[Frontiers in Bioscience 13, 1385-1395, January 1, 2008]

Nucleic acid-based inhibition of flavivirus infections

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1. ABSTRACT

The genus *Flavivirus* in the family *Flaviviridae* contains many arthropod-transmitted human pathogens, including dengue, yellow fever, Japanese encephalitis, West Nile, St. Louis encephalitis, Murray Valley encephalitis, and tick-borne encephalitis viruses. Treatment options for flaviviral diseases are extremely limited, with no effective drugs yet commercially available. Recent advances in virology, synthetic organic chemistry, and the discovery of RNA interference (RNAi), have provided the basis for advances in the development of antisense-based approaches to address flaviviral infections. Oligomers of various antisense structural types, targeted to different locations in the flaviviral RNA genome, have now been used to successfully suppress viral gene expression and thereby inhibit flavivirus replication. Double-stranded RNA, containing viral sequence and designed to induce the endogenous cellular machinery of RNAi, has also been shown capable of potently interfering with flavivirus production and transmission. These studies provide insights into flaviviral molecular biology and the basis for the development of novel therapeutic approaches. The goal of this review is to summarize the findings of many of the significant reports that have appeared on the topic of antisense-mediated strategies for the development of antiviral therapy for flaviviruses.

2. INTRODUCTION

2.1. Flaviviral diseases

The genus *Flavivirus* (of the family *Flaviviridae*) is composed of 53 viruses, several of which cause significant human diseases (1). Dengue virus (DENV), Yellow fever virus (YFV), and Japanese encephalitis virus (JEV) are transmitted to humans by mosquitoes and cause Dengue fever (DF), Yellow fever (YF) and Japanese encephalitis (JE), respectively. These three flaviviral diseases have wide distributions in tropical and subtropical regions and are considered public health threats of great concern. In addition, West Nile virus (WNV), previously found mostly in Africa and the Middle East, has caused significant outbreaks of West Nile (WN) disease in North America and elsewhere in the past ~10 years (1-4). Other notable human-disease-causing flaviviruses with more localized distributions include the mosquitoborne St. Louis encephalitis virus (SLEV) (in North America) and Murray valley encephalitis virus (in Australia). The complex of tickborne encephalitis viruses (TBEV) cause several thousand cases of tickborne encephalitis yearly, mostly in Northern Eurasia (1, 5, 6). Among the US National Institute of Allergy and Infectious Disease Priority Pathogens, DENV is listed in Category A; WNV and JEV in Category B; and YFV and TBEV in Category C.

Despite the existence of effective vaccines against YFV and JEV, the incidence of severe disease and mortality caused by these pathogens is considerable. There are as yet no approved human vaccines against DENV or WNV. No highly effective therapeutic is currently available for any flaviviral disease; and unfortunately, as yet, no potential therapeutic has shown consistent clinical efficacy (1, 2, 7). Virtually any symptom-causing flavivirus infection is a miserable experience for the victim, and although a majority of flavivirus infections do not cause permanent damage, they frequently warrant medical attention and result in extensive economic and social costs (8-10). With flaviviral diseases causing large and increasing disease burdens, an increased effort is being exerted to develop new vaccines and therapeutics to prevent or treat them. Since most flaviviruses are tropical or subtropical in distribution, they affect nations with less developed public health and disease-research infrastructures than many of those in the temperate regions. Likewise, drug development efforts against flaviviruses have not been as vigorously pursued and well-endowed as have efforts against other viral diseases for which the wealthier nations represent ready markets for approved drugs. However, the presence of WN disease in North America, the greatly increased incidence of DF in the Americas over recent years (11), the increased funding opportunities for tropical infectious disease drug development (12), a greater overall public and private sector awareness of the importance of global public health, and an awakening of drug makers to potential benefits, have all helped to bring about an increased utilization of modern drug development resources for pursuit of therapeutics against flaviviruses. Recent advances in molecular virology, immunology, structural biology, synthetic organic chemistry and pharmaceutical science, along with the availability of genetic data, better animal models, and the discovery of RNA interference (RNAi), have helped foster the development of several novel and promising therapeutics against flaviviruses (1, 2, 7, 13-17), including improved strategies for antisense-mediated approaches to intervention in flaviviral infections.

