar reduction was observed at 8 hpi, indicating the viral genome is still being replicated, but fewer viruses are being released. A reduction in genomic RNA was only observed at 8 hpi following sorafenib treatment (Fig. 4C). While changes in viral RNA levels were statistically significant, some were minor (less than a log), indicating that viral RNA production is minimally affected by sorafenib treatment. Analysis of the particle to pfu ratio indicates that sorafenib treatment leads to an increase in defective viral particles (Fig. 4D). Probing for the viral structural proteins, glycoproteins and capsid, showed a reduction in viral proteins in sorafenib treated samples (lanes 7–8) compared to DMSO controls (lanes 3–4) at 8 hpi (Fig. 5). While this could be partly

Table 1
Non-toxic FDA approved compounds that significantly reduced luminescence in TC83-luc infected cells. Table 1 represents a selection of compounds that were found to significantly reduce TC83-luc represented by the percent infection, the p-value of significance, and the accepted function of the compound. Compounds were removed from the screen if they were determined to reduce cellular viability by 20% or more compared to DMSO-treated infected controls.

Target	Drug name	Infection vs. DMSO (%)	*p-Value*
Growth factor receptor inhibitors	Sorafenib (Nexavar)	30	0.0036
	OSI-420 (Desmethyl Erlotinib)	26	0.0074
	Apatinib	48	0.0097
DNA synthesis inhibitors	Gemcitabine HCl (Gemzar)	2	0.0001
	Capecitabine (Xeloda)	40	0.0060
Purine synthesis inhibitors	Mycophenolic acid	3	0.0172
	Mycophenolate mofetil	3	0.0033
Calcium antagonists	Manidipine (Manyper)	4	0.0027
	Manidipine diHCl	16	0.0261
Antifungal agents	Itraconazole (Sporanox)	21	0.0001
	Posaconazole	21	0.0307
Microtubule assembly and disassembly modulator	Vincristine	10	0.0004
Anti-inflammatory	Tranilast (Rizaben)	16	0.0319
Progesterone receptor agonist	Drospirenone	42	0.0340
Angiotensin II receptor antagonist	Candesartan cilexetil (Atacand)	46	0.0290

due to the overall reduction of viral replication, it suggests sorafenib impairs translation of viral proteins, which may result in defective particles being released. These data also indicate that sorafenib is not impacting egress, as it would be expected that the amount of intracellular viral RNA would be similar to or greater than levels seen in DMSO treated controls, indicating a build-up of viral RNA unable to escape host cells, which was not observed (Fig. 4B and C). Pre-treating cells for two hours only had no effect on viral replication compared to cells that were post-treated only or both pre- and post-treated after viral infection (Fig. 4E), indicating that sorafenib does not impact viral entry. Finally, neither sorafenib nor SC1 are viricidal, as incubating VEEV for 2 h with these compounds prior to infection had no effect on viral infectivity (data not shown). These data suggest that sorafenib does not affect entry or egress, or demonstrate any viricidal activity, but rather inhibits a host protein or pathway needed by the virus to replicate its genome or translate its proteins.

3.4. Sorafenib treatment inhibits phosphorylation of eIF4E, p70S6K, and S6 ribosomal protein in VEEV infected cells

Prior research has shown that one mechanism by which cancer cells treated with sorafenib became apoptotic is through inhibition of translation (Rahmani et al., 2005) and the dephosphorylation of the cap binding protein eukaryotic initiation factor 4E (eIF4E) (Liu et al., 2006). To this end, we examined the phosphorylation states over time of several host proteins involved in translation, comparing infected and uninfected Vero cells, both sorafenib treated and treated with the vehicle. Phosphorylation of eIF4E was dramatically reduced in uninfected cells treated with sorafenib (Fig. 6A, lanes 5–8) at all time points analyzed compared to DMSO treated cells (Fig. 6A, lanes 1–4). eIF4E phosphorylation was seen in VEEV infected DMSO-treated cells at 4 and 8 hpi, but phosphorylation was dramatically reduced at 16 and 24 hpi (Fig. 6B, compare lanes 1–2 to 3–4), indicating an infection induced reduction of eIF4E phosphorylation at late times points after infection. In contrast, phosphorylation of eIF4E was at low to undetectable levels in sorafenib treated VEEV infected cells over the entire treatment time-course (Fig. 6B, lanes 5–8). Pixel density was measured as a semi-quantitative representation of concentration of each protein (Fig. 6, bottom panels). In mock-infected cells the phosphorylation status of p70S6K, a classical marker of mTOR activation, was not affected by sorafenib treatment, confirming sorafenib is likely not inhibiting that particular pathway in uninfected cells (Fig. 6A). This is in agreement with previously published results (Rahmani et al., 2005). Likewise in mock-infected cells, phosphorylation levels of ribosomal S6K follows that of its kinase, p70S6K (Fig. 6A). However, in VEEV infected cells decreased phosphorylation of p70S6K and ribosomal S6K was observed at 16 and 24 hpi in sorafenib, but not DMSO treated cells (Fig. 6B), suggesting that a different signaling architecture is present and/or

