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(72) Inventeurs/Inventors:
IVERSEN, PATRICK L., US;
STEIN, DAVID A., US

(73) Propriétaires/Owners:
AVI BIOPHARMA, INC., US;
SAREPTA THERAPEUTICS, INC., US

(74) Agent: GOWLING LAFLEUR HENDERSON LLP

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(54) Title: ANTISENSE ANTIVIRAL COMPOUND AND METHOD FOR TREATING SSRNA VIRAL INFECTION

(57) Abrégé/Abstract:
The invention provides antisense antiviral compounds and methods of their use and production in inhibition of growth of viruses of the Flaviviridae, Picornoviridae, Caliciviridae, Togaviridae, Arteriviridae, Coronaviridae, Astroviridae and Hepeviridae families in the treatment of a viral infection. The antisense antiviral compounds are substantially uncharged morpholino oligonucleotides having a sequence of 12-40 subunits, including at least 12 subunits having a targeting sequence that is complementary to a region associated with stem-loop secondary structure within the 5'-terminal end 40 bases of the positive-sense RNA strand of the virus.

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(71) Applicant *(for all designated States except US)*: **AVI BIOPHARMA, INC.** [US/US]; 4575 S.W. Research Way, # 200, Corvallis, OR 97333 (US).

(72) Inventors; and

(75) Inventors/Applicants *(for US only)*: **IVERSEN, Patrick, L.** [US/US]; 5902 N.W. Fair Oaks Place, Albany, OR 97330 (US). **STEIN, David, A.** [US/US]; 2135 N.W. Evergreen Street, Corvallis, OR 97330 (US).

(74) Agents: **DEHLINGER, Peter, J.** et al.; Perkins Coie LLP, P.O. Box 2168, Menlo Park, CA 94026 (US).

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(54) Title: ANTISENSE ANTIVIRAL COMPOUND AND METHOD FOR TREATING SSRNA VIRAL INFECTION

(57) Abstract: The invention provides antisense antiviral compounds and methods of their use and production in inhibition of growth of viruses of the Flaviviridae, Picornoviridae, Caliciviridae, Togaviridae, Arteriviridae, Coronaviridae, Astroviridae and Hepeviridae families in the treatment of a viral infection. The antisense antiviral compounds are substantially uncharged morpholino oligonucleotides having a sequence of 12-40 subunits, including at least 12 subunits having a targeting sequence that is complementary to a region associated with stem-loop secondary structure within the 5'-terminal end 40 bases of the positive-sense RNA strand of the virus.

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ANTISENSE ANTIVIRAL COMPOUND AND METHOD FOR TREATING ssRNA VIRAL INFECTION

Field of the Invention

This invention relates to antisense oligonucleotide compounds for use in treating a *Flavivirus*, *Hepacivirus*, *Enterovirus*, *Rhinovirus*, *Hepatovirus*, *Apthovirus*, *Hepevirus*, *Coronavirus*, *Arterivirus*, *Vesivirus*, *Norovirus*, *Mamastrovirus*, *Alphavirus*, and *Rubivirus* infection and antiviral treatment methods employing the compounds.

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Background of the Invention

Single-stranded RNA (ssRNA) viruses cause many diseases in wildlife, domestic animals and humans. These viruses are genetically and antigenically diverse, exhibiting broad tissue tropisms and a wide pathogenic potential. The incubation periods of some of the most pathogenic viruses, *e.g.* the caliciviruses, are very short. Viral replication and expression of virulence factors may overwhelm early defense mechanisms (Xu 1991) and cause acute and severe symptoms.

There are no specific treatment regimes for many viral infections. The infection may be serotype specific and natural immunity is often brief or absent (Murray and al. 1998). Immunization against these virulent viruses is impractical because of the diverse serotypes. RNA virus replicative processes lack effective genetic repair mechanisms, and current estimates of RNA virus replicative error rates are such that each genomic replication can be expected to produce one to ten errors, thus generating a high number of variants (Holland 1993). Often, the serotypes show no cross protection such that infection with any one serotype does not protect against infection with another. For example, vaccines against the vesivirus genus of the caliciviruses would have to provide protection against over 40 different neutralizing serotypes (Smith, Skilling *et al.* 1998) and

vaccines for the other genera of the Caliciviridae are expected to have the same limitations.

Thus, there remains a need for an effective antiviral therapy in several virus families, including small, single-stranded, positive-sense RNA viruses in the Flaviviridae, Picornoviridae, Caliciviridae, Togaviridae, Arteriviridae, Coronaviridae, Astroviridae or Hepeviridae families.

Summary of the Invention

The invention includes, in one aspect, a method of producing an anti-viral compound effective in inhibiting replication within a host cell of an RNA virus having a single-stranded, positive-sense genome and selected from one of the Flaviviridae, Picornoviridae, Caliciviridae, Togaviridae, Arteriviridae, Coronaviridae, Astroviridae or Hepeviridae families. The method includes first identifying as a viral target sequence, a region within the 5'-terminal 40 bases of the positive strand of the infecting virus whose sequence is capable of forming internal stem-loop secondary structure. There is then constructed, by step-wise solid-phase synthesis, an oligonucleotide analog compound characterized by:

(i) a nuclease-resistant backbone,

(ii) capable of uptake by mammalian host cells,

(iii) containing between 12-40 nucleotide bases, and

(iv) having a targeting sequence of at least 12 subunits that is complementary to the virus-genome region capable of forming internal duplex structure, and

(v) an ability to form with the viral target sequence, a heteroduplex structure (i) composed of the positive sense strand of the virus and the oligonucleotide compound, and (ii) characterized by a Tm of dissociation of at least 45 °C and disruption of such stem-loop structure.

The target sequence may be identified by obtaining analyzing the 5'-terminal sequences, *e.g.*, the 5'-terminal 40 bases by a computer program capable of performing secondary structure predictions based on a search for the minimal free energy state of the input RNA sequence.

The invention includes, in another aspect, a method of inhibiting in a mammalian host cell, replication of an infecting RNA virus having a single-stranded, positive-sense genome and selected from one of the Flaviviridae, Picornoviridae, Caliciviridae, Togaviridae, Arteriviridae, Coronaviridae, Astroviridae or Hepeviridae families. The method includes administering to the infected host cells, a virus-inhibitory amount of an oligonucleotide analog compound characterized by:

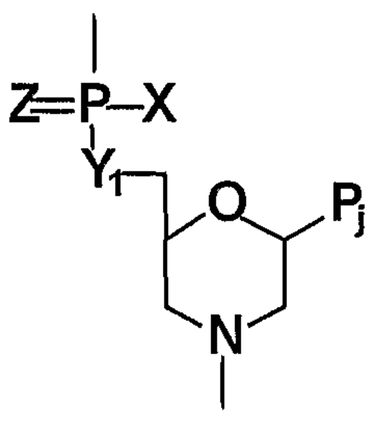
(i) a nuclease-resistant backbone,

(ii) capable of uptake by mammalian host cells,

(iii) containing between 12-40 nucleotide bases, and

(iv) having a targeting sequence of at least 12 subunits that is complementary to a region within the 5'-terminal 40 bases of the positive-strand viral genome that is capable of forming internal stem-loop secondary structure. The compound is effective, when administered to the host cells, to form a heteroduplex structure (i) composed of the positive sense strand of the virus and the oligonucleotide compound, and (ii) characterized by a Tm of dissociation of at least 45 °C and disruption of such stem-loop secondary structure. The compound may be administered to a mammalian subject infected with the virus, or at risk of infection with the virus.

The compound may be composed of morpholino subunits linked by uncharged, phosphorus-containing intersubunit linkages, joining a morpholino nitrogen of one subunit to a 5' exocyclic carbon of an adjacent subunit. In one embodiment, the intersubunit linkages are phosphorodiamidate linkages, such as those having the structure:



where Y_1=O, Z=O, Pj is a purine or pyrimidine base-pairing moiety effective to bind, by base-specific hydrogen bonding, to a base in a polynucleotide, and X is alkyl, alkoxy, thioalkoxy, or alkyl amino, *e.g.*, wherein X=NR_2, where each R is independently hydrogen or methyl.

The compound may be a covalent conjugate of an oligonucleotide analog moiety capable of forming such a heteroduplex structure with the positive sense strand of the virus, and an arginine-rich polypeptide effective to enhance the uptake of the compound into host cells.

In a related aspect, the invention includes a heteroduplex complex formed between:

(a) a region within the 5'-terminal 40 bases of the positive strand RNA of an RNA virus having a single-stranded, positive-sense RNA genome and selected from one of the Flaviviridae, Picornoviridae, Caliciviridae, Togaviridae, Arteriviridae, Coronaviridae, Astroviridae or Hepeviridae families, which region is capable of forming internal stem-loop secondary structure, and

(b) an oligonucleotide analog compound characterized by:

(i) a nuclease-resistant backbone,

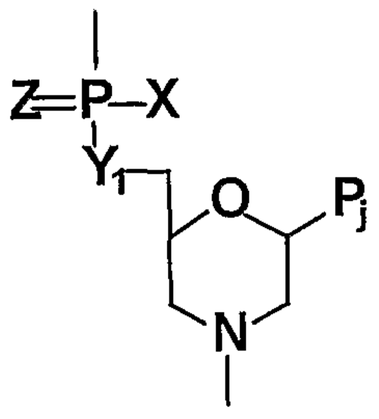
(ii) capable of uptake by mammalian host cells,

(iii) containing between 12-40 nucleotide bases,

(iv) having a targeting sequence of at least 12 subunits that is complementary to a region associated with such stem-loop secondary structure within the 5'-terminal end 40 bases of the positive-sense RNA strand of the virus,

where said heteroduplex complex has a Tm of dissociation of at least 45 °C and disruption of such stem-loop secondary structure.

An exemplary compound is composed of morpholino subunits linked by uncharged, phosphorus-containing intersubunit linkages, joining a morpholino nitrogen of one subunit to a 5' exocyclic carbon of an adjacent subunit. The compound may have phosphorodiamidate linkages, such as in the structure



where Y_1=O, Z=O, Pj is a purine or pyrimidine base-pairing moiety effective to bind, by base-specific hydrogen bonding, to a base in a polynucleotide, and X is alkyl, alkoxy, thioalkoxy, or alkyl amino. In a preferred compound, X=NR_2, where each R is independently hydrogen or methyl. The compound may be the oligonucleotide analog alone or a conjugate of the analog and an arginine-rich polypeptide capable of enhancing the uptake of the compound into host cells.

The invention is also directed to a method for detecting the presence of a viral infection by an RNA virus having a single-stranded, positive-sense RNA genome and selected from one of the Flaviviridae, Picornoviridae, Caliciviridae, Togaviridae, Arteriviridae, Coronaviridae, Astroviridae or Hepeviridae families a in a mammalian subject, or for confirming the presence of an effective interaction between such a virus infecting a mammalian subject and an antisense oligonucleotide analog compound directed against the virus. In practicing the method, the subject is administered an oligonucleotide analog compound having (a) a sequence of 12-40 subunits, including a targeting sequence of at least 12 subunits that is complementary to a region associated with stem-loop secondary structure within the 5'-terminal end 40 bases of the positive-sense RNA strand of the virus, (b) morpholino subunits linked by uncharged, phosphorus-containing intersubunit linkages, each linkage joining a morpholino nitrogen of one subunit to a 5' exocyclic carbon of an adjacent subunit, and (c) capable of forming with the positive-strand viral ssRNA genome, a heteroduplex structure characterized by a Tm of dissociation of at least 45 °C and disruption of the stem-loop secondary structure.

At a selected time after the compound is administered, a sample of a body fluid is obtained from the subject; and assayed for the presence of a nuclease-resistant heteroduplex comprising the antisense oligonucleotide complexed with a complementary-sequence 5'-end region of the positive-strand RNA of the virus.

In still another aspect, the invention includes an oligonucleotide analog compound for use in inhibiting replication in mammalian host cells of an RNA virus having a single-stranded, positive-sense RNA genome and selected from the Flaviviridae, Picornoviridae, Caliciviridae, Togaviridae, or Coronaviridae families and hepatitis E virus. The compound is characterized by:

(i) a nuclease-resistant backbone,

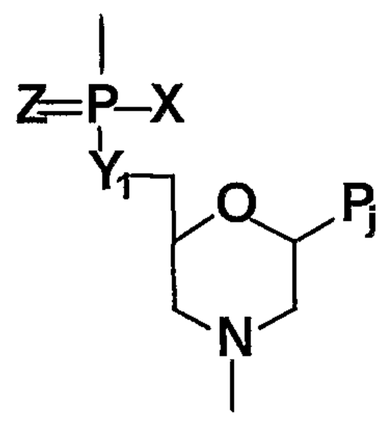
(ii) capable of uptake by mammalian host cells,

(iii) containing between 12-40 nucleotide bases,

(iv) having a targeting sequence of at least 12 subunits that is complementary to a region associated with stem-loop secondary structure within the 5'-terminal end 40 bases of the positive-sense RNA strand of the virus, and

(v) capable of forming with the positive-strand viral ssRNA genome, a heteroduplex structure having a Tm of dissociation of at least 45 °C and disruption of such stem-loop secondary structure.

An exemplary compound is composed of morpholino subunits linked by uncharged, phosphorus-containing intersubunit linkages, joining a morpholino nitrogen of one subunit to a 5' exocyclic carbon of an adjacent subunit. The compound may have phosphorodiamidate linkages, such as in the structure



where Y_1=O, Z=O, Pj is a purine or pyrimidine base-pairing moiety effective to bind, by base-specific hydrogen bonding, to a base in a polynucleotide, and X is alkyl, alkoxy, thioalkoxy, or alkyl amino. In a preferred compound, X=NR_2, where each R is independently hydrogen or methyl. The compound may be the oligonucleotide analog alone or a conjugate of the analog and an arginine-rich polypeptide capable of enhancing the uptake of the compound into host cells.

For treatment of a *Flavivirus* or *Hepacivirus* as given below, the targeting sequence is complementary to a region associated with stem-loop secondary structure within one of the following sequences:

(i) SEQ ID NO. 1, for St Louis encephalitis virus;

(ii) SEQ ID NO. 2, for Japanese encephalitis virus;

(iii) SEQ ID NO. 3, for a Murray Valley encephalitis virus;

(iv) SEQ ID NO. 4, for a West Nile fever virus;

(v) SEQ ID NO. 5, for a Yellow fever virus

(vi) SEQ ID NO. 6, for a Dengue Type-2 virus;

(vii) SEQ ID NO. 7, for a Hepatitis C virus;

(viii) SEQ ID NO. 8, for a tick-borne encephalitis virus;

(ix) SEQ ID NO. 9, for Omsk hemorrhagic fever virus; and

(x) SEQ ID NO. 10, for Powassan virus.

Exemplary targeting sequences for these viruses include the following sequences, or portions of these sequences that overlap with one or more regions of duplex secondary structure in the associated target sequence:

(i) SEQ ID NOS. 41 and 42, for St Louis encephalitis virus;

(ii) SEQ ID NOS. 43 and 44, for Japanese encephalitis virus;

(iii) SEQ ID NOS. 45 and 46, for a Murray Valley encephalitis virus;

(iv) SEQ ID NOS. 47 and 48, for a West Nile fever virus;

(v) SEQ ID NOS. 49 and 50, for a Yellow fever virus

(vi) SEQ ID NOS. 51, 52, for a Dengue virus;

(vii) SEQ ID NOS. 53 and 54, for a Hepatitis C virus;

(viii) SEQ ID NOS. 55 and 56, for a tick-borne encephalitis virus;

(ix) SEQ ID NOS. 57 and 58, for Omsk hemorrhagic fever virus; and

(x) SEQ ID NOS. 59 and 60, for Powassan virus.

For treatment of an *Enterovirus*, *Rhinovirus*, *Hepatovirus* or *Aphthovirus* the targeting sequence is complementary to a region associated with stem-loop secondary structure within one of the following sequences:

(i) SEQ ID NO. 11, for a polio virus of the Mahoney and Sabin strains;

(ii) SEQ ID NO. 12, for a Human enterovirus A;

(iii) SEQ ID NO. 13, for a Human enterovirus B;

(iv) SEQ ID NO. 14, for a Human enterovirus C;

(v) SEQ ID NO. 15, for a Human enterovirus D;

(vi) SEQ ID NO. 16, for a Human enterovirus E;

(vii) SEQ ID NO. 17, for a Bovine enterovirus;

(viii) SEQ ID NO. 18, for Human rhinovirus 89;

(ix) SEQ ID NO. 19, for Human rhinovirus B;

(x) SEQ ID NO. 20, for Foot-and-mouth disease virus; and

(xi) SEQ ID NO. 21, for a hepatitis A virus.

Exemplary targeting sequences for these viruses include the following sequences, or portions of these sequences that overlap with one or more regions of duplex secondary structure in the associated target sequence:

(i) SEQ ID NOS. 61 and 62, for a polio virus of the Mahoney and Sabin strains;

(ii) SEQ ID NOS. 63 and 64, for a Human enterovirus A;

(iii) SEQ ID NOS. 65 and 66, for a Human enterovirus B;

(iv) SEQ ID NOS. 67 and 68, for a Human enterovirus C;

(v) SEQ ID NOS. 69 and 70, for a Human enterovirus D;

(vi) SEQ ID NOS. 71 and 72, for a Human enterovirus E;

(vii) SEQ ID NOS. 73 and 74, for a Bovine enterovirus;

(viii) SEQ ID NOS. 75 and 76, for Human rhinovirus 89;

(ix) SEQ ID NOS. 77 and 78, for Human rhinovirus B;

(x) SEQ ID NOS. 79 and 80, for Foot-and-mouth disease virus; and

(xi) SEQ ID NOS. 81 and 82, for a hepatitis A virus.

For treatment of a *Calicivirus* or *Norovirus* the targeting sequence is complementary to a region associated with stem-loop secondary structure within one of the following sequences:

(i) SEQ ID NO. 22, for a Feline Calicivirus;

(ii) SEQ ID NO. 23, for a Canine Calicivirus;

(iii) SEQ ID NO. 24, for a Porcine enteric calicivirus;

(iv) SEQ ID NO. 25, for Calicivirus strain NB; and

(v) SEQ ID NO. 26, for a Norwalk virus.

Exemplary targeting sequences for these viruses include the following sequences, or portions of these sequences that overlap with one or more regions of duplex secondary structure in the associated target sequence:

(i) SEQ ID NOS. 83 and 84, for a Feline Calicivirus;

(ii) SEQ ID NOS. 85 and 86, for a Canine Calicivirus;

(iii) SEQ ID NOS. 87 and 88, for a Porcine enteric calicivirus;

(iv) SEQ ID NOS. 89 and 90, for Calicivirus strain NB; and

(v) SEQ ID NOS. 91 and 92, for a Norwalk virus.

For treatment of the *Hepevirus*, Hepatitis E virus, the targeting sequence is complementary to a region associated with stem-loop secondary structure within the sequence identified as SEQ ID NO: 27. Exemplary targeting sequences include SEQ ID NOS: 93 and 94, or portions thereof that overlap with one or more regions of secondary structure in the associated target sequence.

For treatment of a *Rubivirus* or *Alphavirus* the targeting sequence is complementary to a region associated with stem-loop secondary structure within one of the following sequences:

(i) SEQ ID NO. 28, for Rubella virus;

(ii) SEQ ID NO. 38, for Eastern equine encephalitis virus;

(iii) SEQ ID NO. 39, for Western equine encephalitis virus; and

(iv) SEQ ID NO. 40, for Venezuelan equine encephalitis virus.

Exemplary targeting sequences for each of these viruses are identified by the following sequence ID numbers, or portions of these sequences that overlap with one or more regions of duplex secondary structure in the associated target sequence:

(i) SEQ ID NOS. 95 and 96, for Rubella virus;

(ii) SEQ ID NOS. 115 and 116, for Eastern equine encephalitis virus;

(iii) SEQ ID NOS. 117 and 118, for Western equine encephalitis virus; and

(iv) SEQ ID NOS. 119 and 120, for Venezuelan equine encephalitis virus

For treatment of a *Coronavirus* or *Arterivirus* the targeting sequence is complementary to a region associated with stem-loop secondary structure within one of the following sequences:

(i) SEQ ID NO. 29, for SARS coronavirus TOR2;

(ii) SEQ ID NO. 30, for Porcine epidemic diarrhea virus;

(iii) SEQ ID NO. 31, for Transmissible gastroenteritis virus;

(iv) SEQ ID NO. 32, for Bovine coronavirus;

(v) SEQ ID NO. 33, for Human coronavirus 229E;

(vi) SEQ ID NO. 34, for Murine hepatitis virus; and

(vii) SEQ ID NO. 35, for Porcine reproductive and respiratory syndrome virus.

Exemplary targeting sequences for each of these viruses are identified by the following sequence ID numbers, or portions of these sequences that overlap with one or more regions of duplex secondary structure in the associated target sequence:

(i) SEQ ID NOS. 97 and 98, for SARS coronavirus TOR2;

(ii) SEQ ID NOS. 99 and 100, for Porcine epidemic diarrhea virus;

(iii) SEQ ID NOS. 101 and 102, for Transmissible gastroenteritis virus;

(iv) SEQ ID NOS. 103 and 104, for Bovine coronavirus;

(v) SEQ ID NOS. 105 and 106, for Human coronavirus 229E;

(vi) SEQ ID NOS. 107 and 108, for Murine hepatitis virus; and

(vii) SEQ ID NOS. 109 and 110, for Porcine reproductive and respiratory syndrome virus.

For treatment of a *Mamastrovirus*, Human astrovirus, the targeting sequence is complementary to a region associated with stem-loop secondary structure within the sequence identified as SEQ ID NO: 37. Exemplary targeting sequences are SEQ ID NOS. 113 and 114, or portions of these sequences that overlap with one or more regions of duplex secondary structure in the associated target sequence.

For treatment of an Equine arteritis virus, the targeting sequence is complementary to a region associated with stem-loop secondary structure within the sequence identified as SEQ ID NO: 36. Exemplary targeting sequences are SEQ ID NOS. 111, 112, or portions of these sequences that overlap with one or more regions of duplex secondary structure in the associated target sequence.

These and other objects and features of the invention will be more fully appreciated when the following detailed description of the invention is read in conjunction with the accompanying figures.

Brief Description of the Drawings

Figs. 1A-1G show the backbone structures of various oligonucleotide analogs with uncharged backbones;

Figs. 2A-2D show the repeating subunit segment of exemplary morpholino oligonucleotides;

Figs. 3A-3E are schematic diagrams of genomes of exemplary viruses and viral

target sites;

Figs. 4A-4E show examples of predicted secondary structures of 5' end terminal positive-strand regions for exemplary viruses; and

Figs. 5A-5D show the inhibition of Dengue virus replication in infected Vero cells in the presence of an antisense oligomer that targets the 5' positive-strand terminal region of Dengue virus types 1-4.

Fig. 6 shows that PMO can reduce PRRSV replication as measured by viral titer.

Fig. 7 shows *in vitro* PMO treatment of CRL11171 cells inoculated with PRRSV have reduced cell pathogenic effects.

Fig. 8 shows the reduction of TBEV replication *in vitro* in the presence PMO targeting the 5' terminal region of TBEV.

Detailed Description of the Invention

I. Definitions

The terms below, as used herein, have the following meanings, unless indicated otherwise:

The terms "oligonucleotide analog" refers to an oligonucleotide having (i) a modified backbone structure, *e.g.*, a backbone other than the standard phosphodiester linkage found in natural oligo- and polynucleotides, and (ii) optionally, modified sugar moieties, *e.g.*, morpholino moieties rather than ribose or deoxyribose moieties. The analog supports bases capable of hydrogen bonding by Watson-Crick base pairing to standard polynucleotide bases, where the analog backbone presents the bases in a manner to permit such hydrogen bonding in a sequence-specific fashion between the oligonucleotide analog molecule and bases in a standard polynucleotide (*e.g.*, single-stranded RNA or single-stranded DNA). Preferred analogs are those having a substantially uncharged, phosphorus containing backbone.

A substantially uncharged, phosphorus containing backbone in an oligonucleotide analog is one in which a majority of the subunit linkages, *e.g.*, between 60-100%, are uncharged at physiological pH, and contain a single phosphorous atom. The analog contains between 8 and 40 subunits, typically about 8-25 subunits, and preferably about 12 to 25 subunits. The analog may have exact sequence complementarity to the target

sequence or near complementarity, as defined below.

