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Inhibition of alphavirus infection in cell culture and in mice with antisense morpholino oligomers

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ABSTRACT

The genus *Alphavirus* contains members that threaten human health, both as natural pathogens and as potential biological weapons. Peptide-conjugated phosphorodiamidate morpholino oligomers (PPMO) enter cells readily and can inhibit viral replication through sequence-specific steric blockade of viral RNA. Sindbis virus (SINV) has low pathogenicity in humans and is regularly utilized as a model alphavirus. PPMO targeting the 5′-terminal and AUG translation start site regions of the SINV genome blocked the production of infectious SINV in tissue culture. PPMO designed against corresponding regions in Venezuelan equine encephalitis virus (VEEV) were likewise found to be effective *in vitro* against several strains of VEEV. Mice treated with PPMO before and after VEEV infection were completely protected from lethal outcome while mice receiving only post-infection PPMO treatment were partially protected. Levels of virus in tissue samples correlated with animal survival. Uninfected mice suffered no apparent ill-effects from PPMO treatment. Thus, PPMO appear promising as candidates for therapeutic development against alphaviruses.

Introduction

The genus *Alphavirus* in the family *Togaviridae* consists of 28 viruses, most of which cycle between mosquito vectors and vertebrate hosts. Several alphaviruses, including Venezuelan equine encephalitis virus (VEEV), Eastern equine encephalitis virus (EEEV), Western equine encephalitis virus (WEEV), O'nyong-nyong virus and Chikungunya virus, can cause severe disease in humans, that typically includes fever and neurological sequelae (Griffin, 2007). Of these, VEEV is the most important human pathogen, with several recent outbreaks consisting of hundreds of thousands of cases occurring mostly in Latin America (Weaver et al., 2004). Furthermore, EEEV, WEEV, and VEEV are considered bioterrorist threats because they cause severe disease in humans, can be produced in large quantity and/or are potentially transmitted by aerosol (Hawley and Eitzen, 2001; Sidwell and Smee, 2003). Veterinary vaccines of varying quality against EEEV, WEEV, and VEEV are commercially available, but only IND preparations are approved for human use and their availability is limited to military and laboratory personnel. No therapeutic for alphavirus-induced disease exists, although supportive treatment and anti-inflammatory drugs may be beneficial. Recently short interfering RNAs (siRNAs) have been shown to be effective against the alphaviruses Semliki Forest virus (Caplen et al., 2002) and VEEV (O'Brien, 2006), in cell cultures, and against O'nyong-nyong virus replication in its natural mosquito vector, *Anopheles gambiae* (Keene et al., 2004).

Alphaviruses have a single positive-stranded RNA genome of approximately 12 kb that codes for two polyproteins that are processed to four nonstructural proteins and three structural proteins, respectively. The open reading frames are flanked by 5′ and 3′ untranslated regions (UTRs) of approximately (~) 60 and ~300 nucleotides, respectively. The nonstructural proteins are translated from the full-length genomic RNA and are utilized to produce a full-length negative-strand antigenomic RNA. The negative-strand intermediate is used as template to produce both full-length positive-strand, and, using a 24 nucleotide internal promoter, a ~4 kb subgenomic RNA is produced, which is identical in sequence to the 3′ terminal third of the genomic RNA. The structural proteins are translated from the subgenomic RNA. Both genomic and subgenomic RNA are 5′ capped and 3′ polyadenylated. Sindbis virus (SINV) has been extensively used as a model alphavirus because of its low

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pathogenicity to humans, easy propagation in a variety of cell lines, and molecular biology that is considered representative of the genus (Strauss and Strauss, 1994).

Antisense oligomers of various structural types have been used to interfere with gene expression of several human viral pathogens (Schubert and Kurreck, 2006), and a phosphorothioate oligonucleotide designed to target mRNA of cytomegalovirus (CMV) that is intended to treat CMV-induced retinitis is an approved drug (De Clercq, 2004). However, antisense therapeutic technology continues to be hampered by limitations in both oligomer stability and delivery to RNA targets within relevant cells (Kurreck, 2003). Phosphorodiamidate morpholino oligomers (PMO) are a class of oligonucleotide-like antisense agents that possess the same four bases as DNA, but contain a different backbone. The deoxyribose ring and phosphodiester linkage of DNA are replaced by a morpholine ring and phosphorodiamidate linkage in PMO (Summerton and Weller, 1997). PMO are nonionic, are stable in cellular extracts and human serum (Nelson et al., 2005; Youngblood et al., 2007) and typically are synthesized to a length of 20–25 subunits. The PMO mechanism of antisense action is via steric-blocking of complementary RNA sequence (Stein et al., 1997), and thus differs from that of antisense agents based on DNA chemistry, which induce RNase H-mediated cleavage of the RNA strand of a RNA–DNA duplex, or RNAi/siRNA involving double-stranded agents that recognize target mRNA and induce its degradation by cellular proteins (Masiero et al., 2007). PMO covalently conjugated to a cell-penetrating peptide (CPP) can be delivered efficiently into cells (Deas et al., 2005; Moulton et al., 2004; Yuan et al., 2006; Zhang et al., 2006). CPP-PMO are water-soluble and have been shown to generate potent inhibition of several RNA viruses, including dengue virus (Holden et al., 2006; Kinney et al., 2005), West Nile virus (Deas et al., 2005), SARS Coronavirus (Neuman et al., 2005), Equine Arterivirus (van den Born et al., 2005) and influenza virus (Ge et al., 2006) in cell culture, and Coxsackievirus B3 (Yuan et al., 2006), Ebola virus (Enterlein et al., 2006), murine Coronavirus (Burrer et al., 2007) and West Nile virus (Deas et al., 2007) both in cell culture and in mouse models. Two different CPP-conjugated PMO (PPMO), one containing oligoarginine (P3) and one containing 6-aminohexanoic acid (P7), have been utilized (Moulton et al., 2004; Abes et al., 2006). A recent report documented that P7-conjugated PPMO are highly stable in human serum for at least 2 hours (h), and moderately stable for 24 h (Youngblood et al., 2007).