2.2. Overview of flavivirus structure and life-cycle

Flavivirus virions are spherical, with an inner nucleocapsid surrounded by a lipoprotein envelope sporting small glycoprotein projections (18). The nucleocapsid is composed of capsid protein (C) and genomic RNA. The single-stranded, plus-sense RNA genome is approximately 11 kilobases and consists of a 5'-untranslated region (UTR) of ~100 nucleotides, a single long open reading frame (ORF), and a non-polyadenylated 3' UTR of ~400-700 nucleotides (Figure 1A). The ORF encodes a single polyprotein that is co- and post-translationally cleaved by viral and host proteases to yield three structural (C, prM and E) and seven nonstructural (NS) proteins (NS1-NS2A-NS2B-NS3-NS4A-NS4B-NS5) (18, 19). The structural proteins are essential components of the virion. E, prM (and its mature form M) are present in the envelope and are important in virus entry, maturation and egress, as well as the immunologic character of the viral particles (20-22). The nonstructural proteins function in viral RNA replication, virion assembly, and evasion of host immune responses (18, 23-25). The 5' end of flavivirus genomes contains a type 1 (m7G) cap, and it is believed that the pre-initiation of translation is usually mediated through cap-dependent events (26). Flaviviral UTRs contain regions of conserved sequence (CS) and conserved stem-loop structures, some of which function as *cis*-acting elements critical to flaviviral replication (18, 27). Various host and viral proteins implicated in the regulation of viral processes have been found to interact specifically with the UTRs of the flavivirus genome (18, 26). The NS5 protein of WNV, which conducts methyltransferase and polymerase activities, binds to the 5'-terminal nucleotides of the viral genome (28). Host protein EF1α, which is required for the minus-strand RNA synthesis, binds specifically to the 3'-terminal stem-loop (29). Additionally, at least three RNA:RNA interactions are predicted to form within the genome: (i) the mosquitoborne-flavivirus-conserved 5'CS (located in the 5' portion of the C-coding region) forms a 12-nucleotide base pairing with the 3'CSI element within the 3' UTR (30, 31); (ii) a 5'UAR (upstream of the AUG region) element likely interacts with a 3'UAR sequence (located in the 3'-terminal stem loop) (32); and (iii) a pseudoknot could be formed within the 3' terminal two stem-loop structures (33). Among these, the 5'CS/3'CSI- and 5'UAR/3'UAR-mediated base pairings cyclize the flavivirus genome and are essential for efficient viral replication (Figure 1B) (18, 31, 32).

Flaviviruses can infect many different cell types in humans, notably those of the immune- and nervous-systems, liver, kidney, and spleen (18). Upon interaction with receptors and other molecules on the surface of a cell, flavivirus are endocytosed and trafficked to endososomal vesicles. The viral nucleocapsid becomes uncoated and viral genomic RNA is released into the cytosol, where it goes on to associate with ribosomes and undergo initial rounds of translation. After sufficient viral proteins are produced, RNA replicase complexes are assembled and the positive-strand viral genome begins to serve as a template for the production of negative-strand antigenome, which is then used in turn to synthesize more positive strand viral genomes. All RNA synthesis is carried out in close association with cytoplasmic membrane structures (34, 35). As in other positive-strand RNA viruses, RNA synthesis is asymmetric, with many times more positive than negative strands being produced. Furthermore, it appears that negative strand RNA is rarely present as an independent species, but rather is typically duplexed, to some extent, with positive strand (36). Positive strand progeny can be either translated or packaged into virions. After ample levels of both viral genomic RNA and viral proteins are produced, virions are assembled and transited through the Golgi system to be secreted from the cell (18). The translation process appears to be the stage of the viral life cycle in which viral RNA is most likely to be single stranded and not highly-complexed with membrane or protein structures, and therefore most accessible to antisense agents.

2.3. Antisense structural types and strategies

Antiviral antisense agents are typically designed to bind to viral mRNA. Flaviviruses utilize their genomic