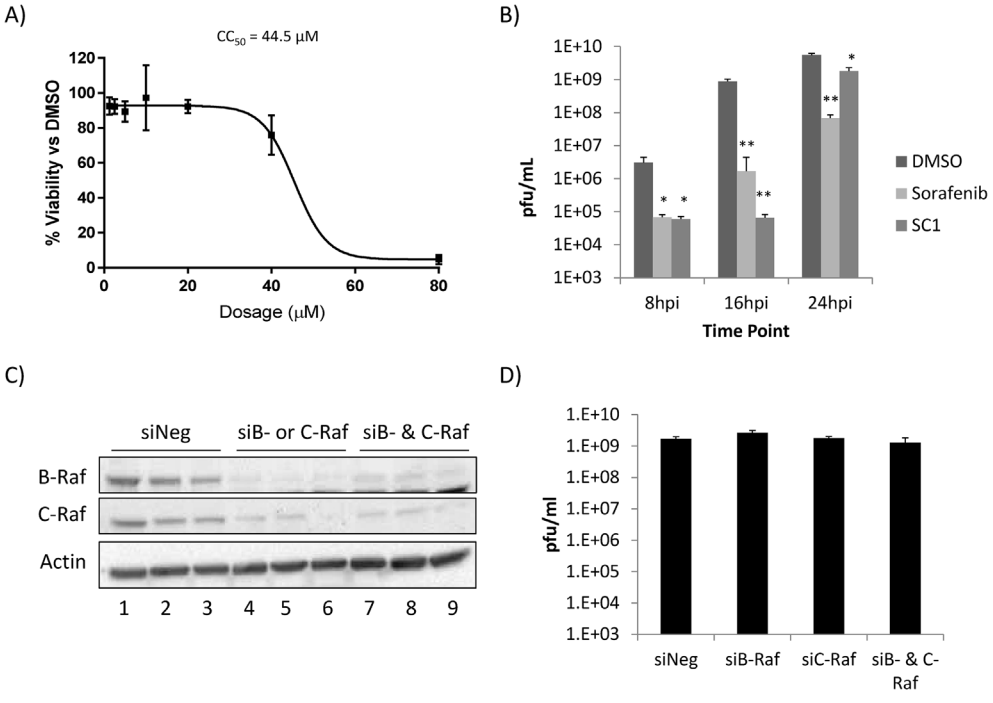


Fig. 3. Sorafenib reduces TC83 replication. (A) Vero cells were treated with different concentrations of SC1 to determine toxicity. After 24 h, Promega's CellTiter-Glo was performed to determine cell viability compared to DMSO treated controls. Data represent biological triplicates, and the experiment was performed twice. (B) Vero cells were treated with DMSO, sorafenib (10 µM), or SC1 (10 µM) for 2 h, infected with TC83 (MOI 0.1), then media containing inhibitors added back to cells. Supernatants were collected at 8, 16, and 24 hpi then plaques were performed to determine titer. Data represent biological triplicates, and the experiment was performed twice. (C) Vero cells were transfected with 10 nM siRNA against B-Raf, C-Raf, or B- and C-Raf combined for 48 h. Protein lysates were analyzed by western blot for B-Raf, C-Raf and actin expression. (D) Cells were transfected as described in (C) then infected with TC83 (MOI 1). Sixteen hours post-infection, supernatants were collected and plaque assays performed to determine viral titers. Data represents biological triplicates, and the experiment was performed twice. *p < 0.05, **p < 0.001.

