Nuclear Import and Export Inhibitors Alter New World Alphavirus Capsid Localization and Reduce Viral Replication in Mammalian Cells

Lindsay Lundberg[1], Chelsea Pinkham[1], Ashwini Benedict[1], Nazly Shafagati[1], Moushimi Amaya[1], Aarthi Narayanan[1], Kylie M. Wagstaff[2], David A. Jans[2], Sharon Tamir[3], Kylene Kehn-Hall[1]

[1]National Center for Biodefense and Infectious Diseases, School of Systems Biology, George Mason University, Manassas, VA, USA

[2]Nuclear Signaling Laboratory, Department of Biochemistry and Molecular Biology, Monash University, Clayton, Victoria, Australia

[3]Karyopharm Therapeutics Inc., Newton, MA, USA

ABSTRACT

New World Alphaviruses, such as Eastern, Venezuelan, and Western Equine Encephalitis Viruses (EEEV, VEEV, and WEEV), cause high mortality and morbidity in equines and humans and are characterized by a febrile illness that may progress into encephalitis. The Centers for Disease Control and Prevention considers all three viruses Category B agents due to their ease of weaponization and the lack of licensed vaccines or therapeutics.[1] The TC-83 live vaccine, which is used to vaccinate equines in Mexico and Colombia and has *Investigational New Drug* status for human use under the FDA, is used extensively in drug research and toxicity assays. It can be handled in BSL-2 conditions, unlike the fully virulent BSL-3 strain TrD, though it occasionally invokes residual pathogenicity in humans and rodent models. TC-83 was generated by passaging the virulent Trinidad donkey strain eighty-three times in guinea pig heart cells, resulting in twelve mutations, only two of which are attenuating.[2] Research into better vaccines and effective antivirals are needed. VEEV was weaponized by both the US and USSR bioweapons programs. It is highly infectious via the aerosol route and may infect most mosquito species, which could prove problematic if the virus was to become enzootic in the US. Safe vaccines for the equine encephalitic viruses are lacking, highlighting the need for broad-spectrum therapeutics. Targeting the host immune response may also have therapeutic potential.

The structural capsid protein of New World Alpahviruses is the primary virulence factor and inhibits host transcription. VEEV capsid also blocks nuclear import in mammalian cells, possibly due to its complexing with the host CRM1 and importin α/β1 nuclear transport proteins.[3] Our lab reasoned that if capsid is using the host's nuclear transport proteins to shuttle between the nucleus and cytoplasm, inhibitors of nuclear import and export should disrupt this interaction, resulting in altered capsid distribution and decreased viral replication.[4]

To confirm capsid and host protein interactions, we used siRNA mediated knockdown of host import or export proteins. Immunofluorescent confocal microscopy revealed altered capsid localization patterns compared to siNeg controls. To chemically inhibit host nuclear transport proteins and alter capsid localization, we used mifepristone and ivermectin[5] to inhibit importin α/β1, and Leptomycin B and the novel CRM1 inhibitors KPT-185, KPT-335, and KPT-350 to inhibit CRM1. Treatment of cells with nuclear import or export inhibitors prior to infection significantly altered capsid localization compared to vehicle treated cells in a pattern similar to the siRNA data. Extracellular viral RNA levels were found to be comparatively less than intracellular RNA levels measured by q-RT-PCR in cells treated with CRM1 inhibitors prior to infection, indicating the inhibitors likely interfered with viral assembly by sequestering capsid to the nucleus. Plaque assays were used to measure infectious titer; cells treated with inhibitors prior to infection with VEEV, EEEV, and WEEV showed significantly reduced titers compared to vehicle treated cells. Our experiments confirmed VEEV's interaction with host CRM1 and importin α/β1 nuclear transport proteins, showed that inhibitor treatment altered capsid distribution in the host cell, and that inhibitor treatment reduced replication of the New World Alphaviruses. We have demonstrated for the first time that the capsid proteins of the New World Alphaviruses likely interact with host proteins in a similar manner, and that a pan-antiviral therapeutic may be possible.

