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Evaluation of synthetic oligonucleotides as inhibitors of West Nile virus replication

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Abstract

A series of synthetic oligonucleotide phosphorothioate 15-mers were generated against specific sequences in the West Nile virus RNA genome. These antisense oligonucleotides targeted (1) conserved features of the West Nile virus RNA genome that may be expected to lead to inhibition of virus replication since such features play essential roles in the virus lifecycle; (2) G-quartet oligonucleotides with potential facilitated uptake properties and that also targeted conserved sequences among a range of West Nile virus strains. Several formulations with significant in vitro antiviral activity were found. Among the active oligonucleotides were examples that targeted both C-rich RNA sequences of the West Nile RNA genome as well as recognized conserved sequences key to West Nile virus replication. Since the antiviral activity of the latter oligonucleotides diminished upon 2′-*O*-methyl substitution, it is likely that their activity involves RNase H-catalyzed RNA degradation. One G-rich oligonucleotide that did not target a West Nile virus RNA sequence also was found. These results suggest the potential of antisense strategies for the control of West Nile virus replication if the attendant problem of oligonucleotide delivery can be adequately addressed.

Keywords: Conserved RNA sequences; G-quartet; Facilitated uptake; Ribonuclease H; 2′-*O*-Methyl-RNA oligonucleotides; Phosphorothioate

1. Introduction

The first described case of disease due to West Nile virus (Dauphin et al., 2004; Murgue et al., 2002) was described in 1937 in West Nile Province, Uganda. The virus has spread throughout other regions of Africa, Europe, and Southwestern Asia. In 1999, it entered the United States (Fauci et al., 2005; Koh and Ng, 2005) through New York City where it spread rapidly and moved into Canada, Central America, Mexico, and the Caribbean (Dauphin et al., 2004; Feldmann et al., 2002; Knudsen et al., 2003; VanDemark, 2004; Watson et al., 2004). In 2004 in the United States, 2535 cases of West Nile virus infection were reported, including 1108 cases of neuroinvasive disease and 98 deaths (US CDC).

West Nile virus is a flavivirus which is transmitted by urban culex mosquitoes (Daszak et al., 2004; Gould and Fikrig, 2004; Guharoy et al., 2004; Hayes et al., 2005). Recent West Nile virus epidemics have occurred during unusually hot and dry periods, thereby driving speculation regarding a strong climate change-disease link (Epstein, 2001; Lazar et al., 2002).

No treatment (Jackson, 2004; Borowski, 2005) yet exists for the serious neuroinvasive form (Anon., 2004; Campbell et al., 2002; Hayes and O'Leary, 2004; Katz et al., 2002; McCarthy, 2003; O'Leary et al., 2004; Ou and Ratard, 2005; Wadei et al., 2004) of West Nile virus infections. Recently, A morpholino-oligonucleotide analogue (Deas et al., 2005, AVI-4020) and a nucleoside analogue, 2-amino-8-(β-D-ribofuranosyl)-imidazo [1,2-a]-s-triazine-4-one (ZX-2401), have been reported to possess potent activity against the virus in vitro (Ojwang et al., 2005).

We have explored the possibility of using phosphorothioate oligonucleotide analogues to block West Nile virus replication using two different drug design approaches.

1.1. Conserved RNA sequences

Antisense targeting of conserved features of the West Nile virus RNA genome may be expected to lead to inhibition of

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virus replication since such conserved features play essential roles in the virus lifecycle. A decreased incidence of drug resistance might be an additional benefit of this approach since evidence shows that such conserved features naturally undergo slow mutation. We have previously used this strategy to generate an effective inhibitor of respiratory syncytial virus (RSV) replication (Player et al., 1998).

In the West Nile virus genome, there exists conserved sequence repeats in the 3′ UTR (untranslated region) (Brinton, 2002). These repeats are employed by the virus for cyclization of viral RNA by base pairing with a complementary sequence at the 5′ terminus. This cyclization mediated by CS1 seems essential for replication (Brinton, 2002). The sequences of interest are ***RCS2*** at the 5′-terminus of the viral RNA (. . .GGACUAGAGGUUAGAGGAGACCCC. . .) and two sequences near the 3′-terminus; namely, ***CS2*** and ***CS1*** arranged as ***CS2*** (. . .GGACUAGAGGUUAGAGGAGACCCC. . .) with an intervening 22 nucleotide sequence followed by ***CS1*** (CAGCAUAUUGAAC CUGGGA UAGAC3′). Since the ***RCS2*** sequence is the same as the ***CS2*** sequence, a single antisense molecule can target both. An added bonus, in the framework of drug design, would be the CCCC repeats in both RCS2 and CS2. The usefulness of the latter is discussed later.