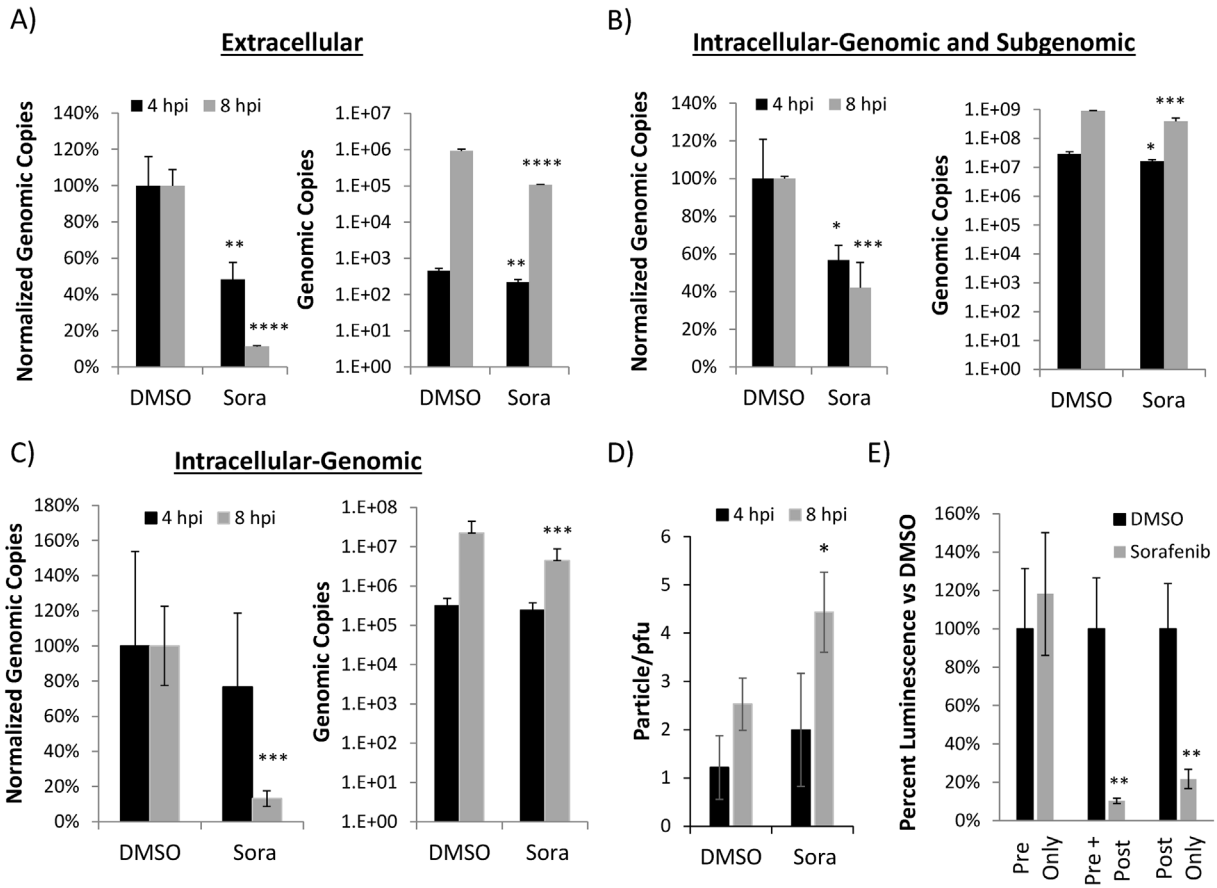


Fig. 4. Sorafenib treatment likely inhibits the VEEV viral life cycle post-entry and upstream of egress. (A) Vero cells were pre-treated with sorafenib (10 μM) or DMSO for 2 h, infected with TC83 (MOI 5) for 1 h, and then media containing compounds added back to cells. Extracellular (A) and intracellular (B and C) RNA was collected at 4 and 8 hpi and genomic copies calculated by RT-qPCR. Primer and probe sets for capsid (panel B) and the viral packaging sequence (panel C) were used for RT-qPCR. Results are displayed as genomic copies in logarithmic scale in the right panels and as normalize genomic copies (relative to the DMSO control) in the left panels. (D) The particle to pfu ratio was calculated at 4 and 8 hpi by dividing the genomic copies/ml to the pfu/ml values of cells treated with sorafenib and infected at an MOI 5. (E) Vero cells were pre-treated with sorafenib (10 μM) or DMSO for 2 h prior to infection (pre-only samples), treated after infection until the time of collection (post-only samples) or both pre- and post-treated (pre and post samples). All samples were infected with TC83luc (MOI 1) for 1 h. Luminescence was measured at 16 hpi and normalized to DMSO controls. Unpaired student t-tests were performed between treated samples compared to corresponding DMSO samples. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, ****$p < 0.0001$.

being impacted by sorafenib following infection. The loss of phosphorylation of eIF4E indicates suppression of translation by sorafenib treatment, including the translation of virally encoded mRNAs, leading to an overall decrease in viral proteins and a reduction in titer.

3.5. Loss of eIF4E reduces VEEV replication

Given the impact of sorafenib treatment on eIF4E phosphorylation we next investigated the importance of eIF4E for VEEV replication. A titration of eIF4E siRNA (10, 25, and 50 nM) indicated that 50 nM was optimal for depleting eIF4E protein levels (data not shown). Vero cells were transfected with 50 nM eIF4E siRNA for 48 h, followed by