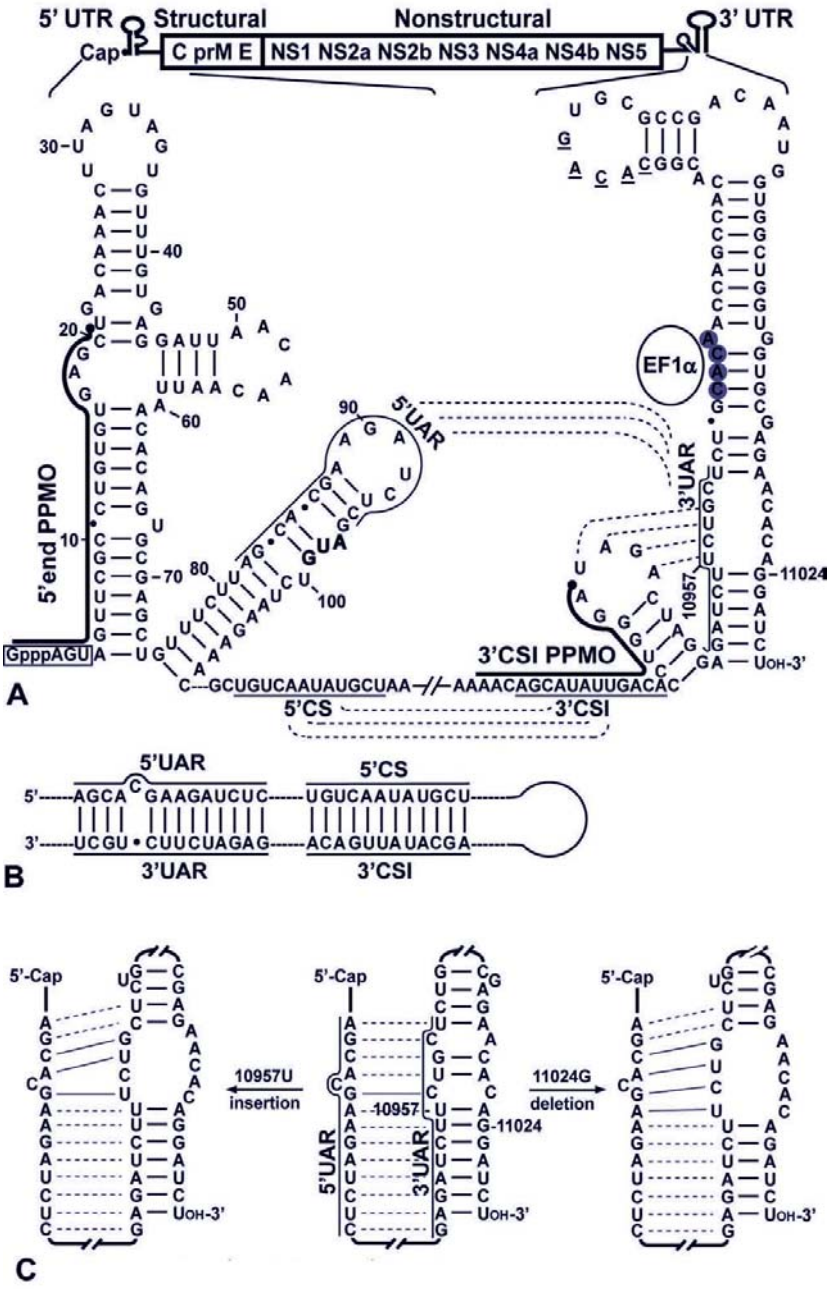

Figure 1. Sequence and structure of the 5'- and 3'-termini of the genomic RNA of WNV, a representative Flavivirus. (A) Terminal stem-loop structures and potential RNA-RNA interactions within the WNV genome. The AUG initiation codon of the open-reading frame (nucleotides 97-99) is in bold. The flavivirus-conserved pentanucleotide (5'-CACAG-3' located at the top of the 3'-stem-loop) is underlined. The major binding site of host protein EF1α (5'-CACA-3') is shaded. Three RNA interactions are labeled by dashed lines: the 5'UAR/3'UAR, 5'CS/3'CSI, and a 3' pseudoknot base pairing. The sequences involved in the 5'UAR/3'UAR and 5'CS/3'CSI interactions are indicated by thin lines. Two PPMO, 5'END and 3'CSI (see text on WNV and DENV for details), are indicated as thick lines with a filled circle [representing a cell-penetrating peptide conjugated to the PMO 5' end]. The 5'-terminal nucleotides of the WNV genome that bind to the NS5 protein are boxed. Nucleotide positions are numbered based on the full-length sequence of the WNV genome (GenBank accession number AF404756). (B) Potential genome cyclization of WNV through the 5'UAR/3'UAR and 5'CS/3'CSI base pairings. (C) Speculative conformational switch between the 5'UAR/3'UAR and the 3'-stem-loop interaction in 3'CSI PPMO-resistant viruses (see text on WNV for details). Adapted from figure 1 of (70).

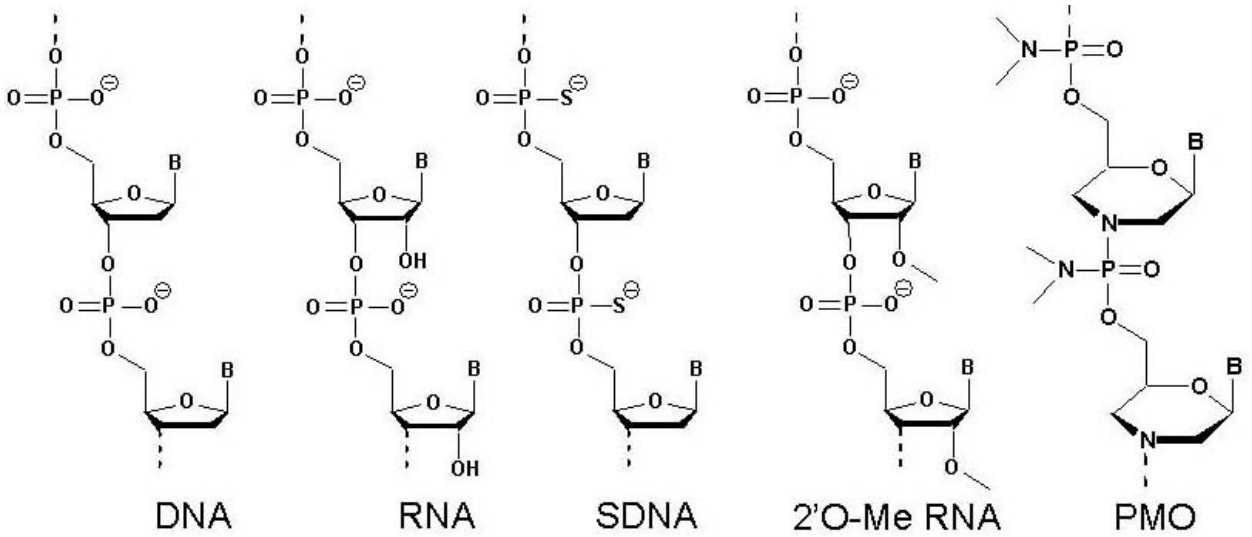

Figure 2. Representative subunits of the various antisense structural types discussed in this review. Various modifications of the chemical backbone of DNA or RNA have been introduced in an effort to improve stability, cellular uptake, and pharmacologic properties. Antisense-mediated strategies all depend on Watson-Crick base-pairing to provide specificity. In the above diagram, B represents a DNA or RNA nucleotide base: A, C, G, T, or U.

