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Nuclear import and export inhibitors alter capsid protein distribution in mammalian cells and reduce Venezuelan Equine Encephalitis Virus replication

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ABSTRACT

Targeting host responses to invading viruses has been the focus of recent antiviral research. Venezuelan Equine Encephalitis Virus (VEEV) is able to modulate host transcription and block nuclear trafficking at least partially due to its capsid protein forming a complex with the host proteins importin α/β1 and CRM1. We hypothesized that disrupting the interaction of capsid with importin α/β1 or the interaction of capsid with CRM1 would alter capsid localization, thereby lowering viral titers *in vitro*. siRNA mediated knockdown of importin α, importin β1, and CRM1 altered capsid localization, confirming their role in modulating capsid trafficking. Mifepristone and ivermectin, inhibitors of importin α/β-mediated import, were able to reduce nuclear-associated capsid, while leptomycin B, a potent CRM1 inhibitor, confined capsid to the nucleus. In addition to altering the level and distribution of capsid, the three inhibitors were able to reduce viral titers in a relevant mammalian cell line with varying degrees of efficacy. The inhibitors were also able to reduce the cytopathic effects associated with VEEV infection, hinting that nuclear import inhibitors may be protecting cells from apoptosis in addition to disrupting the function of an essential viral protein. Our results confirm that VEEV uses host importins and exportins during part of its life cycle. Further, it suggests that temporarily targeting host proteins that are hijacked for use by viruses is a viable antiviral therapy.

1. Introduction

Venezuelan Equine Encephalitis Virus (VEEV), from the genus *Alphavirus* and family *Togaviridae*, can cause a fatal neurological disease in equines and humans. Endemic to northern South America and ranging into Mexico and the southern United States, the virus is transmitted between hosts and mosquitoes (Paredes et al., 2005). Fatal human cases involve pathology in the central nervous system (CNS), the lungs, lymphoid tissue and the liver (Zacks and Paessler, 2010). VEEV effectively infects and replicates in astrocytes and microglial cells *in vitro* and controversially *in vivo* (Schoneboom et al., 1999). Currently, there are no licensed antiviral drugs, vaccines, or FDA-approved treatment for infections (Reichert et al., 2009). The live-attenuated vaccine strain TC83 is only manufactured for use in equines in Central and South America. The live vaccine often produces moderate flu-like symptoms in humans and fails to completely protect non-human primates in aerosol challenges (Paessler and Weaver, 2009).

New World alphaviruses like VEEV are positive-sense, non-segmented, enveloped RNA viruses. VEEV is composed of a 49S RNA molecule and a nucleocapsid made of capsid protein bound by a host-derived lipid bilayer with membrane associated glycoproteins (Paredes et al., 2005). The 5′ two-thirds of the 11.4 kb genome encodes four non-structural proteins, nsP1 through nsP4 (Paessler and Weaver, 2009). The complementary negative template is synthesized by the non-structural proteins (Ryman and Klimstra, 2008). Positive-sense genomic RNA and subgenomic 26S RNA are synthesized from the full-length RNA template. The subgenomic RNA, which corresponds to the other 3′ one-third of the genome, is translated into capsid and the glycoproteins E1 and E2 (Paessler and Weaver, 2009). Replication occurs in the host cytoplasm. Pre-viral nucleocapsid will self-assemble with viral RNA into icosahedral particles in the cytoplasm before undergoing further rearrangement (Lamb et al., 2010).

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Capsid is found in the cytoplasm, nucleus, and nuclear rim, suggesting that it has roles beyond encasing viral RNA. Multiple studies identified capsid as being a key regulator of the host anti-viral defenses (Atasheva et al., 2008, 2010a,b; Ryman and Klimstra, 2008). In cultured cells, capsid antagonizes the induction of stress and innate immune responses, including IFN-α/β. However, a robust IFN-α/β response is seen *in vivo*. Capsid mediates host cell transcription and translation shut-off in both systems (Ryman and Klimstra, 2008a). Capsid also inhibits nuclear trafficking in cultured mammalian cells but not in mosquito cells (Atasheva et al., 2008). Capsid forms a tetrameric complex with the karyopherins importin α/β and CRM1, which associates with the nuclear pore complex (NPC) and hinders its functioning. Capsid contains both a nuclear localization signal (NLS) and nuclear export signal (NES) that associate with the host proteins and NPC. The down-regulation of host cellular transcription could be a consequence of the capsid/nuclear pore complex (Atasheva et al., 2010a).

Nuclear import and export is a tightly-regulated, highly specific process moderated by the cargo-recognizing importin superfamily of proteins. Import is regulated by NLS recognizing importins that interact with nucleoporins of the NPC to transport cargo into the nucleus. Export is controlled by exportins and the cargo's NES (Poon and Jans, 2005). Other RNA viruses that replicate in the host's cytoplasm are known to evade the immune response by modulating nuclear trafficking. The Ebola protein VP24 binds to importin α2 to block STAT1 (Sekimoto and Yoneda, 2012), NS5 of Dengue virus binds to importin α/β1 to access the nucleus to moderate host gene transcription that promotes infection (Rawlinson et al., 2009), and HIV's integrase interacts with importin α/β1 to facilitate the viral genome's transport to the nucleus for host genome integration (Hearps and Jans, 2006). Nuclear protein import and export may be a viable target for antivirals designed to specifically interrupt the interaction between viral proteins and the host's trafficking proteins (Caly et al., 2012).

Previous work demonstrated that ivermectin and mifepristone, both FDA-approved drugs for human use, inhibit HIV's integrase driven nuclear import in cultured cells. Mifepristone appears to be a specific inhibitor of the importin α/β1/integrase interaction (Wagstaff et al., 2011), whereas ivermectin was shown to reduce NLS binding to importin α/β1 generally (Wagstaff et al., 2012). Leptomycin B, a *Streptomyces* metabolite, inhibits nuclear export by binding specifically to CRM1 and is the only commercially available inhibitor of nuclear transport (Kudo et al., 1998). Leptomycin B underwent phase I clinical trials, but the trial was discontinued due to safety issues (Newlands et al., 1996). Its research use as an inhibitor of CRM1 (Kudo et al., 1998) prompted us to test its ability to disrupt the capsid/host protein complex. We hypothesized that the nuclear import inhibitors, mifepristone and/or ivermectin, and nuclear export inhibitor, leptomycin B, would disrupt the interaction between capsid and importin α/β1 or between capsid and CRM1, resulting in altered VEEV capsid localization and activity. Our results demonstrate that all three inhibitors have varying degrees of efficacy and that antivirals targeting viral/host trafficking protein interactions may be a viable treatment for VEEV infection. Mifepristone and ivermectin treated cells displayed reduced nuclear associated capsid, whereas treatment with leptomycin B confined capsid to the nucleus. siRNA mediated knockdown of importin α/β1 and CRM1 demonstrated similar findings, providing additional evidence that the disruption of the capsid, importin α/β1, CRM1 tetrameric complex results in alteration of capsid localization. Inhibitor treatment reduced VEEV induced cytopathic effects, which is largely attributable to capsid. Viral production was also reduced in cells treated with nuclear import inhibitors. Based on our results, capsid's interaction with the host's nuclear import machinery is a viable target for the rational design of antivirals.

2. Materials and methods

2.1. Cell culture

Vero (ATCC, CCL-81) and U87MG (ATCC, HTB-14) cells were maintained at 37 °C, 5% CO_2 in Dulbecco's modified Eagle medium (DMEM) supplemented with 10% fetal bovine serum (FBS), 1% glutamine, and 1% penicillin/streptomycin.

2.2. Nuclear import inhibitor treatment

Mifepristone and ivermectin were purchased from Sigma–Aldrich (M8046 and I8898). The inhibitors were dissolved in sterile DMSO. Unless stated otherwise, cells were treated with 10 μM mifepristone, 1 μM ivermectin, or 0.1% DMSO in supplemented DMEM prior to viral infection. The inhibitor-containing media was replaced after infection unless otherwise noted. Leptomycin B was purchased from Sigma–Aldrich (L2913). Cells were treated at 45 nM in supplemented DMEM prior to viral infection. The inhibitor-containing media was replaced after infection unless otherwise noted.