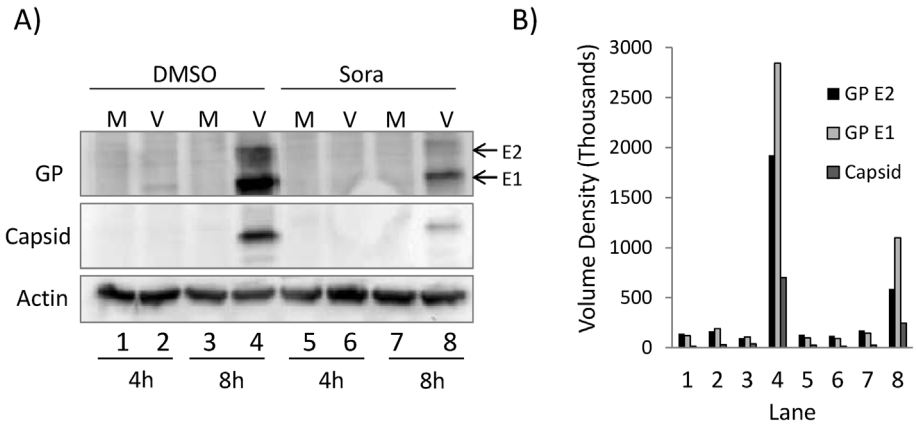


Fig. 5. Sorafenib treatment inhibits VEEV viral structural protein production. (A) Vero cells were pre-treated with sorafenib (10 μM) or DMSO for 2 h, infected with TC83 (MOI 5) for 1 h, and then media containing compounds added back to cells. Protein lysates were collected at 4 and 8 hpi and analyzed by western blotting for VEEV glycoproteins (GP), VEEV capsid, and actin as a control. M = mock infected and V = VEEV infected. (B) The graph represents the average volume density normalized to actin of two separate experiments. Numbers along the X-axis correspond to lane numbers in panel A.

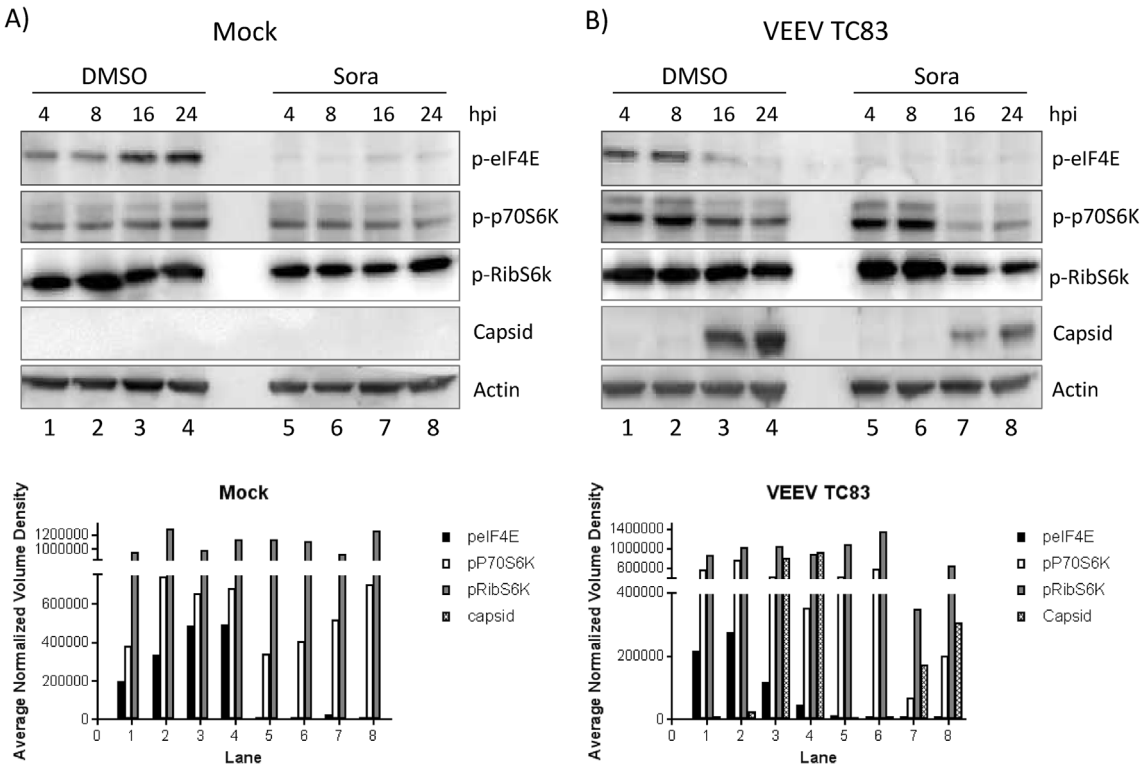


Fig. 6. Sorafenib inhibits phosphorylation of eIF4E, p70S6K, and S6 ribosomal protein in VEEV infected cells. (A) Vero cells were pre-treated with sorafenib (10 μM) or DMSO for 2 h, mock-infected for 1 h, then compound-containing media was added back to cells. Samples were collected 4, 8, 16, and 24 h post-sham infection and lysed then probed for the indicated protein by western blot. Actin was measured as a loading control. (B) Vero cells were treated as described in (A) but infected with VEEV TC83 (MOI 1) instead of mock-infected. Graphs shown below each western blot panel indicate semi-quantitative analysis of the blots. The protein concentration, measured as volume density normalized to actin, from two separate experiments was averaged and graphed. Numbers along the X-axis correspond to lane number.