A "subunit" of an oligonucleotide analog refers to one nucleotide (or nucleotide analog) unit of the analog. The term may refer to the nucleotide unit with or without the attached intersubunit linkage, although, when referring to a "charged subunit", the charge typically resides within the intersubunit linkage (*e.g.* a phosphate or phosphorothioate linkage).

A "morpholino oligonucleotide analog" is an oligonucleotide analog composed of morpholino subunit structures of the form shown in Figs. 2A-2D, where (i) the structures are linked together by phosphorus-containing linkages, one to three atoms long, joining the morpholino nitrogen of one subunit to the 5' exocyclic carbon of an adjacent subunit, and (ii) P_i and P_j are purine or pyrimidine base-pairing moieties effective to bind, by base-specific hydrogen bonding, to a base in a polynucleotide. The purine or pyrimidine base-pairing moiety is typically adenine, cytosine, guanine, uracil or thymine. The synthesis, structures, and binding characteristics of morpholino oligomers are detailed in U.S. Patent Nos. 5,698,685, 5,217,866, 5,142,047, 5,034,506, 5,166,315, 5,521,063, and 5,506.337.

The subunit and linkage shown in Figure 2B are used for six-atom repeating-unit backbones, as shown in Figure 3B (where the six atoms include: a morpholino nitrogen, the connected phosphorus atom, the atom (usually oxygen) linking the phosphorus atom to the 5' exocyclic carbon, the 5' exocyclic carbon, and two carbon atoms of the next morpholino ring). In these structures, the atom Y_1 linking the 5' exocyclic morpholino carbon to the phosphorus group may be sulfur, nitrogen, carbon or, preferably, oxygen. The X moiety pendant from the phosphorus is any stable group which does not interfere with base-specific hydrogen bonding. Preferred X groups include fluoro, alkyl, alkoxy, thioalkoxy, and alkyl amino, including cyclic amines, all of which can be variously substituted, as long as base-specific bonding is not disrupted. Alkyl, alkoxy and thioalkoxy preferably include 1-6 carbon atoms. Alkyl amino preferably refers to lower alkyl (C_1 to C_6) substitution, and cyclic amines are preferably 5- to 7-membered nitrogen heterocycles optionally containing 1-2 additional heteroatoms selected from oxygen, nitrogen, and sulfur. Z is sulfur or oxygen, and is preferably oxygen.

A preferred morpholino oligomer is a phosphorodiamidate-linked morpholino

oligomer, referred to herein as a PMO. Such oligomers are composed of morpholino subunit structures such as shown in Fig. 2B, where X=NH_2, NHR, or NR_2 (where R is lower alkyl, preferably methyl), Y=O, and Z=O, and P_i and P_j are purine or pyrimidine base-pairing moieties effective to bind, by base-specific hydrogen bonding, to a base in a polynucleotide. Also preferred are structures having an alternate phosphorodiamidate linkage, where, in Fig. 2B, X = lower alkoxy, such as methoxy or ethoxy, Y=NH or NR, where R is lower alkyl, and Z=O.

The term "substituted", particularly with respect to an alkyl, alkoxy, thioalkoxy, or alkylamino group, refers to replacement of a hydrogen atom on carbon with a heteroatom-containing substituent, such as, for example, halogen, hydroxy, alkoxy, thiol, alkylthio, amino, alkylamino, imino, oxo (keto), nitro, cyano, or various acids or esters such as carboxylic, sulfonic, or phosphonic. It may also refer to replacement of a hydrogen atom on a heteroatom (such as an amine hydrogen) with an alkyl, carbonyl or other carbon containing group.

As used herein, the term "target", relative to the viral genomic RNA, refers to a viral genomic RNA, and specifically, to a region associated with stem-loop secondary structure within the 5'-terminal end 40 bases of the positive-sense RNA strand of a single-stranded RNA (ssRNA) virus described herein.

The term "target sequence" refers to a portion of the target RNA against which the oligonucleotide analog is directed, that is, the sequence to which the oligonucleotide analog will hybridize by Watson-Crick base pairing of a complementary sequence. As will be seen, the target sequence may be a contiguous region of the viral positive-strand RNA, or may be composed of complementary fragments of both the 5' and 3' sequences involved in secondary structure.

The term "targeting sequence" is the sequence in the oligonucleotide analog that is complementary (meaning, in addition, substantially complementary) to the target sequence in the RNA genome. The entire sequence, or only a portion, of the analog compound may be complementary to the target sequence. For example, in an analog having 20 bases, only 12-14 may be targeting sequences. Typically, the targeting sequence is formed of contiguous bases in the analog, but may alternatively be formed of non-contiguous sequences that when placed together, *e.g.*, from opposite ends of the analog, constitute

sequence that spans the target sequence. As will be seen, the target and targeting sequences are selected such that binding of the analog to a region within the 5'-terminal end 40 bases of the positive-sense RNA strand of the virus acts to disrupt secondary structure, particularly, the most 3' stem loop structure, in this region.

Target and targeting sequences are described as "complementary" to one another when hybridization occurs in an antiparallel configuration. A targeting sequence may have "near" or "substantial" complementarity to the target sequence and still function for the purpose of the present invention, that is, still be "complementary." Preferably, the oligonucleotide analog compounds employed in the present invention have at most one mismatch with the target sequence out of 10 nucleotides, and preferably at most one mismatch out of 20. Alternatively, the antisense oligomers employed have at least 90% sequence homology, and preferably at least 95% sequence homology, with the exemplary targeting sequences as designated herein.

An oligonucleotide analog "specifically hybridizes" to a target polynucleotide if the oligomer hybridizes to the target under physiological conditions, with a T_m substantially greater than 45°C, preferably at least 50°C, and typically 60°C-80°C or higher. Such hybridization preferably corresponds to stringent hybridization conditions. At a given ionic strength and pH, the T_m is the temperature at which 50% of a target sequence hybridizes to a complementary polynucleotide. Again, such hybridization may occur with "near" or "substantial" complementary of the antisense oligomer to the target sequence, as well as with exact complementarity.

A "nuclease-resistant" oligomeric molecule (oligomer) refers to one whose backbone is substantially resistant to nuclease cleavage, in non-hybridized or hybridized form; by common extracellular and intracellular nucleases in the body; that is, the oligomer shows little or no nuclease cleavage under normal nuclease conditions in the body to which the oligomer is exposed.

A "heteroduplex" refers to a duplex between an oligonucleotide analog and the complementary portion of a target RNA. A "nuclease-resistant heteroduplex" refers to a heteroduplex formed by the binding of an antisense oligomer to its complementary target, such that the heteroduplex is substantially resistant to *in vivo* degradation by intracellular and extracellular nucleases, such as RNAseH, which are capable of cutting double-

stranded RNA/RNA or RNA/DNA complexes.

A "base-specific intracellular binding event involving a target RNA" refers to the specific binding of an oligonucleotide analog to a target RNA sequence inside a cell. The base specificity of such binding is sequence specific. For example, a single-stranded polynucleotide can specifically bind to a single-stranded polynucleotide that is complementary in sequence.

An "effective amount" of an antisense oligomer, targeted against an infecting ssRNA virus, is an amount effective to reduce the rate of replication of the infecting virus, and/or viral load, and/or symptoms associated with the viral infection.

As used herein, the term "body fluid" encompasses a variety of sample types obtained from a subject including, urine, saliva, plasma, blood, spinal fluid, or other sample of biological origin, such as skin cells or dermal debris, and may refer to cells or cell fragments suspended therein, or the liquid medium and its solutes.

The term "relative amount" is used where a comparison is made between a test measurement and a control measurement. The relative amount of a reagent forming a complex in a reaction is the amount reacting with a test specimen, compared with the amount reacting with a control specimen. The control specimen may be run separately in the same assay, or it may be part of the same sample (for example, normal tissue surrounding a malignant area in a tissue section).

"Treatment" of an individual or a cell is any type of intervention provided as a means to alter the natural course of the individual or cell. Treatment includes, but is not limited to, administration of *e.g.*, a pharmaceutical composition, and may be performed either prophylactically, or subsequent to the initiation of a pathologic event or contact with an etiologic agent. The related term "improved therapeutic outcome" relative to a patient diagnosed as infected with a particular virus, refers to a slowing or diminution in the growth of virus, or viral load, or detectable symptoms associated with infection by that particular virus.

An agent is "actively taken up by mammalian cells" when the agent can enter the cell by a mechanism other than passive diffusion across the cell membrane. The agent may be transported, for example, by "active transport", referring to transport of agents across a mammalian cell membrane by *e.g.* an ATP-dependent transport mechanism, or by "facilitated transport", referring to transport of antisense agents across the cell membrane

by a transport mechanism that requires binding of the agent to a transport protein, which then facilitates passage of the bound agent across the membrane. For both active and facilitated transport, the oligonucleotide analog preferably has a substantially uncharged backbone, as defined below. Alternatively, the antisense compound may be formulated in a complexed form, such as an agent having an anionic backbone complexed with cationic lipids or liposomes, which can be taken into cells by an endocytotic mechanism. The analog also may be conjugated, *e.g.*, at its 5' or 3' end, to an arginine-rich peptide, *e.g.*, a portion of the HIV TAT protein, or polyarginine, to facilitate transport into the target host cell as described (Moulton, Nelson *et al.* 2004). Exemplary arginine-rich peptides useful in practicing the present invention as listed as SEQ ID NOS:121-126 in the Sequence Listing table.

A sequence is "capable of forming internal stem-loop secondary structure" if it can spontaneously, under physiological conditions, form one or more regions of double-stranded (duplex) RNA separated by one or more regions of single-stranded RNA. The stem and loop in this structure refers to the duplex RNA region (stem) terminating in a looped single-stranded region. The stem-loop structure of the 5'-terminal regions of several of the viruses encompassed by the invention are seen in Figs. 4A-4E. As seen for the West Nile virus (WNV) or HCV, for example (Fig. 4A), the stem-loop structure may comprise a single stem, a single loop, and non-duplexed end regions. In other cases, *e.g.*, Yellow fever virus (YFV) or Dengue-2 virus (Fig. 4A), the stem-loop secondary structure can include two or more double-stranded "stem" regions interspersed by non-duplexed regions and including a single loop.

By "disruption of such stem-loop structure" is meant disruption of any portion of the stem-loop structure in the 5' terminal region of the RNA viral positive-strand genome, by interfering with duplex RNA formation within this region by forming a stable heteroduplex complex between the oligonucleotide analog compound of the invention and duplex-forming sequences within the 5' terminal region of the virus genome. Rules for the selection of targeting sequences capable of disrupting secondary stem-loop structure in the 5'-terminal region of a viral genome are discussed below.

II. Targeted Viruses

The present invention is based on the discovery that effective of single-stranded, positive-sense RNA viruses can be achieved by exposing cells infected with the virus to antisense oligonucleotide analog compounds (i) targeted against the 5' end terminal sequences of the positive-strand viral RNA strand, and in particular, against target sequences that contribute to stem-loop secondary structure in this region, (ii) having physical and pharmacokinetic features which allow effective interaction between the antisense compound and the virus within host cells. In one aspect, the oligomers can be used in treating a mammalian subject infected with the virus.

The invention targets RNA viruses having genomes that are: (i) single stranded, (ii) positive polarity, and (iii) less than 32 kb. The targeted viruses also synthesize a genomic RNA strand with negative polarity, the minus-strand or negative-sense RNA, as the first step in viral RNA replication. In particular, targeted viral families include Flaviviridae, Picornoviridae, Caliciviridae, Togaviridae, Arteriviridae, Coronaviridae, Astroviridae and Hepeviridae families. Targeted viruses organized by family, genus and species are listed in Table 1. Various physical, morphological, and biological characteristics of each of these eight families, and members therein, can be found, for example, in Textbook of Human Virology, R. Belshe, ed., 2nd Edition, Mosby, 1991 and at the Universal Virus Database of the International Committee on Taxonomy of Viruses. Some of the key biological characteristics of each family are summarized below.

A. Flaviviridae. Members of this family include several serious human pathogens, among them mosquito-borne members of the genus *Flavivirus* including yellow fever virus (YFV), West Nile virus (WNV), Japanese encephalitis virus (JEV), St. Louis encephalitis virus (SLEV), Murray Valley encephalitis, Kunjin virus, and the four serotypes of dengue virus (DEN1-4).

Tick-borne members of the *Flavivirus* genus include tick-borne encephalitis virus (TBEV) and related viruses including Omsk hemorrhagic fever virus (OHFV), Louping ill virus, Powassan virus, Kyasanur Forest disease virus and Alkhurma virus.

The Flaviviridae also includes Hepatitis C virus, a member of the genus *Hepacivirus*.

B. Picornaviridae. This medically important family, whose members infect both

humans and animals, can cause severe paralysis (paralytic poliomyelitis), aspectic meningitis, hepatitis, pleurodynia, myocarditis, skin rashes, and colds; unapparent infection is common. Several medically important genera are members of this family; *Enterovirus* including poliovirus (PV) and human enteroviruses (*e.g.* coxsackie viruses); *Hepatovirus* which includes hepatitis A virus (HAV); *Rhinoviruses*; and *Aphthoviruses* which include the foot-and mouth disease virus (FMDV).

Rhinoviruses such as human rhinovirus 89 (HRV-89) and human rhinovirus B (HRV-B) are recognized as the principle cause of the common cold in humans. Serotypes are designated from 1A to 100. Transmission is primarily by the aerosol route and the virus replicates in the nose.

Like all positive-sense RNA viruses, the genomic RNA of Picornaviruses is infectious; that is, the genomic RNA is able to direct the synthesis of viral proteins directly, without host transcription events.

C. Caliciviridae. Members of the Caliciviridae infect both humans and animals. The genus *Vesivirus* produces disease manifestations in mammals that include epithelial blistering and are suspected of being the cause of animal abortion storms and some forms of human hepatitis (non A through E) (Smith *et al.*, 1998). Other genera of the Caliciviridae include the Norwalk-like and Sapporo-like viruses, which together comprise the human caliciviruses, and the Lagoviruses, which include rabbit hemorrhagic disease virus, a particularly rapid and deadly virus.

The human caliciviruses are the most common cause of viral diarrhea outbreaks worldwide in adults, as well as being significant pathogens of infants (O'Ryan 1992). There are at least five types of human caliciviruses that inhabit the gastrointestinal tract. The Norwalk virus is a widespread human agent causing acute epidemic gastroenteritis and causes approximately 10% of all outbreaks of gastroenteritis in man (Murray and al. 1998).

Vesiviruses are now emerging from being regarded as somewhat obscure and host specific to being recognized as one of the more versatile groups of viral pathogens known. For example, a single serotype has been shown to infect a diverse group of 16 different species of animals that include a saltwater fish (opal eye), sea lion, swine, and man.

D. Togaviridae. Members of this family include the mosquito-borne viruses which

infect both humans and animals. The family includes the genera *Alphavirus* and *Rubivirus* (rubella). Representative *Alphaviruses* include Sindbus, Western equine encephalomyelitis virus (WEEV), Eastern equine encephalitis virus (EEEV) and Venezuelan equine encephalitis virus (VEEV).

E. Hepatitis E –like Viruses. Hepatitis E virus (HEV) was initially described in 1987 and first reported in the U.S. in 1991. The virus was initially described as a member of the Caliciviridae based on the small, single-stranded RNA character. Some still classify HEV as belonging to the Caliciviridae, but it has also been recently classified as the only member of the Hepeviridae family. Infection appears to be much like hepatitis A viral infection. The disease is an acute viral hepatitis which is apparent about 20 days after initial infection, and the virus may be observed for about 20 days in the serum. Transmission occurs through contaminated water and geographically the virus is restricted to less developed countries.

F. Coronaviridae, Arteriviridae and Astroviridae. Members of the Coronaviridae include the human coronaviruses that cause 10 to 30% of common colds and other respiratory infections, and murine hepatitis virus. More recently, the viral cause of severe acute respiratory syndrome (SARS) has been identified as a coronavirus. The Arteriviridae include two important animal viruses, Equine arteritis virus (EAV) and porcine reproductive and respiratory syndrome virus (PRRSV). The Astroviridae includes the human astrovirus (HAstV).

Table 1, below, lists the targeted viruses of the invention organized by family and genus.

Family	**Genus**	**Virus**
Flaviviridae	*Flavivirus*	St. Louis encephalitis (SLEV)
		Japanese encephalitis (JEV)
		Murray Valley encephalitis (MVEV)
		West Nile (WNV)
		Yellow fever (YFV)
		Dengue Types 1-4 (DEN1-4)
		Tick-borne encephalitis (TBEV)
		Omsk hemorrhagic fever (OHFV)
		Powassan
	Hepacivirus	Hepatitis C (HCV)
Picornaviridae	*Enterovirus*	Poliovirus (PV)
		Human enterovirus A (HEV-A)
		Human enterovirus B (HEV-B)
		Human enterovirus C (HEV-C)
		Human enterovirus D (HEV-D)

		Human enterovirus E (HEV-E)
		Bovine enterovirus (BEV)
	Rhinovirus	Human Rhinovirus B (HRV-B)
		Human Rhinovirus 89 (HRV-89)
	Apthovirus	Foot and mouth disease (FMDV)
	Hepatovirus	Hepatitis A (HAV)
Caliciviridae	*Vesivirus*	Feline calicivirus (FCV)
		Canine calicivirus (CaCV)
		Porcine enteric calcivirus (PoCV)
		Calicivirus strain NB (CVNB)
	Norovirus	Norwalk(NV)
Hepeviridae	*Hepevirus*	Hepatitis E (HEV)
Togaviridae	*Rubivirus*	Rubella (RUBV)
	Alphavirus	Eastern equine encephalitis (EEEV)
		Western equine encephalitis (WEEV)
		Venezuelan equine encephalitis (VEEV)
Coronaviridae	*Coronavirus*	Porcine epidemic diarrhea (PEDV)
		Transmissible gastroenteritis (TGEV)
		SARS coronavirus (SARS-CoV)
		Bovine coronavirus (BCoV)
		Human coronavirus 229E (HCoV-229E)
		Murine hepatitis (MHV)
Arteriviridae	*Arterivirus*	Equine arteritis (EAV)
		Porcine respiratory and reproductive syndrome (PRRSV)
Astroviridae	*Mamastrovirus*	Human astrovirus (HAstV)

III. Viral Target Regions

Single-stranded, positive-sense RNA viruses, like all RNA viruses, are unique in their ability to synthesize RNA on an RNA template. To achieve this task they encode and induce the synthesis of a unique RNA-dependent RNA polymerases (RdRp) and possibly other proteins which bind specifically to the 3' and 5' end terminal untranslated regions (UTRs) of viral RNA. Since viral RNAs are linear molecules, RdRps have to employ unique strategies to initiate *de novo* RNA replication while retaining the integrity of the 5' end of their genomes. It is generally accepted that positive-strand (+strand) viral RNA replication proceeds via the following pathway:

+strand RNA → –strand RNA synthesis → RF → +strand RNA synthesis

where "–strand RNA" is negative-sense or minus-strand RNA complementary to the "+strand RNA" and "RF" (replicative form) is double-stranded RNA. The minus-strand RNA is used as a template for replication of multiple copies of positive-strand RNA

which is destined for either translation into viral proteins or incorporation into newly formed virions. The ratio of positive to minus-strand RNA in poliovirus-infected cells is approximately 50:1 in Hepatitis C-infected cells indicating each minus-strand RNA serves as a template for the synthesis of many positive-strand RNA molecules.

The present invention is based on the discovery that several classes of positive-strand RNA viruses can be effectively inhibited by exposing the viruses to an anti-sense compound capable of binding to a sequence within the '5 UTR of the virus positive strand, and in particular, to a sequence designed to disrupt one or more of the cis-acting elements (stem-loop structures) within the 5' UTR.

Therefore, as a first step in identifying an effective target region (the sequence in the positive strand 5'-UTR to which the antisense compound will bind), one identifies those regions within the 5'-UTR which are involved in stem-loop secondary structure. This may be done, for example, by computer-assisted secondary structure predictions which are based on a search for the minimal free energy state of the input RNA sequence (Zuker 2003). When this analysis is applied to the terminal 40 bases of the 5'-UTR region of various target viruses, the secondary structures or stem loops shown in Fig. 4A-4E are obtained. As seen, regions of secondary structure (forming the cis-acting elements) are found typically in the terminal 20-25 bases, but in many cases, in bases up to position 40. Therefore, the preferred target sequences are the 5' end terminal regions of the positive-strand RNA that include the end-most 40 nucleotides, typically the 5' terminal 5-35 nucleotides. Preferred target regions include those bases involved in secondary structure in these regions, as indicated in Figs. 4A-4D. In particular, the targeting sequence contains a sequence of at least 12 bases that are complementary to the 5'-end region of the positive-strand RNA, and are selected such that hybridization of the compound to the RNA is effective to disrupt stem-loop secondary structure in this region, preferably the 5'-end most stem-loop secondary structure. By way of example, Fig. 4A shows secondary structure of viral-genome sequences that are available from well known sources, such as the NCBI Genbank databases. Alternatively, a person skilled in the art can find sequences for many of the subject viruses in the open literature, *e.g.*, by searching for references that disclose sequence information on designated viruses. Once a complete or partial viral sequence is obtained, the 5' end-terminal sequences of the virus are identified.

The general genomic organization of each of the eight virus families is discussed below, followed by exemplary target sequences obtained for selected members (genera, species or strains) within each family.

A. Picornaviridae. Typical of the picornaviruses, the human rhinovirus 89 genome (Figure 3A) is a single molecule of single-stranded, positive-sense, polyadenylated RNA of approximately 7.2kb. The genome includes a long 618 nucleotide UTR which is located upstream of the first polyprotein, a single ORF, and a VPg (viral genome linked) protein covalently attached to its 5' end. The ORF is subdivided into two segments, each of which encodes a polyprotein. The first segment encodes a polyprotein that is cleaved subsequently to form viral proteins VP1 to VP4, and the second segment encodes a polyprotein which is the precursor of viral proteins including a protease and a polymerase. The ORF terminates in a polyA termination sequence.

B. Caliciviridae. Figure 3B shows the genome of a calicivirus; in this case the Norwalk virus. The genome is a single molecule of infectious, single stranded, positive-sense RNA of approximately 7.6kb. As shown, the genome includes a small UTR upstream of the first open reading frame which is unmodified. The 3' end of the genome is polyadenylated. The genome includes three open reading frames. The first open reading frame encodes a polyprotein, which is subsequently cleaved to form the viral non-structural proteins including a helicase, a protease, an RNA dependent RNA polymerase, and "VPg", a protein that becomes bound to the 5' end of the viral genomic RNA (Clarke and Lambden, 2000). The second open reading frame codes for the single capsid protein, and the third open reading frame codes for what is reported to be a structural protein that is basic in nature and probably able to associate with RNA.

C. Togaviridae. Figure 3C shows the structure of the genome of a togavirus, in this case, a rubella virus of the Togavirus genus. The genome is a single linear molecule of single-stranded, positive-sense RNA of approximately 9.8 kb, which is infectious. The 5' end is capped with a 7-methylG molecule and the 3' end is polyadenylated. Full-length and subgenomic messenger RNAs have been demonstrated, and post translational cleavage of polyproteins occurs during RNA replication. The genome also includes two open reading frames. The first open reading frame encodes a polyprotein which is subsequently cleaved into four functional proteins, nsP1 to nsP4. The second open

reading frame encodes the viral capsid protein and three other viral proteins, PE2, 6K and E1.

D. Flaviviridae. Figure 3D shows the structure of the genome of the hepatitis C virus of the Hepacivirus genus. The HCV genome is a single linear molecule of single-stranded, positive-sense RNA of about 9.6 kb and contains a 341 nucleotide 5' UTR. The 5' end is capped with an m^7GppAmp molecule, and the 3' end is not polyadenylated. The genome includes only one open reading frame which encodes a precursor polyprotein separable into six structural and functional proteins.

E. Coronaviridae. Figure 3E shows the genome structure of human coronavirus 229E. This coronovirus has a large genome of approximately 27.4 kb that is typical for the Coronoviridae and a 292 nucleotide 5' UTR. The 5'-most ORF of the viral genome is translated into a large polyprotein that is cleaved by viral-encoded proteases to release several nonstructural proteins, including an RdRp and a helicase. These proteins, in turn, are responsible for replicating the viral genome as well as generating nested transcripts that are used in the synthesis of other viral proteins.