1.2. 2. Facilitated uptake sequences conserved across West Nile virus types

Studies using antisense oligonucleotides have revealed that G quartets formation can enhance the uptake of oligonucleotides by an unknown mechanism (Peyman et al., 1995) observed that in Vero cells the cellular uptake of homopolymers decreased in the order $(dG)_{16} \gg (dT)_{16} > (dA)_{16} > (dC)_{16}$. Uptake of $(dG)_{16}$, which aggregated in culture media, was 40 times higher than that of $(dC)_{16}$ (Peyman et al., 1995) compared the uptake and anti-herpes simplex virus activity of two G-rich oligonucleotides, called AO1 and AO2. Both had phosphodiester linkages with protected termini to enhance stability. However, AO1 contained only three GG doublets per molecule, whereas AO2 had two G runs, a GGGG and a GGGGG. AO2, which formed aggregates in medium, possessed a five-fold higher uptake and a three-fold higher level of anti-herpes simplex virus activity than AO1, which did not form aggregates. Altering two Gs to As disrupted one of the two G stretches of the AO2 sequence, and thereby decreased cellular uptake of AO2 by three-fold. Interruption of both G stretches in AO2 abolished its ability to aggregate in culture media, and resulted in a 50-fold decrease in cellular uptake compared with the parent AO2 sequence.

Other studies have shown that PS ONs with four consecutive guanosine residues form a tetraplex and other higher-order structures, i.e., aggregates, particularly if the oligonucleotide has more than one G-quartet per molecule (Wang and Patel, 1993; Stein, 1999). Finally, other reports have found an increase of antisense oligonucleotide nuclease stability and activity related to the presence of G quartet-induced aggregates (Bishop et al., 1996).

2. Materials and methods

The West Nile virus strains (and their GenBank accession numbers) which we searched for common RNA sequences containing three or more C's were: HNY1999 (AF20251); Connecticut 1999 (AF206518); NY99-eghs (AF260967); Eg 101 (AF260968); RO97-50 (AF260969); NY99-Flamingo 382 (AF196835); VLG-4 (AF317203); IS-98 STD (AF4818640); WN Italy 1998 equine (AF404757); NY 2000 Crow 3356 (AF404756); NY 2000 Grouse 3282 (AF404755); NJ 2000 MQ5488 (AF404754); MD 2000 Crow 265 (AF404753).

Phosphorothioate oligonucleotides were synthesized by Midland Certified Reagent Co. (Cuthbert, TX) and made up in sterile water a 1 mM concentrations.

Parameters in the tables are as follows: EC_{50}, effective concentration (μM) required to reduce neutral red-based cytopathogenicity by 50%; IC_{50}, concentration (μM) required to cause 50% cytopathogenic effect in cells treated only with oligonucleotide and not with virus; $CCID_{50}$, cell culture infectious doses; SI, selectivity index determined by dividing IC_{50} by EC_{50}.

A New York West Nile virus isolate from homogenized crow brain dated 8/20/00 and named as prototype NY99 strain of WNV. CDC 996625, V1D3 11/10/1999, Robert Lanciotti, CDC, Division of Vector-Borne Infectious Diseases, Fort Collins, CO)) was the source of virus. Cells used were African green monkey kidney (Vero 76, ATCC CCL1587), which were grown in MEM with 10% FBS and 0.1% $NaHCO_3$. MEM with 1% FBS, 0.1% $NaHCO_3$, and 50 μg/ml gentamycin (Sigma, St. Louis, MO) were used to maintain Vero or MA-104 cells during antiviral experiments. Virus stocks were prepared in MA104 cells and stored at −80 °C. Virus stocks were prepared using MEM with 10% FBS and no gentamycin (Day et al., 2005; Morrey et al., 2002, 2004; Ojwang et al., 2005; Smee et al., 1988a, 1988b). The viruses were titered in subconfluent Vero cells in 96-well microtiter plates.