RNA as mRNA, and most successful antisense-mediated attempts at inhibiting flaviviruses have targeted the plus-strand genome. The studies summarized in this review employed antisense agents composed predominantly of single-stranded DNA, phosphorothioate DNA (PSO or SDNA), 2'-O-methyl RNA (2'OM) or phosphorodiamidate morpholino oligomers (PMO), or utilized RNAi, which requires double-stranded RNA (dsRNA) as starting material. Figure 2 shows a representative subunit of each structural type of antisense chemistry mentioned above. Antisense agents based on DNA chemistry (which includes PSO) function through the action of the ubiquitous cellular endonuclease RNase H, which cleaves the RNA strand of an RNA-DNA duplex, leaving the DNA strand undamaged and able to rehybridize with other RNA targets, in a catalytic manner (37). The sole antisense drug to gain FDA approval to date is a PSO designed against cytomegalovirus (CMV) *IE-2* mRNA, for use in treating CMV-induced retinitis (38). DNAzymes are designed to bind and cleave their target RNA (37), without the aid of cellular proteins. PMO are single-stranded DNA mimics, with each subunit consisting of one DNA base (A, C, G or T) joined to a morpholine ring and phosphorodiamidate intersubunit linkage (39). The mechanism of antisense action of PMO and 2'OM is through steric blocking of complementary RNA (40, 41). By forming a stable duplex with target RNA, steric-blocking antisense agents presumably obstruct access of other biomolecules to the targeted RNA, or disrupt higher order RNA structure necessary for normal function. In order to actuate delivery into cells, PMO have been covalently conjugated to any of a number of Arginine-rich cell-penetrating peptides (CPP), thus producing peptide-PMO (PPMO) (42, 43). PPMO are water-soluble, and relatively stable in biological systems (44).

RNAi refers to a process in which damage to target mRNA is ultimately brought about by a RNA-induced silencing complex (RISC), a molecular consortium made up of RNA and a complex of proteins including an RNase (45). The effector RNA component of RISC is a 20-25 nt strand of single-stranded RNA (ssRNA) (the guide strand) complementary in sequence to the target mRNA, and derived from a double-stranded RNA (dsRNA) precursor of 20-25 basepairs in length often referred to as small interfering RNA (siRNA). Endogenous RNAi activity has been found to occur in cells of many organisms, and functions in the regulation of development and maintenance of genome integrity. It is thought to have evolved, at least in part, as a form of innate immunity against viruses, which often feature dsRNA at some point in their life cycle, unlike most eukaryotic cells (46). Synthetic dsRNA, capable of eliciting damage to specific mRNA through the RNAi process, can be introduced into mammalian cells by traditional plasmid transfection techniques. Alternatively, dsRNA can be produced intracellularly by plasmid or viral expression systems, thereby invoking cellular RNAi machinery (47). As described below, RNAi strategies have been used to inhibit flaviviral replication and to address transmission of flaviviruses by preventing mosquito infections.

3. SUMMARIES OF EXPERIMENTAL RESULTS

3.1. Dengue virus

Of all the flaviviruses, DENV currently causes the most human health problems. An estimated 50-100 million cases of DF occur yearly, mostly in the tropical regions of southern Asia and Latin America (48, 49). Infection with DENV produces a spectrum of possible clinical outcomes, ranging from a mild flu-like syndrome to severe illness. Typical symptoms include high fever, headache and bone pain (50). The fatality rate is relatively low, at about 1% of those showing symptoms, but this amounts to greater than 15,000 deaths per year. More than

500,000 people per year, mostly children, suffer from severe DF, the recommended treatment for which is supportive care in a hospital (51). There has been an increase in both the incidence and severity of DF over the past 30 years, due at least in part to increased human population, urbanization and modern transportation, and decreased mosquito control, allowing vector proliferation (1, 3, 48, 51).

In one of the first reports on the application of antisense to inhibit the replication of a flavivirus, Raviprakash *et al.* (52) used PSO in which the C-5 atoms of uridines and cytidines were replaced with propynyl groups. The authors chose to target purine-rich regions of DENV genomic sequence so that a large proportion of the antisense molecule residues could be propyne substituted. They compared five antisense sequences, by microinjecting them into cultured cells, infecting with DEN-2, and assaying for virus level by immunofluoresence or *in situ* hybridization. Two of the five C-5 propyne-substituted PSO (PrPS), designed against the AUG translation start-site region or sequence in the 3'-terminal stem loop, generated 50-75% reduction in virus level at 24 h post-infection (pi). PrPS compounds targeted against sequence in NS5, or the 3'CS region, were found to be ineffective, as were DNA oligos and unmodified-PSO with identical base sequences. It is uncertain whether the effective PrPS oligomers worked by an RNase-H and/or steric blocking mechanism of action.