In the present study, we first evaluated six SINV-specific PPMO designed to base pair with sequences in the four terminal regions of the full-length genome or antigenome, the AUG translation start site region of the polyprotein coding sequence for the nonstructural proteins, and the subgenomic promoter region of the negative-strand antigenome. We found that two PPMO, one targeting the 5′-terminal sequence and the other targeting the first functional AUG translation start site regions of the genome, were effective in blocking viral production. Subsequently, PPMO were designed to target the two corresponding regions in VEEV. As for the SINV PPMO, VEEV-specific PPMO were found to inhibit the replication of several VEEV strains in cell cultures and were efficacious in a murine model of VEE.

Results

Design of PPMO

Considerations in PPMO sequence design for this study included our current understanding of the function of various alphavirus genetic regions, and PPMO mechanism of action. As with other positive-strand viruses that utilize cap-dependent translation, access of trans-acting proteins to the 5′ terminal region of the genome is critical to alphavirus capping reactions and the process of translation pre-initiation (Vasiljeva et al., 2000). It has also been shown by mutational studies that certain sequence and secondary structure requirements in the 5′ terminal region of the alphavirus genome must be present for efficient viral replication to occur (Frolov et al., 2001; Gorchakov et al., 2004; Niesters and Strauss, 1990; Tsiang et al., 1988). The 3′ terminal region of the antigenome has likewise been shown to play an integral role in positive-strand synthesis (Frolov et al., 2001), perhaps through the presence of a stem-loop structure corresponding to the inverse of that present in the 5′ end of the genome. Specific sequence requirements in the 19 nucleotide 3′ conserved sequence element (3′CSE) immediately preceding the polyA tail are necessary for efficient minus-strand synthesis (Frolov et al., 2001). Another study reported high antiviral activity from PPMO targeting the 5′ terminal region of the Equine Arterivirus genome, and moderate activity with PPMO targeting either the 3′ terminus of the genome or 3′ terminus of the antigenome (van den Born et al., 2005), although the 5′ end of the antigenome was not included as a target. Other groups have also reported high efficacy by PPMO targeting the genomic 5′ terminal region of other positive-strand viruses, including dengue (Kinney et al., 2005), West Nile virus (Deas et al., 2005), porcine reproductive and respiratory syndrome virus (Zhang et al., 2006) and murine Coronavirus (Burrer et al., 2007). The above reports documenting the importance of sequence and structures in terminal regions of the alphavirus genome, along with the

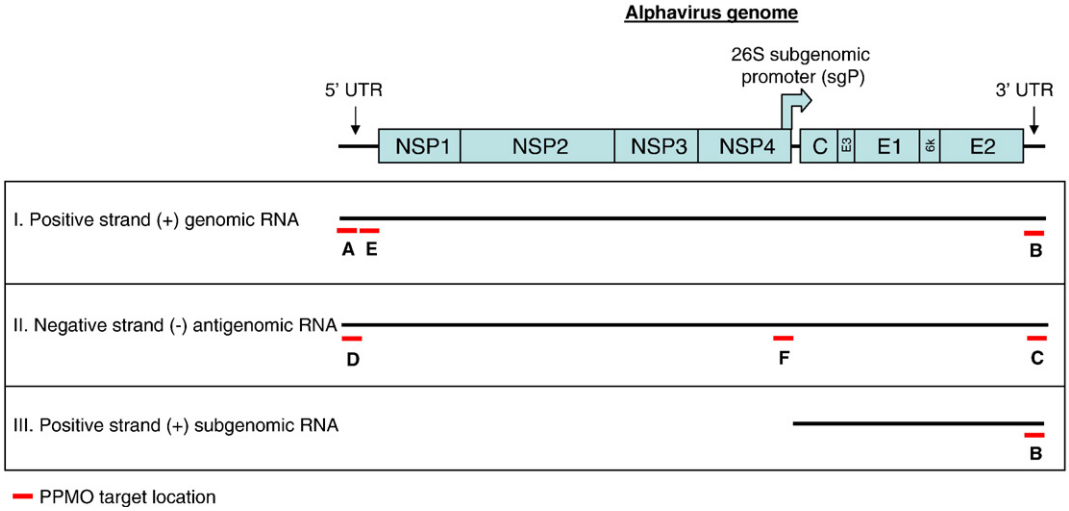

Fig. 1. Location of PPMO target sites in alphavirus genome segments. A schematic representation of the alphavirus genome is shown. PPMO were designed to target the terminal 5′ and 3′ untranslated regulatory regions (UTR) of alphavirus genomic (+) and antigenomic (−) RNA (labeled A–D), as well as the AUG translation start site region of the genomic (labeled E), and subgenomic promoter region of the antigenomic (labeled F), RNA. Sindbis virus (SINV)- and Venezuelan equine encephalitis virus (VEEV)-specific PPMO name designations and sequences are shown in Table 1.