GenBank references for exemplary viral nucleic acid sequences representing the 5' end terminal, positive-strand sequences for the first (most 5'-end) 40 bases for corresponding viral genomes are listed in Table 2 below. The nucleotide sequence numbers in Table 2 are derived from the Genbank reference for the positive-strand RNA. It will be appreciated that these sequences are only illustrative of other sequences in the five virus families, as may be available from available gene-sequence databases of literature or patent resources. The sequences below, identified as SEQ ID NOS:1-40, are also listed in Sequence Listing, Table 4, at the end of the specification.

The target sequences in Table 2 are the first 40 bases at the 5' terminal ends of the positive-strands sequences of the indicated viral RNAs. The sequences shown are in the 5' to 3' orientation so the 3' terminal nucleotide is at the end of the listed sequence. The base designation "N" indicates the nucleotides at these positions are presently unknown. The region within each sequence that is associated with stem-loop secondary structure can be seen from the predicted secondary structures in these sequences, shown in Figs. 4A-4D.

Table 2 Exemplary 5' End Terminal Viral Nucleic Acid Target Sequences

Virus	Gen-Bank No.	Target Sequence (5' to 3')	SEQ ID NO.
St. Louis encephalitis (SLEV)	M18929	GNNGATGTTCGCGTCGGTGAGCGGAGAGGAA ACAGATTTC	1
Japanese encephalitis (JEV)	NC 001437	AGAAGTTTATCTGTGTGAACTTCTTGGCTTAG TATCGTTG	2
Murray Valley encephalitis (MVEV)	NC 000943	AGACGTTCATCTGCGTGAGCTTCCGATCTCA GTATTGTTT	3
West Nile (WNV)	NC 001563	AGTAGTTCGCCTGTGTGAGCTGACAAACTTA GTAGTGTTT	4
Yellow Fever (YFV)	NC 002031	AGTAAATCCTGTGTGCTAATTGAGGTGCATT GGTCTGCAA	5
Dengue –Type 2 (DEN2)	M20558	AGTTGTTAGTCTACGTGGACCGACAAAGACA GATTCTTTG	6
Hepatitis C (HCV)	NC 004102	GCCAGCCCCCTGATGGGGGCGACACTCCACC ATGAATCAC	7
Tick-borne enceph-alitis virus (TBEV)	NC 001672	AGATTTTCTTGCACGTGCATGCGTTTGCTTCG GACAGCAT	8
Omsk hemorrhagic fever (OHFV)	NC 005062	AGATTTTCTTGCACGTGCGTGCGCTTGCTTCA GACAGCAA	9
Powassan	NC 003687	AGATTTTCTTGCACGTGTGTGCGGGTGCTTTA GTCAGTGT	10
Poliovirus-Mahoney strain (PV)	NC 002058	TTAAAACAGCTCTGGGGTTGTACCCACCCCA GAGGCCCAC	11
Human enterovirus A (HEV-A)	NC 001612	TTAAAACAGCCTGTGGGTTGTACCCACCCAC AGGGCCCAC	12
Human enterovirus B (HEV-B)	NC 001472	TTAAAACAGCCTGTGGGTTGTTCCCACCCAC AGGCCCATT	13
Human enterovirus C (HEV-C)	NC 001428	TTAAAACAGCTCTGGGGTTGCTCCCACCCCA GAGGCCCAC	14
Human enterovirus D (HEV-D)	NC 001430	TTAAAACAGCTCTGGGGTTGTTCCCACCCCA GAGGCCCAC	15
Human enterovirus E (HEV-E)	NC 003988	GAGTGTTCCCACCCAACAGGCCCACTGGGTG TTGTACTCT	16
Bovine enterovirus (BEV)	NC 001859	TTAAAACAGCCTGGGGGTTGTACCCACCCCT GGGGCCCAC	17
Human rhinovirus 89 (HRV-89)	NC 001617	TTAAAACTGGGAGTGGGTTGTTCCCACTCAC TCCACCCAT	18
Human rhinovirus B (HRV-B)	NC 001490	TTAAAACAGCGGATGGGTATCCCACCATTCG ACCCATTGG	19

Virus	Gen-Bank No.	Target Sequence (5' to 3')	SEQ ID NO.
Foot-and-mouth disease virus (FMDV)	AY593768	TTGAAAGGGGGCGCTAGGGTTTCACCCCTAG CATGCCAAC	20
Hepatitis A (HAV)	NC 001489	TTCAAGAGGGGTCTCCGGGAATTTCCGGAGT CCCTCTTGG	21
Feline calicivirus (FCV)	NC 001481	GTAAAAGAAATTTGAGACAATGTCTCAAACT CTGAGCTTC	22
Canine calicivirus (CaCV)	NC 004542	GTTAATGAGAAATGGCTTCTGCCATCGCTCT CTCGAGCTC	23
Porcine enteric calicivirus (PoCV)	NC 000940	GTGATCGTGATGGCTAATTGCCGTCCGTTGC CTATTGGGC	24
Calicivirus strain NB (CVNB)	NC 004064	GTGATTTAATTATAGAGAGATAGTGACTTTC ACTTTTCTT	25
Norwalk (NV)	NC 001959	GTGAATGATGATGGCGTCAAAAGACGTCGTT CCTACTGCT	26
Hepatitis E (HEV)	NC 001434	GCCATGGAGGCCCATCAGTTTATTAAGGCTC CTGGCATCA	27
Rubella (RUBV)	NC 001545	ATGGAAGCTATCGGACCTCGCTTAGGACTCC CATTCCCAT	28
SARS coronavirus (SARS-CoV)	NC 004718	ATATTAGGTTTTTACCTACCCAGGAAAAGCC AACCAACCT	29
Porcine epidemic diarrhea (PEDV)	NC 003436	ACTTAAAAAGATTTTCTATCTACGGATAGTTA GCTCTTTT	30
Transmissible gastroenteritis (TGEV)	NC 002306	ACTTTTAAAGTAAAGTGAGTGTAGCGTGGCT ATATCTCTT	31
Bovine coronavirus (BCoV)	NC 003045	GATTGCGAGCGATTTGCGTGCGTGCATCCCG CTTCACTGA	32
Human corona-virus 229E (HCoV-229E)	NC 002645	ACTTAAGTACCTTATCTATCTACAGATAGAAA AGTTGCTT	33
Murine Hepatitis (MHV)	NC 001846	TATAAGAGTGATTGGCGTCCGTACGTACCCT CTCAACTCT	34
Porcine repro-ductive and respiratory syn-drome (PRRSV)	AF 176348	ATGACGTATAGGTGTTGGCTCTATGCCTTGG CATTTGTAT	35
Equine arteritis (EAV)	NC 002532	GCTCGAAGTGTGTATGGTGCCATATACGGCT CACCACCAT	36
Human astro-virus (HAstV)	NC 001943	CCAAGAGGGGGGTGGTGATTGGCCTTTGGCT TATCAGTGT	37
Eastern equine encephalitis (EEEV)	NC 003899	ATAGGGTACGGTGTAGAGGCAACCACCCTAT TTCCACCTA	38
Western equine encephalomyelits (WEEV)	NC 003908	ACCCTACAAACTAATCGATCCAATATGGAAA GAATTCACG	39
Venezuelan equine enceph-alitis (VEEV)	NC 001449	ATGGGCGGCGCAAGAGAGAAGCCCAAACCAA TTACCTACC	40

To select a targeting sequence, one looks for a sequence that, when hybridized to a complementary sequence in the 5'-end region of the positive-strand RNA (SEQ ID NOS:1-40), will be effective to disrupt stem-loop secondary structure in this region, and preferably, the initial stem structure in the region. By way of example, a suitable targeting sequence for the West Nile Virus (WNV in Fig. 4A) is a sequence that will disrupt the stem loop structure shown in the figure. Three general classes of sequences would be suitable (exemplary 12-14 base targeting sequences are shown for illustrative purposes):

(1) a sequence such as 5'-ACAGGCGAACTACT-3' that targets the most 5' bases (1-14) of the stem and surrounding bases;

(2) a sequence such as 5'-GTCAGCTCACAC-3' that targets the complementary bases of the stem and surrounding bases (13-24);

(3) a sequence such as 5'-GCTCACACAGGCGA- 3'), that targets a portion of one or both "sides" of a stem loop and surrounding bases (7-20); typically, the sequence should disrupt all but at least 2-4 of the paired bases forming the stem structure;

It will be appreciated how this selection procedure can be applied to the other sequences shown in Table 2, For example, for the yellow fever virus (YFV) shown in Fig. 4A, exemplary 14-18 base sequences patterned after the three general classes above, might include:

(1) a sequence such as 5'-GCACACAGGATTTACT-3' that targets the most 5' bases (1-16) of the intial stem and surrounding bases;

(2) a sequence such as 5'-GTCCAATGCACCTC-3' that targets the complementary bases of the initial stem and surrounding bases (22-35);

(3) a sequence such as 5'-CAATGCACCTCAATTAGC-3' that targets a portion of both sides of a stem and surrounding bases (15-32);

It will be understood that targeting sequences so selected can be made shorter, *e.g.*, 12 bases, or longer, *e.g.*, 20 bases, and include a small number of mismatches, as long as the sequence is sufficiently complementary to disrupt the stem structure(s) upon hybridization with the target, and forms with the virus positive-strand, a heteroduplex having a Tm of 45°C or greater.

More generally, the degree of complementarity between the target and targeting sequence is sufficient to form a stable duplex. The region of complementarity of the

antisense oligomers with the target RNA sequence may be as short as 8-11 bases, but is preferably 12-15 bases or more, *e.g.* 12-20 bases, or 12-25 bases. An antisense oligomer of about 14-15 bases is generally long enough to have a unique complementary sequence in the viral genome. In addition, a minimum length of complementary bases may be required to achieve the requisite binding T_m, as discussed below.

Oligomers as long as 40 bases may be suitable, where at least the minimum number of bases, *e.g.*, 8-11, preferably 12-15 bases, are complementary to the target sequence. In general, however, facilitated or active uptake in cells is optimized at oligomer lengths less than about 30, preferably less than 25, and more preferably 20 or fewer bases. For PMO oligomers, described further below, an optimum balance of binding stability and uptake generally occurs at lengths of 14-22 bases.

The oligomer may be 100% complementary to the viral nucleic acid target sequence, or it may include mismatches, *e.g.*, to accommodate variants, as long as a heteroduplex formed between the oligomer and viral nucleic acid target sequence is sufficiently stable to withstand the action of cellular nucleases and other modes of degradation which may occur *in vivo*. Oligomer backbones which are less susceptible to cleavage by nucleases are discussed below. Mismatches, if present, are less destabilizing toward the end regions of the hybrid duplex than in the middle. The number of mismatches allowed will depend on the length of the oligomer, the percentage of G:C base pairs in the duplex, and the position of the mismatch(es) in the duplex, according to well understood principles of duplex stability. Although such an antisense oligomer is not necessarily 100% complementary to the viral nucleic acid target sequence, it is effective to stably and specifically bind to the target sequence, such that a biological activity of the nucleic acid target, *e.g.*, expression of viral protein(s), is modulated.

The stability of the duplex formed between the oligomer and the target sequence is a function of the binding T_m and the susceptibility of the duplex to cellular enzymatic cleavage. The T_m of an antisense compound with respect to complementary-sequence RNA may be measured by conventional methods, such as those described by Hames *et al.*, Nucleic Acid Hybridization, IRL Press, 1985, pp.107-108 or as described in Miyada C.G. and Wallace R.B., 1987, Oligonucleotide hybridization techniques, *Methods Enzymol.* Vol. 154 pp. 94-107. Each antisense oligomer should have a binding T_m, with respect to a

complementary-sequence RNA, of greater than body temperature and preferably greater than 50°C. T_m's in the range 60-80°C or greater are preferred. According to well known principles, the T_m of an oligomer compound, with respect to a complementary-based RNA hybrid, can be increased by increasing the ratio of C:G paired bases in the duplex, and/or by increasing the length (in base pairs) of the heteroduplex. At the same time, for purposes of optimizing cellular uptake, it may be advantageous to limit the size of the oligomer. For this reason, compounds that show high T_m (50°C or greater) at a length of 20 bases or less are generally preferred over those requiring greater than 20 bases for high T_m values.

Table 3 below shows exemplary targeting sequences, in a 5'-to-3' orientation, that are complementary to upstream and downstream portions of the 5' terminal 40 base regions of the positive strand of the viruses indicated. The sequences here provide a collection of targeting sequence or sequences from which targeting sequences may be selected, according to the general class rules discussed above. Thus, for example, in selecting a target against St. Louis encephalitis virus, one might select either SEQ ID NOS: 40 or 41, or a portion of either sequence effective to block secondary structure formation in the virus' 5' terminal UTR.

Table 3.

Exemplary Antisense Sequences Targeting the 5' End Terminal Positive-Strand Regions

Virus	GenBank Acc. No.	Ncts.	Sequences (5' to 3')	SEQ ID NO.
St. Louis encephalitis	M16614	1-18 11-30	ACCGACGCGAACATCNNC TCCTCTCCGCTCACCGACGC	41 42
Japanese encephalitis	NC 001437	1-18 11-30	TCACACAGATAAACTTCT AAGCCAAGAAGTTCACACAG	43 44
Murray Valley encephalitis	NC 000943	1-18 11-30	TCACGCAGATGAACGTCT GAGATCGGAAGCTCACGCAG	45 46
West Nile	NC 001563	1-20 11-31	GCTCACACAGGCGAACTACT TAAGTTTGTCAGCTCACACAG	47 48
Yellow Fever	NC 002031	1-22 21-40	CAATTAGCACACAGGATTTACT TTGCAGACCAATGCACCTCA	49 50
Dengue -Type 2	M20558	1-20 11-30	GTCCACGTAGACTAACAACT GTCTTTGTCGGTCCACGTAG	51 52
Hepatitis C	NC 004102	1-17 10-29	CCCATCAGGGGGCTGGC TGGAGTGTCGCCCCCATCAG	53 54
Tick-borne encephalitis	NC 001672	1-20 21-40	ATGCACGTGCAAGAAAATCT ATGCTGTCCGAAGCAAACGC	55 56
Omsk hemorrhagic fever	NC 005062	1-21 13-32	CACGCACGTGCAAGAAAATCT TGAAGCAAGCGCACGCACGT	57 58

Virus	GenBank Acc. No.	Ncts.	Sequences (5' to 3')	SEQ ID NO.
Powassan	NC 003687	1-20 21-40	ACACACGTGCAAGAAAATCT ACACTGACTAAAGCACCCGC	59 60
Poliovirus-Mahoney strain	NC 002058	1-24 21-40	GGTACAACCCCAGAGCTGTTTTAA GTGGGCCTCTGGGGTGGGTA	61 62
Human enterovirus A	NC 001612	1-20 21-40	CAACCCACAGGCTGTTTTAA GTGGGCCCTGTGGGTGGGTA	63 64
Human enterovirus B	NC 001472	1-20 21-40	CAACCCACAGGCTGTTTTAA AATGGGCCTGTGGGTGGGAA	65 66
Human enterovirus C	NC 001428	1-20 21-40	CAACCCCAGAGCTGTTTTAA GTGGGCCTCTGGGGTGGGAG	67 68
Human enterovirus D	NC 001430	1-20 21-40	CAACCCCAGAGCTGTTTTAA GTGGGCCTCTGGGGTGGGAA	69 70
Human enterovirus E	NC 003988	1-20 21-40	CCTGTTGGGTGGGAACACTC AGAGTACAACACCCAGTGGG	71 72
Bovine enterovirus	NC 001859	1-20 21-40	CAACCCCCAGGCTGTTTTAA GTGGGCCCCAGGGGTGGGTA	73 74
Human rhinovirus 89	NC 001617	1-20 21-40	CAACCCACTCCCAGTTTTAA ATGGGTGGAGTGAGTGGGAA	75 76
Human rhinovirus B	NC 001490	1-20 21-40	ATACCCATCCGCTGTTTTAA CCAATGGGTCGAATGGTGGG	77 78
Foot-and-mouth disease	AY593768	1-21 21-40	AACCCTAGCGCCCCCTTTCAA GTTGGCATGCTAGGGGTGAA	79 80
Hepatitis A	NC 001489	1-20 21-40	TCCCGGAGACCCCTCTTGAA CCAAGAGGGACTCCGGAAAT	81 82
Feline calicivirus	NC 001481	1-20 21-40	TTGTCTCAAATTTCTTTTAC GAAGCTCAGAGTTTGAGACA	83 84
Canine calicivirus	NC 004542	1-20 21-40	AGAAGCCATTTCTCATTAAC GAGCTCGAGAGAGCGATGGC	85 86
Porcine enteric calicivirus	NC 000940	1-20 13-32	CAATTAGCCATCACGATCAC GGCAACGGACGGCAATTAGC	87 88
Calicivirus strain NB	NC 004064	1-20 11-30	TCTCTCTATAATTAAATCAC AAAGTCACTATCTCTCTATA	89 90
Norwalk	NC 001959	1-20 21-40	TTGACGCCATCATCATTCAC AGCAGTAGGAACGACGTCTT	91 92
Hepatitis E	NC 001434	1-20 21-40	AACTGATGGGCCTCCATGGC TGATGCCAGGAGCCTTAATA	93 94
Rubella	NC 001545	1-20 21-40	CGAGGTCCGATAGCTTCCAT ATGGGAATGGGAGTCCTAAG	95 96
SARS coronavirus TOR2	NC 004718	1-20 21-40	GGTAGGTAAAAACCTAATAT AGGTTGGTTGGCTTTTCCTG	97 98
Porcine epidemic diarrhea	NC 003436	1-20 21-40	GATAGAAAATCTTTTTAAGT AAAAGAGCTAACTATCCGTA	99 100
Transmissible gastroenteritis	NC 002306	1-20 11-30	ACTCACTTTACTTTAAAAGT GCCACGCTACACTCACTTTA	101 102
Bovine coronavirus	NC 003045	1-20 21-40	CACGCAAATCGCTCGCAATC TCAGTGAAGCGGGATGCACG	103 104
Human coronavirus 229E	NC 002645	1-20 21-40	GATAGATAAGGTACTTAAGT AAGCAACTTTTCTATCTGTA	105 106
Murine Hepatitis	NC 001846	1-21 18-39	CGGACGCCAATCACTCTTATA GAGTTGAGAGGGTACGTACGGA	107 108
Porcine reproductive & respiratory syndrome	AF176348	5-25 21-40	CATAGAGCCAACACCTATACG ATACAAATGCCAAGGCATAG	109 110

Virus	GenBank Acc. No.	Ncts.	Sequences (5' to 3')	SEQ ID NO.
Equine arteritis	NC 002532	1-20 21-40	GCACCATACACACTTCGAGC ATGGTGGTGAGCCGTATATG	111 112
Human astrovirus	NC 001943	1-20 11-30	AATCACCACCCCCCTCTTGG GCCAAAGGCCAATCACCACC	113 114
Eastern equine encephalitis	NC 003899	1-20 21-40	GCCTCTACACCGTACCCTAT TAGGTGGAAATAGGGTGGTT	115 116
Western equine encephalomyelitis	NC 003908	1-20 21-40	GATCGATTAGTTTGTAGGGT CGTGAATTCTTTCCATATTG	117 118
Venezuelan equine encephalitis	NC 001449	1-20 21-40	TTCTCTCTTGCGCCGCCCAT GGTAGGTAATTGGTTTGGGC	119 120

IV. Antisense Oligonucleotide analog compounds

A. Properties

As detailed above, the antisense oligonucleotide analog compound (the term "antisense" indicates that the compound is targeted against the virus' sense or positive-sense strand RNA) has a base sequence targeting a region of the 5' end 40 bases that are associated with secondary structure in the negative-strand RNA. In addition, the oligomer is able to effectively target infecting viruses, when administered to a host cell, *e.g.* in an infected mammalian subject. This requirement is met when the oligomer compound (a) has the ability to be actively taken up by mammalian cells, and (b) once taken up, form a duplex with the target ssRNA with a T_m greater than about 45°C.

As will be described below, the ability to be taken up by cells requires that the oligomer backbone be substantially uncharged, and, preferably, that the oligomer structure is recognized as a substrate for active or facilitated transport across the cell membrane. The ability of the oligomer to form a stable duplex with the target RNA will also depend on the oligomer backbone, as well as factors noted above, the length and degree of complementarity of the antisense oligomer with respect to the target, the ratio of G:C to A:T base matches, and the positions of any mismatched bases. The ability of the antisense oligomer to resist cellular nucleases promotes survival and ultimate delivery of the agent to the cell cytoplasm.

Below are disclosed methods for testing any given, substantially uncharged backbone for its ability to meet these requirements.

A1. Active or facilitated uptake by cells

The antisense compound may be taken up by host cells by facilitated or active transport across the host cell membrane if administered in free (non-complexed) form, or by an endocytotic mechanism if administered in complexed form.

In the case where the agent is administered in free form, the antisense compound should be substantially uncharged, meaning that a majority of its intersubunit linkages are uncharged at physiological pH. Experiments carried out in support of the invention indicate that a small number of net charges, *e.g.*, 1-2 for a 15- to 20-mer oligomer, can in fact enhance cellular uptake of certain oligomers with substantially uncharged backbones. The charges may be carried on the oligomer itself, *e.g.*, in the backbone linkages, or may be terminal charged-group appendages. Preferably, the number of charged linkages is no more than one charged linkage per four uncharged linkages. More preferably, the number is no more than one charged linkage per ten, or no more than one per twenty, uncharged linkages. In one embodiment, the oligomer is fully uncharged.

An oligomer may also contain both negatively and positively charged backbone linkages, as long as opposing charges are present in approximately equal number. Preferably, the oligomer does not include runs of more than 3-5 consecutive subunits of either charge. For example, the oligomer may have a given number of anionic linkages, *e.g.* phosphorothioate or N3'→P5' phosphoramidate linkages, and a comparable number of cationic linkages, such as N,N-diethylenediamine phosphoramidates (Dagle, 2000). The net charge is preferably neutral or at most 1-2 net charges per oligomer.

In addition to being substantially or fully uncharged, the antisense agent is preferably a substrate for a membrane transporter system (*i.e.* a membrane protein or proteins) capable of facilitating transport or actively transporting the oligomer across the cell membrane. This feature may be determined by one of a number of tests for oligomer interaction or cell uptake, as follows.

A first test assesses binding at cell surface receptors, by examining the ability of an oligomer compound to displace or be displaced by a selected charged oligomer, *e.g.*, a phosphorothioate oligomer, on a cell surface. The cells are incubated with a given quantity of test oligomer, which is typically fluorescently labeled, at a final oligomer concentration of between about 10-300 nM. Shortly thereafter, *e.g.*, 10-30 minutes

(before significant internalization of the test oligomer can occur), the displacing compound is added, in incrementally increasing concentrations. If the test compound is able to bind to a cell surface receptor, the displacing compound will be observed to displace the test compound. If the displacing compound is shown to produce 50% displacement at a concentration of 10X the test compound concentration or less, the test compound is considered to bind at the same recognition site for the cell transport system as the displacing compound.

A second test measures cell transport, by examining the ability of the test compound to transport a labeled reporter, *e.g.*, a fluorescence reporter, into cells. The cells are incubated in the presence of labeled test compound, added at a final concentration between about 10-300 nM. After incubation for 30-120 minutes, the cells are examined, *e.g.*, by microscopy, for intracellular label. The presence of significant intracellular label is evidence that the test compound is transported by facilitated or active transport.

The antisense compound may also be administered in complexed form, where the complexing agent is typically a polymer, *e.g.*, a cationic lipid, polypeptide, or non-biological cationic polymer, having an opposite charge to any net charge on the antisense compound. Methods of forming complexes, including bilayer complexes, between anionic oligonucleotides and cationic lipid or other polymer components, are well known. For example, the liposomal composition Lipofectin® (Felgner *et al.*, 1987), containing the cationic lipid DOTMA (N-[1-(2,3-dioleyloxy)propyl]-N,N,N-trimethylammonium chloride) and the neutral phospholipid DOPE (dioleyl phosphatidyl ethanolamine), is widely used. After administration, the complex is taken up by cells through an endocytotic mechanism, typically involving particle encapsulation in endosomal bodies.