2.3. Viruses and infections

VEEV TC83 and VEEV Trinidad Donkey (TrD) were obtained from BEI Resources. VEEV TC83-GFP was a kind gift from Ilya Frolov of the University of Alabama at Birmingham. All experiments with VEEV TC83 were performed under BSL-2 conditions, whereas experiments with VEEV TrD were performed 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. For infections, virus was added to supplemented DMEM to achieve an MOI of 0.1 or 1. Cells were infected for one hour at 37 °C and rotated every 15 min to ensure adequate coverage. Cells were then washed with sterile 1× PBS and inhibitor-treated media was added unless otherwise stated.

To determine titers, crystal violet plaque assays were performed. Infected supernatants were serially diluted in 2× Eagle's Minimal Essential Medium (EMEM) supplemented with 5% FBS, 1% minimum essential amino acids, 1% sodium pyruvate, and 2% penicillin/streptomycin, and added to confluent six well plates of Vero cells. After one hour incubation at 37 °C, 3 mL of a 1:1 mixture of supplemented EMEM with 0.6% agarose in diH_2O were added to each well. Plates were fixed with 10% formaldehyde in diH_2O after 48 h. Cells were stained using 1% crystal violet in 20% ethanol and diH_2O. Plaques were counted and the duplicate values of each dilution averaged. Each experiment was performed in duplicate or triplicate.

2.4. Cell viability assays

Cellular viability was measured using Promega's CellTiter Luminescent Cell Viability Assay (G7571), which generates a luminescent signal proportional to the amount of ATP present. Ninety-six well, white-wall plates (Corning, 3610) were seeded with 10,000 cells/well 24 h prior to treatment. The assay was performed according to the manufacturer's protocol 24 h post treatment. The luminescent signal was measured using Beckman Coulter's DTX880 Multimode Detector with an integration time of 100 ms per well.

2.5. Immunofluorescence analysis

Vero cells were grown on coverslips in a six-well plate, infected with VEEV TC83 as described above, and washed with PBS (without Ca and Mg) then fixed with 4% formaldehyde. Cells were permeabilized with 0.5% Triton X-100 in PBS for 20 min then washed twice with PBS. The cells were blocked for 10 min at room temperature in 3% BSA in PBS. The primary antibody, VEEV-capsid (BEI Resources, NR-9403) diluted 1:600, was incubated in fresh blocking buffer at 37 °C for 1 h and washed three times for 3 min in 300 mM NaCl with 0.1% Triton X-100. Alexa Fluor 568 donkey anti-goat secondary (Invitrogen, A11057) dilution 1:400 was used as a secondary antibody and treated in the same manner as the primary antibody. For siRNA experiments, an Alexa Fluor 488 donkey anti-mouse secondary (Invitrogen, A21202) was also used. DAPI, dilution 1:1000, was used to visualize nuclei. Cover slips were mounted to glass slides using 10 μL of Fluoromount G (Southern Biotech, 0100-01). A Nikon Eclipse TE 2000-U was used for fluorescence microscopy. Images were viewed using an oil-immersion 60× objective lens. Each sample was subjected to at least five images, with a representative image shown. All images were subjected to four line averaging. The images were processed through Nikon NIS-Elements AR Analysis 3.2 software.

Digitized images were analyzed using the ImageJ version 1.47 public domain software (NIH) to determine the ratio of nuclear (Fn) to cytoplasmic (Fc) fluorescence (Fn/c) according to the formula: $Fn/c = (Fn - Fb)/(Fc - Fb)$, where Fb is background autofluorescence. Statistical analysis was performed using Welch's test and the GraphPad Prism 5.0 software.

2.6. siRNA knockdown experiments

U87MG were transfected with 50 nM of FlexiTube siRNA (Qiagen) against importin α (Hs_KPNA2_28, SI05151468) using HiPerFect Transfection Reagent (Qiagen). For importin β1 and CRM1, U87MG cells were transfected with 50 nM of SMARTpool ON-TARGETplus siRNA against importin β (L-017523-00-0005) and 10 nM of CRM1 (L-003030-00-0005) using DharmaFECT Transfection Reagent (Thermo Scientific). A negative control siRNA (Qiagen) was included as a control. Twenty-four hours post-transfection, cells were infected with VEEV TC83 (MOI 0.1). Mock infected and mock transfected cells were included as a control. Twenty-four hours after infection, cells were collected and processed for confocal microscopy or Western blot analysis. For whole cell protein preparation, cells were washed with 1× PBS and collected in 200 μL of lysis buffer (1:1 mixture of T-PER reagent (Pierce, IL), 2× Tris–glycine SDS sample buffer (Novex, Invitrogen), 33 mM DTT, and protease and phosphatase inhibitor cocktail (1× Halt cocktail, Pierce)) then boiled for 10 min. Whole cell lysates were boiled for three minutes prior to Western blot analysis.

2.7. Western blot analysis

Whole cell lysates were separated on NuPAGE 4–12% Bis–Tris gels (Invitrogen) and transferred to PVDF membranes (Invitrogen, IB4010-01) using Invitrogen's iBlot (IB1001) system. The membranes were blocked in 3% dry nonfat milk solution in 1× PBS and 0.1% Tween 20 (Promega, H5151) for one hour rocking at room temperature. Primary antibodies were diluted in 3% milk solution according to the manufacturer and incubated rocking overnight at 4 °C. The membranes were washed four times for five minutes with PBS-T and incubated rocking at room temperature for 2 h with secondary HRP-conjugated antibody diluted in 3% milk solution according to the manufacturer's instructions. Membranes were washed with PBS-T four times for 5 min and visualized by chemiluminescence using SuperSignal West Femto Maximum Sensitivity Substrate Kit (ThermoScientific) and a Bio-Rad Molecular Imager ChemiDoc XRS system. The primary and secondary antibodies used were: capsid (BEI Resources, NR-9403), importin α (Bethyl Laboratories, A300-484A), importin β (Bethyl Laboratories, A300-482A), CRM1 (Bethyl Laboratories, A300-469A), actin (Abcam, ab49900), goat anti-rabbit (Thermo Scientific, 32460) and donkey anti-goat (Santa Cruz Biotechnologies, sc-2020).

2.8. Flow cytometry

Vero or U87MG cells were plated in 12-well plates at 500,000 cells per well 24 h prior to treatment. Cells were pre-treated with nuclear import inhibitors for two hours unless stated otherwise. Cells were infected with VEEV TC83-GFP at a MOI of 1 for an hour, washed with 1× PBS, and media containing the inhibitors was replaced. At the appropriate collection time, cells were trypsinized and washed with supplemented DMEM. Cells were then washed with FACS stain (1× PBS, 2% FBS, 0.05% sodium azide) followed by a wash with 1× phosflow lyse/fix buffer (1× PBS, 5× phosflow lyse/fix buffer BT 558049). After another wash with FACS stain, Accuri's C6 Flow Cytometer was used to analyze the fluorescent signal in the FL1 channel to quantitate the level of GFP signal. The mean fluorescent signal was normalized to inhibitor-treated, mock infected samples. Samples were measured in triplicate and experiments replicated at least twice unless otherwise stated.

2.9. Statistical analysis

Unless otherwise stated, all statistical analysis was calculated with the unpaired, two-tailed Student *T*-test using GraphPad's free online software, QuickCalcs.