This cytopathic effect (CPE) inhibitory assay was described elsewhere (Smee et al., 1988a, 1988b) with the following modifications. One-half log serial dilutions of test compounds were added to subconfluent Vero cells in 96-well microplates, after which dilutions of viral stocks were added to the cells, which were assayed to be five $CCID_{50}$ of WNV New York isolate. The oligonucleotide dosages were applied to uninfected cells. Infected cells with no oligonucleotide application and uninfected oligonucleotide-treated cells were used as controls. Duplicates of toxicity controls at each oligonucleotide concentration and triplicates of test samples were performed. After 6 days post-infection, cells were visually scored for CPE. The 50% effective concentration (EC_{50}) and the 50% inhibitory cytotoxic concentration (IC_{50}) were calculated by regression analysis using the means of the CPE ratings at each concentration of the compound. A selectivity index (SI) was determined as the IC_{50}/EC_{50}. Neutral red (NR) vital stain was used to verify the visual CPE assay (Player et al., 1998); however, these data are not shown as they were congruent with the CPE assays. The CPE and NR assays were repeated at least two times in different experiments for compounds having anti-WNV activity.

3. Results and discussion

3.1. Design strategy

To target conserved RNA sequences, we generated a total of four antisense oligonucleotides (15–17 nucleotides in length). Two targeted the ***RCS2/CS2*** sequence (**WNV21S**, **WNV22S**) and two targeted the ***CS*** sequence (**WNV23S**, **WNV24S**). All of these were prepared as phosphorothioates to provide enhanced resistance to degradation. Their sequences were as follows (deoxyguanosines underlined): **WNV21S**, 5′GGG GTC TCC TCT AAC3′; **WNV22S**, 5′TCT AAC CTC TAG TCC3′; **WNV23S**, 5′GTC TAT CCC AGG TCG3′; **WNV24S**, 5′AGG TCG TCA ATA TGC TG3′.

To provide sequences capable of facilitated uptake by virtue of G sequences, we have examined a variety of West Nile virus strains (found in GenBank) to discover RNA sequences that (a) contain runs of three or more C's and (b) are conserved across as many strains of West Nile virus as possible. The deoxycytidine nucleotide was chosen in the target RNA so that the antisense would be the complement G. In this way, an anti-West Nile virus active oligonucleotide would retain activity against the commonly occurring strains. Specifically, the West Nile virus strains which we searched for common RNA sequences containing three of more C's were: HNY1999; Connecticut 1999; NY99-eghs; Eg101; RO97-50; NY99-Flamingo 382; VLG-4; IS-98 STD; WN Italy 1998 equine; NY 2000 Crow 3356; NY 2000 Grouse 3282; NJ 2000 MQ5488; MD 2000 Crow 265.

We found six West Nile virus RNA genome sequences, containing a single sequence of four G residues, that were 30–40 nucleotides in length with complete sequence conservation across all of the above examined strains. The G sequence length of four was chosen to reduce the number of sequence "hits". We used just four of the conserved GGGG-containing sequences to select five different sub-sequences (which would partly overlap in some cases) to generate a total of 20 antisense complement oligonucletides 15 nucleotides long. The choice of 15-mers for antisense oligonucleotide length was based upon to well-established fact that this would provide a resulting sense–antisense duplex of sufficient thermal stability to exist under physiological conditions. It is always possible, once an active "lead" or "hit" has been found, to optimize the length parameter.

In order to ensure sufficient resistance to degradative enzymes such as phosphodiesterases or endonucleases, each oligonucleotide was synthesized containing all phosphorothioate internucleotide linkages. Phosphorothioates are well known (Chen et al., 2005; Park et al., 2004; Sanghvi and Schulte, 2004) for their relative resistance to degradative enzymes that quickly destroy phosphodiester-based oligonucleotides.

Candidate oligonucleotides were screened for West Nile antiviral activity in Vero 76 cells infected with a very low MOI (five cell culture infectious doses per well) as described previously.

Initial antiviral testing was performed on oligonucleotides generated as described above. For screening purposes, initially only two concentrations (10 and 100 μM) of each candidate oligonucleotide were evaluated against West Nile virus. The first antiviral screening revealed 15 of 24 oligonucleotides with sufficient activity to warrant extended evaluation through a greater range of concentrations. These were **WNV1S**, **WNV3S**, **WNV4S**, **WNV6S**, **WNV7S**, **WNV10S**, **WNV11S**, **WNV12S**, **WNV13S**, **WNV17S**, **WNV21S**, **WNV22S**, **WNV23S**, **WNV24S** (Table 1). The remaining nine oligonucleotides were devoid of significant activity. Significant activity was defined as an EC_{50} of less than 30 μM.