Two studies have shown PPMO capable of profoundly inhibiting DENV amplification in cell culture experiments. Kinney *et al.* (53) used plaque assays to demonstrate multi-log reductions of representative strains from the four serotypes of DENV, lasting several days, by pre- and post-infection treatment of cells with an 18mer PPMO (named 3'CS) designed against sequence in the 3'CS1 region that is perfectly conserved in all 4 DENV serotypes. Similar results against DENV-1, -2, and -3 were obtained using a 20mer PPMO (named 5'SL) targeted to the genomic 5'-terminal sequence that is perfectly conserved between these three DENV serotypes. Substantially lower efficacy was seen with the 5'SL PPMO against DENV-4, which is likely because of the two nucleotide difference in sequence between DENV-4 and the other 3 DENV serotypes in the PPMO-target region. This study also tested the effect of delaying the onset of PPMO treatment of cells in culture until after infection, and showed that treatment beginning 3 or 6 h pi was still highly efficacious, although not as effective as a pre-infection treatment. Delaying PPMO treatment until two or more days pi generated little or no effect on viral titers through the 10-day duration of the experiments. Holden *et al.* (54) later showed that the two highly effective PPMO used in the Kinney *et al.* study above work by different mechanisms of action. A DENV reporter replicon that generates two distinct peaks of luciferase activity in cultured cells, at 4-8 h and 2-3 days post-transfection, was constructed. The authors show that the first peak of activity results from translation of transfected replicon RNA, whereas the presence of a second peak requires that the input replicon RNA serve as a template for RNA synthesis; thus providing a tool to distinguish between the processes of viral translation and RNA synthesis. The 3'CS PPMO had little effect on the amplitude of the first peak of activity, but greatly diminished the second peak, indicating that it acts by interfering with RNA synthesis. The 5'SL PPMO reduced the level of the first peak almost completely, indicating it works by interfering with translation. As translation of the input replicon RNA is required to achieve the RNA synthesis necessary to generate the second peak, it is not possible to know if 5'SL affected RNA synthesis, as the oligomer so prevented input replicon translation. Another PPMO (3'SLT), designed to target the top of the 3' terminal stem loop, was also observed to be highly active against DENV-1, -2, and -3, as assessed by viral plaque assays. Using the two-peak DENV replicon described above, along with real time RT-PCR and Western immunoblotting, the 3'SLT PPMO was shown to have considerable impact on both translation and synthesis of viral RNA.

A number of approaches have been used to invoke RNAi cellular machinery for the purpose of reducing DENV infection and transmission. Gaines *et al.* (55) used double subgenomic Sindbis (dsSIN) virus transducing vectors, for transient expression of full-length prM coding sequence in either sense or antisense orientation in mosquito cell culture. The dsSIN viruses are produced by transcribing infectious SIN genome RNA *in vitro* from cDNA clones, and inserting a second subgenomic promoter capable of driving expression of a cloned insert. Cells expressing prM RNA sequence in either orientation were completely protected from subsequent DENV-2 challenge, when measured at 5 days pi. Olson *et al.* (56) administered DENV-2 and a dsSIN vector with an insert of ~500 nt of DENV prM-coding sequence as an intrathoracic co-injection and observed inhibition of DENV-2 replication in mosquito salivary glands at 14 days pi. Adelman *et al.* (57) then showed that dsSIN vectors designed to express a variety of inserts derived from either sense or antisense RNA of all four DENV serotypes induced resistance to homologous DENV challenge in mosquitoes, even if DENV inoculation did not occur until 2-8 days after dsSIN vector infection. A similar approach by the same authors was used against yellow-fever virus in mosquito cells and mosquitoes (see below). Adelman *et al.* (58) later used plasmid vectors that expressed inverted-repeat DENV RNA transcripts to achieve stable transformation of cultured mosquito cells and thereby establish a DENV-2 resistant cell line. ssRNAs of 21-25 nt in length, which specifically hybridized to both sense and antisense probes corresponding to the DENV-2 genomic sequence used for transformation, were detected in these DENV-2-resistant cells, providing evidence of dsRNA-induced and RNAi-mediated silencing as the mechanism of resistance. Remarkably, this same group recently developed a transgenic line of the mosquito *Aedes aegypti*, the principal vector of DENV, that is highly refractory to DENV-2 infection (59, 60). By using a nonautonomous *mariner* transposable element construct, containing inverted-repeat DENV prM coding-sequence driven by a promoter that becomes activated in the mosquito midgut upon ingestion of a bloodmeal, the 'Carb77' transgenic line was created. The authors present evidence that these insects' resistance to infection and

diminished ability to transmit DENV-2 are a result of RNAi activity. The production of mosquitoes with heritable impaired vector competence raises the prospect of developing population replacement strategies to control transmission of DENV. Caplen *et al.* (61) similarly demonstrated inhibition of DENV-1 replication by RNAi in mosquito cells transiently transfected with dsRNAs containing sequence corresponding to DENV E, NS1, or NS5 coding sequence. The level of inhibition peaked at about 1 $\log_{10}$ reduction on day 4 or 5 pi. Zhang *et al.* (62) demonstrated that adeno-associated virus (AAV)-vector mediated delivery of siRNA, when applied to cultured cells two days before virus infection, can generate a reduction in the subsequent DENV infection level measured at five days pi. Transfected AAV capable of expressing an siRNA cassette containing highly conserved sequence from the DENV 3'UTR, suppressed replication of DENV-2 in Vero or human dendritic cells. This demonstration may have particular significance, in that dendritic cells are crucial in the early stages of DENV infection, and AAV vectors have been used in human clinical trials.