3. Results

3.1. Loss of importin α, importin β1 and CRMI alter viral capsid cellular distribution

Frolova and colleagues demonstrated that capsid binds to importin α, importin β1 and CRM1 forming a tetrameric complex (Atasheva et al., 2010a). This tetrameric complex results in obstructing the nuclear pore and thus blocking nuclear export and import. Capsid is found not only at the nuclear rim, but also in the cytoplasm and nucleus; therefore the binding of capsid to these proteins likely also mediates capsid nuclear import and export. To determine if importin α and importin β1 are important for capsid nuclear import, siRNA studies were performed. U87MG astrocytoma cells were transfected with siRNA against importin α or importin β1, followed by infection with VEEV. Negative control siRNA (siNeg) transfected cells were included as controls. siRNA titration experiments indicated that 50 nM of importin α siRNA (siImpα) was sufficient to reduce protein expression by greater than 80% (Fig. 1A, lane 5). Preliminary siRNA titration experiments with siRNA against importin β1 (siImpβ1) indicated that 50 nM of siRNA resulted in the best reduction in protein expression (data not shown). A titration of DharmaFECT transfection reagent resulted in an optimal knockdown of ~80% when using the lowest amount of reagent (Fig. 1B, lane 5), without any toxicity due to the transfection reagent. Next, capsid localization was examined by confocal microscopy in cells transfected with siRNA against importin α, importin β1, or a combination of the two (siImp α/β1). Capsid localization was quantitated by measuring the fluorescence signal present in the nucleus and the cytoplasm and generating an Fn/c value (see methods). An Fn/c value of less than one denotes predominant cytoplasmic fluorescence,

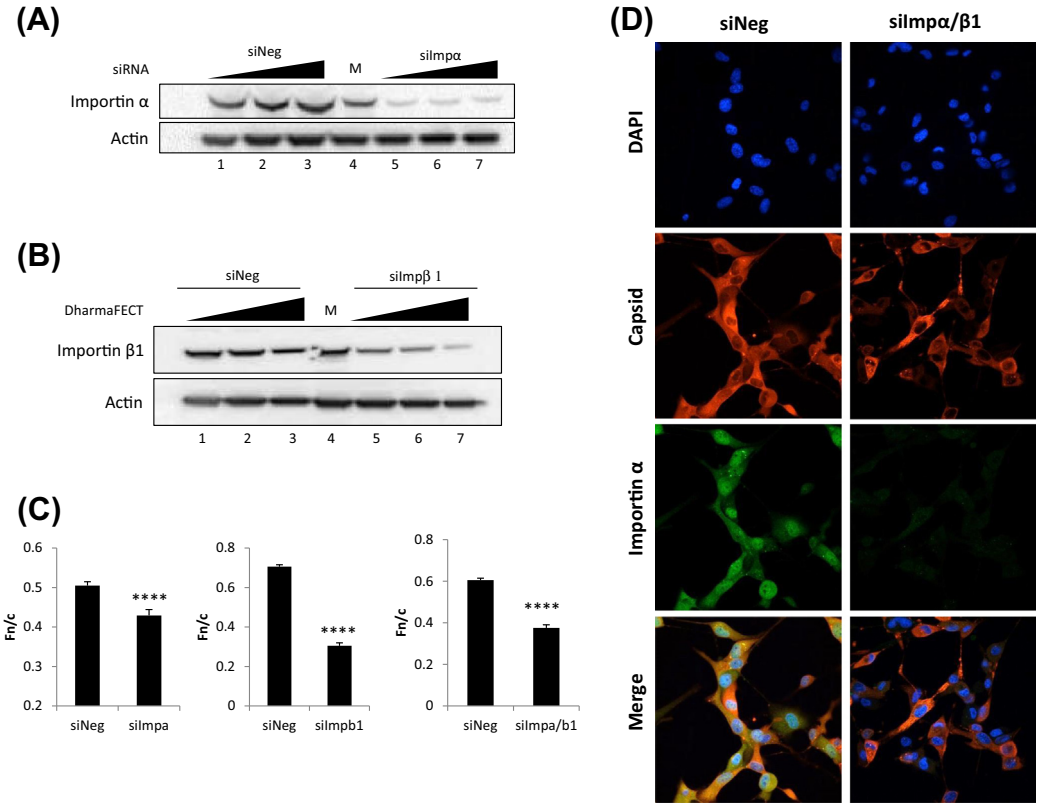

Fig. 1. Loss of importin α and importin β results in increased capsid cytoplasmic localization. (A) U87MG cells were transfected with siRNA (50, 100, or 200 nM) against importin α (siImpα) or negative control siRNA (siNeg) using HiPerFect (Qiagen). Twenty-four hours post-transfection, cells were infected with VEEV TC83 (MOI 0.1). Twenty-four hours after infection, cells were collected and processed for Western blot analysis. M = mock infected cells, included as a control. (B) U87MG cells were transfected with 50 nM siNeg or siRNA against importin β1 (siImpβ1) using various amounts of DharmaFECT Transfection Reagent (1, 5 and 10 μl). Twenty-four hours post-transfection, cells were infected with VEEV TC83 (MOI 0.1). Twenty-four hours after infection, cells were collected and processed for Western blot analysis. M = mock infected cells, included as a control. (C) U87MG cells were transfected with siRNA and infected with VEEV as described above. Cells were fixed at 24 h and probed with DAPI, anti-VEEV capsid antibody and an Alexa Fluor 568 labeled secondary antibody. Slides were imaged on a Nikon Eclipse TE2000-U after immunofluorescent staining. Digitized images were analyzed using the ImageJ version 1.47 public domain software (NIH) to determine the ratio of nuclear (Fn) to cytoplasmic (Fc) fluorescence (Fn/c). The numbers of cells analyzed are as follows: left panel: siNeg (50 nM) $N = 116$, siImpα (50 nM) $N = 74$, middle panel: siNeg (50 nM) $N = 79$, siImpβ1 (50 nM) $N = 73$, right panel: siNeg (100 nM) $N = 95$, siImpα/β1 (100 nM) $N = 106$. (D) Representative images of U87MG cells infected with VEEV and transfected with 100 nM siNeg or 50 nM of siImpα and 50 nM siImpβ1 (siImpα/β1). $^{****}p \leqslant 0.0001$ (compared to siNeg).

whereas an Fn/c value of greater than one denotes predominant nuclear fluorescence. Loss of either importin α or importin β1 resulted in a significant increase in cytoplasmic capsid accumulation (Fig. 1C). Cells transfected with both importin α and importin β1 siRNA (siImpα/β1) also displayed an increase in cytoplasmic capsid localization (Fig. 1C and D).

A similar set of experiments were performed to determine if CRM1 mediates capsid nuclear export. siRNA titration (10, 25, and 50 nM of siRNA) and transfection reagent titration (1, 5, and 10 μl) experiments were performed. These results indicated that 10 nM of CRM1 siRNA in combination with the lowest amount of transfection reagent tested, reduced CRM1 protein levels by ~85% (Fig. 2A) and was thus selected for additional experiments. Capsid localization was measured in the presence of CRM1 siRNA as described above. Results indicated that the loss of CRM1 resulted in a significant increase in nuclear accumulation of capsid (Fig. 2B and C). The set of data described in Figs. 1 and 2 demonstrated that importin α/importin β1 and CRM1 mediate capsid nuclear import and export, respectively.

3.2. Nuclear import and export inhibitors alter viral capsid cellular distribution

As we have confirmed that importin α, importin β1, and CRM1 are involved in capsid localization, we hypothesized that the importin α/β1 inhibitors, mifepristone and ivermectin, could alter capsid's localization. As leptomycin B treatment has previously been demonstrated to confine capsid localization mainly to the nucleus (Atasheva et al., 2010a), leptomycin B treatment was analyzed in parallel as a positive control of altered capsid distribution. As a first step, cell viability was measured to establish inhibitor toxicity and working concentrations. Serial dilutions of each inhibitor diluted in DMEM were incubated with the cell lines of interest for 24 h, following which Promega's CellTiter-Glo Luminescent Cell Viability Assay was used to measure viability. Vero cells were chosen for their ease in producing virus, and the human astrocyte line U87MG was chosen as a relevant line to model the infection of nervous tissue (Kehn-Hall et al., 2012). Ivermectin proved toxic at higher concentrations in both cell lines; therefore 1 μM was selected as the concentration to be used in all future assays. The CC_{50} (concentration of ivermectin that was cytotoxic to half the population of cells) was approximately 8 μM in Vero cells and 22 μM in U87MG cells. Mifepristone was less toxic to both cell lines; therefore 10 μM was chosen (Fig. 3A and B). The CC_{50} of mifepristone was approximately 165 μM in Vero cells and greater than 50 μM in U87MG cells. The same conditions were tested for leptomycin B, which also proved to be toxic at higher concentrations in both lines (Fig. 3C and D). A working dilution of 45 nM was chosen based on the published literature (Atasheva et al., 2010b) and little to no toxicity was observed at this dose. The CC_{50} of leptomycin B was approximately 80 nM in Vero cells and greater than 100 nM in U87MG.