The second, more extensive, screen examined eight concentrations and used visual cytopathogenicity as an antiviral endpoint (Table 1). In this round, four oligonucleotides emerged with significant anti-West Nile virus activity. They were: **WNV7S** (EC_{50} = 4.3 μM, SI = 23); **WNV12S**

Table 1
Antiviral testing against West Nile virus in vero cells

Drug name	Sequence[a]	CPE inhibition (visual)		
		EC_{50}[b]	IC_{50}[b]	SI_{50}[c]
WNV1S	5′CCA CTG GGG TTT TG3′	100	100	1
WNV3S	5′TTT CTC CAC TGG GG3′	52	>93	>1.8
WNV4S	5′CTT CCA TCC ATT CA3′	>100	>100	–
WNV6S	5′CAT GTG GGG TCC TC3′	>100	>100	–
WNV7S	5′GGG GTC CTC CTT CC3′	4.3	100	23
WNV10S	5′GCT TTG AAG TTA CA3′	>100	>100	–
WNV11S	5′CCT CCT GGG GCA CTA3′	56	>100	>1.8
WNV12S	5′GGG GCA CTA TCG CA3′	13	>100	>7.7
WNV13S	5′CCA GTC CTC CTG GGG3′	56	>100	>1.8
WNV17S	5′GGG GTC TCC ACT AA3′	7.5	>100	>13.3
WNV21S	5′GGG GTC TCC TCT AAC3′	>100	>100	–
WNV22S	5′TCT AAC CTC TAG TCC3′	>100	>100	–
WNV23S	5′GTC TAT CCC AGG TCG3′	>100	>100	–
WNV24S	5′AGG TCG TCA ATA TGC TG3′	18	>100	>5.6

[a] All phosphorothioate, G's underlined for clarity vs. C's.
[b] Stationary cells.
[c] IC_{50}/EC_{50}.

Table 2
Antiviral testing against West Nile virus in vero cells

Drug name	Sequence	CPE inhibition (visual)		
		EC_{50}[a]	IC_{50}[a]	SI_{50}[b]
WNV91S	5′GGG GAA AAA AAG GGG3′	100	100	1
WNV92S	5′GGG GTT TTT TTG GGG3′	100	100	1
WNV93S	5′GGG GCC CCC CCG GGG3′	56	>100	>1.8
WNV94S	5′GGG GAA AAA AAA AAA3′	32	>100	>3.1
WNV95S	5′GGG GTT TTT TTT TTT3′	4	100	25
WNV96S	5′GGG GCC CCC CCC CCC3′	32	>100	>3.1
WNV127S	5′GmGmGm GmTCm CmTCm CmTT CmCm3′	>100	>100	0
WNV128S	5′GmGmGm GmCmAm CmTAm TCmGm CmAm3′	32	>100	>3.1
WNV129S	5′GmGmGm GmTCm TCmCm AmCmT AmAm3′	56	>100	>1.8

Gm: 2′-*O*-methyl RNA (m) guanine; Cm: 2′-*O*-methyl RNA (m) cytosine; Am: 2′-*O*-methyl RNA (m) adenine. Positive control was interferon-alpha.
[a] Stationary cells.
[b] IC_{50}/EC_{50}.

($EC_{50} = 13$, $SI > 7.7$); **WNV17S** ($EC_{50} = 7.5$, $SI > 13.3$); **WNV24S** ($EC_{50} = 18$, $SI > 5.6$).

Next we addressed several questions related to the above results.

