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Phosphorodiamidate morpholino targeting the 5′ untranslated region of the ZIKV RNA inhibits virus replication

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ABSTRACT

Background: Zika virus (ZIKV) infection has been associated with microcephaly in infants. Currently there is no treatment or vaccine. Here we explore the use of a morpholino oligonucleotide targeted to the 5′ untranslated region (5′-UTR) of the ZIKV RNA to prevent ZIKV replication.
Methods: Morpholino DWK-1 inhibition of ZIKV replication in human glomerular podocytes was examined by qRT-PCR, reduction in ZIKV genome copy number, western blot analysis, immunofluorescence and proinflammatory cytokine gene expression.
Results: Podocytes pretreated with DWK-1 showed reduced levels of both viral mRNA and ZIKV E protein expression compared to controls. We observed suppression in proinflammatory gene expression for IFN-β (interferon β) RANTES (regulated on activation, normal T cell expressed and secreted), MIP-1α (macrophage inflammatory protein-1α), TNF-α (tumor necrosis factor-α) and IL1-α (interleukin 1-α) in ZIKV-infected podocytes pretreated with DWK-1.
Conclusions: Morpholino DWK-1 targeting the ZIKV 5′-UTR effectively inhibits ZIKV replication and suppresses ZIKV-induced proinflammatory gene expression.

1. Introduction

Zika virus (ZIKV) is a member of the Flaviviridae family, genus Flavivirus, which also includes dengue virus, West Nile virus, Japanese encephalitis virus, and yellow fever virus (Faye et al., 2014; Hayes, 2009). ZIKV is a mosquito-borne arbovirus transmitted primarily by vectors from the Aedes family, in particular *Aedes aegypti* and *Aedes albopictus* (Vorou, 2016). ZIKV has quickly spread to more than 70 countries in the Americas and the Caribbean infecting more than 2 million people (Tappe et al., 2016; Center for Disease Control and Prevention, 2015; Center for Disease Control and Prevention, 2016). Infection with ZIKV results in asymptomatic disease in 70–80% of infected individuals, however ZIKV infection has been strongly associated with increased incidence of Guillain-Barré syndrome and microcephaly in infants. (Center for Disease Control and Prevention, 2015, Calvet et al., 2016; Adibi et al., 2016). Clinical presentations of ZIKV infection include skin rash, headache, myalgia, joint pain, and conjunctivitis but are largely self-limiting.

Currently there is no specific treatment or FDA approved vaccine for ZIKV infection. This represents an urgent unmet medical need for efficacious therapeutics for ZIKV. Vivo morpholinos have been shown to be effective at suppression of flavivirus replication in vitro and in vivo. Morpholino nucleotide oligomers are uncharged molecules that bind to cognate RNA sequences that can effectively block translation, inhibit miRNA maturation, or modify pre-mRNA splicing (Blum et al., 2015; Subbotina et al., 2016). The vivo-morpholino is composed of a morpholino oligo with a unique covalently linked delivery moiety, which is comprised of an octaguanidine dendrimer. The active component, namely the arginine rich delivery peptide of the guanidinium group facilitates delivery of the modified morpholino into the cytosol. Once introduced into cells morpholinos freely diffuse between cytoplasmic and nuclear compartments to effectively bind complementary RNA sequences. Morpholinos are soluble, have low-toxicity, stable at room temperature, and are currently in clinical trials.

Mukherjee et al., used a Retinoic acid inducible gene 1 (RIG 1) morpholino to suppress RIG-1 expression in a mouse model to understand neurogenesis caused by Japanese encephalitis virus (JEV) infection (Mukherjee, 2017). The RIG 1 morpholino showed a marked reduction in RIG I protein expression in mice after intracerebral injection. Nazmi et al., explored the effects of Toll-like receptor 7 (TLR7)

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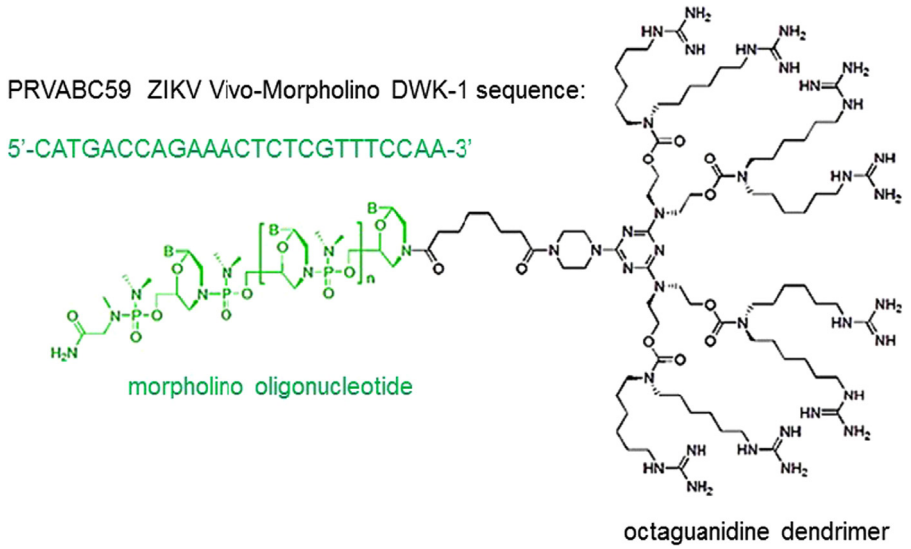