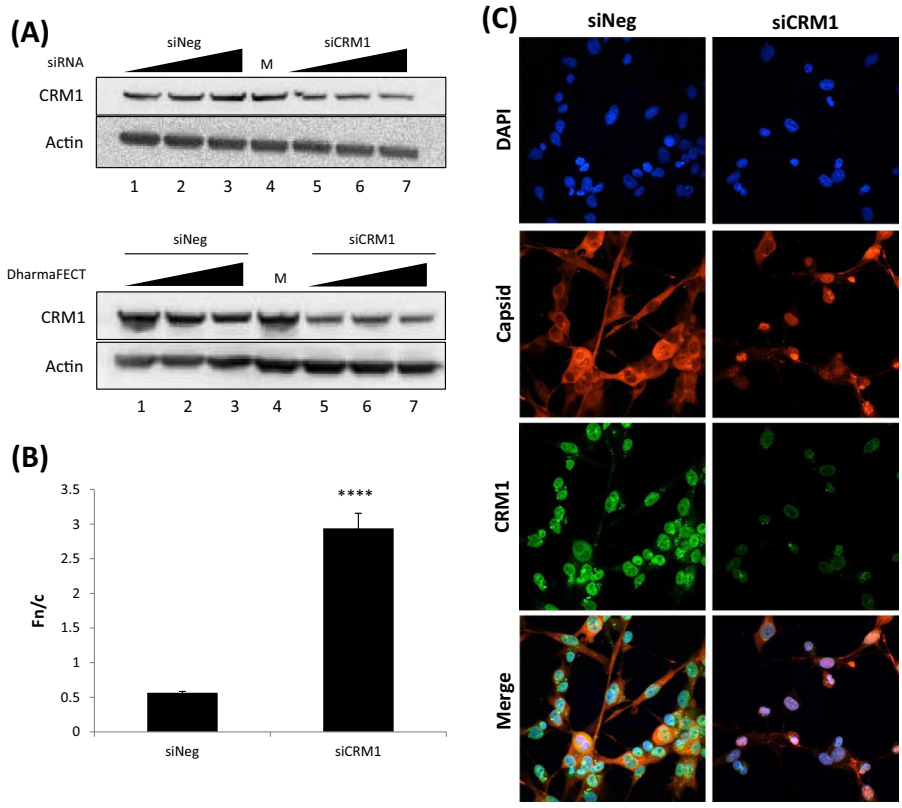

Fig. 2. Loss of CRMI results in increased capsid nuclear localization. (A) Top panel: U87MG cells were transfected with siRNA (10, 25, or 50 nM) against CRM1 or negative control siRNA (siNeg) using DharmaFECT Transfection Reagent (Thermo Scientific). Twenty-four hours post-transfection, cells were infected with VEEV TC83 (MOI 0.1). Twenty-four hours after infection, cells were collected and processed for Western blot analysis. M = mock infected cells, included as a control. Bottom panel: U87MG cells were transfected with siRNA (10 nM) and varying amounts of DharmaFECT transfection reagent (1, 5, and 10 μl) and processed as described above. (B) U87MG cells were transfected with siRNA (10 nM) using 1 μl of DharmaFECT transfection reagent and infected with VEEV as described above. Cells were fixed at 24 h and probed with DAPI, anti-VEEV capsid antibody, and an Alexa Fluor 568 labeled secondary antibody. Slides were imaged on a Nikon Eclipse TE2000-U after immunofluorescent staining. Digitized images were analyzed using the ImageJ version 1.47 public domain software (NIH) to determine the ratio of nuclear (Fn) to cytoplasmic (Fc) fluorescence (Fn/c). The numbers of cells analyzed are as follows: siNeg (10 nM) $N = 122$, siCRM1 (10 nM) $N = 78$. (C) Representative images of U87MG cells infected with VEEV and transfected with 50 nM siNeg or 50 nM siCRM1. $^{****}p \leqslant 0.001$ (siCRM1 compared to siNeg).

The ability of these inhibitors to alter VEEV capsid localization in the context of VEEV infection was assessed. Leptomycin B treatment was analyzed first as a control. Vero cells seeded on microscope slides were treated with DMSO or leptomycin B (45 nM) for two hours prior to infection with VEEV TC83 (MOI 1). Twenty-four hours after infection, the slides were fixed and capsid localization was analyzed by confocal fluorescent microscopy. DMSO treated cells displayed capsid localized throughout the cytoplasm and nucleus. In contrast, leptomycin B treatment induced a dramatic rearrangement of capsid distribution with capsid predominantly localized to the nucleus (Fig. 4A). The results were statistically significant; the ratio of nuclear to cytoplasmic fluorescence (Fn/c) increased with leptomycin B treatment (Fig. 4C).

A similar set of experiments were performed to determine the influence of nuclear import inhibitors (mifepristone and ivermectin) on capsid localization compared to DMSO treated cells. Fig. 4B shows that Vero cells treated with DMSO two hours prior to infection with VEEV TC83 (MOI 1) have capsid distributed in both the cytoplasm and nucleus as previously seen. When treated with mifepristone (10 μM) or ivermectin (1 μM), capsid is mainly excluded from the nucleus and found predominantly in the cytoplasm (Fig. 4B).The change in the ratio of nuclear to cytoplasmic fluorescence is significant as well. Compared to DMSO, treatment with mifepristone and ivermectin reduce Fn/c by nearly half (Fig. 4D). These results demonstrate that mifepristone and ivermectin are capable of modulating VEEV capsid cellular localization.

3.3. Nuclear import and export inhibitors aid in cell survival following VEEV infection

Capsid is responsible for the massive cytopathic effect (CPE) that is observed following VEEV infection (Garmashova et al., 2007a,b). The N-terminal region (aa 30–68), which is also the same area involved in binding to importin α, importin β1, and CRM1 (Atasheva et al., 2010a), plays the most critical role in CPE development (Garmashova et al., 2007a). Therefore, experiments were performed to determine if altered VEEV capsid localization, as modulated by mifepristone and ivermectin, would affect cell survival following VEEV infection. Vero cells were pre-treated with inhibitors, infected with VEEV TC83 and viability was measured using Promega's CellTiterGlo Luminescent Cell Viability Assay. Both mifepristone and ivermectin increased the viability of infected cells compared to DMSO-treated infected cells (Fig. 5A and B). Results were statistically significant with the lowest concentration of mifepristone. Mifepristone, ivermectin and leptomycin B treatment were also all capable of increasing cell survivability following VEEV infection in U87MG cells (Fig. 5C), confirming the results observed in Vero cells. These results demonstrate the ability of mifepristone and ivermectin to aid in cell survival following VEEV infection.

3.4. Mifepristone, ivermectin, and leptomycin B treatment results in decreased VEEV TC83 replication

Since treatment with nuclear import inhibitors alters the localization of capsid as well as reduced VEEV induced cell death, we