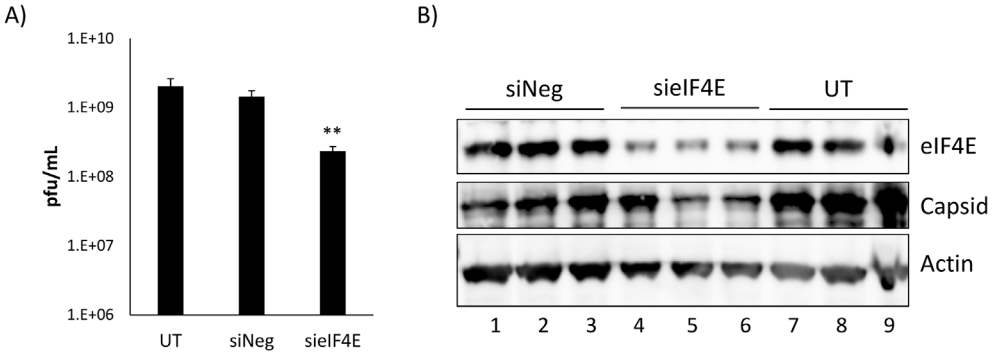
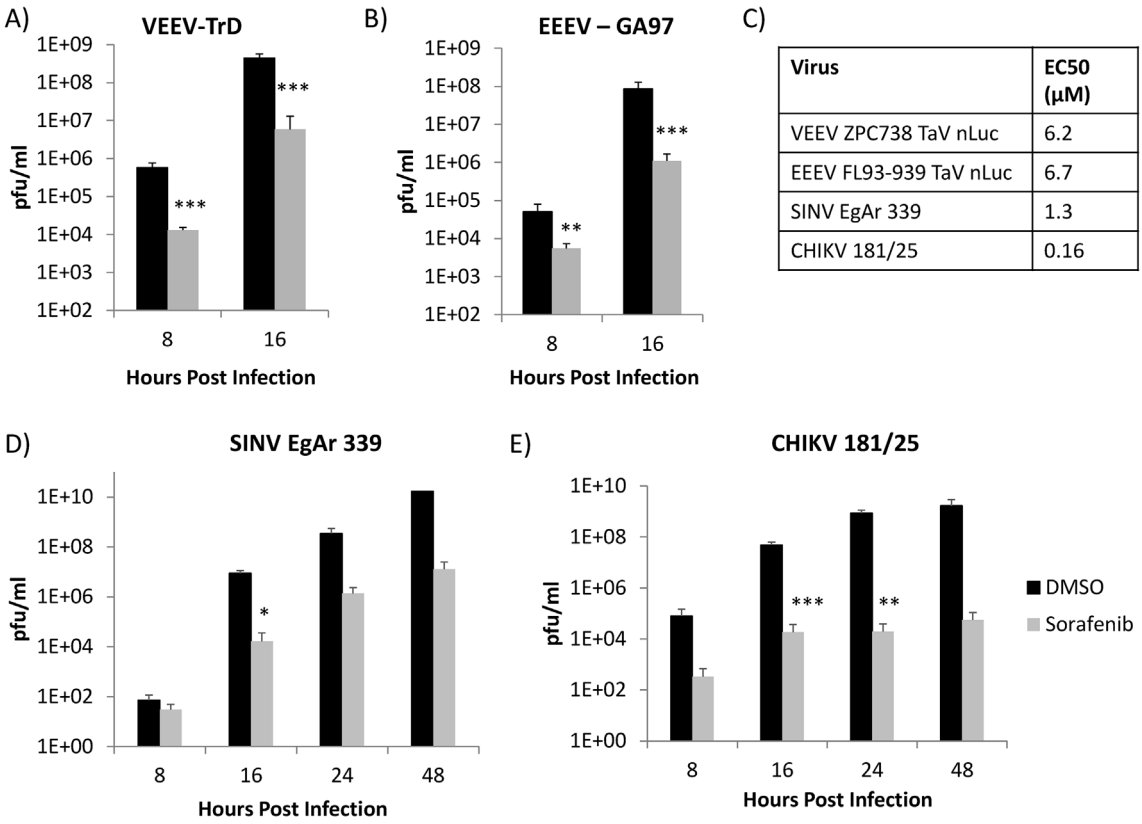


