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

Lindsay Lundberg [a,1], Chelsea Pinkham [a,1], Alan Baer [a], Moushimi Amaya [a], Aarthi Narayanan [a], Kylie M. Wagstaff [b], David A. Jans [b], Kylene Kehn-Hall [a,*]

[a] National Center for Biodefense and Infectious Diseases, George Mason University, Manassas, VA, USA
[b] Nuclear Signalling Laboratory, Department of Biochemistry and Molecular Biology, Monash University, Clayton, Victoria, Australia

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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.

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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).

* Corresponding author. Address: National Center for Biodefense and Infectious Diseases, George Mason University, Biomedical Research Lab, 10650 Pyramid Place, MS 1J5, Manassas, VA 20110, United States. Tel.: +1 703 993 8869, lab: +1 703 993 9493; fax: +1 703 993 4280.
E-mail address: kkehnhal@gmu.edu (K. Kehn-Hall).
[1] These authors contributed equally to this work.

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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.

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