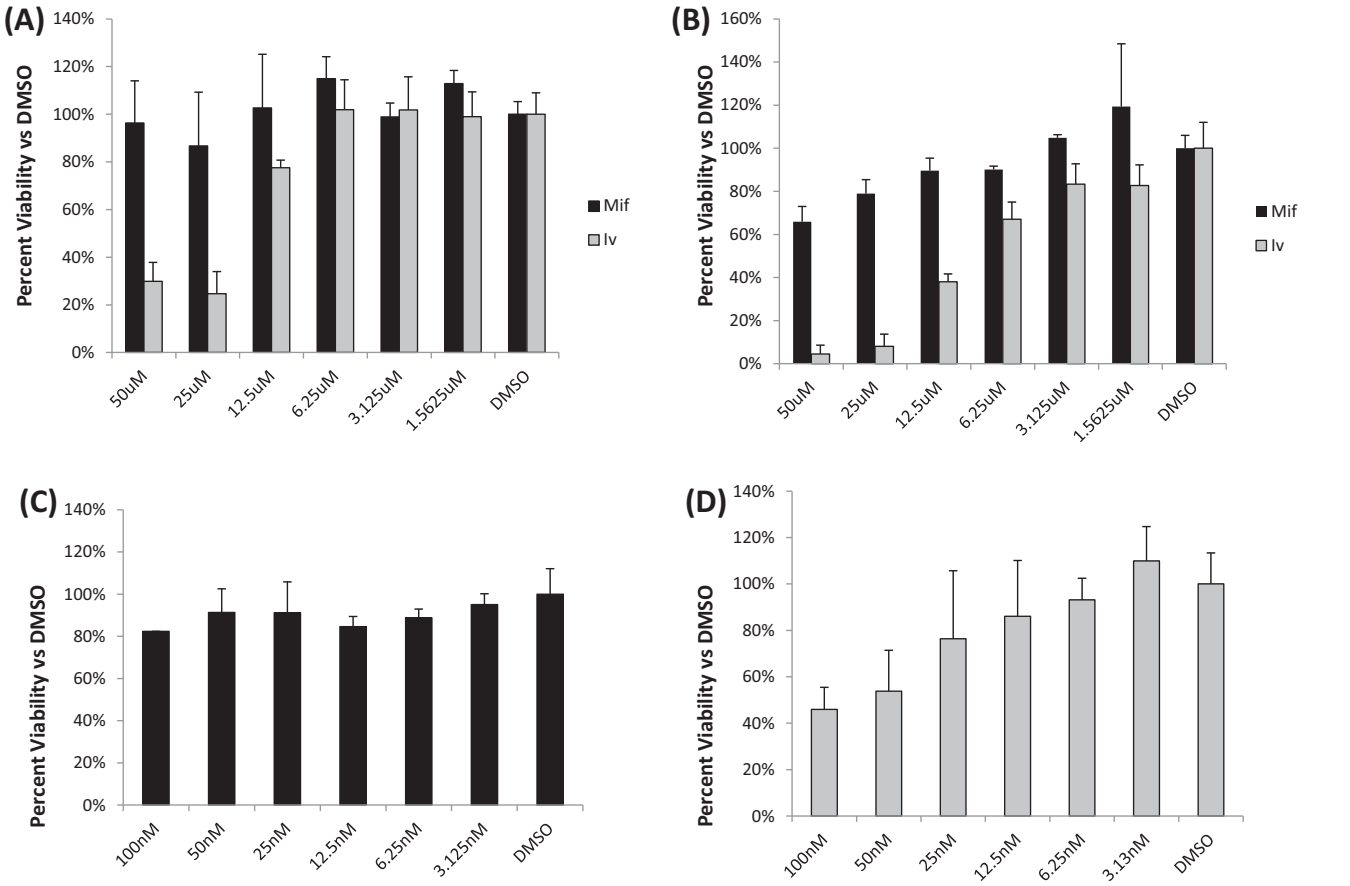

Fig. 3. Cytotoxicity screens of mifepristone, ivermectin, and leptomycin B. (A) Cellular viability was measured using Promega's CellTiter-Glo Luminescent Cell Viability Assay. U87MG cells were treated for 24 h with serial dilutions of mifepristone and ivermectin to establish the CC_{50}. Luminescence was measured using Promega's protocol. (B) The same method from (A) was used to determine mifepristone and ivermectin cytotoxicity in Vero cells. (C) Cellular viability of U87MG cells treated with serial dilutions of leptomycin B was measured using Promega's CellTiter-Glo Luminescent Cell Viability Assay 24 h after treatment to establish CC_{50}. (D) The same method from (C) was used to determine leptomycin B cytotoxicity in Vero cells.

aimed to determine the effect of these inhibitors on VEEV replication. To this end, we utilized VEEV TC83 with GFP inserted downstream of a duplicated subgenomic promoter (Atasheva et al., 2010a). Using flow cytometry, the fluorescent signal produced by the replicating virus can be measured. Pilot experiments indicated that the signal was robust enough to measure using this system around 16 h post-infection (data not shown). To determine the influence of nuclear import inhibitors on VEEV-GFP, cells were pre-treated with each compound or DMSO for two hours prior to infection with VEEV-GFP (MOI 1) or mock infection. Inhibitor-treated media was replaced after an hour-long infection. Cells were fixed for flow cytometry 16 or 24 h post-infection.

Nuclear import and export inhibitor treatment reduced the fluorescent signal in U87MG cells at both 16 and 24 h post-infection. By 16 h, mifepristone had reduced fluorescence by 40% (Fig. 6A), ivermectin had reduced fluorescence by 30% (Fig. 6B), and leptomycin B had reduced fluorescence to a level equivalent to that of mock infected cells (Fig. 6C). Twenty-four hours post infection, mifepristone had reduced fluorescence by 60% (Fig. 6A), ivermectin had reduced fluorescence by 30% (Fig. 6B), and leptomycin B has reduced fluorescence by 80% (Fig. 6C). Leptomycin B was also shown to reduce GFP production at 24 h in a dose-dependent manner in both U87MG and Vero cells (Supplemental Fig. 1A and B). A similar pattern was observed in Vero cells. Mifepristone reduced fluorescence by nearly 40% as compared to DMSO at 16 h (Fig. 6D), and leptomycin B reduced fluorescence almost to mock infected levels (Fig. 6F). By 24 h post-infection, mifepristone had reduced fluorescence by 40% (Fig. 6D), and leptomycin B had effectively reduced the fluorescence signal by over 90% (Fig. 6F). Ivermectin treatment displayed limited inhibition at both 16 and 24 h post infection, neither of which were statistically significant (Fig. 6E). These results indicate that mifepristone, ivermectin, and leptomycin B inhibit VEEV replication but with varying efficacies.

We next sought to determine the efficacy of these inhibitors in reducing infectious virions. To measure the reduction in viral titer, U87MG cells were pre-treated with mifepristone, ivermectin, leptomycin B, or DMSO, infected with VEEV TC83 (MOI 0.1) for an hour, post-treated with inhibitor media, and the viral supernatants collected at 8, 16 and 24 h after infection. Mifepristone treatment resulted in a constant decrease of VEEV replication, with ~1 log loss of virus at all time points examined (Fig. 7A). In contrast, ivermectin treatment displayed a delayed growth curve, inhibiting VEEV at 8 and 16 h post-infection, but no inhibition being observed by 24 h. Leptomycin B treatment had a more dramatic effect on VEEV, reducing viral titers by 4 logs by 24 h (Fig. 7B). Similar results were observed in Vero cells, where at 24 h post-infection, no inhibition was observed with ivermectin (Fig. 7C). Mifepristone and leptomycin B treatment did inhibit VEEV replication in Vero cells, but to a lesser extent than what was observed in U87MG cells (Fig. 7C and D). These results mirror the data obtained with VEEV-GFP (Fig. 6), where leptomycin B was the most effective in reducing GFP expression followed by mifepristone. Ivermectin was the least effective in reducing GFP expression and little to no inhibition was observed by 24 h.