The antisense compound may also be administered in conjugated form with an arginine-rich peptide linked covalently to the 5' or 3' end of the antisense oligomer. The peptide is typically 8-16 amino acids and consists of a mixture of arginine, and other amino acids including phenyalanine and cysteine. The use of arginine-rich peptide-PMO conjugates can be used to enhance cellular uptake of the antisense oligomer (See, *e.g.* (Moulton, Nelson *et al.* 2004).

In some instances, liposomes may be employed to facilitate uptake of the antisense oligonucleotide into cells. (See, *e.g.*, Williams, S.A., Leukemia 10(12):1980-1989, 1996;

Lappalainen *et al.*, Antiviral Res. 23:119, 1994; Uhlmann *et al.*, ANTISENSE OLIGONUCLEOTIDES: A NEW THERAPEUTIC PRINCIPLE, Chemical Reviews, Volume 90, No. 4, pages 544-584, 1990; Gregoriadis, G., Chapter 14, Liposomes, Drug Carriers in Biology and Medicine, pp. 287-341, Academic Press, 1979). Hydrogels may also be used as vehicles for antisense oligomer administration, for example, as described in WO 93/01286. Alternatively, the oligonucleotides may be administered in microspheres or microparticles. (See, *e.g.*, Wu, G.Y. and Wu, C.H., J. Biol. Chem. 262:4429-4432, 1987). Alternatively, the use of gas-filled microbubbles complexed with the antisense oligomers can enhance delivery to target tissues, as described in US Patent No. 6,245,747.

Alternatively, and according to another aspect of the invention, the requisite properties of oligomers with any given backbone can be confirmed by a simple *in vivo* test, in which a labeled compound is administered to an animal, and a body fluid sample, taken from the animal several hours after the oligomer is administered, assayed for the presence of heteroduplex with target RNA. This method is detailed in subsection D below.

A2. Substantial resistance to RNaseH

Two general mechanisms have been proposed to account for inhibition of expression by antisense oligonucleotides. (See *e.g.*, Agrawal *et al.*, 1990; Bonham *et al.*, 1995; and Boudvillain *et al.*, 1997). In the first, a heteroduplex formed between the oligonucleotide and the viral RNA acts as a substrate for RNaseH, leading to cleavage of the viral RNA. Oligonucleotides belonging, or proposed to belong, to this class include phosphorothioates, phosphotriesters, and phosphodiesters (unmodified "natural" oligonucleotides). Such compounds expose the viral RNA in an oligomer:RNA duplex structure to hydrolysis by RNaseH, and therefore loss of function.

A second class of oligonucleotide analogs, termed "steric blockers" or, alternatively, "RNaseH inactive" or "RNaseH resistant", have not been observed to act as a substrate for RNaseH, and are believed to act by sterically blocking target RNA nucleocytoplasmic transport, splicing or translation. This class includes methylphosphonates (Toulme *et al.*, 1996), morpholino oligonucleotides, peptide nucleic acids (PNA's), certain 2'-O-allyl or 2'-O-alkyl modified oligonucleotides (Bonham, 1995), and N3'→P5' phosphoramidates (Gee, 1998; Ding, 1996).

A test oligomer can be assayed for its RNaseH resistance by forming an

RNA:oligomer duplex with the test compound, then incubating the duplex with RNaseH under a standard assay conditions, as described in Stein *et al.* After exposure to RNaseH, the presence or absence of intact duplex can be monitored by gel electrophoresis or mass spectrometry.

A3. *In vivo* uptake

In accordance with another aspect of the invention, there is provided a simple, rapid test for confirming that a given antisense oligomer type provides the required characteristics noted above, namely, high T_m, ability to be actively taken up by the host cells, and substantial resistance to RNaseH. This method is based on the discovery that a properly designed antisense compound will form a stable heteroduplex with the complementary portion of the viral RNA target when administered to a mammalian subject, and the heteroduplex subsequently appears in the urine (or other body fluid). Details of this method are also given in co-owned U.S. Patent Application Serial No. 09/736,920, entitled "Non-Invasive Method for Detecting Target RNA" (Non-Invasive Method).

Briefly, a test oligomer containing a backbone to be evaluated, having a base sequence targeted against a known RNA, is injected into a mammalian subject. The antisense oligomer may be directed against any intracellular RNA, including a host RNA or the RNA of an infecting virus. Several hours (typically 8-72) after administration, the urine is assayed for the presence of the antisense-RNA heteroduplex. If heteroduplex is detected, the backbone is suitable for use in the antisense oligomers of the present invention.

The test oligomer may be labeled, *e.g.* by a fluorescent or a radioactive tag, to facilitate subsequent analyses, if it is appropriate for the mammalian subject. The assay can be in any suitable solid-phase or fluid format. Generally, a solid-phase assay involves first binding the heteroduplex analyte to a solid-phase support, *e.g.*, particles or a polymer or test-strip substrate, and detecting the presence/amount of heteroduplex bound. In a fluid-phase assay, the analyte sample is typically pretreated to remove interfering sample components. If the oligomer is labeled, the presence of the heteroduplex is confirmed by detecting the label tags. For non-labeled compounds, the heteroduplex may be detected by immunoassay if in solid phase format or by mass spectroscopy or other known methods

if in solution or suspension format.

When the antisense oligomer is complementary to a virus-specific region of the viral genome (such as 5' end terminal region of the viral RNA, as described above, the method can be used to detect the presence of a given ssRNA virus, or reduction in the amount of virus during a treatment method.

B. Exemplary oligomer backbones

Examples of nonionic linkages that may be used in oligonucleotide analogs are shown in Figs. 1A-1G. In these figures, B represents a purine or pyrimidine base-pairing moiety effective to bind, by base-specific hydrogen bonding, to a base in a polynucleotide, preferably selected from adenine, cytosine, guanine and uracil. Suitable backbone structures include carbonate (Fig. 1A, R=O) and carbamate (Fig. 1A, R=NH_2) linkages (Mertes and Coats 1969; Gait, Jones *et al.* 1974); alkyl phosphonate and phosphotriester linkages (Fig. 1B; R=alkyl or -O-alkyl) (Lesnikowski, Jaworska *et al.* 1990); amide linkages (Fig. 1C) (Blommers, Pieles *et al.* 1994); sulfone and sulfonamide linkages (Fig. 1D, R_1, R_2 = CH_2) (Roughten, 1995; McElroy, 1994); and a thioformacetyl linkage (Fig. 1E) (Matteucci, 1990; Cross, 1997). The latter is reported to have enhanced duplex and triplex stability with respect to phosphorothioate antisense compounds (Cross, 1997). Also reported are the 3'-methylene-N-methylhydroxyamino compounds of the structure in Fig. 1F (Mohan, 1995).

Peptide nucleic acids (PNAs) (Fig. 1G) are analogs of DNA in which the backbone is structurally homomorphous with a deoxyribose backbone, consisting of N-(2-aminoethyl) glycine units to which pyrimidine or purine bases are attached. PNAs containing natural pyrimidine and purine bases hybridize to complementary oligonucleotides obeying Watson-Crick base-pairing rules, and mimic DNA in terms of base pair recognition (Egholm *et al.*, 1993). The backbone of PNAs are formed by peptide bonds rather than phosphodiester bonds, making them well-suited for antisense applications. The backbone is uncharged, resulting in PNA/DNA or PNA/RNA duplexes which exhibit greater than normal thermal stability. PNAs are not recognized by nucleases or proteases.

A preferred oligomer structure employs morpholino-based subunits bearing base-pairing moieties, joined by uncharged linkages, as described above. Especially preferred is a substantially uncharged phosphorodiamidate-linked morpholino oligomer, such as

illustrated in Figs. 2A-2D. Morpholino oligonucleotides, including antisense oligomers, are detailed, for example, in co-owned U.S. Patent Nos. 5,698,685, 5,217,866, 5,142,047, 5,034,506, 5,166,315, 5,185, 444, 5,521,063, and 5,506,337.

Important properties of the morpholino-based subunits include: the ability to be linked in a oligomeric form by stable, uncharged backbone linkages; the ability to support a nucleotide base (*e.g.* adenine, cytosine, guanine or uracil) such that the polymer formed can hybridize with a complementary-base target nucleic acid, including target RNA, with high T_m, even with oligomers as short as 10-14 bases; the ability of the oligomer to be actively transported into mammalian cells; and the ability of the oligomer:RNA heteroduplex to resist RNAse degradation.

Exemplary backbone structures for antisense oligonucleotides of the invention include the β-morpholino subunit types shown in Figs. 2A-2D, each linked by an uncharged, phosphorus-containing subunit linkage. Fig. 2A shows a phosphorus-containing linkage which forms the five atom repeating-unit backbone, where the morpholino rings are linked by a 1-atom phosphoamide linkage. Fig. 2B shows a linkage which produces a 6-atom repeating-unit backbone. In this structure, the atom Y linking the 5' morpholino carbon to the phosphorus group may be sulfur, nitrogen, carbon or, preferably, oxygen. The X moiety pendant from the phosphorus may be fluorine, an alkyl or substituted alkyl, an alkoxy or substituted alkoxy, a thioalkoxy or substituted thioalkoxy, or unsubstituted, monosubstituted, or disubstituted nitrogen, including cyclic structures, such as morpholines or piperidines. Alkyl, alkoxy and thioalkoxy preferably include 1-6 carbon atoms. The Z moieties are sulfur or oxygen, and are preferably oxygen.

The linkages shown in Fig. 2C and 2D are designed for 7-atom unit-length backbones. In Structure 3C, the X moiety is as in Structure 3B, and the moiety Y may be methylene, sulfur, or, preferably, oxygen. In Structure 2D, the X and Y moieties are as in Structure 2B. Particularly preferred morpholino oligonucleotides include those composed of morpholino subunit structures of the form shown in Fig. 2B, where $X=NH_2$ or $N(CH_3)_2$, Y=O, and Z=O.

As noted above, the substantially uncharged oligomer may advantageously include a limited number of charged linkages, *e.g.* up to about 1 per every 5 uncharged linkages,

more preferably up to about 1 per every 10 uncharged linkages. Therefore a small number of charged linkages, *e.g.* charged phosphoramidate or phosphorothioate, may also be incorporated into the oligomers.

The antisense compounds can be prepared by stepwise solid-phase synthesis, employing methods detailed in the references cited above. In some cases, it may be desirable to add additional chemical moieties to the antisense compound, *e.g.* to enhance pharmacokinetics or to facilitate capture or detection of the compound. Such a moiety may be covalently attached, typically to a terminus of the oligomer, according to standard synthetic methods. For example, addition of a polyethyleneglycol moiety or other hydrophilic polymer, *e.g.*, one having 10-100 monomeric subunits, may be useful in enhancing solubility. One or more charged groups, *e.g.*, anionic charged groups such as an organic acid, may enhance cell uptake. A reporter moiety, such as fluorescein or a radiolabeled group, may be attached for purposes of detection. Alternatively, the reporter label attached to the oligomer may be a ligand, such as an antigen or biotin, capable of binding a labeled antibody or streptavidin. In selecting a moiety for attachment or modification of an antisense oligomer, it is generally of course desirable to select chemical compounds of groups that are biocompatible and likely to be tolerated by a subject without undesirable side effects.

V. Inhibition of Viral Replication

The antisense compounds detailed above are useful in inhibiting replication of ssRNA viruses of the Flaviviridae, Picornoviridae, Caliciviridae, Togaviridae, Arteriviridae, Coronaviridae, Astroviridae and Hepeviridae virus families. In one embodiment, such inhibition is effective in treating infection of a host animal by these viruses. Accordingly, the method comprises, in one embodiment, contacting a cell infected with the virus with an antisense agent effective to inhibit the replication of the specific virus. In this embodiment, the antisense agent is administered to a mammalian subject, *e.g.*, human or domestic animal, infected with a given virus, in a suitable pharmaceutical carrier. It is contemplated that the antisense oligonucleotide arrests the growth of the RNA virus in the host. The RNA virus may be decreased in number or eliminated with little or no detrimental effect on the normal growth or development of the

host.

A. Identification of the Infective Agent

The specific virus causing the infection can be determined by methods known in the art, *e.g.* serological or cultural methods, or by methods employing the antisense oligomers of the present invention.

Serological identification employs a viral sample or culture isolated from a biological specimen, *e.g.*, stool, urine, cerebrospinal fluid, blood, *etc.*, of the subject. Immunoassay for the detection of virus is generally carried out by methods routinely employed by those of skill in the art, *e.g.*, ELISA or Western blot. In addition, monoclonal antibodies specific to particular viral strains or species are often commercially available.

Culture methods may be used to isolate and identify particular types of virus, by employing techniques including, but not limited to, comparing characteristics such as rates of growth and morphology under various culture conditions.

Another method for identifying the viral infective agent in an infected subject employs one or more antisense oligomers targeting broad families and/or genera of viruses, *e.g.*, Picornaviridae, Caliciviridae, Togaviridae and Flaviviridae. Sequences targeting any characteristic viral RNA can be used. The desired target sequences are preferably (i) common to broad virus families/genera, and (ii) not found in humans. Characteristic nucleic acid sequences for a large number of infectious viruses are available in public databases, and may serve as the basis for the design of specific oligomers.

For each plurality of oligomers, the following steps are carried out: (a) the oligomer(s) are administered to the subject; (b) at a selected time after said administering, a body fluid sample is obtained from the subject; and (c) the sample is assayed for the presence of a nuclease-resistant heteroduplex comprising the antisense oligomer and a complementary portion of the viral genome. Steps (a) – (c) are carried for at least one such oligomer, or as many as is necessary to identify the virus or family of viruses. Oligomers can be administered and assayed sequentially or, more conveniently, concurrently. The virus is identified based on the presence (or absence) of a heteroduplex comprising the antisense oligomer and a complementary portion of the viral genome of the given known virus or family of viruses.

Preferably, a first group of oligomers, targeting broad families, is utilized first, followed by selected oligomers complementary to specific genera and/or species and/or strains within the broad family/genus thereby identified. This second group of oligomers includes targeting sequences directed to specific genera and/or species and/or strains within a broad family/genus. Several different second oligomer collections, *i.e.* one for each broad virus family/genus tested in the first stage, are generally provided. Sequences are selected which are (i) specific for the individual genus/species/strains being tested and (ii) not found in humans.

B. Administration of the Antisense Oligomer

Effective delivery of the antisense oligomer to the target nucleic acid is an important aspect of treatment. In accordance with the invention, routes of antisense oligomer delivery include, but are not limited to, various systemic routes, including oral and parenteral routes, *e.g.*, intravenous, subcutaneous, intraperitoneal, and intramuscular, as well as inhalation, transdermal and topical delivery. The appropriate route may be determined by one of skill in the art, as appropriate to the condition of the subject under treatment. For example, an appropriate route for delivery of an antisense oligomer in the treatment of a viral infection of the skin is topical delivery, while delivery of an antisense oligomer for the treatment of a viral respiratory infection is by inhalation. The oligomer may also be delivered directly to the site of viral infection, or to the bloodstream.

The antisense oligomer may be administered in any convenient vehicle which is physiologically acceptable. Such a composition may include any of a variety of standard pharmaceutically accepted carriers employed by those of ordinary skill in the art. Examples include, but are not limited to, saline, phosphate buffered saline (PBS), water, aqueous ethanol, emulsions, such as oil/water emulsions or triglyceride emulsions, tablets and capsules. The choice of suitable physiologically acceptable carrier will vary dependent upon the chosen mode of administration.

In some instances, liposomes may be employed to facilitate uptake of the antisense oligonucleotide into cells. (See, *e.g.*, Williams, S.A., Leukemia 10(12):1980-1989, 1996; Lappalainen *et al.*, Antiviral Res. 23:119, 1994; Uhlmann *et al.*, ANTISENSE OLIGONUCLEOTIDES: A NEW THERAPEUTIC PRINCIPLE, Chemical Reviews, Volume 90, No. 4, pages 544-584, 1990; Gregoriadis, G., Chapter 14, Liposomes, Drug Carriers in

Biology and Medicine, pp. 287-341, Academic Press, 1979). Hydrogels may also be used as vehicles for antisense oligomer administration, for example, as described in WO 93/01286. Alternatively, the oligonucleotides may be administered in microspheres or microparticles. (See, *e.g.*, Wu, G.Y. and Wu, C.H., J. Biol. Chem. 262:4429-4432, 1987). Alternatively, the use of gas-filled microbubbles complexed with the antisense oligomers can enhance delivery to target tissues, as described in US Patent No. 6,245,747.

Sustained release compositions may also be used. These may include semipermeable polymeric matrices in the form of shaped articles such as films or microcapsules.

In one aspect of the method, the subject is a human subject, *e.g.*, a patient diagnosed as having a localized or systemic viral infection. The condition of a patient may also dictate prophylactic administration of an antisense oligomer of the invention, *e.g.* in the case of a patient who (1) is immunocompromised; (2) is a burn victim; (3) has an indwelling catheter; or (4) is about to undergo or has recently undergone surgery. In one preferred embodiment, the oligomer is a phosphorodiamidate morpholino oligomer, contained in a pharmaceutically acceptable carrier, and is delivered orally. In another preferred embodiment, the oligomer is a phosphorodiamidate morpholino oligomer, contained in a pharmaceutically acceptable carrier, and is delivered intravenously (i.v.).

In another application of the method, the subject is a livestock animal, *e.g.*, a chicken, turkey, pig, cow or goat, etc, and the treatment is either prophylactic or therapeutic. The invention also includes a livestock and poultry food composition containing a food grain supplemented with a subtherapeutic amount of an antiviral antisense compound of the type described above. Also contemplated is, in a method of feeding livestock and poultry with a food grain supplemented with subtherapeutic levels of an antiviral, an improvement in which the food grain is supplemented with a subtherapeutic amount of an antiviral oligonucleotide composition as described above.

The antisense compound is generally administered in an amount and manner effective to result in a peak blood concentration of at least 200-400 nM antisense oligomer. Typically, one or more doses of antisense oligomer are administered, generally at regular intervals, for a period of about one to two weeks. Preferred doses for oral administration are from about 1-100 mg oligomer per 70 kg. In some cases, doses of greater than 100 mg oligomer/patient may be necessary. For i.v. administration, preferred doses are from

about 0.5 mg to 100 mg oligomer per 70 kg. The antisense oligomer may be administered at regular intervals for a short time period, *e.g.*, daily for two weeks or less. However, in some cases the oligomer is administered intermittently over a longer period of time. Administration may be followed by, or concurrent with, administration of an antibiotic or other therapeutic treatment. The treatment regimen may be adjusted (dose, frequency, route, *etc.*) as indicated, based on the results of immunoassays, other biochemical tests and physiological examination of the subject under treatment.

C. Monitoring of Treatment

An effective *in vivo* treatment regimen using the antisense oligonucleotides of the invention may vary according to the duration, dose, frequency and route of administration, as well as the condition of the subject under treatment (*i.e.*, prophylactic administration versus administration in response to localized or systemic infection). Accordingly, such *in vivo* therapy will often require monitoring by tests appropriate to the particular type of viral infection under treatment, and corresponding adjustments in the dose or treatment regimen, in order to achieve an optimal therapeutic outcome. Treatment may be monitored, *e.g.*, by general indicators of infection, such as complete blood count (CBC), nucleic acid detection methods, immunodiagnostic tests, viral culture, or detection of heteroduplex.

The efficacy of an *in vivo* administered antisense oligomer of the invention in inhibiting or eliminating the growth of one or more types of RNA virus may be determined from biological samples (tissue, blood, urine *etc.*) taken from a subject prior to, during and subsequent to administration of the antisense oligomer. Assays of such samples include (1) monitoring the presence or absence of heteroduplex formation with target and non-target sequences, using procedures known to those skilled in the art, *e.g.*, an electrophoretic gel mobility assay; (2) monitoring the amount of viral protein production, as determined by standard techniques such as ELISA or Western blotting, or (3) measuring the effect on viral titer, *e.g.* by the method of Spearman-Karber. (See, for example, Pari, G.S. *et al.*, Antimicrob. Agents and Chemotherapy 39(5):1157-1161, 1995; Anderson, K.P. *et al.*, Antimicrob. Agents and Chemotherapy 40(9):2004-2011, 1996, Cottral, G.E. (ed) in: Manual of Standard Methods for Veterinary Microbiology, pp.60-93, 1978).

A preferred method of monitoring the efficacy of the antisense oligomer treatment is by detection of the antisense-RNA heteroduplex. At selected time(s) after antisense oligomer administration, a body fluid is collected for detecting the presence and/or measuring the level of heteroduplex species in the sample. Typically, the body fluid sample is collected 3-24 hours after administration, preferably about 6-24 hours after administering. As indicated above, the body fluid sample may be urine, saliva, plasma, blood, spinal fluid, or other liquid sample of biological origin, and may include cells or cell fragments suspended therein, or the liquid medium and its solutes. The amount of sample collected is typically in the 0.1 to 10 ml range, preferably about 1 ml or less.

The sample may be treated to remove unwanted components and/or to treat the heteroduplex species in the sample to remove unwanted ssRNA overhang regions, *e.g.* by treatment with RNase. It is, of course, particularly important to remove overhang where heteroduplex detection relies on size separation, *e.g.*, electrophoresis of mass spectroscopy.

A variety of methods are available for removing unwanted components from the sample. For example, since the heteroduplex has a net negative charge, electrophoretic or ion exchange techniques can be used to separate the heteroduplex from neutral or positively charged material. The sample may also be contacted with a solid support having a surface-bound antibody or other agent specifically able to bind the heteroduplex. After washing the support to remove unbound material, the heteroduplex can be released in substantially purified form for further analysis, *e.g.*, by electrophoresis, mass spectroscopy or immunoassay.

VI. Heteroduplex complex

In another aspect, the invention includes a heteroduplex complex formed between:

(a) a region within the 5'-terminal 40 bases of the positive strand RNA of an RNA virus having a single-stranded, positive-sense RNA genome and selected from one of the Flaviviridae, Picornoviridae, Caliciviridae, Togaviridae, Arteriviridae, Coronaviridae, Astroviridae or Hepeviridae families, which region is capable of forming internal stem-loop secondary structure, and

(b) an oligonucleotide analog compound characterized by:

(i) a nuclease-resistant backbone,

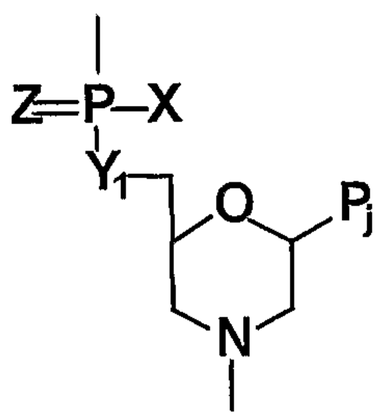
(ii) capable of uptake by mammalian host cells,

(iii) containing between 12-40 nucleotide bases,

(iv) having a targeting sequence of at least 12 subunits that is complementary to a region associated with such stem-loop secondary structure within the 5'-terminal end 40 bases of the positive-sense RNA strand of the virus,

where said heteroduplex complex has a Tm of dissociation of at least 45 °C and disruption of such stem-loop secondary structure.

An exemplary compound is composed of morpholino subunits linked by uncharged, phosphorus-containing intersubunit linkages, joining a morpholino nitrogen of one subunit to a 5' exocyclic carbon of an adjacent subunit. The compound may have phosphorodiamidate linkages, such as in the structure



where Y_1=O, Z=O, Pj is a purine or pyrimidine base-pairing moiety effective to bind, by base-specific hydrogen bonding, to a base in a polynucleotide, and X is alkyl, alkoxy, thioalkoxy, or alkyl amino. In a preferred compound, X=NR_2, where each R is independently hydrogen or methyl. The compound may be the oligonucleotide analog alone or a conjugate of the analog and an arginine-rich polypeptide capable of enhancing the uptake of the compound into host cells.

In one embodiment, the compound is effective, when administered to the host cells, to form a heteroduplex structure (i) composed of the positive sense strand of the virus and the oligonucleotide compound, and (ii) characterized by a Tm of dissociation of at least 45 °C and disruption of such stem-loop secondary structure.

EXAMPLES

The following examples illustrate but are not intended in any way to limit the invention.

Materials and Methods

Standard recombinant DNA techniques were employed in all constructions, as described in Ausubel, FM *et al.*, in CURRENT PROTOCOLS IN MOLECULAR BIOLOGY, John Wiley and Sons, Inc., Media, PA, 1992 and Sambrook, J. *et al.*, in MOLECULAR CLONING: A LABORATORY MANUAL, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY, Vol. 2, 1989).

