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Short communication

Effective siRNA targeting of the 3′ untranslated region of the West Nile virus genome

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ABSTRACT

West Nile virus (WNV) is an emerging human pathogen for which specific antiviral therapy has not been developed. The therapeutic potential of RNA interference (RNAi) as a sequence-specific inhibitor of WNV has been well demonstrated. Although multiple siRNA targets have been identified within the genomic coding region, targets within the untranslated regions (UTR), which encode cis-acting regulatory elements, remain relatively unknown. In WNV and other flaviviruses, UTRs are located at the genomic termini. These regions form complex secondary structures, which pose difficulty when designing effective siRNA targets. In this study, we report the identification of siRNA targets in the WNV 3′ UTR. These targets were selected by siRNA predictor algorithms, and synthesized as short hairpin RNA sequences from a plasmid-based expression system. Vero cells stably expressing these sequences had greatly diminished ability to support WNV replication but not the related dengue virus, demonstrating that the siRNAs were effective and suppressed WNV viral replication in a sequence-specific manner. The siRNAs developed in this study could function as potential antiviral therapeutics and as genetic tools to investigate the role of 3′ UTR in WNV pathogenesis.

West Nile virus is a member of the genus flavivirus of the family flaviviridae, which include significant human pathogens such as dengue, yellow fever, Japanese encephalitis, Murray Valley encephalitis and tick-borne encephalitis viruses (Brinton, 2002). No specific therapy or vaccine has been approved for treatment or prophylaxis of WNV infections in humans, which range from mild febrile illness to life-threatening meningitis and encephalitis (Solomon and Vaughn, 2002).

RNA interference (RNAi), the cellular process that specifically degrades RNA in a sequence-specific manner (Meister et al., 2004), has been explored as an antiviral therapy for WNV infections (Bai et al., 2005; Geiss et al., 2005; McCown et al., 2003; Ong et al., 2006, 2008; Kumar et al., 2006; Yang et al., 2008). Numerous siRNA targets have been identified in the genomic coding region, which encodes three structural (C, prM, and E) and seven non-structural proteins (NS1, NS2A, NS2B, NS3, NS4A, NS4B, and NS5). siRNAs targeting the ten viral genes have been shown to effectively diminish viral replication in mammalian cell lines and in a mouse model (Bai et al., 2005; Geiss et al., 2005; Kumar et al., 2006; McCown et al., 2003; Ong et al., 2006, 2008; Yang et al., 2008). Effective siRNA targets that lie outside of the coding region, specifically in the genomic UTRs have not been previously described.

Like all flaviviral genomes, the WNV genome is a single stranded plus-strand RNA of approximately 11-kb (Lindenbach and Rice, 2003; Brinton, 2002). Both termini of the genome, flanking the coding region, consist of UTRs, which in the epidemic strain WNV NY99, extend to 96 nucleotides at the 5′ end and 632 nucleotides at the 3′ end (Markoff, 2003). Flaviviral UTRs are essential, as they have been shown to modulate gene expression and genome replication. A number of cis-elements have been mapped to these regions, though their precise roles in the viral life cycle have not been fully characterized. The 5′ UTR contains a methylated cap for translational initiation, a stem loop, and a highly conserved cyclization sequence that base pairs with complementary sequences at the 3′ UTR to form a replication intermediate (Markoff, 2003). The distal 96 nucleotides of the 3′ UTR form two adjacent stem loop structures, which bind viral and cellular replication proteins and appear to function as a promoter for minus strand synthesis (Davis et al., 2007). Extensive RNA–RNA interactions have been predicted to form within the genome, including genome cyclization through long-range interaction between the 5′ and 3′ UTRs and pseudoknot formation between the two 3′ UTR stem loop structures (Stein and Shi, 2008; Bredenbeek et al., 2003; Khromykh et al., 2001; Zhang et al., 2008). The presence of these complex higher ordered structures become problematic when designing effective siRNA targets