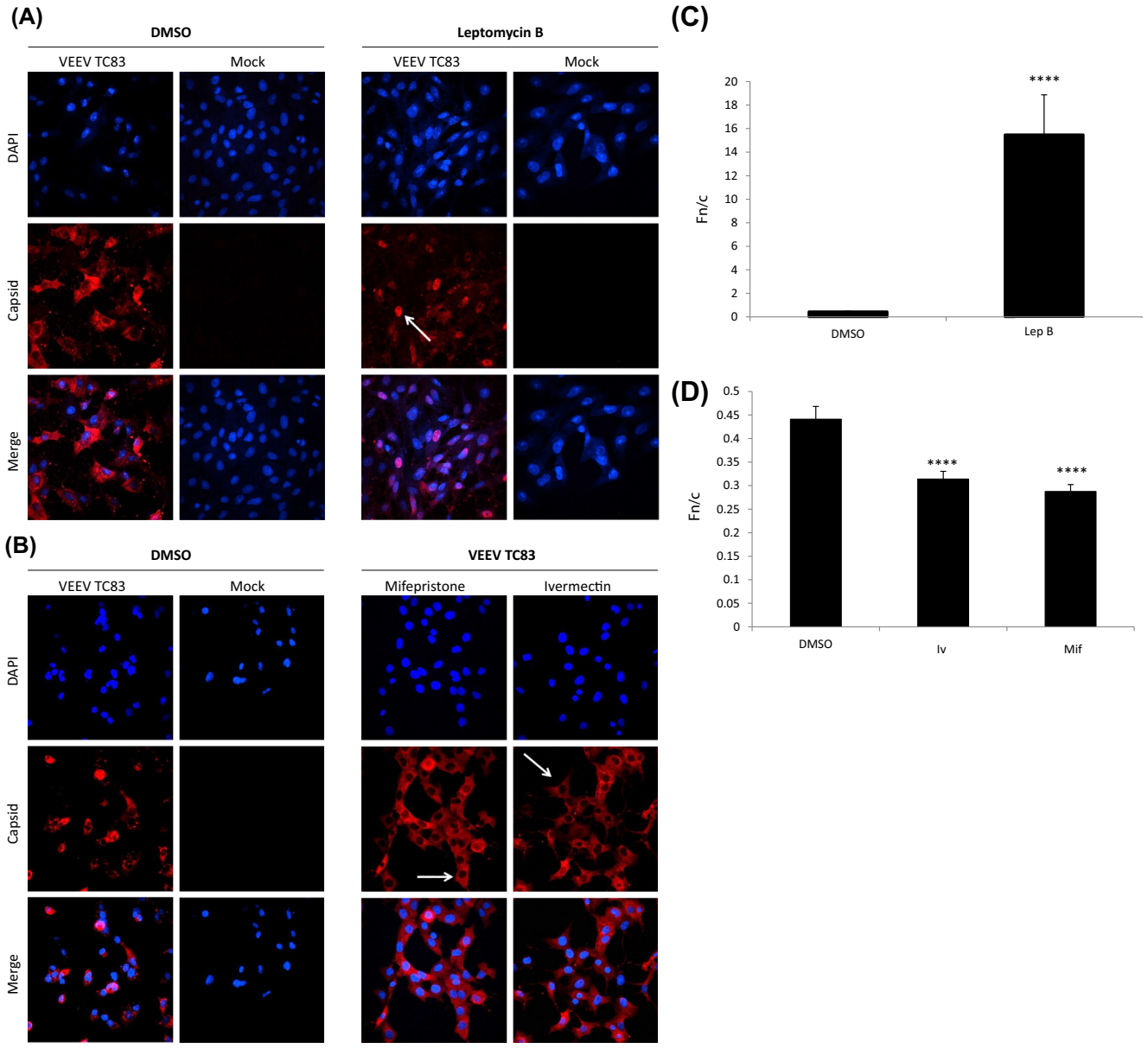

Fig. 4. Nuclear import and export inhibitors alter viral capsid distribution in the host cytoplasm and nucleus. (A) Vero cells were pre-treated with leptomycin B (45 nM) or DMSO for two hours, infected with VEEV TC83 (MOI 0.1), media with inhibitors replaced, and fixed at 24 h. Slides were probed with DAPI, anti-VEEV capsid antibody and an Alexa Fluor 568 labeled secondary antibody. Slides were then imaged on a Nikon Eclipse TE2000-U after immunofluorescent staining. (B) The same method from panel A was used to treat Vero cells with DMSO, mifepristone (10 μM), or ivermectin (1 μM). (C) Digitized images were analyzed using the ImageJ version 1.47 public domain software (NIH) to determine the ratio of nuclear (Fn) to cytoplasmic (Fc) fluorescence (Fn/c). Greater than 55 cells were analyzed per sample. (D) Digitized images were analyzed as described above. Greater than 75 cells were analyzed per sample. $^{****}p \leqslant 0.0001$ (compared to DMSO treated VEEV infected cells).

3.5. Mifepristone and leptomycin B treatment results in decreased viral replication of fully virulent VEEV

VEEV is a BSL-3 select agent pathogen and therefore many studies are performed with TC83, which is the live attenuated vaccine derivative of VEEV Trinidad Donkey (TrD). To confirm that the results observed with VEEV TC83 are relevant to the fully virulent VEEV TrD, experiments were performed to test the ability of mifepristone, ivermectin and leptomycin B to inhibit VEEV TrD viral replication. U87MG cells were treated with inhibitors as described for Fig. 7 and viral replication measured by plaque assays. Results from these experiments indicate that VEEV TrD is inhibited in a similar manner to VEEV TC83 (Fig. 8). Leptomycin B dramatically inhibited VEEV TrD in U87MG cells (~5 logs of inhibition). Mifepristone treated cells displayed a decrease of 0.7 logs in viral replication. The reduced viral replication in leptomycin B and mifepristone treated cells was found to be statistically significant when compared to DMSO treated controls. In contrast, ivermectin treatment did not result in any significant decrease in viral replication. These results confirm the ability of mifepristone and leptomycin B to inhibit VEEV replication.

4. Discussion

Targeting host mechanisms rather than the virus to control viral propagation has been previously explored for alphavirus treatment (Tan et al., 2007). Treatment of VEEV-infected BALB/c mice with polyethylene glycol-conjugated alpha interferon increases macrophage activation, dampens tumor necrosis factor production, and

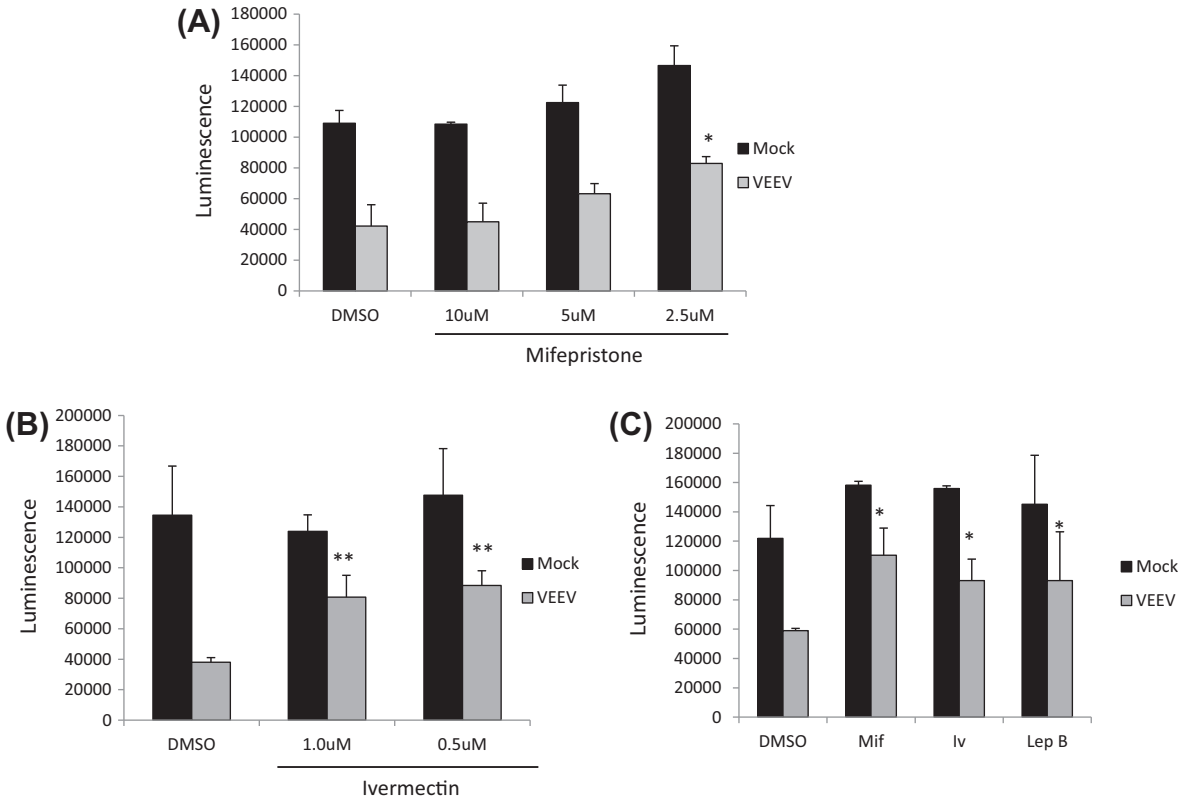

Fig. 5. Mifepristone, ivermectin and leptomycin B treatment increases cell survival following VEEV infection. (A) Vero cells were pre-treated with a serial dilution of mifepristone in supplemented DMEM for two hours then infected at MOI 1 with VEEV TC83. Media containing the inhibitors was replaced and cell viability measured after 24 h using Promega's CellTiter Luminescent Cell Viability Assay. Luminescence was normalized to a mock-infected, DMSO-treated control. (B) The same method from panel A was used for ivermectin treatment. (C) U87MG cells were pre-treated mifepristone (10 μM), ivermectin (1 μM), or leptomycin B (45 nM) in supplemented DMEM for two hours then infected at MOI 5 with VEEV TC83. Media containing the inhibitors was replaced and cell viability measured after 24 h using Promega's CellTiter Luminescent Cell Viability Assay. Luminescence was normalized to a mock-infected, DMSO-treated control. $^{*}p \leqslant 0.05$, $^{**}p \leqslant 0.01$ (compared to DMSO treated VEEV infected cells).