RESULTS

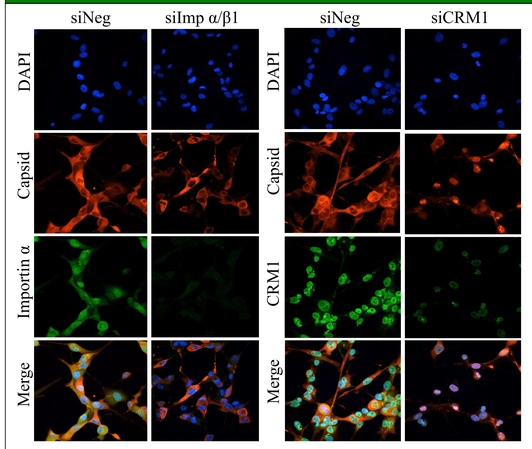

Figure 1: Loss of importin α, β, and CRM1 alters capsid localization. U87MG cells seeded on slides were transfected with 50nM siNeg, 50nM siImpα, 50nM siImpβ1, or 50nM siCRM1 using HiPerFect or DharmaFect. Twenty-four hours post-transfection, cells were infected with VEEV-TC83 (MOI 0.1), then fixed 24 hours post-infection. Slides were probed with DAPI, anti-VEEV capsid antibody, anti-importin α or anti-CRM1, and an Alexa Fluor 568 labeled secondary antibody. After immunofluorescent staining, slides were imaged on a Nikon Eclipse TE2000-U. Data not shown: the ratio of nuclear to cytoplasmic fluoresnce was found to significantly alter with the loss of import or export proteins using ImageJ software (NIH, version 1.47).

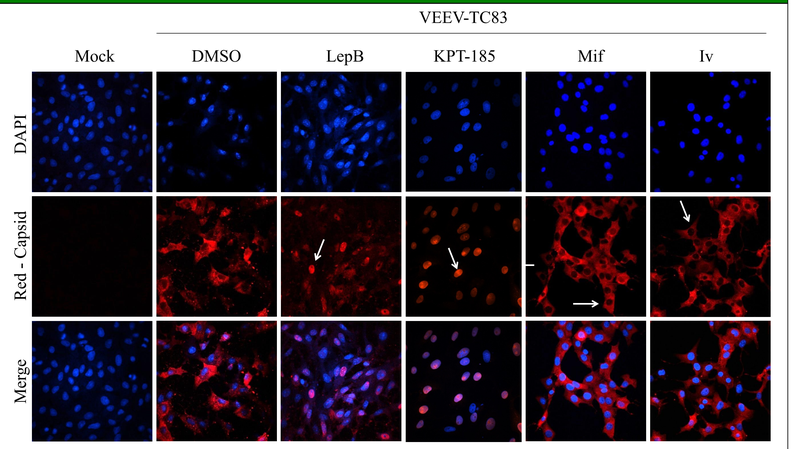

Figure 2: Nuclear import and export inhibitors alter viral capsid distribution in the host cytoplasm and nucleus. Vero cells seeded on slides were pre-treated with DMSO (1%), Leptomycin B (45nM), KPT-185 (2.5μM), Mifepristone (10μM), or Ivermectin (1μM) for two hours, infected with VEEV-TC83 (MOI 0.1 or 1), media with inhibitors replaced, then fixed at 24 hours post-infection. Slides were probed with DAPI, anti-VEEV capsid antibody, and an Alexa Fluor 568 labeled secondary antibody. After immunofluorescent staining, slides were imaged on a Nikon Eclipse TE2000-U. Data not shown: KPT-335, and -350.