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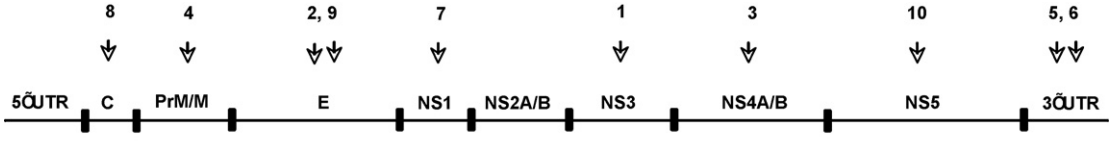

1. 5'-GATCCG **TTATGGCAATGGAGTCATA** TTCAAGAGA **TATGACTCCATTGCCATAAGC** TTTTTTGGAAA-3′(5039)
2. 5'-GATCC **AGAGGTCCGCAGTTATTGC** TTCAAGAGA **GCAATAACTGCGGACCTCTGC** TTTTTTGGAAA-3′(1110)
3. 5'-GATCCG **TGACCTCAATAAACGTTCA** TTCAAGAGA **TGAACGTTTATTGAGGTCAAT** TTTTTTGGAAA-3′(7102)
4. 5'-GATCC **AAAGCACTCTAGCGAACAA** TTCAAGAGA **TTGTTCGCTAGAGTGCTTTCT** TTTTTTGGAAA-3′(749)
5. 5'-GATCCG **TTAGTGGTGTTAGTGTAAA** TTCAAGAGA **TTTACACTAACACCACTAAAT** TTTTTTGGAAA-3′(10444)
6. 5'-GATCCG **CCGCGAAGTGATCCATGTA** TTCAAGAGA **TACATGGATCACTTCGCGGCT** TTTTTTGGAAA-3′(10572)
7. 5'-GATCC **GAACGGCTGTCAAGAACAA** TTCAAGAGA **TTGTTCTTGACAGCCGTTCCA** TTTTTTGGAAA-3′(3005)
8. 5'-GATCC **ACAAACAAACAGCGATGAA** TTCAAGAGA **TTCATCGCTGTTTGTTTGTTC** TTTTTTGGAAA-3′(312)
9. 5'-GATCC **GCTGCGTGACTATCATGTC** TTCAAGAGA **GACATGATAGTCACGCAGCTG** TTTTTTGGAAA-3′(1053)
10. 5'-GATCC **AGAGCCATTTGGTTCATGT** TTCAAGAGA **ACATGAACCAAATGGCTCTGC** TTTTTTGGAAA-3′(9099)

Fig. 1. siRNA targets in WNV genome. The horizontal line represents the WNV genome with 5′ and 3′ UTRs flanking the structural (C, PrM and E) and non-structural (NS1, NS2A/B, NS3, NS4A/B, and NS5) genes. Arrows indicate the location of the individual siRNA targets. Oligonucleotide sequences encoding the siRNA templates (boldface) and their precise genomic location (in parenthesis) are shown below the diagram. Each siRNA template oligonucleotide contained BamHI and HindIII overhangs, 19-mer hairpin sequences specific to the target, a loop sequence separating the two complementary domains, and a polythymidine tract to terminate transcription.

in these regions. So far, only one siRNA target has been identified in the flaviviral UTR. This siRNA, which recognizes a sequence in the dengue virus 3′ UTR, inhibits viral replication only marginally as compared to siRNAs targeting the coding region (Zhang et al., 2004). Effective siRNA targets in the homologous WNV genome UTRs have not been previously reported.

In the present work, we report the identification of two siRNA targets within the WNV 3′ UTR. Using siRNA predictor algorithms, we designed ten distinct siRNAs targeting various regions of the genome, eight in the coding sequence and two in the 3′ UTR. We synthesized DNA encoding short hairpin RNA sequences, cloned them into a plasmid-based expression system, and transfected the DNA into Vero cells to derive stable siRNA expressor cell lines. We compared the effectiveness of the 3′ UTR siRNAs at suppressing WNV replication and progeny virus production to the efficacy of siRNAs targeting the coding region. Of the two, the siRNA targeting the proximal region of the 3′ UTR inhibited WNV viral replication and production as effectively as the most robust siRNA targeting the coding region. Collectively, these data demonstrate the presence of effective siRNA targets in the 3′ UTR of WNV genome.