increases survival of infected animals by modulating immunological mechanisms targeted by the virus (Lukaszewski and Brooks, 2000). The tetracycline derivative minocycline inhibits microglial activation by neuroadapted Sindbis Virus and blocks local interleukin-1β production in C57BL/6 mice, protecting mice from paralysis and death even after a viral challenge (Irani and Prow, 2007). Artificial interferon treatment protected hamsters challenged with lethal doses of western equine encephalitis virus, implying that synthetic stimulation of the immune response could also be a viable treatment (Julander et al., 2007). For alphaviruses and other RNA viruses that encode viral proteins that traffic to the nucleus, host proteins are often needed to shuttle these viral proteins, providing another avenue for host-based therapeutics. The VEEV capsid is no exception and actually goes a step further by forming a tetrameric complex with host importins α/β1 and the exportin CRM1, which blocks the nuclear pore (Atasheva et al., 2010a).

Our results provide evidence that the nuclear pore components importins α/β1 and the exportin CRM1 modulate capsid localization. Previous studies have demonstrated that VEEV capsid is found in a tetrameric complex with these proteins, however these results were demonstrated with capsid peptide (aa 30–68) fused with GFP (Atasheva et al., 2010b). Our results are the first report to demonstrate the regulation of capsid in the context of infection and with the full length non-tagged protein. Moreover, we have demonstrated that importin α/β1 inhibitors can block the nuclear import of capsid and confine it to the cytoplasm. Our results also demonstrated that the CRM1 inhibitor, leptomycin B, dramatically increased the amount of nuclear associated capsid. These data are consistent with a model of capsid nuclear trafficking whereby capsid must first interact with importin αβ1 to mediate its nuclear entry, the interaction of which if blocked results in capsid being stuck in the cytoplasm. Once inside the nucleus, capsid must then also associate with CRM1 at which point it migrates to and associates with the NPC to cause blockage. If the interaction with CRM1 is disrupted then capsid is found throughout the nucleoplasm as it cannot return through the NPC.

Treatment of VEEV infected cells with mifepristone, ivermectin, and leptomycin B resulted in a decrease in VEEV/capsid induced CPE. These results provide further evidence that the ability of capsid to form a tetrameric complex with importin α, importin β1, and CRM1 contributes to CPE development. It is of interest that infected cells treated with the lowest concentration of mifepristone displayed an increase in cell viability in Vero cells. It is likely that at higher concentrations of mifepristone there are multiple cellular components, in addition to importin αβ1 that are being inhibited. This could provide a situation where both pro- and anti-apoptotic proteins are being altered, thereby masking the pro-survival feature of preventing the interaction between capsid and importin αβ1. While our studies were performed *in vitro*, they have clear implications for *in vivo* applications, as VEEV induced cell death has been documented *in vivo* (Sharma et al., 2008). Apoptotic genes such as caspase 3 are upregulated in cynomolgus macaques infected with VEEV (Hammamieh et al., 2007). In mouse models of infection, VEEV infection of the CNS results in an intense inflammatory response, active gliosis, and neuronal cell death. Microarray analysis indicated that VEEV infection was characterized by an increase in expression of antigen presentation, inflammation, viral response, and apoptotic genes (Sharma et al., 2008). Disrupting

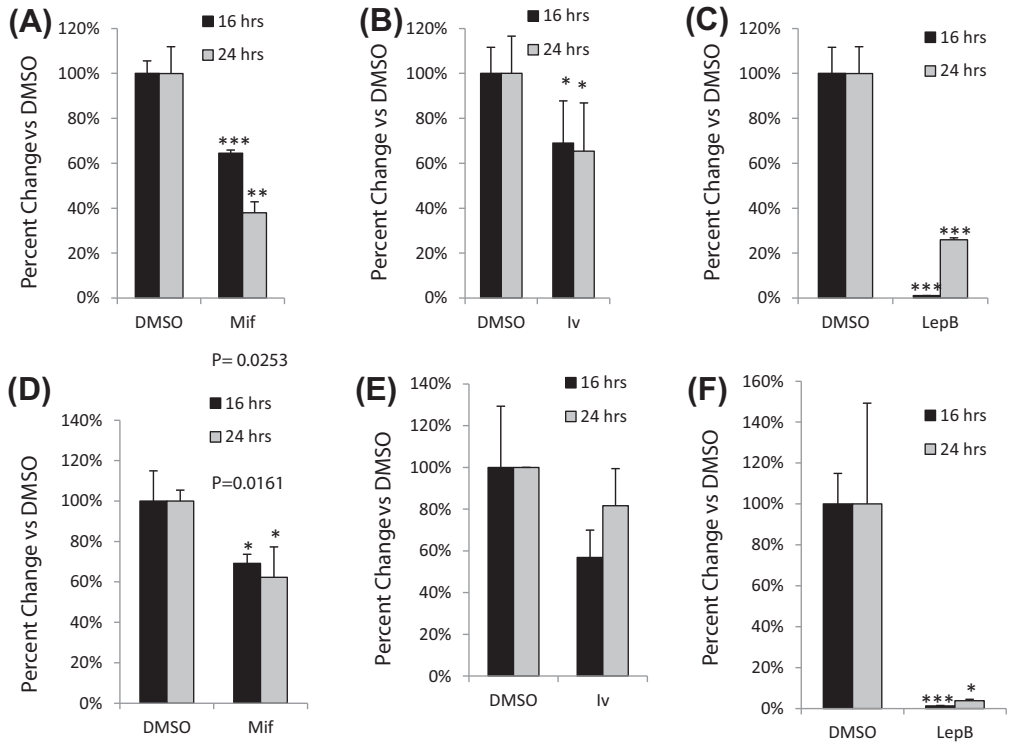

Fig. 6. Reduction of virus production at 16 and 24 h post-infection with mifepristone, ivermectin, and leptomycin B treatment. (A) U87MG cells were pre-treated with mifepristone or DMSO for two hours, infected with VEEV TC83-GFP (MOI 1), and collected at 16 and 24 h. The mean fluorescent signal was normalized to inhibitor-treated, mock infected samples. Experiments were performed in triplicate. Figures are representative of three separate experiments. (B) The same method from panel A was used to treat U87MG cells with ivermectin. (C) The same method from panel A was used to treat U87MG cells with leptomycin B. (D) The same method from panel A was used to treat Vero cells with mifepristone. (E) The same method from panel A was used to treat Vero cells with ivermectin. (F) The same method from panel A was used to treat Vero cells with leptomycin B. $^{*}p \leqslant 0.05$, $^{**}p \leqslant 0.01$, $^{***}p \leqslant 0.001$ (compared to DMSO treated cells at the corresponding time point).