All peptides were custom synthesized by Global Peptide Services (Ft. Collins, CO) or at AVI BioPharma (Corvallis, OR) and purified to >90% purity (see Example 2 below). PMOs were synthesized at AVI BioPharma in accordance with known methods, as described, for example, in (Summerton and Weller 1997) and U.S. Patent No. 5,185,444.

PMO oligomers were conjugated at the 5' end with an arginine-rich peptide (R_9F_2C-5'-PMO) to enhance cellular uptake as described (US Patent Application 60/466,703 and (Moulton, Nelson *et al.* 2004).

Example 1: Antisense Inhibition of Flaviviridae (yellow fever virus) *in vitro*

Although an effective vaccine for yellow fever virus (YFV) has been available for many years, this virus continues to be a leading cause of hemorrhagic fever with mortality rates as high as 50%. Worldwide, there are 200,000 estimated cases of yellow fever (with 30,000 deaths) annually. Small numbers of imported cases also occur in countries free of yellow fever (WHO, Fact Sheet 100, 2001).

A PMO antisense oligomer targeted to the 5' positive strand terminus of YFV (SEQ ID NO:49) was evaluated in a 4-concentration test. The standard CPE test used an 18 h monolayer (80-100% confluent) of Vero cells, medium was drained and each of the concentrations of PMO or scramble control sequence was added, followed within 15 min by virus or virus diluent. Two wells are used for each concentration of compound for both antiviral and cytotoxicity testing. The plate was sealed and incubated the standard time period required to induce near-maximal viral CPE. The plate was then stained with neutral red by the method described below and the percentage of uptake indicating viable

cells read on a microplate autoreader at dual wavelengths of 405 and 540 nm, with the difference taken to eliminate background. An approximated virus-inhibitory concentration, 50% endpoint (EC50) and cell-inhibitory concentration, 50% endpoint (IC50) was determined from which a general selectivity index (S.I.)was calculated: S.I. = (IC50) / (EC50). An SI of 3 or greater indicates significant antiviral activity. The PMO targeting the 5' positive-strand terminal region (SEQ ID NO:49) produced an SI of 21 in this assay.

Example 2: Antisense Inhibition of Flaviviridae (Dengue virus Serotypes 1-4) *in vitro*

Dengue Fever/Dengue Hemorrhagic Fever (DF/DHF) has become a major global health problem over the past 20 years. Geographic distribution of the dengue virus (DEN), its mosquito vectors and the disease burden it causes continue to increase. The World Health Organization estimates that there are 50-100 million new infections yearly. DF/DHF is now a leading cause of hospitalization and death among children in southern Asia, and its incidence is sharply rising in the Americas. There is currently no vaccine or effective therapeutic. One requirement of a successful vaccine or therapeutic is that it be effective against all 4 human serotypes of DEN. The purpose of this study was to evaluate the efficacy and specificity of PMO that target the 5' positive-strand terminal stem loop at inhibiting the replication of four serotypes of DEN in Vero cells in culture. The PMO was designed to target the sequence element in the positive-strand DEN2 RNA that may be important in viral transcription and/or translation (Markoff 2003). The PMO in this study were conjugated to an arginine-rich peptide in order to facilitate entry into Vero E6 cells (Moulton, Nelson *et al.* 2004; Neuman, Stein *et al.* 2004).

A PMO, 5'SL, (SEQ ID NO:51) designed to hybridize to the 5' positive strand terminal region of Dengue 2 virus (DEN2), were evaluated for their ability to inhibit Dengue virus replication in mammalian cell culture. The PMOs were conjugated to a short arginine-rich peptide (R_9F_2C-5'-PMO) to facilitate their entry into cells in culture. Vero E6 cells were incubated with the PMO agents, inoculated with DEN serotypes 1-4 (DEN1, DEN2, DEN3, DEN4, respectively), and viral titer determined by plaque-assay 5-8 days later. The compound targeting the 5' positive strand terminus (5'SL) reduced the titer of DEN2 by over 4 orders of magnitude, compared to controls, in a dose-dependent

and sequence-specific manner over a 4 day period as shown in Fig. 5A. Ten μM solutions of the 5'SL PMO reduced the titer of all four Dengue serotypes by over two to four orders of magnitude, in some cases below detectable limits as shown in Figs. 5A-5B. The 5'SL PMO was less effective against DEN4 (two log reduction) than it was against DEN1, DEN2 and DEN3 (four log reductions) due to a two base pair mismatch between the 5'SL PMO and it's target sequence in DEN4. The effective anti-DEN compounds did not alter the titer of West Nile Virus (WNV) grown in Vero E6 cells. This data indicates that the 5'SL PMO compound is a potential DEN 1-4 therapeutic.

Example 3: Antisense Inhibition of Coronaviridae (Porcine Reproductive and Respiratory Syndrome Virus, PRRSV) *in vitro*

Porcine reproductive and respiratory syndrome (PRRS) is a contagious viral disease that is characterized by reproductive failure in sows and respiratory disease in young pigs. The causative agent, PRRSV, is a single-stranded RNA virus with genome organization similar to that of other members of the Coronaviridae. PRRS causes heavy economic losses to the swine industry though a vaccine has been widely used for years. Specific anti-PRRSV drugs are urgently needed as one of the integrated strategies to prevent and control PRRSV infection. A PMO (PRRSV-1a, SEQ ID NO:109) that targets the 5' positive strand terminal region of PRRSV was tested for its ability to inhibit viral replication as described below.

The first test was designed to determine whether the PRRSV-1a PMO could inhibit the development of virus-induced, cell pathogenic effect (CPE). ATCC CRL11171 cells were used for this experiment as previously reported. The CRL11171 monolayer cells were treated with the PRRSV-1a PMO (SEQ ID NO:109) in DMEM for 4 h at 37°C. The PMOs were removed from the cells and inoculated with PRRSV strain VR2385 at a multiplicity of infection (MOI) of one. The cells were cultured and observed daily for CPE development. A blank control and the control PMO (DSscr, a scramble sequence PMO) were included as negative controls. The cell culture medium was also collected and titrated in CRL11171 cells to determine PRRSV titer.

The PRRSV-1a PMO targeting the 5' positive-strand end-terminus of the untranslated region (UTR), was found to be effective in inhibiting PRRSV replication (Fig.

6). The cells treated with PRRSV-1a PMO at 16 μM had much less cell pathogenic effect (CPE) development than controls (Fig. 7). CPE is clearly visible after PRRSV infection (positive), while uninfected control cells remain an intact monolayer (blank). PMO PRRSV-1a reduced CPE development, while other PMOs including control PMO did not have much effect in blocking CPE. The cells and medium were harvested for titration of PRRSV yield. Tissue culture infectious dose (TCID50) was calculated based on CPE development of different dilutions. PRRSV-1a reduced virus yield by more than 90% (not shown).

The inhibition of PRRSV replication by the PRRSV-1a PMO was also shown to be dose-dependent. Using the PMO-treatment and virus culture conditions described above, three different concentrations of PRRSV-1a and DSscr control PMO (4, 8 and 16 mM) were tested on PRRSV-infected CRRL11171 cells for the ability to inhibit viral replication as measured by viral titer. As shown in Fig. 6, the PRRSV-1a PMO inhibits PRRSV replication in a dose-dependent manner.

Example 4: Antisense Inhibition of Tick Borne Encephalitis Virus

This example describes a study that was devised to test the antiviral activity of antisense PMO compounds of the present invention against two flaviviruses; Tick Borne Encephalitis virus (TBE) and West Nile virus (WNV). Two PMO oligomers were evaluated for antiviral activity; TBE 5'SL, SEQ ID NO:57 and; a scramble control sequence DSscr (5'-AGTCTCGACTTGCTACCTCA-3' SEQ ID NO:133). Both PMO oligomers were conjugated at the 5' end with an arginine-rich peptide (R_9F_2C-5'-PMO) to enhance cellular uptake as described (US Patent Application 60/466,703 and (Moulton, Nelson *et al.* 2004). The WNV infection provided a negative control infection as there is no homology between WNV and the TBE 5'SL targeting PMO. This control indicates the level of non-specific viral suppression of each of the PMOs. The PMO compounds were prepared to provide a 2mM stock solution, which were then titrated against a standard dose of virus on tissue culture cells. Cells were infected with a multiplicity of infection (MOI) of 1 and the virus yield was assessed in samples of supernatant medium taken at 18 hours post infection.

The two virus strains used in this example:

1) TC 401 West Nile 99-34940-31A (New York strain) Passage 2

2) TC 339 Tick Borne Encephalitis virus (Hypr strain) Passage 49

Four T175 tissue culture flasks (NUNC) of SW 13 cells (human caucasian adrenal cortex adenocarcinoma cell line ECAAC 87031801 grown in RPMI 1640 medium plus 5% FBS) at passage 130 were washed twice with trypsin-EDTA (1X) and incubated for 2-3 minutes at 37°C. The cells were resuspended in 11.5 ml growth medium per flask and pooled. A cell count was performed on the pooled cell suspension and the result was 1.74 x 10^6 cells/ml with 99% viability. Six mls of the cell suspension was used to seed four T175 flasks and 40 ml of the cell suspension was diluted to 270 ml. This was dispensed in 3 ml aliquots per well in 15 six-well plates. The plates were incubated overnight to form confluent cell monolayers.

Each of the PMO compounds was diluted to 25, 20, 15, 10 and 5 μM in 4 ml serum-free RPMI 1640 medium. The medium was removed from the wells of two six-well plates. 2 ml of the appropriate compound dilution was dispensed in all wells of a plate and this was repeated on separate plates for both PMO compounds. The plates were incubated at 37°C for 5 hours. The two viruses were removed from the -70°C freezer and thawed rapidly. Each virus was diluted to 2 x 10^6pfu/ml to produce 42 ml serum-free medium. The six-well plates were removed from the incubator and the pre-treatment medium aspirated from all the wells. 1 ml of medium was added to each well of the control plate (no compound). Each set of plates received 1 ml/well of either TBE or WN diluted to 2 x 10^6pfu/ml. The plates were incubated at room temperature for 1 hour and the medium was then removed and replaced with 2 ml RPMI 1640 plus 1% FBS plus the same concentration of test compound as used to pre-treat the cells. The plates were incubated at 37°C for 18 hours.

To prepare 24 well plates for determining virus titers, eight T175 tissue culture flasks (NUNC) of SW 13 cells at passage 131 were washed twice with trypsin-EDTA (1X) and incubated for 2-3 minutes at 37°C. The cells were resuspended in 11.5 ml growth medium per flask and pooled. A cell count was performed on the pooled cell suspension and the result was 1.7 x 10^6 cells/ml with 99% viability. 80 ml of the cell suspension was diluted to 680 ml. These cells were dispensed as 1 ml per well aliquots in eight 24-well plates. The plates were incubated overnight to form confluent monolayers.

At 18 hours post-infection the supernatant media from the PMO-treated, virus-infected six-well plates were harvested from each individual wells. Thirty microliters of each harvest was placed in a single cup of a 96-well plate with 270 microliters serum-free medium. The remainder of the sample was placed in cryotube and stored at -70°C. The medium was removed from the 24-well plates and 250 µl of the titration dilutions were transferred from the 96-well plates to the 24 well plates which were incubated at 37°C for one hour. One ml agarose overlay medium was added to each well and after allowing the agarose to set at room temperature the plates were incubated at 37°C for 5 days. After 5 days the plates were removed from the incubator, 1 ml 10% Formol saline was added to each well and the plates were left at room temperature for 3 hours. The plates were washed under running water to remove the agarose medium and left to drain inverted while the remaining plates were washed. Each well then received 1 ml of 0.1% Naphthalene black stain and the plates were left for 30 minutes before the stain was removed and the plates washed under running water. They were then left to dry (inverted) for 3 hours. Viral plaques were counted to determine the titer.

Fig. 8 shows the viral titer obtained from the PMO-treated infections as a percentage of untreated control, with virus-infected cells infected with either TBEV or WNV and treated with either the TBEV antisense compound where the PMO compound is either 5'SL (SEQ ID NO:57) or control PMO (DSscr, a scrambled base sequence). As seen from a comparison of the viral titers in Fig. 8, significant there is a reduction in viral titre in all cells (treated and control) with increasing concentrations of compound, thought to be due to a cell-toxicity effect of the attached arginine-rich peptide present in both antisense and control compounds. However, at compound concentrations of 5 µM and above, there is seen a sequence-specific increase in TBEV inhibition, both relative to WNV (Fig. 8), and relative to the DSscr scrambled control sequence.

Example 5: Effect of PMO on West Nile Virus (WNV) Infection in Mice

PMOs are uncharged, water-soluble, nuclease-resistant antisense agents that are typically synthesized to a length of about 20 subunits and contain purine and pyrimidine bases attached to a backbone composed of morpholine rings joined by phosphorodiamidate intersubunit linkages. In experiments in support of the invention, it

was shown that conjugation of an Arg-rich peptide (designated as P007; SEQ ID NO:122) to the 5'-end of the PMOs greatly facilitates the delivery of the PMO into cultured cells. P007-PMOs targeting different regions of the viral genome have inhibited WNV virus infection to various degrees. Among them, PMOs targeting the 5'-terminal 20 nucleotides (5'End; SEQ ID NO:47) showed potent antiviral activity.

Experiments were conducted in mice to extend the observations to *in vivo* conditions. Female BALB/c mice were used. The mice were obtained from Simonsen Laboratories (Gilroy, CA). At the time that the experiment was started, the animals had been in the animal facility for one week and they were greater than 6 weeks of age. The mice weighed 12.3 to 19.8 g with an average of 16.1 g. Experiments were conducted in the BSL-3 animal suite at Utah State University Laboratory Animal Research Center (LARC). Two PMO compounds were used: 1) a PMO targeting the 5' terminus (NG040006; SEQ ID NO:47) and; 2) the same PMO conjugated at its 5' end with the P007 arginine-rich peptide (SEQ ID NO:122) and named NG040005. NG040007 is an unconjugated, scramble control PMO. Ampligen™ was used as a positive control, antiviral compound and was obtained from William M. Mitchell (School of Medical Pathology, Vanderbilt University, Nashville, TN 37240). Since ampligen is an RNA-like molecule, care was used to prevent contamination with RNase by using RNase-free materials and DEPC-treated water.

Ten animals were randomly assigned to each treatment group, except for the placebo group 11, which had 20 animals. Intraperitoneal treatments were initiated 24 hours before subcutaneous WNV challenge. PMO treatments continued qd, -4 hours before viral challenge, 1, 2, 3, 4, 5 and 5 days post-viral injection (dpi). Ampligen was treated i.p., qd, -1, 1, 3, and 5 dpi. Dosages and treatment groups are indicated in the table below along with Survival and mean day to death (MDD). NG040005 and ampligen increased the MDD of WNV-infected mice as compared to the placebo control (Table 4) .

Table 4: Effect of PMOs on West Nile virus infection in mice

Animals: Female BALB/c mice, >6 wk old
Virus: West Nile virus, NY crow brain homogenate, $10^{6.3}$ infectious units, s.c. injection
Drug diluent: saline

Treatment schedule: qd, -1d,-4h, 1,2,3,4,5,6d
Treatment Route: i.p.
Duration of experiment: 21 days

Drug	Dose	Schedule	% survival (alive/total)	MDD[a] ± SD	Survival analysis[b]
NG040005	250 µg/inj	qd, -1d,-4h, 1,2,3,4,5,6d	50% (5/10)	14.0 ± 3.8***	P = 0.20
NG040006	750 µg/inj	qd, -1d,-4h, 1,2,3,4,5,6d	40% (4/10)	8.2 ± 1.0	P = 0.95
ampligen	14 mg/kg	qd, -1d,1,3,5d	80% (8/10)	13.5 ± 4.9	P ≥ 0.01**
placebo	--	qd, -1d,-4h, 1,2,3,4,5,6d	35% (7/20)	8.6 ± 1.3	--

[a] Mean day to death of mice dying prior to day 21. Student's t-test was used for analysis.
[b] Log-rank survival analysis.
Toxicity controls were not run in this first experiment because of limited amounts of compounds.
*P ≤ 0.05, **P ≤ 0.01, ***P ≤ 0.001 compared to placebo.

Table 5. Sequence Listing Table

SEQ ID NO	Sequence, 5' to 3'
1	GNNGATGTTCGCGTCGGTGAGCGGAGAGGAAACAGATTTC
2	AGAAGTTTATCTGTGTGAACTTCTTGGCTTAGTATCGTTG
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7	GCCAGCCCCCTGATGGGGGCGACACTCCACCATGAATCAC
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47	GCTCACACAGGCGAACTACT
48	TAAGTTTGTCAGCTCACACAG
49	CAATTAGCACACAGGATTTACT
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Name	Peptide Sequences (NH_2 to COOH)	SEQ ID NO
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P007	$(RAhxR)_4$AhxβAla	122
P008	$(RAhx)_8$βAla	123
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RXR2	$(RAhxR)_2$AhxβAla	125
RXR3	$(RAhxR)_3$AhxβAla	126

SEQ ID NO	Sequence, 5' to 3'
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129	GCTCACACAGGCGA
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132	CAATGCACCTCAATTAGC
133	AGTCTCGACTTGCTACCTCA

UNITED STATES PATENT AND TRADEMARK OFFICE
DOCUMENT CLASSIFICATION BARCODE SHEET

New International Application

Claim(s)

7

Index 1.1.5.2
Version 1.0
Rev 12/06/01

Section

DEMANDES OU BREVETS VOLUMINEUX

LA PRÉSENTE PARTIE DE CETTE DEMANDE OU CE BREVETS COMPREND PLUS D'UN TOME.

CECI EST LE TOME __1___ DE __2___

NOTE: Pour les tomes additionels, veillez contacter le Bureau Canadien des Brevets.

JUMBO APPLICATIONS / PATENTS

THIS SECTION OF THE APPLICATION / PATENT CONTAINS MORE THAN ONE VOLUME.

THIS IS VOLUME __1___ OF __2___

NOTE: For additional volumes please contact the Canadian Patent Office.

CLAIMS:

1. A method of producing an anti-viral compound that inhibits the replication of an RNA virus comprising a single-stranded, positive-sense genome selected from Flaviviridae and Coronaviridae virus, said method comprising:

synthesizing an oligonucleotide analog compound characterized by:

(i) a nuclease-resistant backbone,

(ii) uptake by mammalian host cells,

(iii) containing from 12-40 nucleotide bases,

(iv) comprising a targeting nucleic acid sequence of at least 12 subunits that is complementary to a region within the 5'-terminal 40 bases of the positive strand viral genome that forms an internal stem-loop secondary structure, and

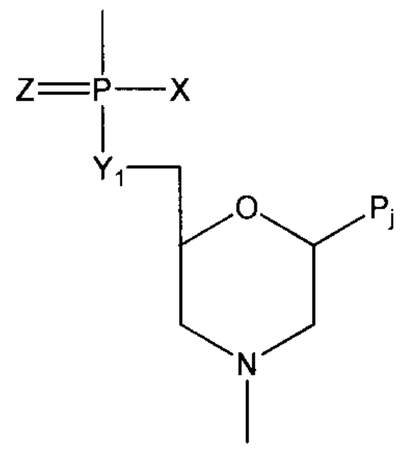
(v) formation of a heteroduplex structure with a viral target nucleic acid that disrupts the internal stem-loop secondary structure, wherein the heteroduplex structure is (a) composed of the virus positive-sense strand and the oligonucleotide analog compound, and (b) characterized by a Tm of dissociation of at least 45^0C.

2. The method of claim 1, further comprising an identifying step comprising (i) analyzing the 5'-terminal 40 bases of the positive strand of the selected virus by a computer program that performs secondary structure predictions based on a search for the minimal free energy state of an input RNA sequence, and (ii) identifying at least a portion of a viral target nucleic acid that forms an internal stem-loop secondary RNA structure.

3. The method of claim 1, wherein said oligonucleotide analog compound is composed of morpholino subunits linked by uncharged, phosphorus-containing intersubunit linkages, joining a morpholino nitrogen of one subunit to a 5' exocyclic carbon of an adjacent subunit.

4. The method of claim 3, wherein said intersubunit linkages are phosphorodiamidate linkages.

5. The method of claim 4, wherein said morpholino subunits are joined by phosphorodiamidate linkages, in accordance with the structure:



where Y_1=O, Z=O, Pj is a purine or pyrimidine base-pairing moiety effective to bind, by base-specific hydrogen bonding, to a base in a polynucleotide, and X is alkyl, alkoxy, thioalkoxy, or alkyl amino.

6. The method of claim 1, wherein the oligonucleotide analog compound is conjugated to an arginine-rich peptide to promote uptake of the compound into infected mammalian host cells.

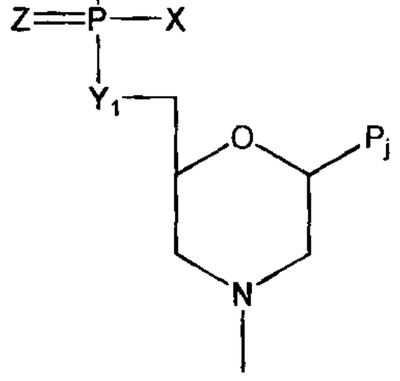
7. The method of claim 6, wherein the arginine rich peptide is selected from SEQ ID NOS: 121-126.

8. The oligonucleotide analog compound as defined in claim 1 for use in inhibiting replication of an RNA virus in a mammalian host cell, wherein the virus comprises a single-stranded, positive-sense genome selected from Flaviviridae and Coronaviridae virus.

9. The oligonucleotide analog compound of claim 8, which is composed of morpholino subunits linked by uncharged, phosphorus-containing intersubunit linkages, joining a morpholino nitrogen of one subunit to a 5' exocyclic carbon of an adjacent subunit.

10. The oligonucleotide analog compound of claim 9, wherein said intersubunit linkages are phosphorodiamidate linkages.

11. The oligonucleotide analog compound of claim 10, wherein said morpholino subunits are joined by phosphorodiamidate linkages, in accordance with the structure:



where Y_1=O, Z=O, Pj is a purine or pyrimidine base-pairing moiety effective to bind, by base-specific hydrogen bonding, to a base in a polynucleotide, and X is alkyl, alkoxy, thioalkoxy, or alkyl amino.

12. The oligonucleotide analog compound of claim 11, wherein X=NR_2, where each R is independently hydrogen or methyl.

13. The oligonucleotide analog compound of claim 8, wherein the oligonucleotide analog compound is covalently conjugated to an arginine-rich peptide to enhance uptake of the oligonucleotide analog compound into host cells.

14. The oligonucleotide analog compound of claim 13, wherein the arginine rich peptide is selected from SEQ ID NOS: 121-126.

15. The oligonucleotide analog compound of claim 8, for use in treating a mammalian subject infected with a Flaviviridae or Coronaviridae virus, or at risk of infection with a Flaviviridae or Coronaviridae virus, wherein said oligonucleotide analog compound is for intravenous or oral administration.

16. The oligonucleotide analog compound of claim 8, wherein the oligonucleotide analog compound is formulated as a cocktail comprising such oligonucleotide analog compounds directed against two or more of said positive-sense, single-strand RNA viruses.

17. A heteroduplex complex, comprising:

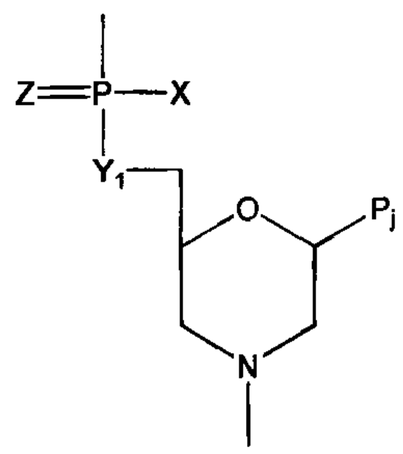
(a) a region within the 5'-terminal 40 bases of the positive strand RNA of an RNA virus that forms an internal stem-loop structure, wherein the 5'-terminal 40 bases are located in a single-stranded, positive-sense RNA genome selected from a Flaviviridae and Coronaviridae virus, and

(b) an oligonucleotide analog compound according to claim 1.

18. The complex of claim 17, wherein said oligonucleotide analog compound is composed of morpholino subunits linked by uncharged, phosphorus-containing intersubunit linkages, joining a morpholino nitrogen of one subunit to a 5' exocyclic carbon of an adjacent subunit.

