Antiviral Research 112 (2014) 26–37

Contents lists available at ScienceDirect

Antiviral Research

journal homepage: www.elsevier.com/locate/antiviral

Small molecule inhibitors of Ago2 decrease Venezuelan equine encephalitis virus replication

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ARTICLE INFO

Article history:
Received 19 May 2014
Revised 1 October 2014
Accepted 7 October 2014
Available online 18 October 2014

Keywords:
Venezuelan equine encephalitis virus
Ago2
miRNA
Acriflavine
Therapeutic
Alphavirus

ABSTRACT

Venezuelan equine encephalitis virus (VEEV) is classified as a Category B Select Agent and potential bioterror weapon for its severe disease course in humans and equines and its potential for aerosol transmission. There are no current FDA licensed vaccines or specific therapies against VEEV, making identification of potential therapeutic targets a priority. With this aim, our research focuses on the interactions of VEEV with host microRNA (miRNA) machinery. miRNAs are small non-coding RNAs that act as master regulators of gene expression by downregulating or degrading messenger RNA, thus suppressing production of the resultant proteins. Recent publications implicate miRNA interactions in the pathogenesis of various viral diseases. To test the importance of miRNA processing for VEEV replication, cells deficient in Ago2, an important component of the RNA-induced silencing complex (RISC), and cells treated with known Ago2 inhibitors, notably acriflavine (ACF), were utilized. Both conditions caused decreased viral replication and capsid expression. ACF treatment promoted increased survival of neuronal cells over a non-treated, infected control and reduced viral titers of fully virulent VEEV as well as Eastern and Western Equine Encephalitis Viruses and West Nile Virus, but not Vesicular Stomatitis Virus. ACF treatment of VEEV TC-83 infected mice resulted in increased *in vivo* survival, but did not affect survival or viral loads when mice were challenged with fully virulent VEEV TrD. These results suggest that inhibition of Ago2 results in decreased replication of encephalitic alphaviruses *in vitro* and this pathway may be an avenue to explore for future therapeutic development.

1. Introduction

Venezuelan equine encephalitis virus (VEEV) is a new-world alphavirus that is a significant pathogen of humans and equines. The virus is normally spread by an arthropod vector, but can also be transmitted by aerosol exposure. The disease course in humans ranges from an acutely febrile yet mild illness (Aguilar et al., 2011) to a debilitating condition clinically indistinguishable from Dengue (Casals et al., 1943), and in rare cases can progress to coma or death (Zacks and Paessler, 2010). The disease course is more severe and frequently fatal in equines. Due to its severity and ability to be spread by aerosol exposure, VEEV is classified as a Category B Select Agent and potential bioterror weapon. There is currently no FDA-approved vaccine or specific treatment against VEEV, making identification of therapeutic targets a priority. Recent research has identified multiple microRNAs (miRNA) that are differentially regulated during VEEV infection of mouse brain (Bhomia et al., 2010), suggesting that such interactions could be important in a human-cell model of VEEV pathogenesis.

miRNA are small, non-coding strands of RNA approximately 22 nucleotides in length, which can serve as master regulators of the cell by directly interacting with, and causing downregulation or degradation of target messenger RNA (mRNA). miRNA regulation was first described in *Caenorhabditis elegans*in 2001

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http://dx.doi.org/10.1016/j.antiviral.2014.10.002
0166-3542/

(Lau et al., 2001; Lee and Ambros, 2001), and appears to be evolutionarily conserved among eukaryotes. Canonical processing of miRNA begins with transcription from the host genome and assembly of a primary miRNA containing a stem-loop or hairpin structure, which is processed from the primary transcript by a series of enzymes to produce an asymmetrical miRNA duplex (Cullen, 2005). One strand associates with Argonaute (Ago) proteins and is incorporated into the RNA-induced silencing complex (RISC), which in turn associates with a target mRNA. The other strand is degraded. Perfect complementarity targets the mRNA for degradation, while imperfect complementarity has inhibitory activity, reducing production of the encoded protein (Cullen, 2005). Many viruses have shown significant interaction with host miRNA, including Epstein–Barr Virus (Lin et al., 2010), Pseudorabies Virus (Wu et al., 2012), and Rabies Virus (Zhao et al., 2012); the last of which has been shown, as with VEEV, to affect host miRNA expression in the brains of infected mice.

Viruses can also make use of miRNA for positive regulation, as has been well-studied with Hepatitis C virus (HCV), which uses a liver-specific miRNA called miR-122 to enhance replication by enabling a more favorable association between viral RNA and the host cell ribosomes (Henke et al., 2008; Jopling et al., 2005, 2006). More recent research showed a complex interaction between uncapped RNA, miR-122, and the viral internal ribosome entry site (IRES), in which translation activation is mediated by the presence of Ago2, a component of the RISC (Roberts et al., 2011). While the complete mechanism remains unknown, it is clear that miR-122 provides both a valuable biomarker and a viable therapeutic target for the treatment of HCV infection.