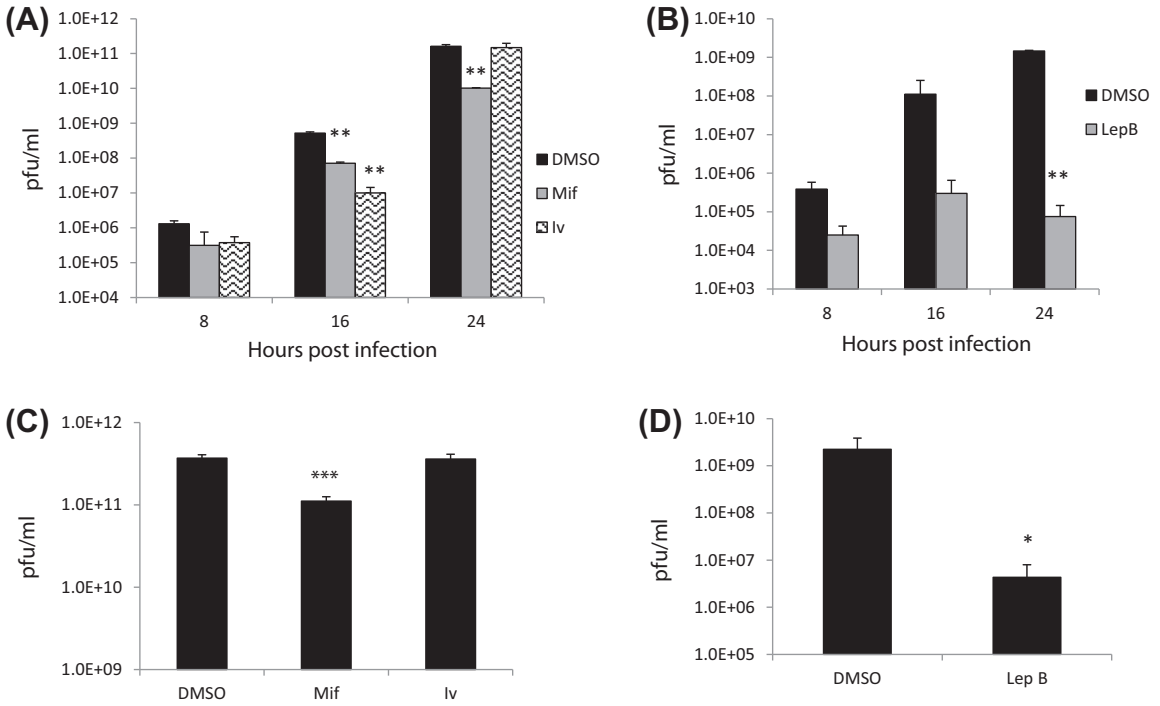

Fig. 7. Viral titer reduction of mifepristone, ivermectin, and leptomycin B. (A) U87MG cells were pre-treated with mifepristone (10 μM), ivermectin (1 μM), or DMSO (0.1%), and then infected with VEEV TC83 (MOI 0.1). After infection, cells were post-treated with the inhibitors. Supernatants were collected at 8, 16, and 24 h post-infection and plaque assays performed. (B) U87MG cells were pretreated with leptomycin B (45 nM) or DMSO (0.1%), and then infected with VEEV TC83 (MOI 0.1). After infection, cells were post-treated with the inhibitor or DMSO. Supernatants were collected at 8, 16, and 24 h post-infection and plaque assays performed. (C) The same method from panel A was used in Vero cells and cell supernatants collected at 24 h post-infection. (D) The same method from panel B was used in Vero cells and cell supernatants collected at 24 h post-infection. $^{*}p \leqslant 0.05$, $^{**}p \leqslant 0.01$, $^{***}p \leqslant 0.001$ (compared to DMSO treated cells at the corresponding time point).

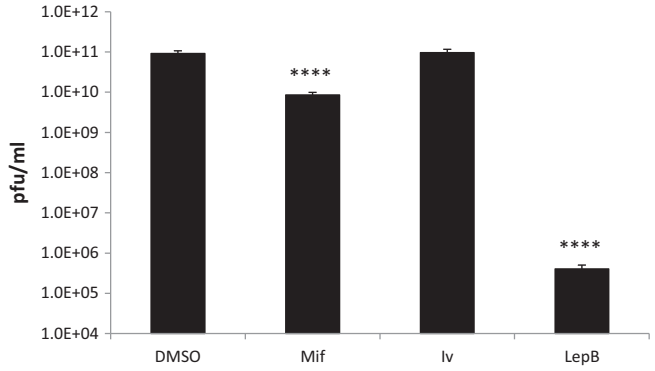

Fig. 8. Mifepristone and leptomycin B inhibit VEEV TrD replication. U87MG cells were pre-treated with mifepristone (10 μM), ivermectin (1 μM), leptomycin B (45 nM), or DMSO (0.1%), and then infected with VEEV TrD (MOI 0.1). After infection, cells were post-treated with the inhibitors. Supernatants were collected at 24 h post-infection and plaque assays performed. $^{****}p \leqslant 0.0001$ (compared to DMSO treated cells).

the interaction of capsid with the nuclear pore (either through importin α/β or CRM1 inhibitors) may result in altered gene expression, thereby contributing to cell survival. Future studies will focus on the modulation of apoptotic pathways by these nuclear import inhibitors.

All three compounds tested were able to inhibit VEEV replication, but with varying efficiencies. Leptomycin B was the most potent inhibitor, decreasing viral replication by 5 logs with the BSL-3 pathogen, VEEV TrD. These results are in agreement with the ability of leptomycin B treatment to dramatically decrease the cytoplasmic levels of capsid. Capsid restricted to the nucleus will not be available to form viable virions or help tether the viral replication complex to the host's envelope structures. Leptomycin B is very broad CRM1 mediated export inhibitor that influences the export of the majority of host proteins, thus it may also be restricting the export of host machinery needed by the virus to aid in replication. While leptomycin B is not in therapeutic use (Newlands et al., 1996), semisynthetic derivatives are better tolerated *in vivo* as anticancer agents (Mutka et al., 2009) and should be tested for antiviral abilities as well. In addition, a more specific inhibitor of the interaction between CRM1 and capsid would be needed to be therapeutically useful, and it an important area of research.

Mifepristone and ivermectin were recently identified as importin α/β1 inhibitors (Wagstaff et al., 2011, 2012), from a panel of US Food and Drug Administration (FDA) approved drugs. The benefits of using mifepristone and ivermectin for VEEV treatment is that they are already licensed for human use and safe doses are known. Mifepristone, marketed as Mifeprex, was approved by the FDA to terminate early-term pregnancies in 2000 (www.accessdata.fda.gov). It has shown promise in treating depression and psychosis due to antagonizing glucocorticoid receptors, in addition to its anti-progestin properties (DeBattista and Belanoff, 2006). Ivermectin is used to treat livestock for scabies and other parasitic infestations, and it is marketed as Stromectol and was approved to treat intestinal nematodes in humans in 1996 (www.fda.gov). This anti-parasitic compound has antiviral promise *in vitro* as well; it inhibited yellow fever virus replication by targeting the NS3 protein's helicase activity (Mastrangelo et al., 2012) and inhibits HIV-1 and dengue virus replication by inhibiting host-mediated nuclear import (Wagstaff et al., 2012). In invertebrates, ivermectin opens γ-aminobutyric acid (GABA)-gated Cl^- channels and glutamate channels, and in mammals, the GABA channels only (Geary, 2005). These two neurotransmitters are found exclusively in the mammalian CNS, and there is some literature concerned with potential neurotoxicity of ivermectin crossing the blood–brain barrier (Burkhart and Burkhart, 1999), which may prove useful for treatment of VEEV infection.

Throughout our experiments we observed differences in the response of Vero cells and U87MG cells to the inhibitors. Overall, VEEV replication was inhibited to a greater extent in U87MG cells as compared to Vero cells. One explanation for these observed differences is that VEEV replicates to higher levels and more rapidly in Vero cells and thus the inhibitors have less of an affect. Likewise, the absence of interferon production in Vero cells, which aids in viral replication, could also be playing a role. Interfering with viral processes through nuclear import inhibitors may allow cells like U87MG to mount an immune response which in turn reduces viral replication. However, preliminary experiments did not detect any change in interferon production following mifepristone or ivermectin treatment of VEEV infected U87MG cells (data not shown). Elucidating the difference in antiviral efficacy between mifepristone, ivermectin, and leptomycin B presents an intriguing avenue for future research.

5. Conclusion

Nuclear-cytoplasmic trafficking inhibitors represent a potential class of VEEV therapeutics and a novel approach to studying virus–host interactions. The inhibitors not only changed the distribution of capsid *in vitro* but also protected cells from VEEV/capsid induced cell death. Further, the inhibitors were able to reduce viral replication in both VEEV TC83 and TrD.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.antiviral.2013.10.004.

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