1. Might the oligonucleotides active against West Nile virus and bearing runs of G nucleotides have a sequence-independent anti-West Nile virus effect? Such non-antisense effects of G-rich oligonucleotides have been well documented (Anselmet et al., 2002; Benimetskaya et al., 1997; Jing et al., 2003, 2004, 2005; McMicken et al., 2003; Redell and Tweardy, 2005; Stein and Krieg, 1994; Dapic et al., 2003; Stein, 2001) Thus, **WNV91S**, **WNV 92S**, **WNV 93S**, **WNV94S**, **WNV95S**, and **WNV96S** were synthesized (with phosphorothioate internucleotide linkages) and evaluated (Table 2). Of this set of modifications, only one (**WNV95S**) possessed significant antiviral activity ($EC_{50} = 4\ \mu M$, $SI = 25$).
2. Is it possible to enhance the antiviral activity of the more active oligonucleotides from Table 1 by chemical modification with 2′-*O*-methyl ribonucleoside substitution? Generally, 2′-*O*-methyl ribonucleoside substitution brings about an increase in affinity of the antisense oligonucleotide for the target (sense) RNA without any loss of nuclease resistance (Iribarren et al., 1990; Sproat et al., 1991; Yoo et al., 2004).
3. Is the antiviral activity related to RNase H degradation of the targeted RNA? RNase H is generally acknowledged as an important contributor to antisense efficacy (Achenbach et al., 2003; Goodchild, 2004; Tauser and Stoica, 2003). RNase H is able to degrade the RNA of DNA/RNA hybrids; however, it requires only a relatively short stretch (five nucleotides) of uninterrupted DNA to anneal with the RNA substrate in order to bring about cleavage. Thus, it is possible to provide "boxes" of intact DNA in the antisense oligonucleotide but still increase affinity for target RNA using 5′- and 3′-terminal 2′-*O*-methyl nucleotides.

Oligonucleotides **WNV127S**, **WNV128S**, and **WNV129S** (Table 2) incorporate 2′-*O*-methyl nucleotides in motifs that would *not* allow activity for RNase H cleavage; however, a steric blocking mode of antisense action would still be possible. Oligonucleotides **WNV137S**, **WNV138S**, and **WNV139S** all contain a "box" of deoxyribonucleotides imbedded between termini of 2′-*O*-methyl ribonucleotides and would therefore permit *both* RNase H-catalyzed RNA cleavage as well as a steric blocking mechanism. Thus, these six oligonucleotides allow both questions 2 and 3 above to be addressed.

The results (Table 3) revealed that none of the potential steric blocking candidates (**WNV127S**, **WNV128S**, **WNV129S**) possessed significant antiviral activity, thus suggesting that steric blocking alone is insufficient to provide anti-West Nile virus activity, at least for these sequences. For the RNase H-active oligonucleotides (**WNV137S**, **WNV138S**, **WNV139S**), one (**WNV138S**) displayed significant activity, suggesting the possible involvement of RNase H in the activity of this particular sequence.

Table 3
Antiviral testing against West Nile virus in vero cells

Drug name	Sequence	CPE inhibition (visual)		
		EC_{50}[a]	IC_{50}[a]	SI_{50}[b]
WNV137S	5′GmGmGm Gm *TC CTC* CmTT CmCm3′	>100	>100	0
WNV138S	5′GmGmGm GmCm *A CTA T* CmGm CmAm3′	7.5	>100	>13.3
WNV139S	5′GmGmGm Gm *TC TCC* AmCmT AmAm3′	100	100	1

DNA backbone is indicated by italics. Otherwise, linkages are phosphorothioates. Gm: 2′-*O*-methyl RNA (m) guanine; Cm: 2′-*O*-methyl RNA (m) cytosine; Am: 2′-*O*-methyl RNA (m) adenine. Positive control was interferon-alpha.
[a] Stationary cells.
[b] IC_{50}/EC_{50}.

To sum up, screening phosphorothioate antisense oligonucleotides designed to target C-rich regions or key conserved elements of the West Nile virus genome has resulted in several formulations with low micromolar antiviral activity. The most active oligonucleotides were **WNV7S** (EC_{50} = 4.3 μM, SI = 23); **WNV12S** (EC_{50} = 13 μM, SI > 7.7); **WNV17S** (EC_{50} = 7.5, SI > 13.3); **WNV24S** (EC_{50} = 18, SI > 5.6), and **WNV138S** (EC_{50} = 7.5 μM, SI > 100). One G-rich oligonucleotide that did not target a West Nile virus RNA sequence was also found; namely, **WNV95S**, a phosphorothioate oligomer or sequence 5′GGG GTT TTT TTT TTT3′ and displaying an EC_{50} = 4 μM) with an SI of 100. Guanosine-thymidine oligonucleotides have been shown previously to possess antiviral activity against HIV, herpes virus, and Friend leukemia virus (Fennewald et al., 1995; Ojwang et al., 1994a, 1994b). Since conversion of **WNV7**, **WNV12S**, and **WNV17S** to all 2′-*O*-methyl phosphorothioate oligonuceotides resulted in a loss of anti-West Nile virus activity, it is possible that these former oligonucleotides may operate through a RNase H-dependent action.

These sequence formulations may provide leads for the generation of more potent inhibitors of West Nile virus replication.

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