The siRNA target sequences in the genome of WNV isolate 2741 (GenBank AF206518) were selected using Dhamacon's siDESIGN Center (Dharmacon, Lafayette, CO). Each genomic region corresponding to the 5′ UTR, 3′ UTR, and C, M, E, NS1, NS2, NS3, NS4, and NS5 genes was analyzed individually. The algorithm selects siRNA sequences by searching for AA dinucleotides in the mRNA where the GC content is between 30 and 50%. Approximately 14 sequences were selected and compared to the GenBank database to exclude sequences with homology to cellular genes. Ten siRNAs with a high likelihood of success were selected. Eight of these were in the coding region (one in each of C, M, NS1, NS3, NS4, NS5 and two in E) and two in the 3′ UTR; none was identified in the 5′ UTR. The nucleotide sequences and their respective locations in the WNV genome are shown in Fig. 1. Oligonucleotide pairs corresponding to short hairpins (Fig. 1) were synthesized (Department of Pathology, Yale University). After annealing, these oligonucleotide pairs generate double-stranded DNA fragments containing a 5′ overhang, 19-nucleotides identical to the targeted transcript, a 9-nucleotide hairpin loop, 19-nucleotides complementary to the target transcript sequence, and a RNA polymerase III terminator. The annealed DNA oligonucleotides were cloned into the BamHI/HindIII digested pSilencer 2.1-U6 hygro vector (Ambion, Austin, TX). This plasmid-based system generates unimolecular hairpin siRNAs transcribed from the U6 human RNA polymerase III promoter. Following DNA sequence confirmation, plasmids bearing the individual siRNAs were transfected into Vero cells (ATCC CCL-81). Populations of transfected cells stably expressing the siRNAs were selected in the presence of hygromycin (300 μg/ml). Genomic integration of the plasmid was confirmed by PCR amplification of the vector sequences from the genomic DNA (data not shown). Ten stable Vero cell populations, each expressing different individual siRNAs, were created.

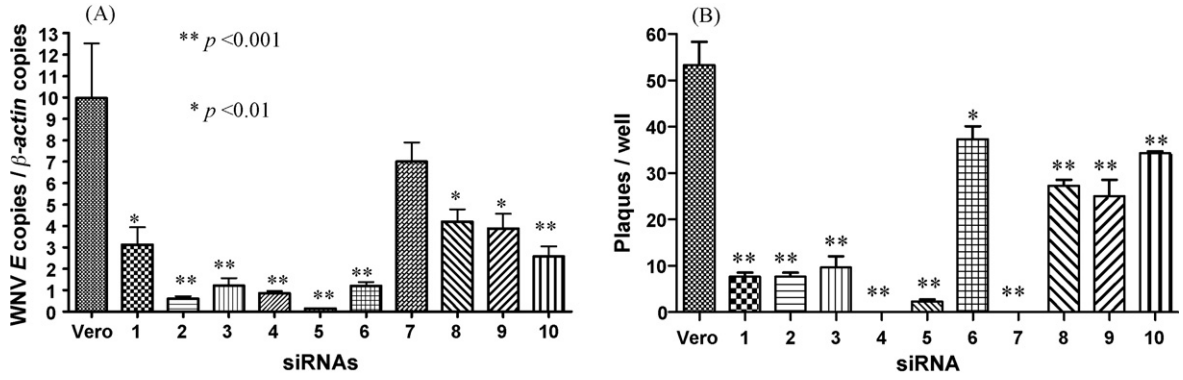

Fig. 2. siRNA-mediated inhibition of WNV infection in Vero cells. Inhibition of viral replication and progeny virus formation were determined 24 h after infection with WNV. (A) Total RNA was isolated and the amounts of viral E and cellular β-actin gene transcripts were determined using sequence-specific primers and quantitative RT-PCR as previously reported (Bai et al., 2005). The amount of viral RNA was normalized to the amount of cellular β-actin transcripts. (B) The number of progeny virus in the Vero cell culture medium was determined by plaque assays as previously reported. Tukey's test with one-way analysis of variance was used for statistical analysis. The numbers were compared to Vero cells without siRNA. A p value of less than 0.05 was considered significant. The data presented represents an average of three independent infections.