Fig. 7. Loss of eIF4E reduces VEEV replication. (A) Vero cells were transfected with 50 nM Neg control siRNA (siNeg) or eIF4E siRNA (sieIF4E) for 48 h. Cells were then infected with TC83 (MOI 0.1). Sixteen hours post-infection, supernatants were collected and plaque assays performed to determine viral titers. UT = untransfected samples. Data represents biological triplicates. **p < 0.001. (B) Protein lysates prepared from samples described in panel A were analyzed by western blot for eIF4E, capsid and actin expression. Biological triplicates are displayed.

infection with VEEV TC83 (MOI 0.1) and collection of supernatants and protein lysates at 16 hpi. Loss of eIF4E via siRNA treatment resulted in ~1 log decrease in VEEV replication (Fig. 7A). Western blot analysis confirmed the loss of eIF4E and showed the reduction of capsid protein in 2 out of 3 samples (Fig. 7B). These results indicate that loss of eIF4E reduces VEEV replication and correlate with the reduction in eIF4E phosphorylation observed with sorafenib treatment.

3.6. Sorafenib reduces viral titers of other alphaviruses

Prior experiments were performed using the attenuated BSL-2 strain of VEEV, TC83, as it is safer and easier to work with than virulent strains. However, we next investigated if treating cells with sorafenib would also protect against the fully virulent BSL-3 strain of VEEV, TrD. Since sorafenib is well-tolerated in Vero cells, an increased concentration, 20 μM, was used in these experiments. Sorafenib treatment significantly reduced the titers of fully virulent VEEV at both 8 and 16 hpi (Fig. 8A) by nearly two logs. Identical experiments were also performed with EEEV, another New World alphavirus and potential emerging threat (Slobodan Paessler, 2011) lacking vaccines and antiviral therapeutics. Again, pre- and post-treatment of cells with sorafenib significantly reduced viral titers of EEEV GA97 (Fig. 8B) by at least a log at both time points. Similarly, pre- and post-treatment of cells with sorafenib also reduced the titers of two Old World alphaviruses, SINV EgAr 339 (Fig. 8D) and CHIKV 181/25 (Fig. 8E), up to 48 hpi. The EC_{50} of sorafenib against all four of these viruses was determined using either Nano-luciferase (nLuc) reporter viruses (Sun et al., 2014) (VEEV and EEEV) or via plaque assays (SINV and CHIKV). Sorafenib displayed an EC_{50} of 6.2 μM against VEEV ZPC738 TaV nLuc, 6.7 μM against EEEV FL93-939 TaV nLuc, 1.3 μM against SINV EgAr 339, and 0.16 μM against CHIKV 181/25 (Fig. 8C). The lower EC_{50} against CHIKV is in agreement with the plaque assay results, where a dramatic and consistent reduction of CHIKV titers following sorafenib treatment was observed up to 48 h. In contrast, a less dramatic decrease in viral titers



Virus	EC50 (μM)
VEEV ZPC738 TaV nLuc	6.2
EEEV FL93-939 TaV nLuc	6.7
SINV EgAr 339	1.3
CHIKV 181/25	0.16

Fig. 8. Sorafenib reduces New World alphavirus replication over time. (A) Vero cells were pretreated with sorafenib (20 μM) or DMSO for 2 h, infected with VEEV-TrD (MOI 0.1) for 1 h, then drug-containing media added back to cells. Supernatants were collected at 8 and 16 hpi, and plaque assays performed to determine titer. Data represent biological triplicates, and the experiments were performed twice. (B) Vero cells were treated as described in (A) and infected with EEEV-GA97 (MOI 0.1). (C) Vero cells were treated with varying concentrations of sorafenib and infected with either VEEV ZPC738 TaV nLuc, EEEV FL93-939 TaV nLuc, SINV-EgAr 339 or CHIKV-181/25 (MOI 0.1) for EC_{50} determination at 16 hpi. (D) Vero cells were treated as described in (A) and infected with SINV-EgAr 339 (MOI 0.1). (E) Vero cells were treated as described in (A) and infected with CHIKV-181/25 (MOI 0.1) *$p < 0.05$, **$p < 0.005$, ***$p < 0.0005$.

was observed in the presence of sorafenib at later time points with VEEV, EEEV, and SINV, suggesting that higher amounts of sorafenib may be needed to completely suppress replication of these viruses and that these viruses are not as sensitive to sorafenib treatment. These results indicate that sorafenib can inhibit multiple alphavirus family members and is a more potent inhibitor of Old World alphaviruses.