Based on these studies, it was hypothesized that VEEV interacts with cellular miRNA and miRNA processing machinery during infection of mammalian cell lines. To test this hypothesis, cells deficient in Ago2, a key miRNA processing enzyme, were infected with VEEV. The absence of Ago2 produced a decrease in viral genomic copies as monitored by qRT-PCR, as well as a decrease in replication as measured by plaque assay. Treatment with a known Ago2 inhibitor, acriflavine (ACF), inhibited viral replication. The effects of ACF on viral transcription, protein expression, and pathogenicity during VEEV infection, as well as its inhibitory effects on other viruses are presented herein.

2. Materials and methods

2.1. Cell culture

Mouse embryonic fibroblasts (MEFs), U87MG, and Vero cells were cultured in Dulbecco's modified Eagle's media (DMEM), supplemented and maintained as described in Lundberg et al. (2013). MEFs deficient in Ago2 ($Ago2^{-/-}$ MEFs) were obtained through Dr. G. Hannon of Cold Spring Harbor (Liu et al., 2004). AP-7 olfactory neuronal cells, a generous gift of Dr. D.E. Griffin (Johns Hopkins University), were cultured as described by Amaya et al. (2014).

2.2. Viruses

VEEV strains Trinidad Donkey (TrD), Mena II (Mena), 3880, and TC-83 were obtained from BEI Resources (Manassas, VA). The TrD strain, considered the model organism for the fully virulent strains, is serological subtype IAB and is associated with periodic epidemic and epizootic outbreaks. The Mena and 3880 strains are subtypes ID and IE, respectively, and, although fully virulent, are not associated with such outbreaks (Powers et al., 1997). The TC-83 virus is a live attenuated vaccine derivative of the TrD strain propagated by 83 serial passages in guinea pig heart cells (Berge et al., 1961), resulting in 12 nucleotide substitutions which confer attenuation principally through changes within the 5′-noncoding region and E2 envelope glycoprotein (Kinney et al., 1993). The TC-83 reporter virus, VEEV-GFP, was provided by Dr. I. Frolov (Atasheva et al., 2010) at the University of Alabama. Working stocks of WNV-NY strain 3356 were generated as previously described (Hussmann et al., 2013) All work with VEEV-TrD, VEEV-Mena, VEEV-3880, WEEV, EEEV, and WNV-NY was performed at BSL-3.

2.3. Protein extraction

Cell pellets were lysed with 50 μl clear lysis buffer [50 mM Tris–HCl (pH 7.5), 120 mM NaCl, 5 mM EDTA, 0.5% NP-40, 50 mM NaF, 0.2 mM Na_3VO_4, 1 mM DTT, and one complete protease cocktail tablet/50 mL]. Protein concentration was determined colorimetrically by a standard curve, using Bradford reagent (Sigma) and a Beckman-Coulter DTX-800 Multimode Detector/Multimode Analysis Software. For the ACF treatment assays, cell lysates were collected in blue lysis buffer as described in Lundberg et al. (2013).

2.4. Cell viability

Following treatment, cells were treated with the Celltiter Glo reagent (Promega) and viability was determined via fluorescence as compared to the control sample, using a Beckman-Coulter DTX-800 Multimode Detector with Multimode Analysis Software platform.

2.5. Western blot

Protein samples of 50 μg (clear lysis buffer) or 20 μl (blue lysis buffer) were separated, transferred, and subsequently processed as described in Austin et al. (2012), using a 5% bovine serum albumin solution for loss of Ago2 assays, and 5% milk for ACF treatment assays. Both the polyclonal Anti-Venezuelan equine encephalitis virus, TC-83 (Subtype IA/B) Capsid Protein (antiserum, Goat), NR-9403 and polyclonal Anti-Venezuelan equine encephalitis virus, TC-83 (Subtype IA/B) Glycoprotein (antiserum, Goat) antibodies were obtained through the NIH Biodefense and Emerging Infections Research Resources Repository. Rabbit monoclonal antibodies to Ago2 were obtained from Abcam and Cell Signaling Technologies. Beta actin HRP conjugated monoclonal antibody was used as a loading control and was obtained from Abcam.

2.6. Plaque assays

Viral supernatants were serially diluted, and applied at 400 μl in duplicate to confluent cultures of Vero cells in 6-well plates, or 200 μl to confluent cultures of Vero cells in 12-well plates, for 1 h prior to immobilization. Immobilization and staining were performed as described in Narayanan et al. (2012) for neutral red staining, or Kehn-Hall et al. (2012) for crystal violet staining. Plaques were assessed at two days post infection. For WNV-NY, monolayers of Vero cells in six-well plates were washed once with Dulbecco's phosphate buffered saline (DPBS) (Hyclone) followed by the addition of serial dilutions of viral samples (200 μl). The cells were incubated in a 5% CO_2 incubator for 1 h at 37 °C with rocking, the inocula removed, and a 0.9% agarose-complete DMEM overlay added. Cell monolayers were incubated for 48 h and a second overlay of agarose-complete DMEM containing 0.003% neutral red (MP Biomedicals) was added. Plaques were counted at days 3 and 4.

2.7. qRT-PCR

U87MG cells were infected with VEEV at an MOI of 0.1 and supernatants collected after 24 h. Viral RNA was extracted using