3.2. Yellow fever virus

Despite the existence of a safe and highly effective vaccine, there are still an estimated 200,000 symptomatic cases and about 30,000 deaths annually from YFV, primarily in tropical Africa (63). YFV remains a major public health problem in parts of South America as well (64). Irregular epidemics produce a considerably higher incidence of disease. Like DENV, and for many of the same reasons, the incidence of YFV has increased over the past 30 or so years (65). YFV infection often causes severe disease, with hemorrhage in multiple tissues and damage to the liver, kidney and heart (66).

Tolou *et al.* (67) evaluated several PSO in Vero cell culture experiments. A PSO targeted to sequence in the 3' UTR produced about 50% reductions in YFV titer. However, results with other PSO, including control compounds, were variable between experiments. Furthermore, results varied depending on the source and history of the cells used. These inconsistent results led the authors to question the specificity and utility of PSO as a structural type of antisense.

dsSIN virus transducing vectors have also been used to generate an RNAi-based inhibition of YFV in mosquitoes, through transient production of dsRNA with sequence derived from YFV prM and NS5 coding regions (68). *Aedes aegypti* cells transduced with these vectors and then infected with YFV did not support an active infection. Likewise, transduction of adult *Aedes aegypti* with YFV sequence-containing dsSIN vectors resulted in a much-diminished capacity to transmit the two geographically distinct strains of YFV tested.

3.3. West Nile virus

WNV has a wide distribution throughout Africa, Europe, Asia, Australia, and since 1999 North America as well. Most human WNV infections are asymptomatic, but about 20% result in a self-resolving flu-like illness characterized by fever, headache and bodyache. Less than 1% of symptomatic patients develop severe neuroinvasive disease characterized by meningitis, encephalitis and/or flaccid limb paralysis (2).

Deas *et al.* (69) evaluated a panel of PPMO designed to target various sites in WNV genomic RNA, mostly in the 5' and 3' UTRs, with a stable cell line expressing a WNV reporter replicon, a full length WNV-luciferase construct, and wild-type virus. They documented that PPMO named 5'END and 3'CSI (see Figure 2A), designed to target the 5' terminal- or the 3'cyclization sequence-region, respectively, were the most potent. These two PPMO target the same respective regions of the viral genome as the 5'SL and 3'CS DENV PPMO described above. The WNV 5'END and 3'CSI PPMO produced specific multi-log reductions in titer at 42 h pi when 5 micromolar of either was present in the cell culture medium around the time of infection with an epidemic strain of WNV. To carry out a mechanism-of-action analysis, a replicon capable of differentiating PPMO-mediated inhibition of translation from inhibition of RNA synthesis was used. The results were similar to that of Holden *et al.*, described above. A recent report from this lab has shown that specific 5' terminal nucleotides are required for WNV 7mG capping reactions to proceed efficiently (see Figure 2A), and that the 5'END PPMO is capable of interfering specifically in those reactions (28). In a second antisense paper from this group (70), the sequence of the 3'CSI PPMO was redesigned to make it fully complementary to its target in all flaviviruses of the JEV serocomplex. The revised 3'CSI PPMO showed high efficacy in cell culture experiments against JEV-serocomplex members WNV, JEV and SLEV, but not against a DENV or YFV, which belong to different serocomplexes, and are divergent in sequence from JEV serocomplex members at this location. This report also mentions that PPMO with sequence customized to target either the 5' terminal-region of YFV or SLEV were highly effective, in concordance with similar demonstrations mentioned above for WNV and DENV. The authors note, however, that the 5' terminal-region of the flavivirus genome contains considerable sequence variation between species.

In an effort by these authors to explore the issue of viral resistance to PPMO, resistant WNVs were selected in the presence of increasing concentrations of 5'END or 3'CSI PPMO (70). Sequencing of the PPMO-resistant WNVs showed that the 5'END PPMO-resistant viruses contained 2-3 mismatches within the PPMO-binding site, whereas the 3'CSI PPMO-resistant viruses accumulated mutations outside the PPMO-targeted region. Mutagenesis of a WNV infectious clone was used to demonstrate that the mutations within the PPMO-binding site were responsible for the 5'END PPMO-resistance, whereas a 10957U-insertion or a 11024G-deletion, located within the 3' terminal stem-loop of the viral genome (Figure 1C) and outside of the 3'CSI PPMO target site, were determinants for 3'CSI PPMO-resistance. Mfold-based RNA secondary structure prediction indicates that the 10957U-insertion or 11024G-deletion results in an internal loop within the 3'-terminal stem-loop structure. Since one strand of the internal loop is located within the 3'UAR sequence, the