4. Discussion

The purpose of this study was to identify FDA-approved drugs that had antiviral activity against VEEV. No FDA-approved vaccines or therapeutics for VEEV for human use, or closely related viruses, WEEV and EEEV, currently exist. From the library screen, three growth factor receptor inhibitors, sorafenib, OSI-420, and apatinib, significantly inhibited VEEV TC83-luc replication (Fig. 2A and Table 1). While OSI-420 and apatinib were both non-toxic in Vero cells, only sorafenib significantly inhibited VEEV TC83 replication via plaque assay (Supplemental Fig. 1), therefore the other receptor inhibitors were dropped from further consideration. This reinforced the conclusion that sorafenib's VEGFR and EGFR inhibitory activities are likely not responsible for its antiviral efficacy against VEEV. Knockdown of B-Raf, C-Raf, or both simultaneously did not reduce VEEV titer (Fig. 3D), nor were the Raf kinases necessary for RVFV replication (Benedict et al., 2015a), indicating Raf inhibition is not the mechanism by which sorafenib exerts its antiviral effects for these two viruses. Like VEEV, RVFV is an RNA virus that replicates in the host cell's cytoplasm and causes an acute, febrile-like illness, but unlike VEEV, RVFV is negative-sense and has a segmented genome. In addition to decreasing viral titer, sorafenib is well-tolerated *in vitro* in several cell lines in our hands, including Vero, human small airway epithelial cells (HSAEC), and Huh 7 (Benedict et al., 2015a), making it an intriguing therapeutic option.

As its EGFR/VEGFR and Raf kinases inhibitory activities are likely not contributing to its antiviral effects, we decided to focus on the stages of the VEEV replication cycle sorafenib may be effecting. Pretreating cells alone failed to reduce viral titer, indicating sorafenib does not affect viral binding or entry. Looking at viral genomic RNA levels inside and outside host cells narrowed the life cycle window. An increase in intracellular viral RNA compared to DMSO controls was not seen, indicating sorafenib is not blocking egress of fully-formed virions. A reduction in viral proteins was observed at early time points compared to DMSO controls. Extracellular viral RNA continued to decrease over time, but the same rate of decrease was not seen intracellularly, indicating the VEEV genome was still being replicated. Moreover the particle to pfu ratio increased following sorafenib treatment, indicating that defective viral particles were being produced, which could be partially attributed to the observed decrease in structural proteins. These data suggest that translation of the viral proteins as at least one stage in the viral life cycle targeted by sorafenib.

Previous studies have shown that sorafenib can inhibit translation independently of the mTOR (Rahmani et al., 2005) and MEK/ERK (Liu et al., 2006) pathways. We found a striking decrease in phosphorylation levels of the translational scaffold and cap-binding protein eIF4E (Rahmani et al., 2005) between sorafenib treated and vehicle treated cells (Fig. 6). Protein levels of eIF4E do not seem to change with sorafenib treatment (Rahmani et al., 2005), but a translation target, the antiapoptotic Mcl-1, decreases (Liu et al., 2006) and is likely the mechanism by which sorafenib induces apoptosis in cancer cells (Huber et al., 2011). Further, eIF4E phosphorylation has been shown to

correspond to increased translation of CHIKV proteins, a closely related Old World alphavirus. Despite CHIKV infection inhibiting mTOR, a metabolic sensor that regulates cap-dependent translation, viral proteins were translated preferentially to those of the host, and chemical inhibition of mTOR increased viral replication, specifically by enhancing the translation of viral proteins. Upon mTOR inhibition, Mnk1/2, MAP kinase-activated protein kinases, become activated, leading to the hyper-phosphorylation of eIF4E independently of S6K activity. Hyper-phosphorylation led to an increase in eIF4E-dependent translation of viral over host proteins (Joubert et al., 2015), indicating activated eIF4E is important for the replication of RNA viruses. This finding is reminiscent of what has been observed in cancer cells, where eIF4E phosphorylation is required for only a subset of mRNAs that contribute to their oncogenic properties (Truitt et al., 2015). Further, eIF4E even interacts directly with some viruses, such as the cap-mimic VPg protein of the *Caliciviridae* family (Royall et al., 2015; Hosmillo et al., 2014; Zhu et al., 2015), as would be expected from positive-sense RNA viruses. Many food crops – such as peppers, barley, melon, and potatoes - are being genetically engineered to express mutant eIF4E that does not interact with *Potyviridae* (a family of plant RNA viruses) VPg proteins, conferring viral resistance to the plant without affecting fitness, underscoring the importance of eIF4E in viral replication (reviewed in (Bastet et al., 2017; Gal-On et al., 2017)). Given that eIF4E phosphorylation is not required for global cellular protein synthesis (McKendrick et al., 2001) it is an attractive therapeutic target for acute viral infections, where treatment would be limited to only a few days to 2 weeks. Moreover, as sorafenib is targeting a cellular protein, rather than a viral protein, the likelihood of developing viral resistance to the drug is significantly decreased (Schwegmann and Brombacher, 2008; Khattab, 2009).