19. The complex of claim 18, wherein said intersubunit linkages are phosphorodiamidate linkages.

20. The complex of claim 19, wherein said morpholino subunits are joined by phosphorodiamidate linkages, in accordance with the structure:



where Y_1=O, Z=O, Pj is a purine or pyrimidine base-pairing moiety effective to bind, by base-specific hydrogen bonding, to a base in a polynucleotide, and X is alkyl, alkoxy, thioalkoxy, or alkyl amino.

21. The complex of claim 20, wherein $X=NR_2$, where each R is independently hydrogen or methyl.

22. A method of detecting the presence of an RNA virus infection or a heteroduplex complex between an RNA virus and an antisense oligonucleotide, comprising:

(a) assaying a body fluid sample from a mammalian subject at a selected time after an uncharged morpholino oligonucleotide has been administered to the subject, wherein the sample is from a subject infected with an RNA virus comprising a single-stranded, positive-sense RNA genome selected from a Flaviviridae or Coronaviridae virus,

wherein the uncharged morpholino oligonucleotide is comprised of

(i) a nucleic acid of 12-40 subunits, including a targeting sequence of at least 12 subunits that is complementary to a region associated with an internal stem-loop secondary structure within the 5'-terminal end 40 bases of the positive-sense RNA strand of the virus, and

(ii) morpholino subunits linked by uncharged, phosphorus-containing intersubunit linkages, each linkage joining a morpholino nitrogen of one subunit to a 5' exocyclic carbon of an adjacent subunit, and

wherein the uncharged morpholino oligonucleotide forms a heteroduplex structure that disrupts the internal stem-loop secondary structure within the 5'-terminal end 40 bases of the positive-sense RNA strand of the virus genome when administered to the subject, wherein the heteroduplex is characterized by a Tm of dissociation of at least 45^0C; and

(b) detecting the presence of a nuclease-resistant heteroduplex comprising the antisense oligonucleotide and a complementary-sequence 5'-end region of the positive-strand RNA of the virus.

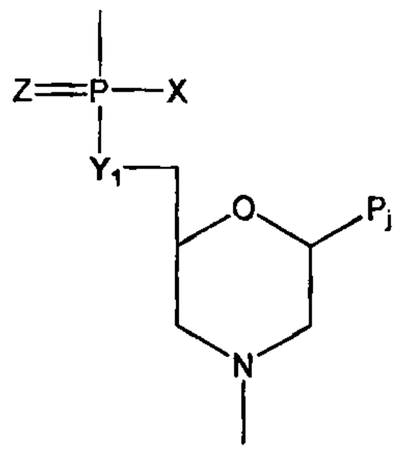
23. The method of claim 22, for use in determining the effectiveness of treating a Flaviviridae or Coronaviridae infection after the uncharged morpholino oligonucleotide has been administered to the subject, wherein said assaying, and detecting is to be conducted at periodic intervals throughout a treatment period.

24. An oligonucleotide analog compound according to claim 8, wherein the 5'-terminal 40 bases are selected from SEQ ID NOS:1-10, 29-34 and 37.

25. The oligonucleotide analog compound of claim 24, composed of morpholino subunits linked by uncharged, phosphorus-containing intersubunit linkages, joining a morpholino nitrogen of one subunit to a 5' exocyclic carbon of an adjacent subunit.

26. The oligonucleotide analog compound of claim 25, wherein said intersubunit linkages are phosphorodiamidate linkages.

27. The oligonucleotide analog compound of claim 26, wherein said morpholino subunits are joined by phosphorodiamidate linkages, in accordance with the structure:



where Y_1=O, Z=O, Pj is a purine or pyrimidine base-pairing moiety effective to bind, by base-specific hydrogen bonding, to a base in a polynucleotide, and X is alkyl, alkoxy, thioalkoxy, or alkyl amino.

28. The oligonucleotide analog compound of claim 27, wherein X=NR_2, where each R is independently hydrogen or methyl.

29. The oligonucleotide analog compound of claim 24, wherein said oligonucleotide analog compound has a T_m, with respect to binding to a viral target nucleic acid, of greater than 50°C, and said compound is actively taken up by mammalian cells.

30. The oligonucleotide analog compound of claim 24, wherein the oligonucleotide analog compound is covalently conjugated with an arginine-rich peptide to enhance uptake into host cells.

31. The oligonucleotide analog compound of claim 30, wherein the arginine rich peptide is selected from any one of SEQ ID NOS: 121-126.

32. The oligonucleotide analog compound of claim 24, for use in inhibiting replication of a Flaviviridae virus.

33. The oligonucleotide analog compound of claim 32, wherein the Flaviviridae virus is a West Nile virus, and the oligonucleotide analog compound comprises a targeting sequence of at least 12 subunits that is complementary to a region of SEQ ID NO:4.

34. The oligonucleotide analog compound of claim 32, wherein the Flaviviridae virus is a Yellow Fever virus, and the oligonucleotide analog compound comprises a targeting sequence of at least 12 subunits that is complementary to a region of SEQ ID NO:5.

35. The oligonucleotide analog compound of claim 32, wherein the Flaviviridae virus is a Dengue virus, and the oligonucleotide analog compound comprises a targeting sequence of at least 12 subunits that is complementary to a region of SEQ ID NO:6.

36. The oligonucleotide analog compound of claim 35, consisting of 22 nucleotide bases that are 100% complementary to a region of SEQ ID NO:6.

37. The oligonucleotide analog compound of claim 32, wherein the Flaviviridae virus is a Tick-borne encephalitis virus, and the oligonucleotide analog compound comprises a targeting sequence of at least 12 subunits that is complementary to a region of SEQ ID NO:8.

38. The oligonucleotide analog compound of claim 24, for use in inhibiting replication of a Coronaviridae virus.

39. The oligonucleotide analog compound of claim 38, wherein the Coronaviridae virus is a SARS coronavirus, and the oligonucleotide analog compound comprises a targeting sequence of at least 12 subunits that is complementary to a region of SEQ ID NO:29.

40. The oligonucleotide analog compound of claim 38, wherein the Coronaviridae virus is a human coronavirus 229E, and the oligonucleotide analog compound comprises a targeting sequence of at least 12 subunits that is complementary to a region of SEQ ID NO:33.

41. The oligonucleotide analog compound according to any one of claims 24-40 for use in treating RNA virus infection in a host, wherein the RNA virus comprises a single-stranded, positive-sense genome selected from a Flaviviridae or Coronaviridae virus.