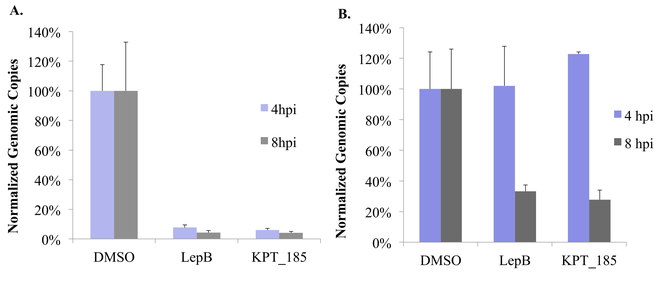

Figure 3: KPT-185 reduces released viral RNA. (A) Vero cells were treated for two hours prior to infection with DMSO; 2.5μM of KPT-185; or 45nM Leptomycin B. After an hour infection with VEEV-TC83 (MOI 1), drug media was replaced. At 4 and 8 hours post-infection (hpi), supernatants were collected and extracellular viral RNA extracted and analyzed by q-RT-PCR. Graphs represent biological triplicates. Genomic copies were normalized to a percentage of the DMSO control. (B) Vero cells were treated as described above, and total intracellular RNA was extracted from lysed cells and analyzed by q-RT-PCR.

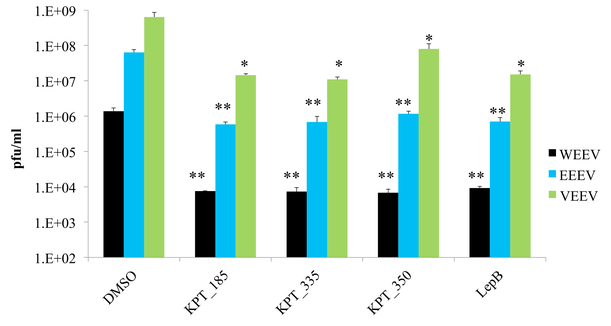

Figure 4: CRM1 inhibitors reduce viral titer of wild-type WEEV, EEEV, and VEEV. Vero cells were treated for two hours prior to infection with DMSO; 2.5μM of KPT-185, -335, or -350; or 45nM Leptomycin B. After an hour infection with WEEV (California 1930), EEEV (GA97), or VEEV-TrD (MOI 1), drug media was replaced. Supernatants were collected 16 hours post-infection and plaque assays performed using Vero cells. Biological triplicates are presented. * $P \leq 0.05$, ** $P \leq 0.005$ (compared to DMSO treated cells)

CONCLUSIONS

These series of experiments demonstrated that:

- **Minimal cytotoxicity in relevant cell types**: (data not shown) Cell viability post-treatment with the KPT compounds, Mifepristone, Ivermectin, and Leptomycin B was measured in Veros and U87MGs. Working concentrations of each drug was established.
- **Nuclear import and export inhibitors alter capsid localization**: Treatment of Vero cells with Mifepristone and Ivermectin prior to infection with VEEV-TC83 keeps capsid primarily cytoplasmic. Treatment of Vero cells with Leptomycin B, KPT-185, KPT-335, and KPT-350 prior to infection with VEEV-TC83 retains the capsid in the nucleus.
- **Nuclear export inhibitors interfere with viral assembly**: Treatment of Vero cells with KPT-185 or Leptomycin B prior to infection with VEEV-TC83 for an hour reduced extracellular viral RNA at 4 and 8 hpi compared to DMSO controls, indicating assembled virus was not being released. Intracellular viral RNA levels were comparable to DMSO controls at 4 hpi but not 8hpi, indicating subsequent rounds of viral replication were halted.
- **Nuclear export inhibitors reduce viral replication of New World Alphaviruses**: Treatment of Vero cells with KPT-185, -335, -350, and Leptomycin B prior to infection with fully virulent WEEV (California 1930), EEEV (GA97), and VEEV (Trinidad Donkey) reduced viral titers by approximately two logs compared to DMSO controls. Titer reduction was confirmed at multiple time points with VEEV-TrD (data not shown).

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