As stated previously, there are no vaccines or therapeutics against VEEV or the other New World alphaviruses. However pre- and post-treatment of infected cells reduced the titers of two fully virulent alphaviruses: VEEV and EEEV (Fig. 8A–C). Due to their emerging status, history as bioweapons, and the possibility of use in a terrorist event, a pan-alphavirus therapeutic or vaccine is desperately needed. Furthermore, pre- and post-treatment of cells with sorafenib decreased titers of two Old World alphaviruses, SINV and CHKIV (Fig. 8D–E). The Old World alphaviruses are closely related to the New World alphaviruses but cause an arthralgic (reviewed in (Suhrbier et al., 2012)) rather than neurological disease course and also utilize different viral proteins as virulence factors (nsP 2 versus capsid (Garmashova et al., 2007)). Additional analysis is beyond the scope of this paper, but it is likely that sorafenib decreases viral titers of these alphaviruses through the same mechanism owing to their relatedness and similar life cycles (Strauss and Strauss, 1994; Lundberg et al., 2016; Powers et al., 2001).

Interestingly enough, sorafenib also inhibits RVFV (Benedict et al., 2015a), another cytoplasmically replicating RNA virus, though a different mechanism. In RVFV infected cells treated with sorafenib, p97/valosin-containing protein, which is involved in membrane remodeling in the secretory pathway and a non-classical target of sorafenib, was inhibited, leading to a buildup of virions in endoplasmic reticulum vesicles and the blockage of viral egress (Brahms et al., 2017). Sorafenib has also been shown to inhibit the flavivirus HCV, a positive-sense RNA virus like VEEV, *in vitro* (Bürckstümmer et al., 2006; Himmelsbach et al., 2009; Himmelsbach and Hildt, 2013) at multiple steps in the viral life cycle, including entry and egress. Claudin-1, a component of cellular tight junctions, had altered localization upon sorafenib treatment, and may be partly responsible for the partial block in viral entry, but sorafenib's block of viral egress was not examined further (Descamps et al., 2015). Like RVFV, HCV is a hepatocentric virus, and with both viruses, egress was targeted by sorafenib. It is of note, then, that sorafenib has many effects beyond its canonical role of inhibiting Raf kinases and suppresses replication of viruses with different life cycles and cellular affinities, while remaining relatively non-toxic in noncancerous cell lines. This makes it an attractive prospect as a potential pan-antiviral therapeutic, and certainly warrants additional investigation, both mechanistically and across a variety of viruses.

An important future aspect of this work would be to extend the testing of sorafenib to *in vivo* efficacy studies using animal models of infection. There are only a few published studies of antiviral compounds achieving protection of mice from lethal challenge of VEEV-TC83, but none to date for an aerosol challenge with VEEV-TrD. A quinazoline compound, CID15997213, targets the viral nsP2 N-terminal domain and protected mice from lethal TC83 challenge at 50 mg/kg/day (Chung et al., 2014). Acriflavine, an Ago2 inhibitor, at 10 mg/kg/day protected approximately 60% of mice from a TC83 LD_{90} challenge (Madsen et al., 2014). Pre-treatment of mice daily with 200 mg/kg of (−)-cabordine, a carbocyclic analog of cytidine, significantly improved survival and mean day-to-death from a lethal TC83 challenge (Julander et al., 2008). The GSK-3β inhibitor and its derivative, BIO and BIOder, administered at 50 and 20 mg/kg pre- and post-infection protected 30% and 50% of mice, respectively, from a lethal TC83 challenge (Kehn-Hall et al., 2012). ML336, an amidine administered at 2.5 mg/kg/twice daily for 4 days, significantly protected 71% of mice infected with a lethal TC83 challenge (Schroeder et al., 2014). The recommended dose of sorafenib is 400 mg twice a day for Hepatocellular Carcinoma, Renal Cell Carcinoma, and Differentiated Thyroid Carcinoma, with some adverse side-effects requiring a reduction in dose after a few months of treatment (Strumberg et al., 2005; Reiss et al., 2017). In mice, sorafenib is well tolerated from 30 to 60 mg/kg per day (Wilhelm et al., 2004b; Benedict et al., 2015b). While it is difficult to predict the dose needed for *in vivo* efficacy based on *in vitro* EC_{50} data, all the compounds mentioned above (CID15997213, ML336, Bioder, (−)-cabordine and acriflavine) displayed nM EC_{50} values against VEEV TC83. These results suggest that protecting against a lethal challenge of VEEV with sorafenib may be challenging, but we have been successful in using sorafenib to prevent RVFV induced disease (Benedict et al., 2015b). Sorafenib can cross the blood-brain barrier (Kim et al., 2012), which is a favorable feature for any potential VEEV therapeutic. The influence of sorafenib treatment on peripheral versus central nervous system complications of VEEV infection would be critical to dissect, including the impact of a delayed treatment regimen. Given sorafenib's potent inhibitory activity against CHIKV (Fig. 8E), future studies should also investigate the efficacy of sorafenib against CHIKV *in vivo*.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.antiviral.2018.07.005.

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