formation of the internal loop appears to favor the 5'UAR/3'UAR interaction (Figure 1C). The resistant viruses may therefore compensate for the 3'CSI PPMO-mediated blockage of the 5'CS/3'CSI interaction by enhancing genome cyclization through the 5'UAR/3'UAR-mediated interaction, although this explanation remains to be supported experimentally. Importantly, this paper also reported the evaluation of PPMO in a mouse model of WNV disease. First, an evaluation of PMO and PPMO toxicity was carried out with uninfected mice. Daily intraperitoneal doses of 10 mg/kg PPMO or 150 mg/kg PMO for 9 days resulted in no weight loss, whereas 5 doses of 15 mg/kg PPMO resulted in greater than 5% total body weight loss and was therefore considered toxic. Next, *in vivo* evaluation of antiviral efficacy was performed. Non-toxic regimens of 5'END and 3'CS PPMO, starting on the same day as subcutaneous WNV-infection, produced mouse survival rates (at day 28 pi) of 60-70% compared to less than 20% for the mock-treated group. In another experiment where the 5'END PPMO was administered starting at day 6 pi, a survival rate of about 50% was observed. PPMO treatments that produced enhanced survivorship also reduced the severity of illness, as measured by scoring of clinical symptoms. The authors suggest that modification of the CPP component of PPMO, to reduce its toxicity while maintaining its PMO transporting ability, could allow more aggressive dosing and improve *in vivo* outcomes.

Torrence *et al.* (71) evaluated PSO, 2'OM and PSO/2'OM hybrid molecules designed to target conserved regions of the WNV genome 3'UTR, in cell culture experiments. The authors purposely targeted C-quartet containing viral sequences in an attempt to benefit from enhanced cellular uptake of G-quartet-containing PSO. These authors used a cytopathic effect-inhibition assay to derive a selectivity index score (defined as the 50% cytotoxic concentration divided by the 50% effective concentration) for each of the of 15-17 residue oligomers. Three WNV-specific PSO were found to have an SI of over 7 and were further investigated. The authors found that 2'OM oligomers with base sequences identical to the PSO did not produce significant anti-WNV activity. However, if the same sequences were synthesized such that a central core of 5 PSO residues was flanked on both ends by 2'OM residues, one of the resulting compounds recovered significant antiviral activity. The authors conclude that RNase H activity, made possible by the 5 or more contiguous PSO residues, was necessary for antiviral activity.

McCown *et al.* (72) used plasmid vectors in murine cell culture to transiently express hairpin siRNA designed against WNV C- or NS5-coding sequence. When plasmid transfection occured from 96 h before to 1 h after infection, about 50-80% reduction in WNV amplification was observable at 24 h pi. Geiss *et al.* (73) found, in a similar experimental system, that transfection of siRNA-generating plasmids with lipid reagent at 10 h pi had little antiviral effect. However, in a human liver cell line expressing a stably integrated WNV reporter replicon, siRNA transfection by electroporation generated a 7-10-fold reduction in reporter activity. The authors speculate that electroporation opens pores in cellular membranes thereby allowing the siRNA access to replicating WNV. Ong *et al.* (74) also showed the antiviral efficacy and low cytotoxicity of an NS5-sequence siRNA-producing plasmid vector which was capable of long-lasting expression in mammalian cells. Bai *et al.* (75) obtained highly positive cell culture results using siRNA targeting WNV E-coding sequence, and they also reported that injection of the same siRNA provided partial protection from death in a murine model of West Nile disease. Hydrodynamic tail vein injection of 180 micrograms of E dsRNA at 24 h before intraperitoneal WNV infection reduced viral presence in spleen, liver and brain at days 3 or 6 pi, and produced a survivorship rate of 68%, compared to 21% in the mock-treated and 37% of the control-siRNA-treated groups of mice. No protection was obtained when administration of the siRNA was delayed until 24 h pi. Although hydrodynamic injection is not considered a viable method of delivery for a human therapeutic, this study provided evidence that siRNA can inhibit WNV replication *in vivo* and alter the course of the infection in mice.

3.4. Japanese encephalitis virus.

JEV is distributed throughout Asia, with about 50,000 cases and 10,000 deaths reported annually (3, 76). Symptoms typically include fever and headache, but other incapacitating manifestations also usually result, and frequently involve neurologic involvement, including brain damage. Fatalities usually result from cardiopulmonary complications (77). As with other flavivirus diseases that occur in developing countries, it is likely that the incidence of JE is much-underreported. Effective vaccines exist, and are in regular use in Japan and other parts of Asia; nevertheless, the disease remains a significant public health problem.

Murakami *et al.* (78) showed RNAi machinery can inhibit JEV replication in cell lines and in mice. Initially, they evaluated several commercially purchased small hairpin RNA (shRNA) (from which siRNA are produced through the action of endogenous cellular machinery) designed to target different sites in JEV RNA. They observed about a 1 $\log_{10}$ reduction of JEV titer in cultured cells at 24 h pi by pre-infection treatment with a shRNA designed against C-coding sequence. They then showed that a plasmid designed to express shRNA against M-coding sequence injected IP immediately after IP JEV infection protected up to 80% of mice from an otherwise-lethal outcome. Protection from body-weight loss due to the infection reflected the increased survivorship.