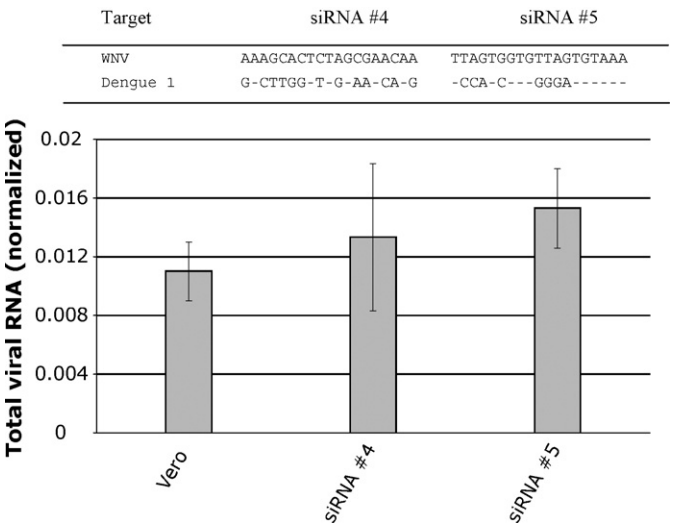

Fig. 3. The effect of siRNA #4 and siRNA #5 on dengue virus RNA expression. Sequence alignment of the WNV siRNA target with the corresponding dengue virus serotype 1 sequences; dashes indicate identical residues. Vero cells were infected with dengue virus serotype 1 at MOI of 1. RNA was isolated 24 h post-infection, and quantified as described for Fig. 2. Samples were run in triplicates and the data reflect the average of three different infections. Untransfected Vero cells were used as the baseline control.

The ability of the siRNAs to block WNV infection was evaluated. Stable siRNA expressor cells were infected with WNV strain 2741 at a multiplicity of infection (MOI) of 0.1. Twenty-four hours following infection, RNA was isolated from the infected cells for viral genome quantification by quantitative RT-PCR, and the culture medium was collected to quantify progeny virus production by plaque assay (Bai et al., 2005). The results of these analyses are summarized in Fig. 2. As compared to an untransfected Vero cell control, all ten siRNA-expressing cell populations exhibited diminished viral load. The eight siRNAs targeting the coding region had 10 to >90% reduced viral loads, with the siRNA in the M region (#4) being the most potent (>90%). Of the two siRNAs targeting the 3′ UTR, siRNA #5 decreased the viral load by >90%, comparable to the level exerted by the most potent siRNA that targets the coding region (#4). In contrast, 3′ UTR siRNA #6, which maps to a region distal to siRNA #5, was less effective.

To confirm that the effects of the siRNAs were sequences-specific, we assessed the abilities of the potent siRNAs to inhibit replication of the related dengue virus, which shares between 40 and 53% overall sequence homology (Pugachev et al., 2003). As shown in Fig. 3, neither of the siRNAs significantly reduces dengue virus replication, indicating that their effect is sequence-specific.

In conclusion, we have identified two effective siRNA targets in the 3′ UTR of the WNV genome. Both targets are located at the 5′ proximal region of the 3′ UTR and away from the terminal stem loops. This study illustrates that the 3′ UTR is amenable to targeting for RNAi despite its highly structured nature. The newly discovered siRNAs may prove useful in developing RNAi-based antiviral therapies for WNV infection, and as genetic tools to probe into the mechanistic function of the 3′ UTR in the viral life cycle.

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