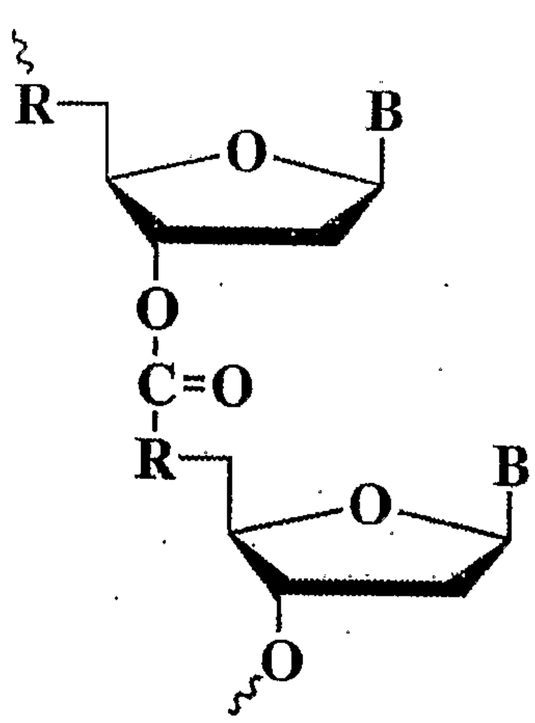


Fig. 1A

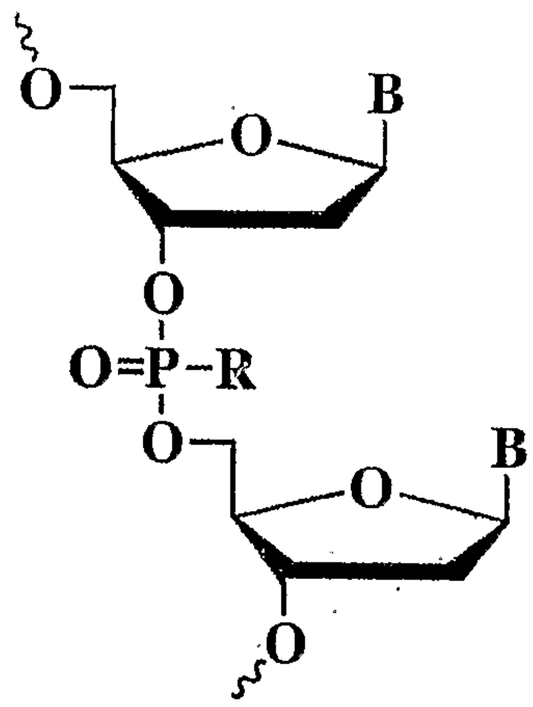


Fig. 1B

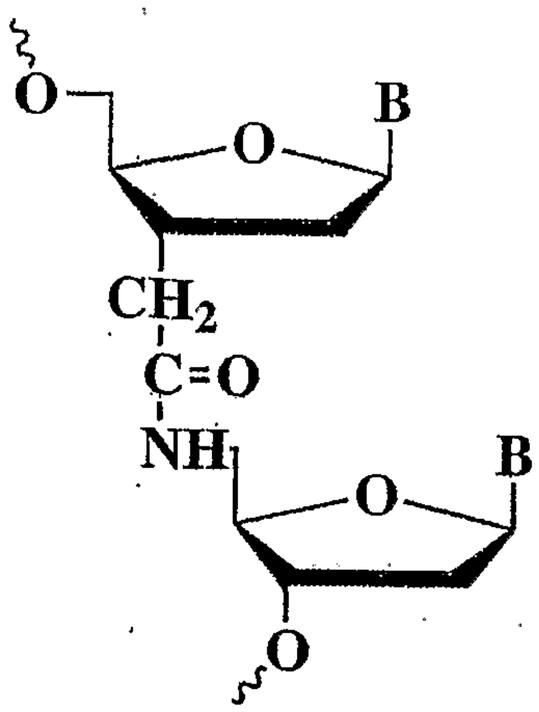


Fig. 1C

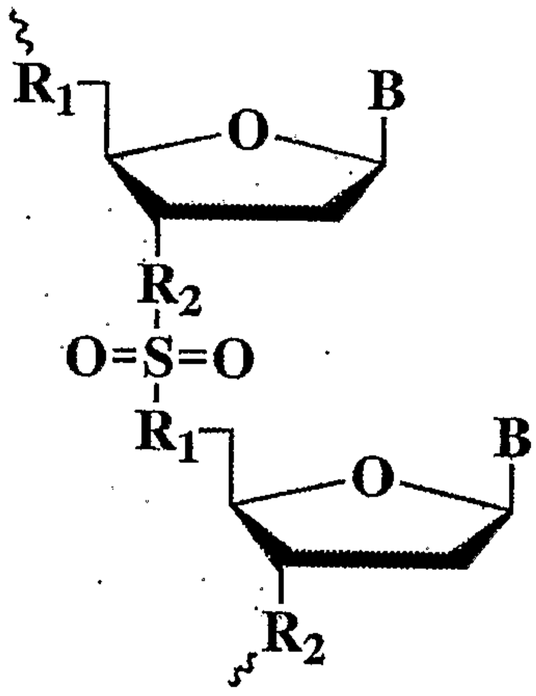


Fig. 1D

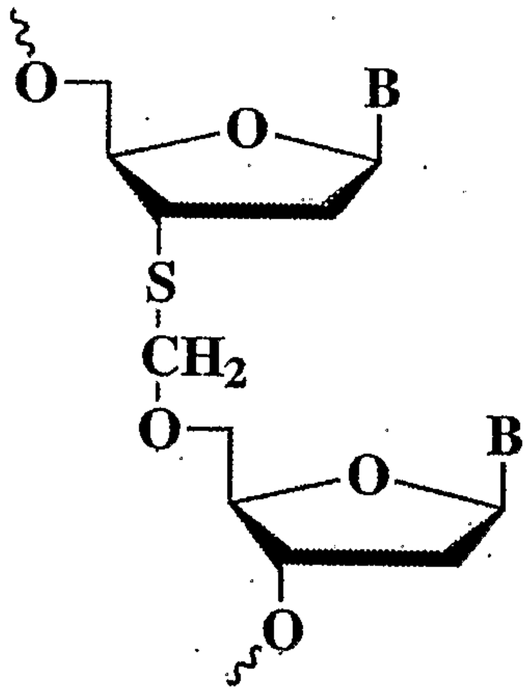


Fig. 1E

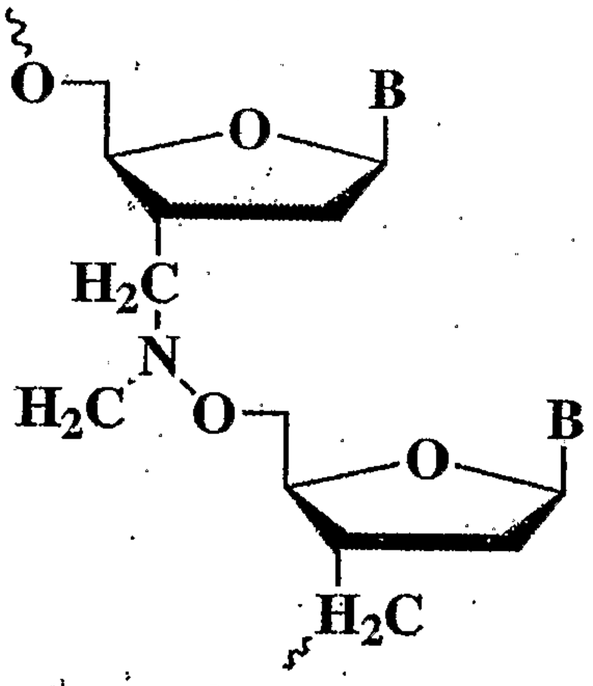


Fig. 1F

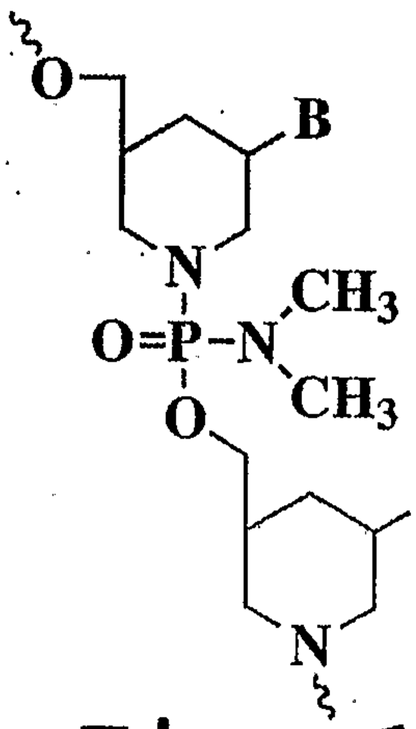


Fig. 1G

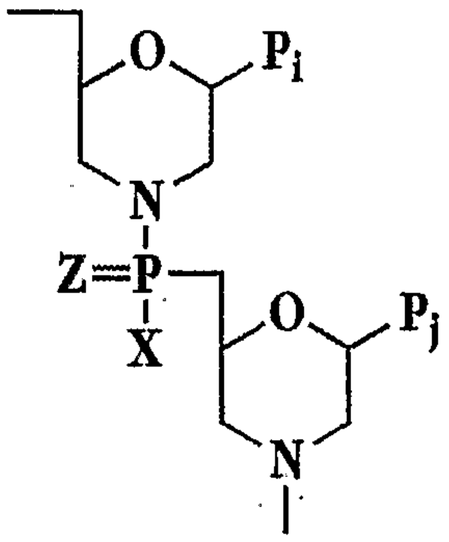


Fig. 2A

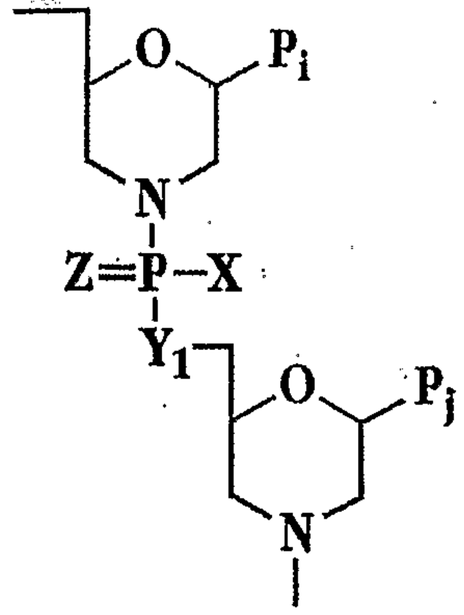


Fig. 2B

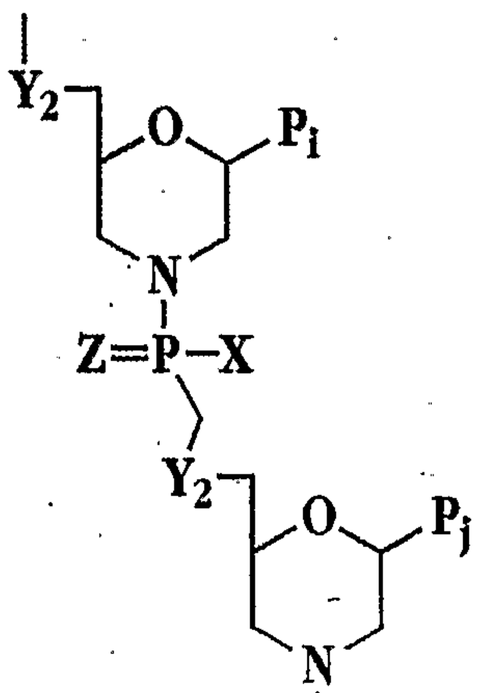


Fig. 2C

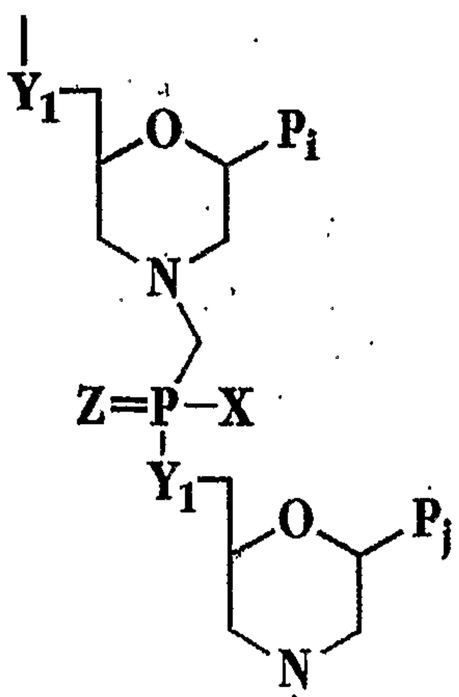


Fig. 2D

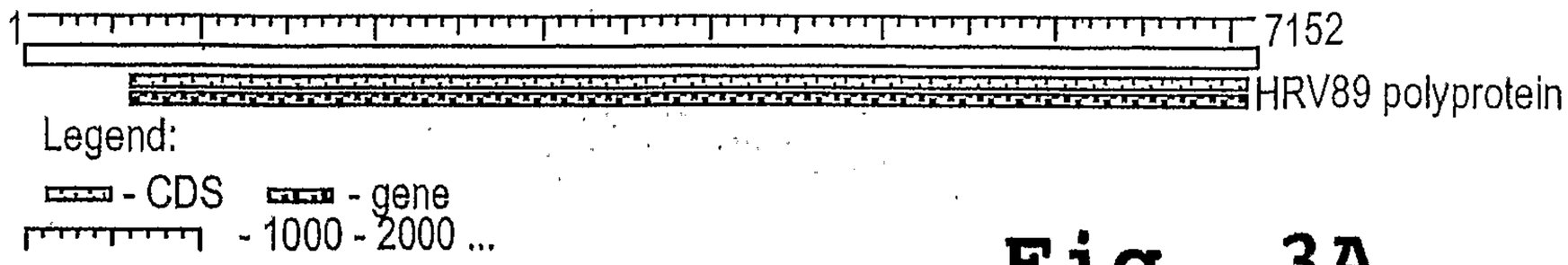


Fig. 3A

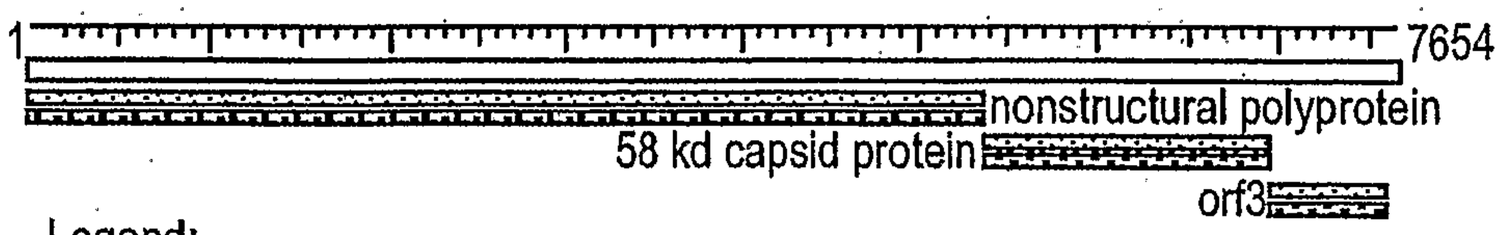


Fig. 3B

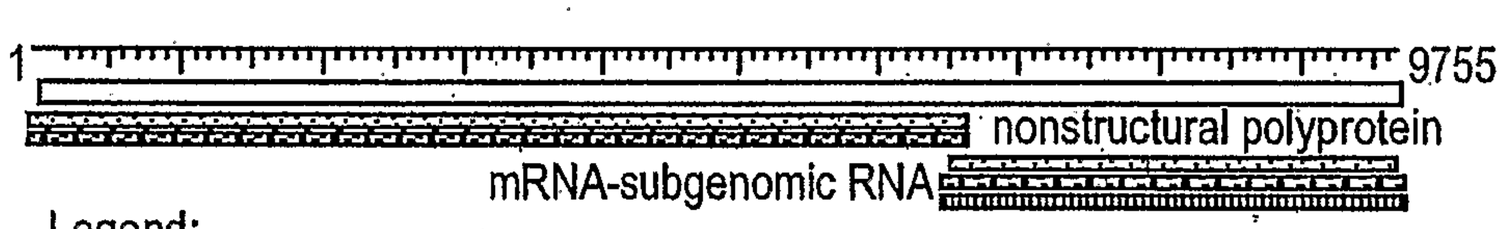


Fig. 3C

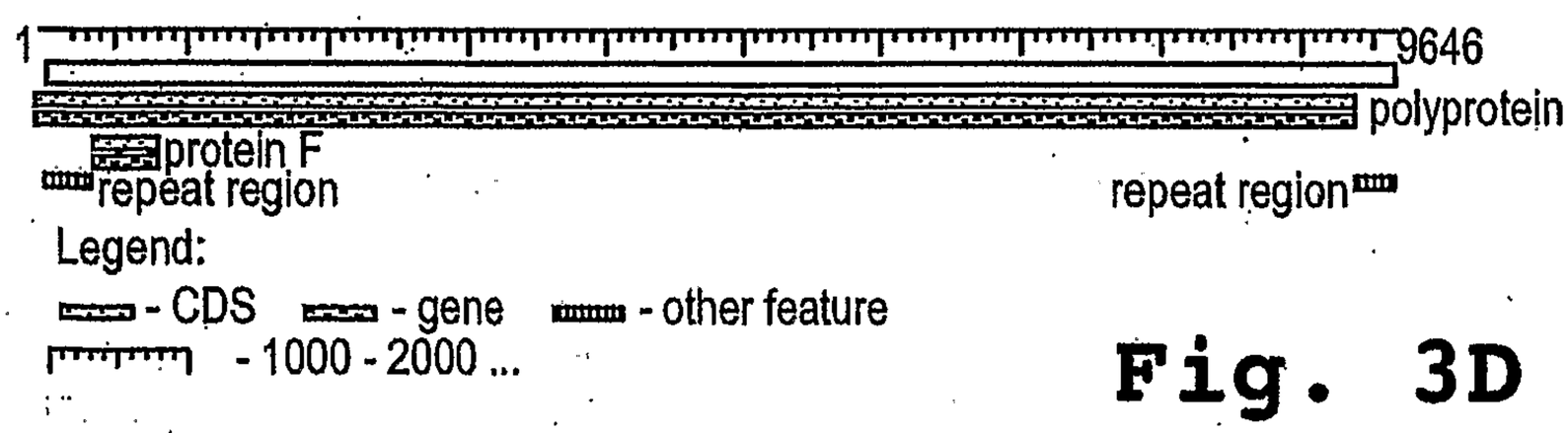


Fig. 3D

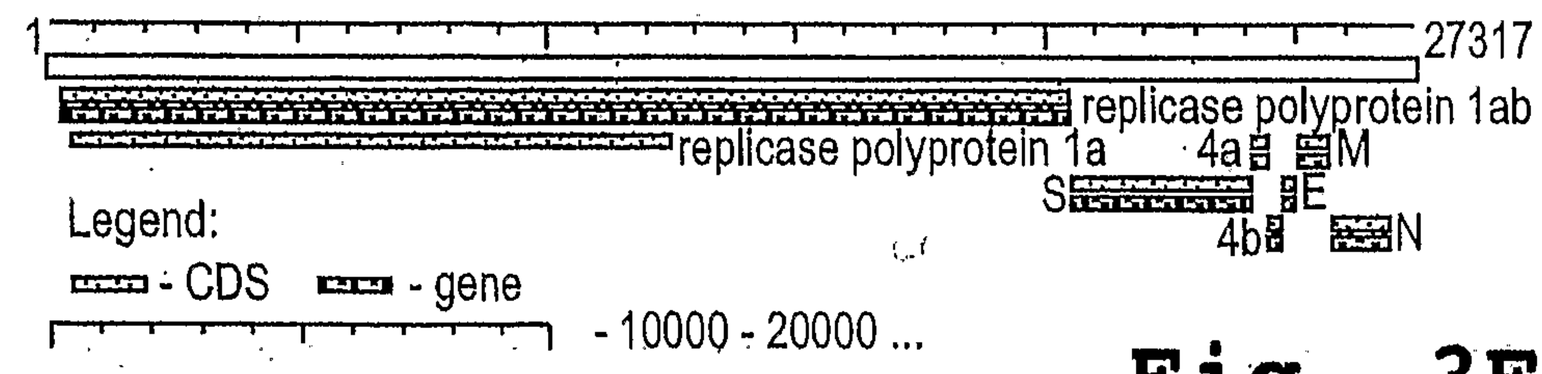


Fig. 3E

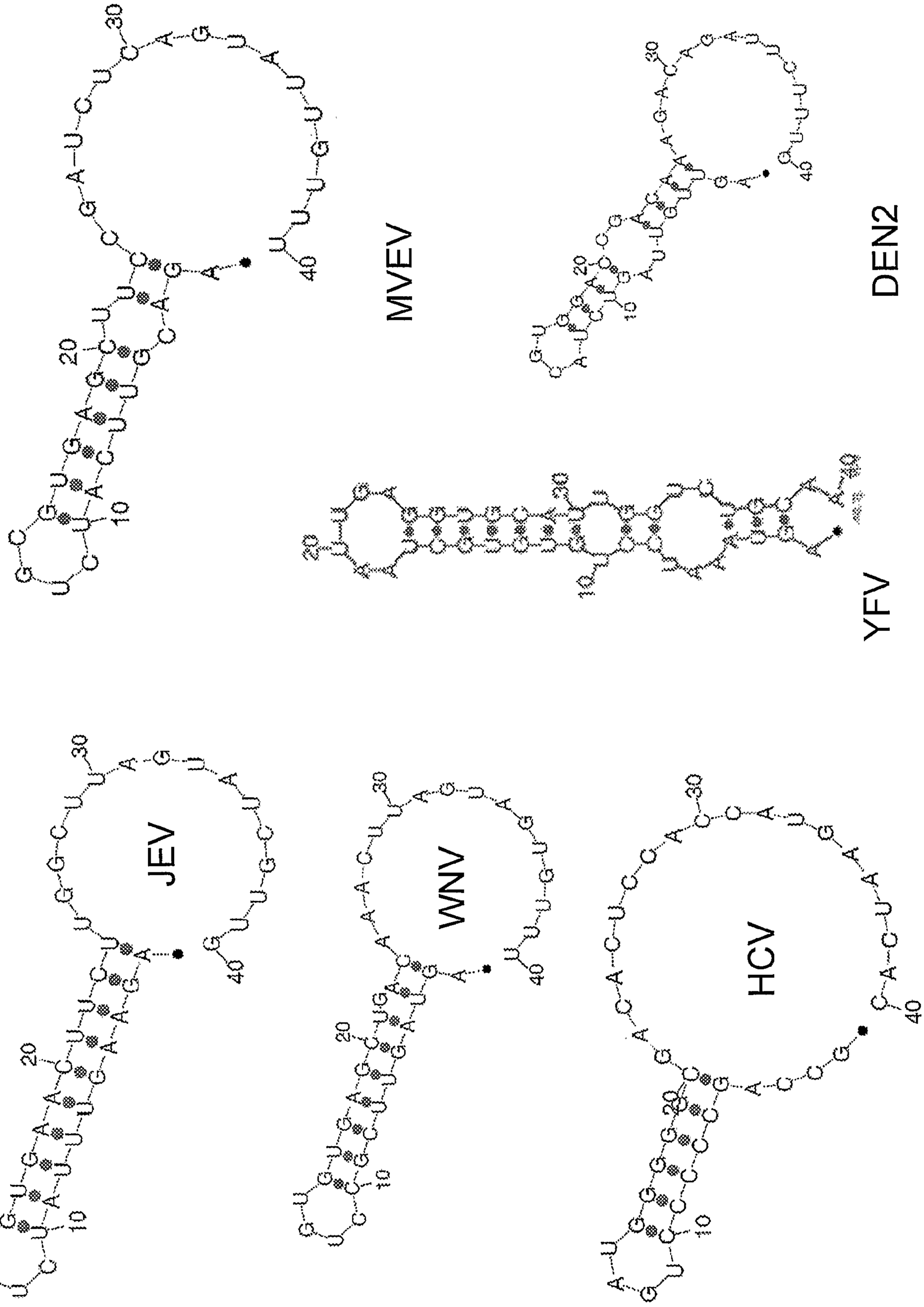


Fig. 4A

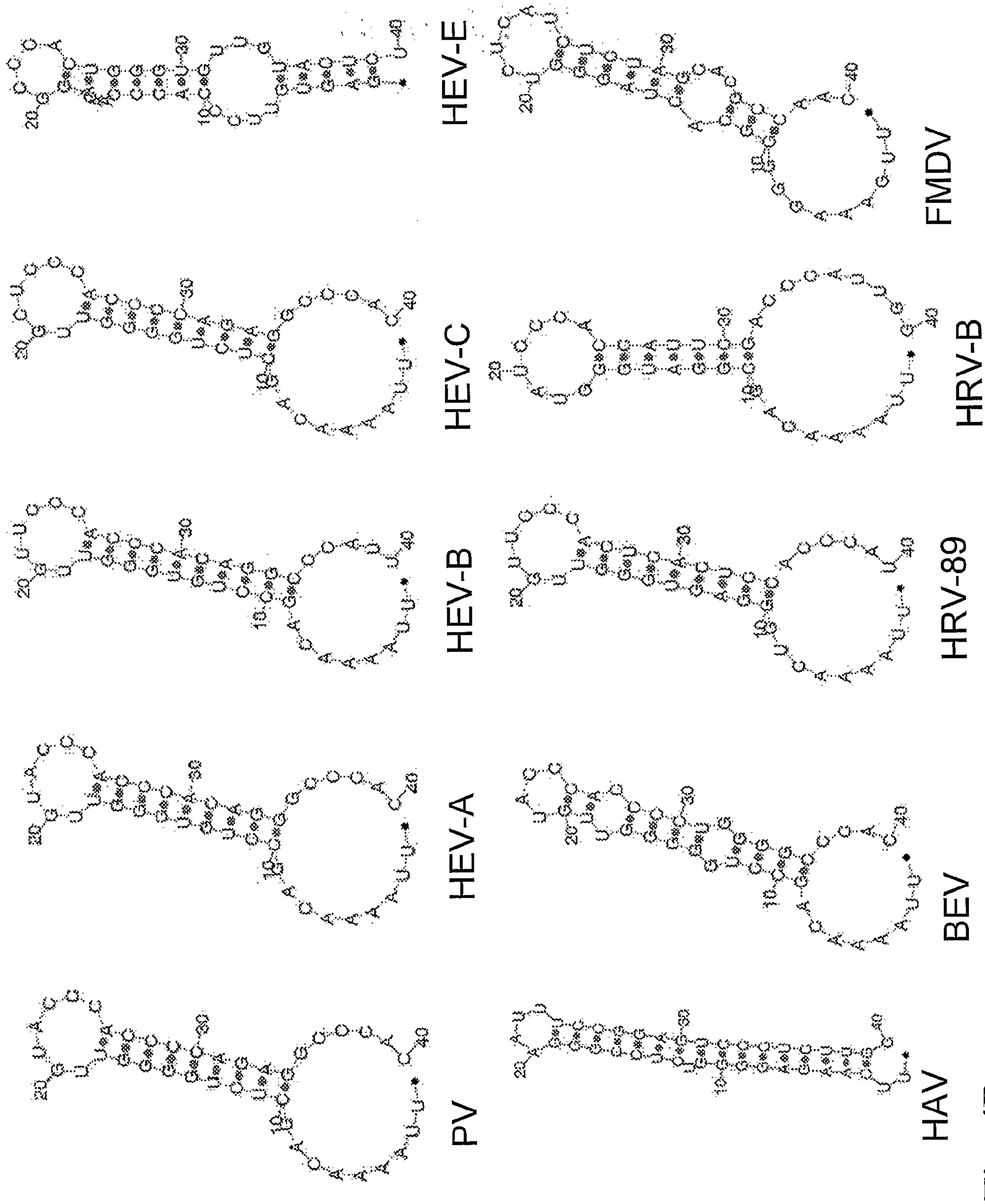


Fig. 4B

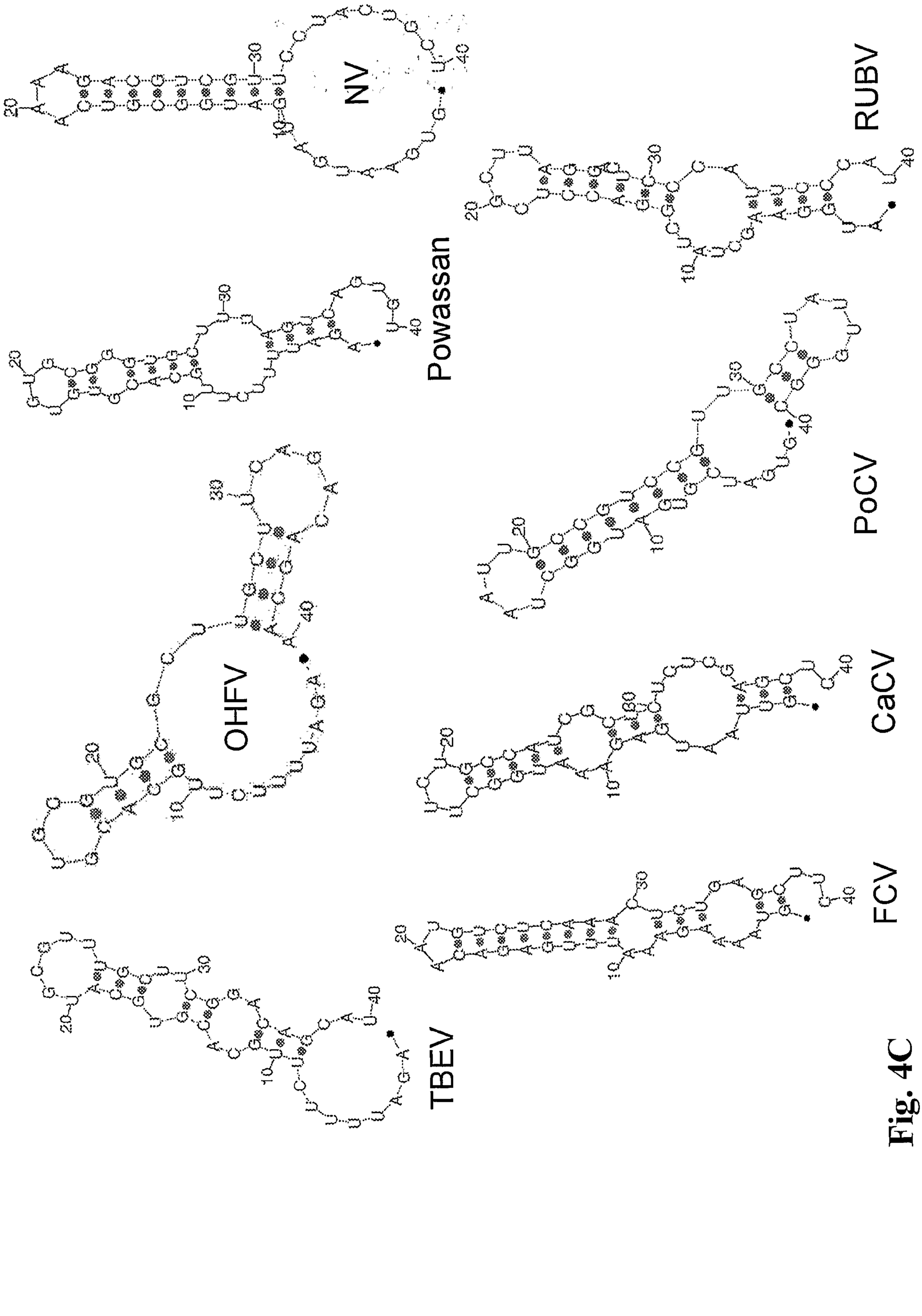