Kumar *et al.* (79) used either synthetic siRNA or lentiviral vectors expressing shRNA to protect against JEV and/or WNV. The authors first showed that direct transfection of siRNA targeting JEV E-coding sequence provided robust protection from JEV infection in cell cultures. They went on to establish stable expression of JEV-specific E-directed shRNA from a lentiviral vector in a neuronal cell line, and showed protection from subsequent JEV infection. As few as one pre-infection intracranial injection of this lentiviral vector completely

protected mice from a lethal JEV infection. Absence of viral titer and histopathology in brain tissue at day 5 pi reflected the greatly enhanced survival benefit. Similar results were obtained with E-siRNA complexed with a cationic lipid transfection reagent and injected intracranially. This strategy was able to offer 100% protection from death when administered before or immediately after a lethal dose of JEV, and 60% protection if administered at 18 h pi. Similarly impressive results were obtained in mouse experiments where WNV E-specific siRNA complexed with a transfection reagent offered a high degree of protection from a usually-lethal WNV challenge. In a stringent demonstration of the sequence specificity of these results, the JEV E-sequence-siRNA lentiviral vector was ineffective against WNV, even though target sequences in the E gene of the respective viruses differ at only 5 positions. The authors proceeded to show that a siRNA, designed to target E-coding sequence with only 2 nucleotides of disagreement between JEV and WNV, was completely protective against either virus in mice, when the siRNA was administered 30 minutes after infection. If the siRNA was administered at 6 h after inoculation, 80% and 100 % protection from JEV and WNV, respectively, was observed. This study provides additional optimism that RNAi may become a viable clinical strategy.

Appaiahgari and Vrati (80) recently showed that a DNAzyme targeting sequence in the JEV 3' UTR reduced JEV titer in mouse brains and extended the survival period of infected animals. The DNAzyme 27-nt target sequence is repeated twice in JEV and is highly conserved amongst mosquitoborne flaviviruses. The DNAzyme was composed of PSO linkages and was tethered to a polyG-tract at the 3'-end to enhance intracellular delivery. The compound was efficiently taken up by mouse neuronal and glial cells in culture and generated 1-2 $\log_{10}$ titer reductions. Intracranial co-injection of the compound and JEV resulted in an up to 4-$\log_{10}$ titer reduction of viral titer in brain at 72 h pi. Furthermore, all animals receiving treatment survived until at least day 14 pi, compared to no survival past day 7 pi for untreated animals.

4. PERSPECTIVE

The research findings summarized above provide useful guidance for targeting flaviviruses with the various antisense technologies, and also contribute to advances in the understanding of flavivirus biology. Further, the potent inhibition of virus production in some of the studies described here fosters optimism that antisense-mediated intervention may someday lead to a therapeutically useful product against flaviviral disease. There is considerable evidence that the level of viral load in a patient affects disease severity (81-83), and a reduction in virus replication can reasonably be expected to be beneficial. The steric-blocking PPMO have thus far been most productive when targeted to the genomic 5'-terminal- or 3' cyclization-sequence-regions, whereas RNase H-competent oligomers were most successful when directed at a variety of locations in the 3' UTR. A DNAzyme against conserved sequence in the 3' UTR of JEV, and RNAi targeting conserved structural-protein- or NS5-coding-sequence of a number of flaviviruses, has also generated marked inhibitions of virus replication. The efforts thus far represent preclinical steps in the right direction; yet further developmental advances will likely be necessary to achieve clinical success with any of the antisense-based approaches. The results with PPMO, DNAzyme and siRNA demonstrate that they can generate safe and effective inhibition of flaviruses in mouse models, and suggest that these technologies could produce candidates that merit pursuit as potential human therapeutics. Challenges clearly remain, however, to the feasibility of developing any of these technologies as a viable anti-flaviviral therapeutic strategy for humans. Although some of the viral vectors show promise, a safe and practical method for delivery of siRNA, or DNAzyme, to sites of flaviviral replication *in vivo* has yet to be convincingly demonstrated. Furthermore, limited siRNA expression profiles (46, 84), as well as off-target effects (85-87) produced from RNAi induction in mammalian systems, continue to be of prominent concern. Although the current formulation of PPMO was significantly protective at a non-toxic dose in a WNV mouse model, it appears that improvements in the composition of the peptide component of the PPMO will be necessary, to reduce toxicity (70), to allow parenteral administration to humans. More *in vivo* experimentation will be needed, both to evaluate new antisense compounds and strategies that have shown substantial efficacy in cell culture studies, and to explore and improve pharmacologic characteristics of the various antisense chemistries. Cost for all antisense therapeutic strategies is also a notable concern, as the vast majority of the victims of flaviviral disease reside in less-affluent countries.

Applying the advances in modern biology and chemistry towards development of drugs against currently untreatable major infectious diseases is imperative for global public health (88). The results reviewed above demonstrate that intervention in flaviviral disease by antisense-mediated strategies holds considerable promise. Continuing efforts to extend the current research onwards towards development of safe and effective antiflaviviral therapies is clearly warranted.

5. ACKNOWLEDGEMENTS

The authors wish to thank Drs. Carol Blair, Robert Jordan, and Richard Bestwick for their critical reading of the manuscript, and Derek Youngblood for help with preparation of Figure 2.

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Key Words: Flavivirus, antisense, RNAi, Antiviral, Drug Development, Review

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