Fig. 1. Schematic structure of a vivo-morpholino. A vivo-morpholino is composed of a 25-mer long morpholino oligonucleotide (green) covalently linked to an octaguanidine dendrimer (black), which serves as a delivery moiety. A nucleotide sequence of ZIKV PRVABC59 vivo-morpholino DWK-1 is shown.

induction of antiviral responses against single stranded RNA viruses in an animal model (Nazmi et al., 2014). They developed systemic and brain specific TLR7 knock-down mice (TLR7 KD) using vivo-morpholinos. They observed differences in susceptibility and survival of wild-type and systemic TLR7 (KD) mice to Japanese encephalitis virus (JEV) but no difference in susceptibility in brain-specific TLR7 (KD) animals. Both TLR7 KD showed reductions in antiviral response and increased viral loads in the brain. In addition, Nazmi et al., also showed that vivo-morpholinos against specific regions of 3′ or 5′ untranslated regions of Japanese encephalitis virus (JEV) genome resulted in increased survival and neuroprotection in a challenged murine model of JEV (Nazmi et al., 2010). Anantpadma et al., demonstrated that peptide-conjugated phosphorodiamidate morpholino oligomers (PPMOs) directed against the JEV 3′ cyclization sequence (3′CSI) had significant antiviral activity in Vero cells and Neuro2A cells (Anantpadma et al., 2010). They also demonstrated 60–80% protection with the PPMO after lethal challenge in a mouse model with no detectable virus in brain tissue 2 days post infection. Zhang et al., demonstrated antiviral activity against West Nile virus (WNV) using PPMOs designed to interfere with the 5′CS/3′CSI or 5′UAR/3′UAR base pairings (Zhang et al., 2008). They showed that a single-nucleotide change within the 3′UAR-PPMO-target site conferred resistance to the PPMO. They have related this resistance to the blocking RNA/RNA interaction required for genome cyclization. Stein et al., has shown increased survival of AG129 mice infected with dengue 2 virus (DENV-2) after prior treatment with phosphorodiamidate morpholino oligomers (PMO) and PPMO (Stein et al., 2008). Deas et al.; evaluated vivo efficacy of two PPMOs conjugated with an arginine-rich peptide against West Nile virus (WNV) (Deas et al., 2007). They observed antiviral activity against WNV, Japanese encephalitis virus, and St. Louis encephalitis virus by in vitro assays. Analysis of viruses resistant to PPMOs revealed the accumulation of mutation within the 5′ and outside the 3′ regions of the PPMO-target binding sites respectively. The designated PPMOs provided partial protection in animals and marginal toxicity at low doses.

Here we employ morpholino DWK-1 that is designed to bind to the ZIKV 5′-UTR RNA to block translation of the ZIKV polyprotein precursor (Harris et al., 2006). Here we utilize ZIKV-targeted vivo-morpholino to inhibit ZIKV replication. We demonstrate morpholino DWK-1 effectively inhibits ZIKV replication and suppresses ZIKV induced inflammatory gene expression in vitro in human glomerular podocytes and primary human retinal endothelial cells that we have identified as being highly permissive for ZIKV infection (Alcendor, 2017 and Roach, 2017). DWK-1 has unique features of a potential antiviral in humans that could be employed for ZIKV acute disease when ZIKV prophylaxis in the form of a vaccine is unwarranted as a treatment option.

2. Materials and methods

2.1. Morpholino oligomers

The ZIKV-targeted morpholino oligomer DWK-1 was designed to be complementary to the 25-mer nucleotide sequence within the ZIKV 5′ untranslated region (5′-UTR) (bolded in brackets) that includes the first ATG translation start codon (bolded, underlined) of the Zika virus strain PRVABC59 (GenBank mRNA transcript KU501215.1, PRVABC59/Puerto-Rico/2015):

5′-GTATCAACAGGTTTTATTTTGGAT **[TTGGAAACGAGAGTTTCTG GTC<u>AUG</u>]**AAAAACC

CAAAAAAGAAATCCG-3′. The 5′-UTR of the ZIKV PRVABC59 RNA sequence targeted by DWK-1 is highly conserved among ZIKV strains. The sequence of DWK-1 complementary to the 25-mer of ZIKV 5′-UTR is as follows: 5′-CATGACCAGAAACTCTCGTTTCCAA-3′. The control oligo used in this study is a standard control oligo that targets a human beta-globin intron mutation that causes beta-thalassemia. This oligo, designated as Co DWK-1, causes little change in phenotype in any known test system except human beta-thalessemic hematopoetic cells and is appropriate negative control for custom vivo-morpholino oligos (Moulton, 2017). The sequence of Co DWK-1 is as follows: 5′-CCTCTTACCTCAG TTACAATTTATA-3′. Morpholino oligonucleotides used in all experiments (vivo-morpholinos) were conjugated to a delivery moiety consisting of an eight-branched dendrimer carrying a guanidinium moiety at each branch tip (see Fig. 1) for efficient delivery of morpholino to the cytosol and nuclear compartments of the cell. The vivo-morpholinos DWK-1 and Co DWK-1 were synthesized by Gene Tools, LLC. The rationale for using 25-mers which is the longest commercially available morpholino is that they are recommended for most applications. This is because efficacies increase substantially with increasing length and because long oligos best assure access to a single-stranded region in the target RNA, as is required for nucleation of pairing by the oligo (Summerton and Weller,1997; Heasman 2002). This length versus activity study was carried out by Gene Tools with morpholino oligos and 25 mers were found to be the optimal length for sequence specific knockdown of genes in mammalian cells.