Fig. 4C

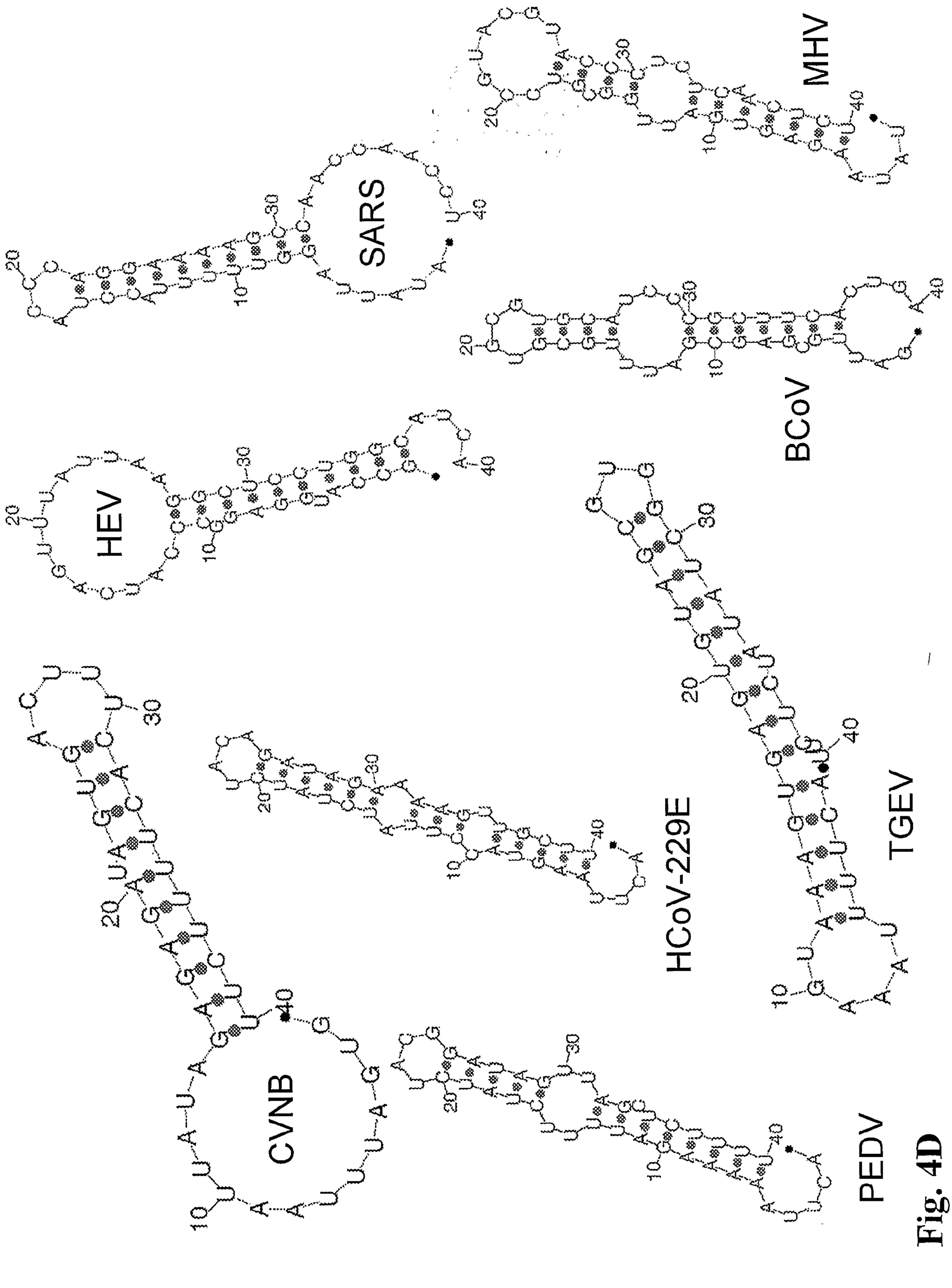


Fig. 4D

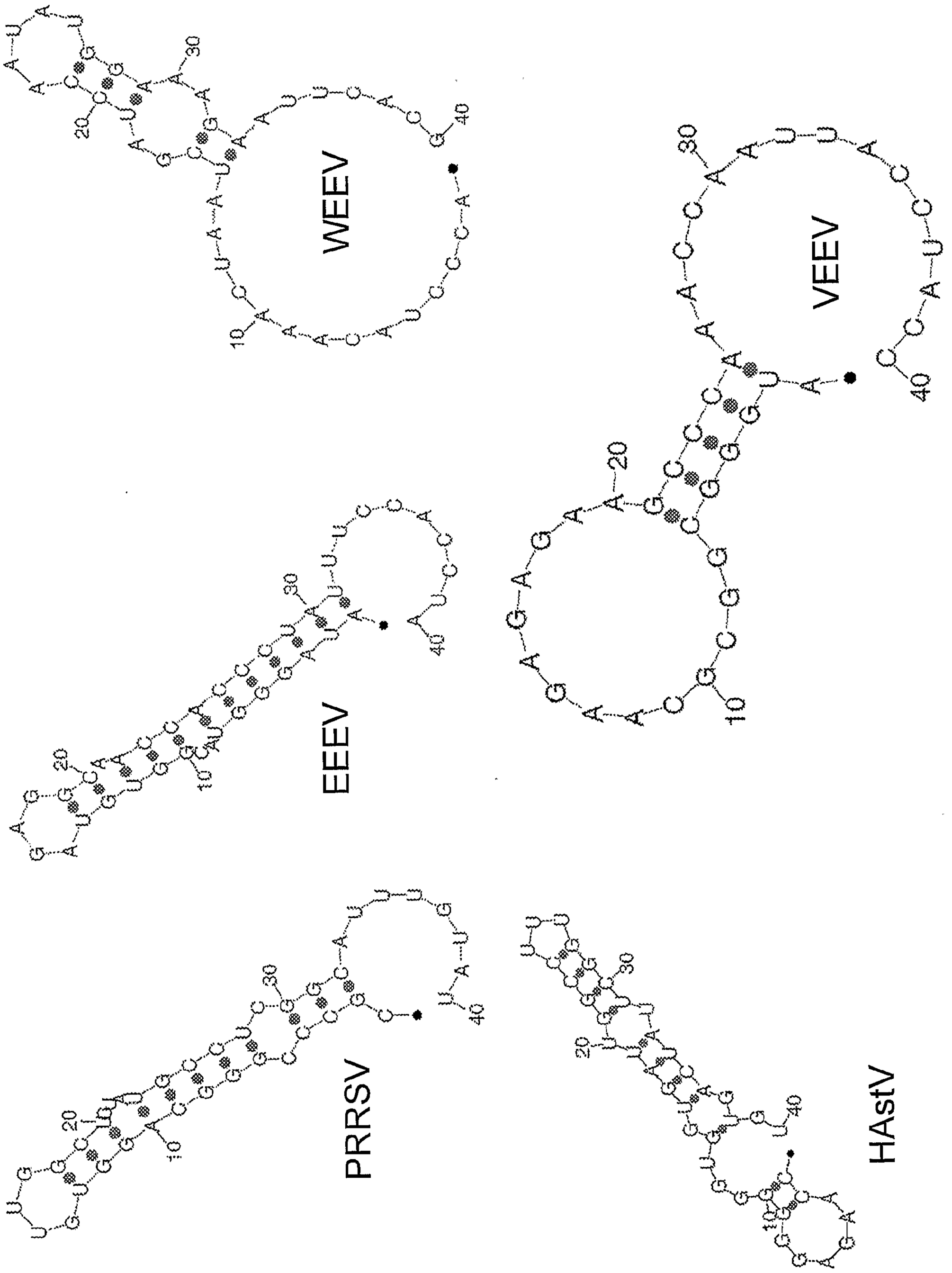


Fig. 4E

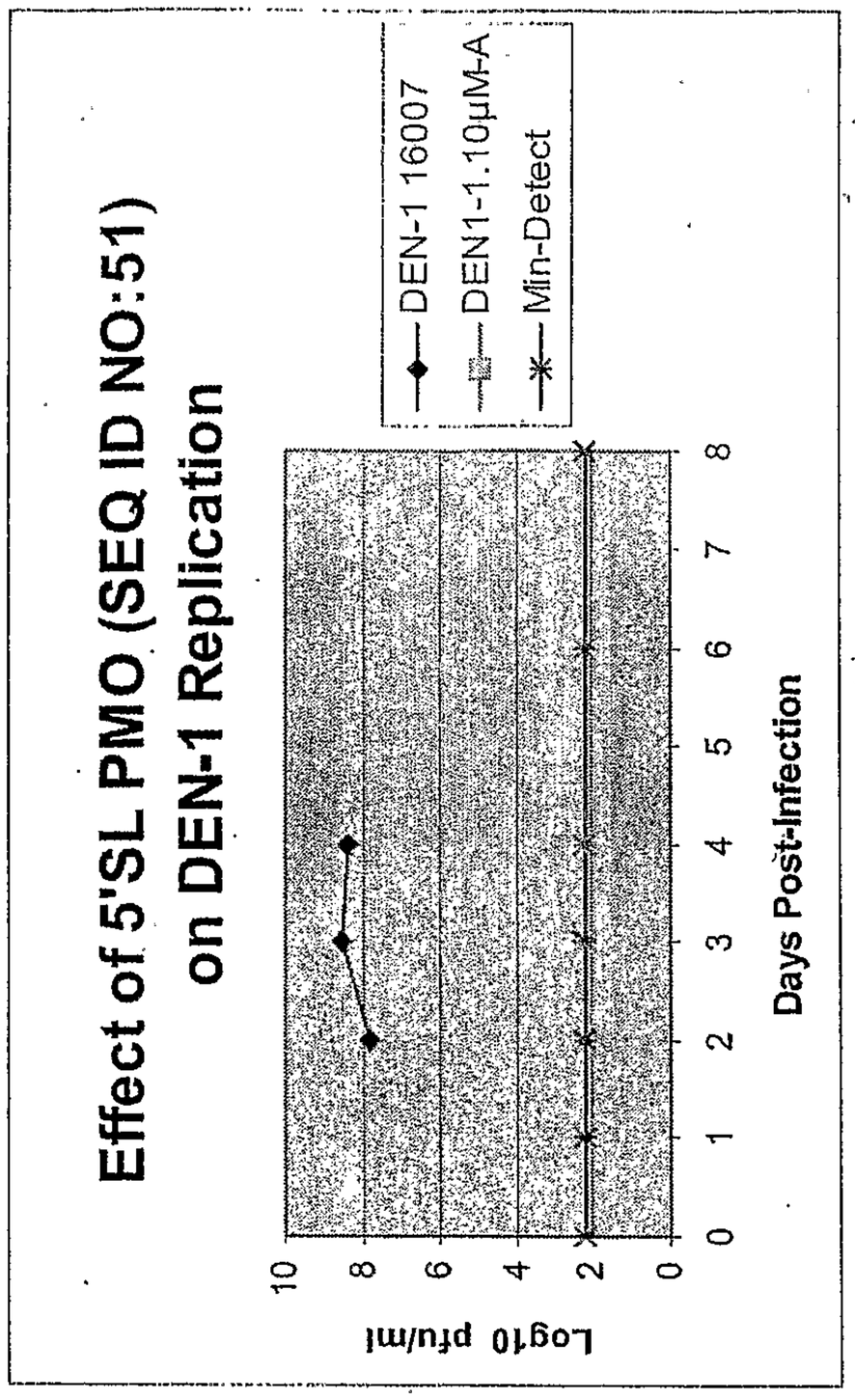


Fig. 5B

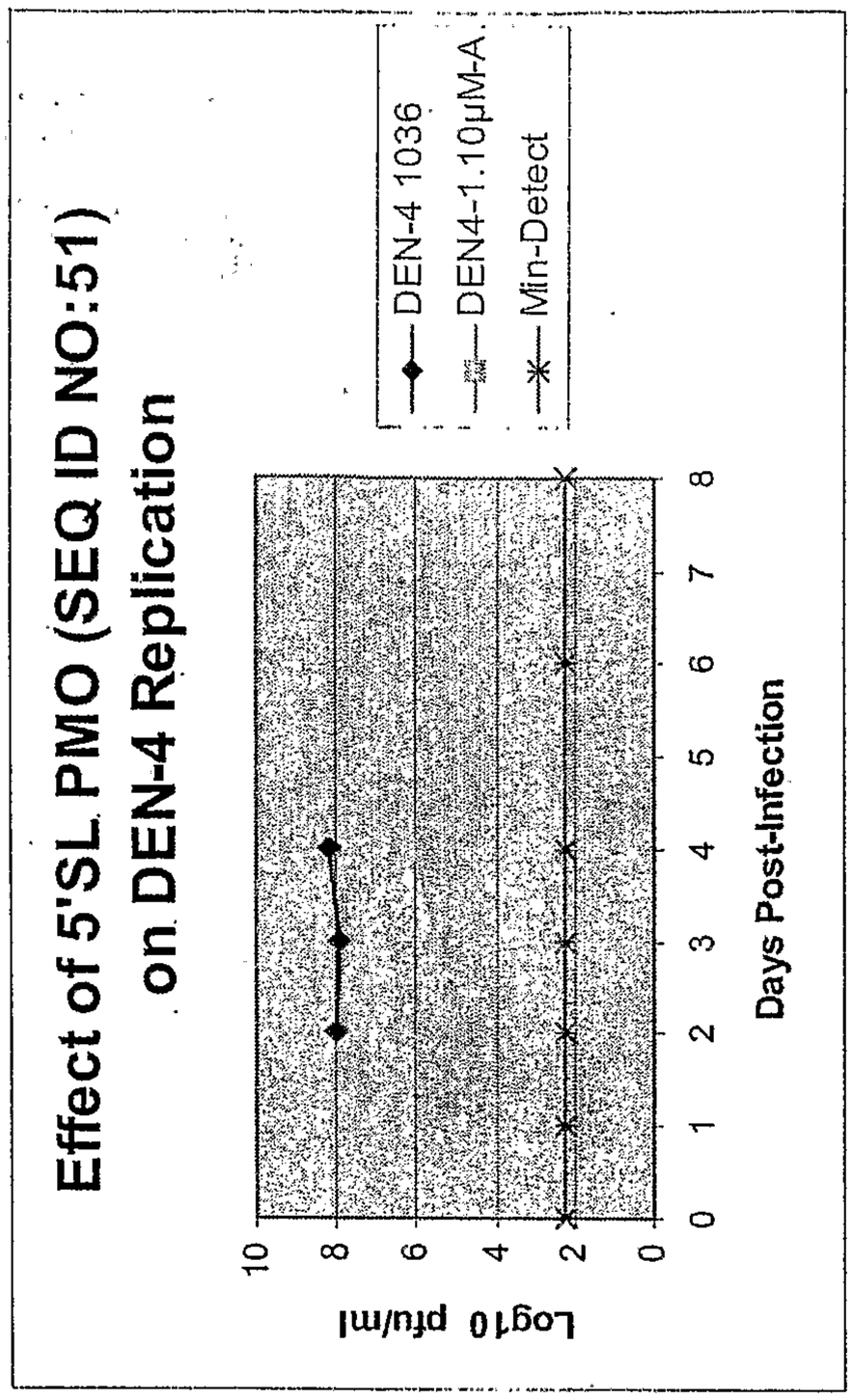


Fig. 5D

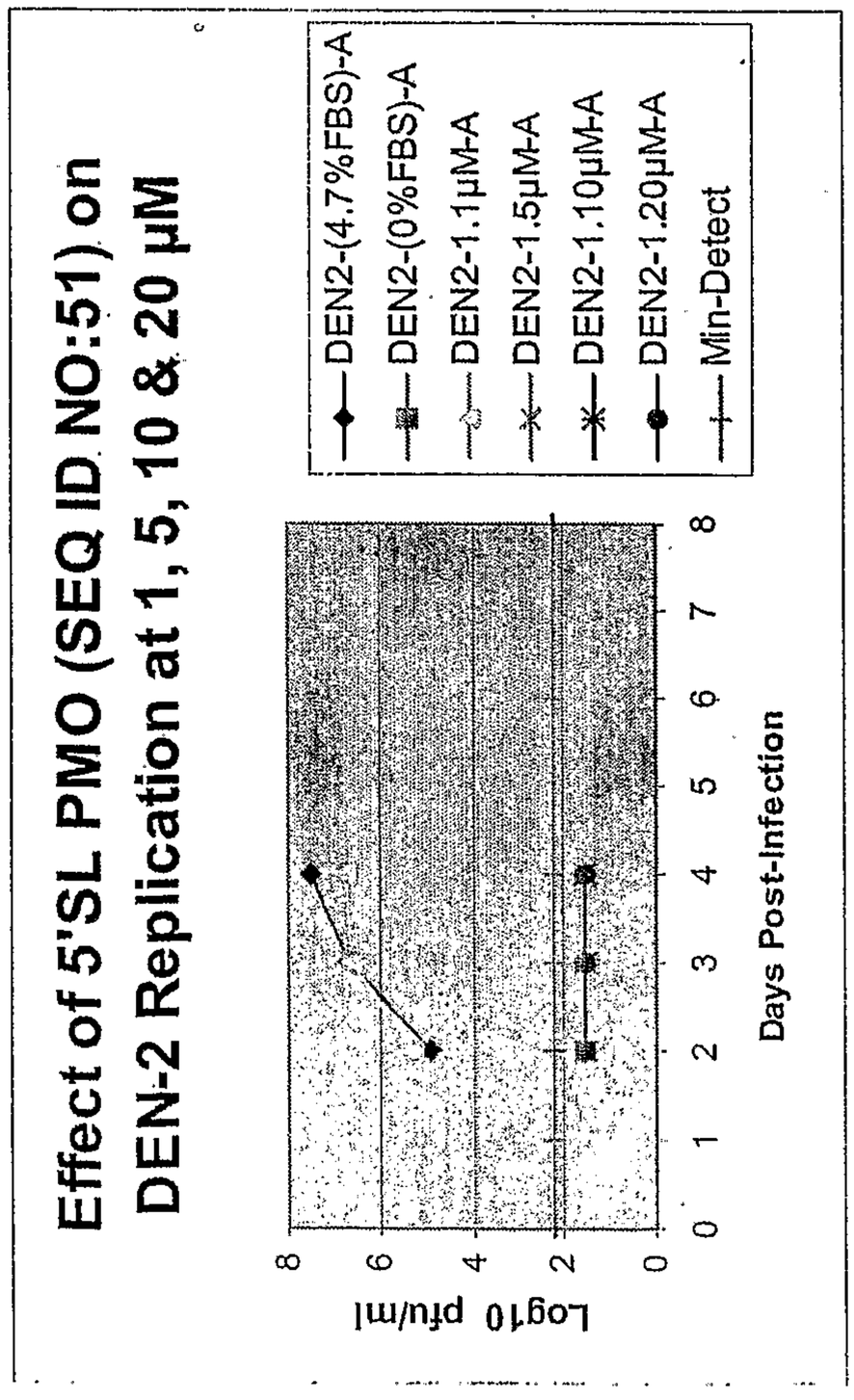


Fig. 5A

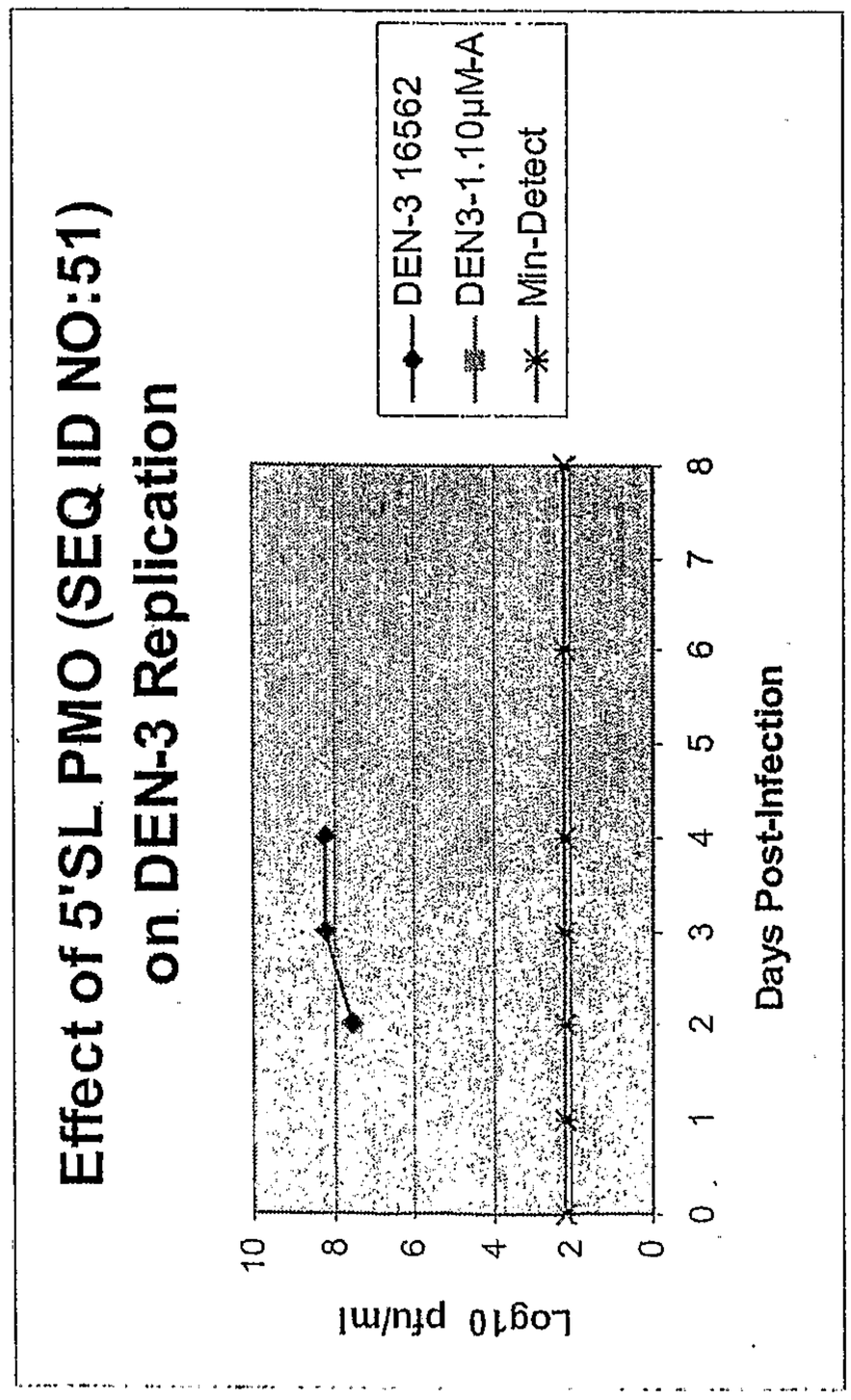


Fig. 5C

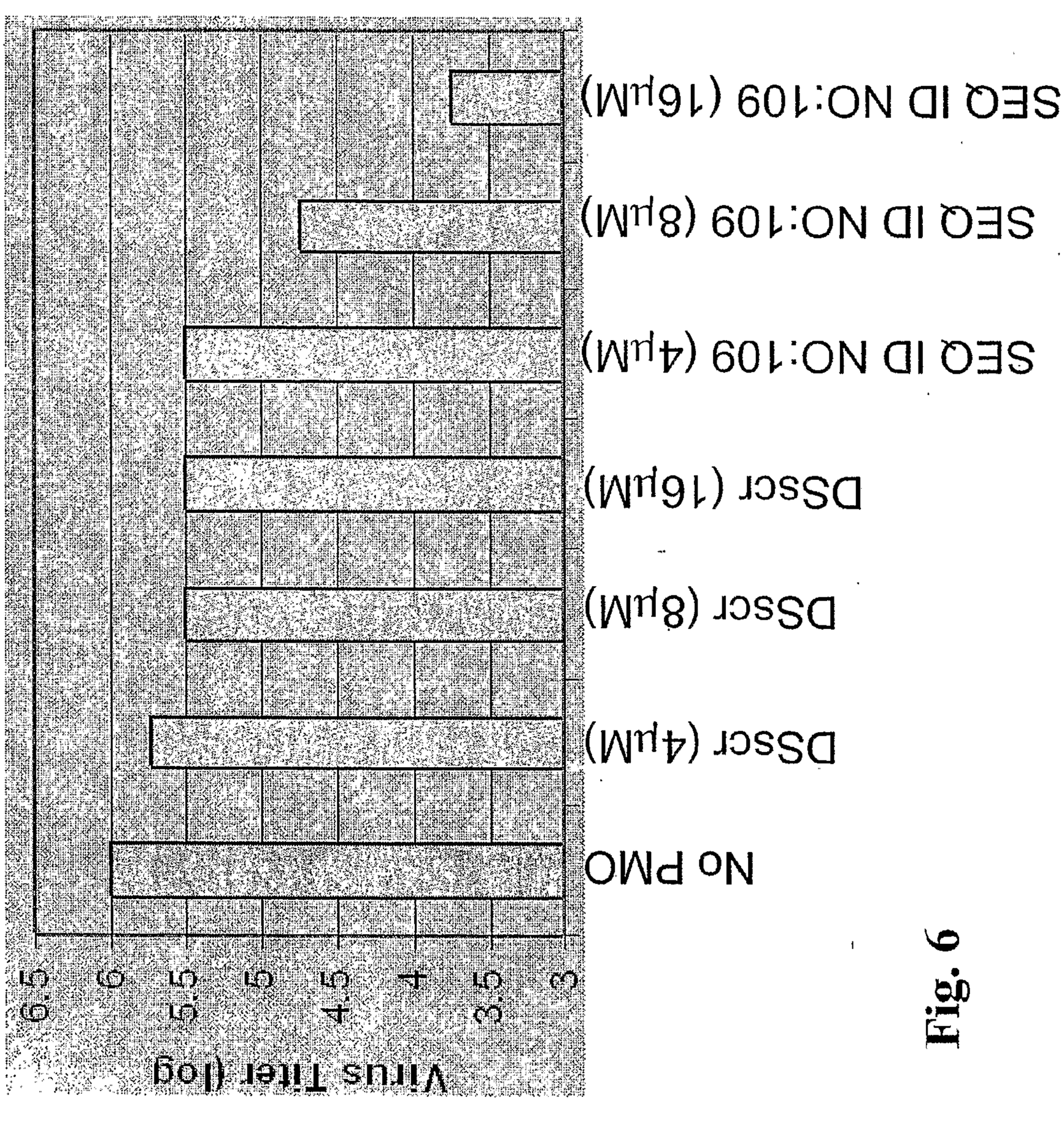

PMO PRRSV-1a (SEQ ID NO:109)
Inhibits PRRSV Replication
Virus Titer (log
6.5
6
5.5
5
4.5
4
3.5
3
No PMO
DSscr (4μM)
DSscr (8μM)
DSscr (16μM)
SEQ ID NO:109 (4μM)
SEQ ID NO:109 (8μM)
SEQ ID NO:109 (16μM)

Fig. 6

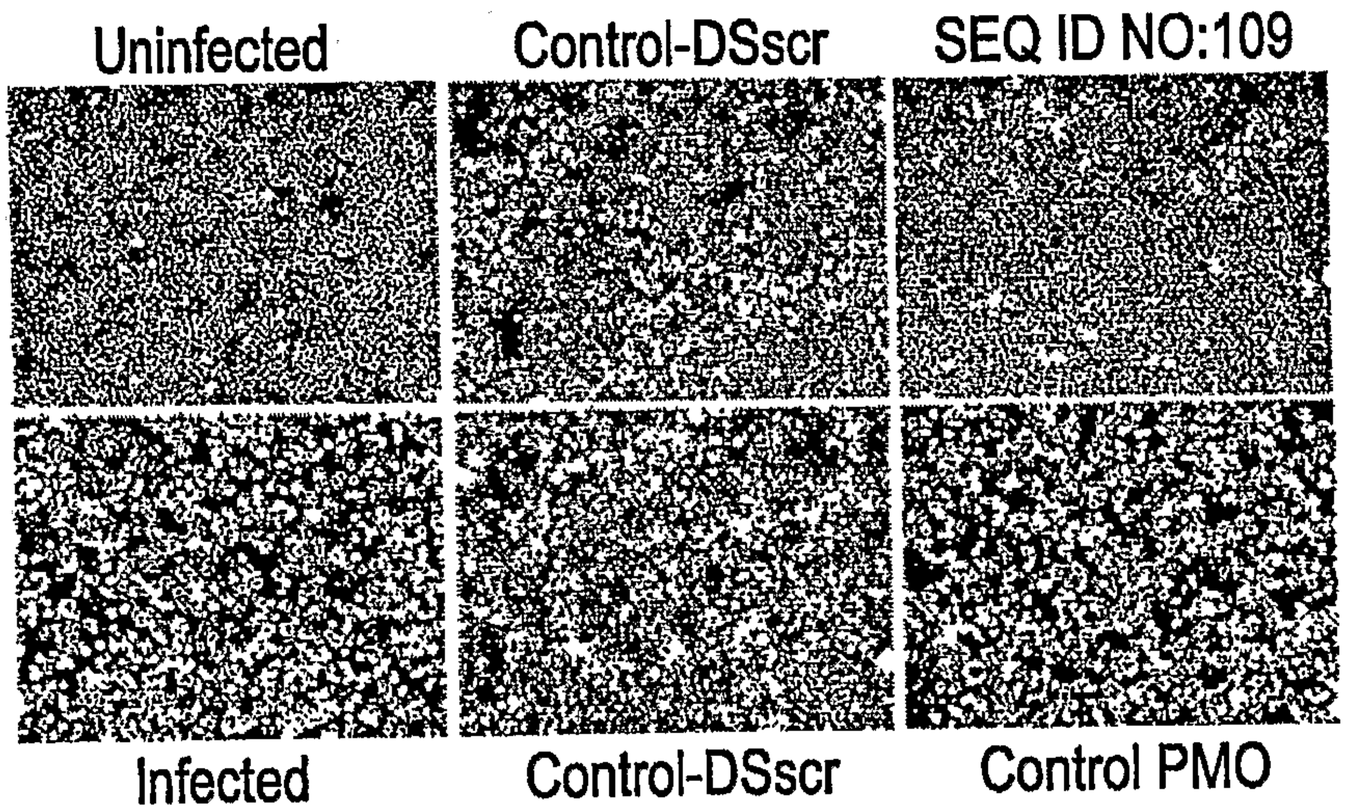

Uninfected
Control-DSscr
SEQ ID NO:109
Infected
Control-DSscr
Control PMO

Fig. 7

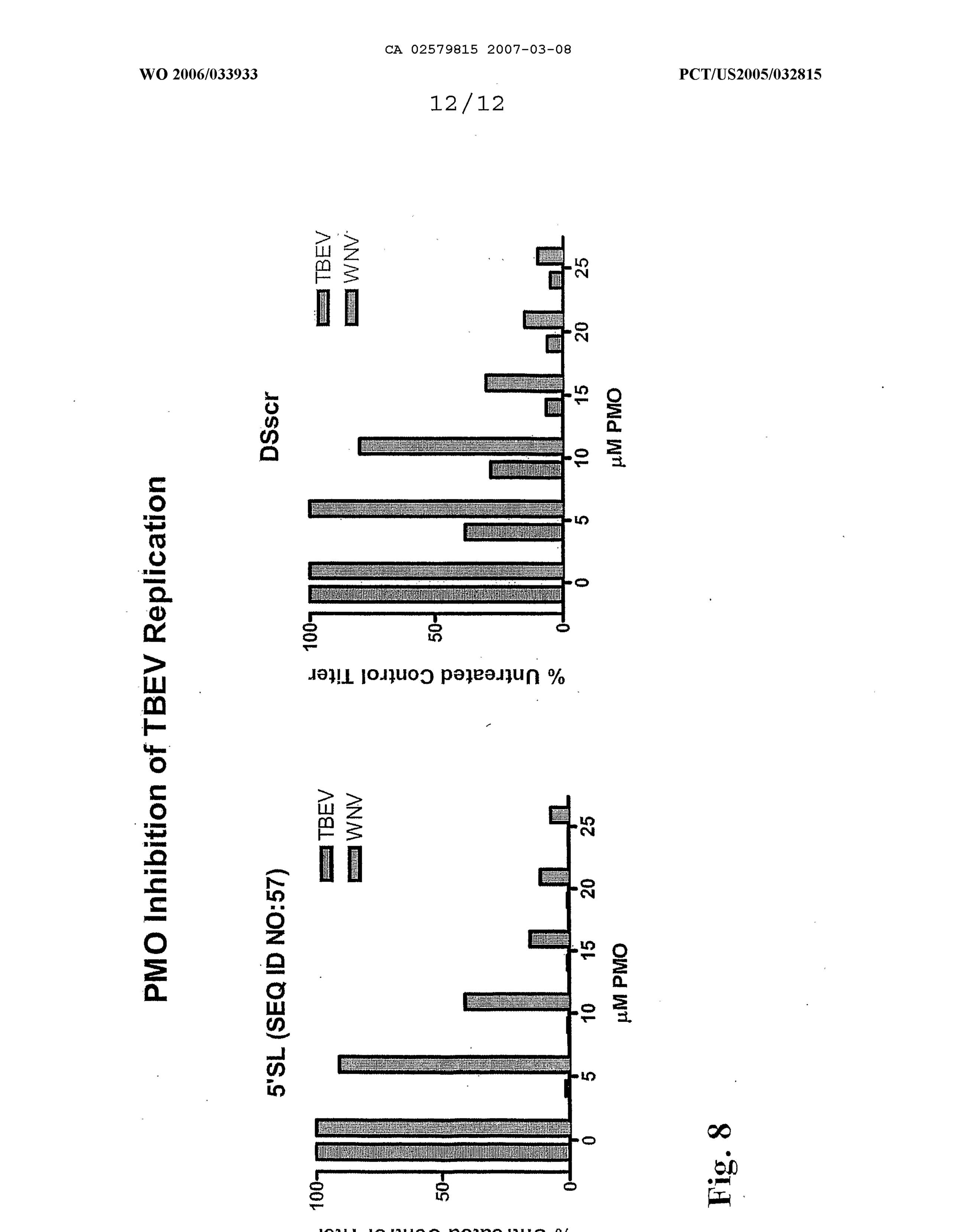

PMO Inhibition of TBEV Replication
5'SL (SEQ ID NO:57)
TBEV
WNV
% Untreated Control Titer
100
50
0
0
5
10
15
20
25
μM PMO
DSscr
TBEV
WNV
% Untreated Control Titer
100
50
0
0
5
10
15
20
25
μM PMO

Fig. 8