



INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification ⁶ : C07H 21/02, 21/04, 19/04, 19/20, 19/048, 19/10, 19/167, 19/173, 19/06, 19/09	A1	(11) International Publication Number: WO 00/06590 (43) International Publication Date: 10 February 2000 (10.02.00)
(21) International Application Number: PCT/US99/16541 (22) International Filing Date: 21 July 1999 (21.07.99) (30) Priority Data: 09/123,108 27 July 1998 (27.07.98) US (63) Related by Continuation (CON) or Continuation-in-Part (CIP) to Earlier Application US 09/123,108 (CON) Filed on 27 July 1998 (27.07.98) (71) Applicant (for all designated States except US): ISIS PHARMACEUTICALS, INC. [US/US]; 2292 Faraday Avenue, Carlsbad, CA 92008 (US). (72) Inventors; and (75) Inventors/Applicants (for US only): MANOHARAN, Muthiah [US/US]; 7634 Reposado Drive, Carlsbad, CA 92009 (US). MOHAN, Venkatraman [IN/US]; 7042 Via Cabana, Carlsbad, CA 92009 (US). BOSWELL, Herb [US/US]; 977 Hawthorne Court, San Marcos, CA 92069 (US).	(74) Agents: CALDWELL, John, W. et al.; Woodcock Washburn Kurtz Mackiewicz & Norris LLP, 46th floor, One Liberty Place, Philadelphia, PA 19103 (US). (81) Designated States: AE, AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, CA, CH, CN, CU, CZ, DE, DK, EE, ES, FI, GB, GD, GE, GH, GM, HR, HU, ID, IL, IN, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MD, MG, MK, MN, MW, MX, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, SL, TJ, TM, TR, TT, UA, UG, US, UZ, VN, YU, ZA, ZW, ARIPO patent (GH, GM, KE, LS, MW, SD, SL, SZ, UG, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, GW, ML, MR, NE, SN, TD, TG). Published <i>With international search report.</i>	
(54) Title: RNA TARGETED 2'-MODIFIED OLIGONUCLEOTIDES THAT ARE CONFORMATIONALLY PREORGANIZED (57) Abstract 2'-O-modified ribosyl nucleosides and modified oligonucleotides containing such nucleotides are disclosed. Oligonucleotides are disclosed that have increased binding affinity as shown by molecular modeling experiments. The 2'-O-modified nucleosides of the invention include ring structures that position the sugar moiety of the nucleosides preferentially in 3' endo geometries.		

FOR THE PURPOSES OF INFORMATION ONLY

Codes used to identify States party to the PCT on the front pages of pamphlets publishing international applications under the PCT.

AL	Albania	ES	Spain	LS	Lesotho	SI	Slovenia
AM	Armenia	FI	Finland	LT	Lithuania	SK	Slovakia
AT	Austria	FR	France	LU	Luxembourg	SN	Senegal
AU	Australia	GA	Gabon	LV	Latvia	SZ	Swaziland
AZ	Azerbaijan	GB	United Kingdom	MC	Monaco	TD	Chad
BA	Bosnia and Herzegovina	GE	Georgia	MD	Republic of Moldova	TG	Togo
BB	Barbados	GH	Ghana	MG	Madagascar	TJ	Tajikistan
BE	Belgium	GN	Guinea	MK	The former Yugoslav Republic of Macedonia	TM	Turkmenistan
BF	Burkina Faso	GR	Greece			TR	Turkey
BG	Bulgaria	HU	Hungary	ML	Mali	TT	Trinidad and Tobago
BJ	Benin	IE	Ireland	MN	Mongolia	UA	Ukraine
BR	Brazil	IL	Israel	MR	Mauritania	UG	Uganda
BY	Belarus	IS	Iceland	MW	Malawi	US	United States of America
CA	Canada	IT	Italy	MX	Mexico	UZ	Uzbekistan
CF	Central African Republic	JP	Japan	NE	Niger	VN	Viet Nam
CG	Congo	KE	Kenya	NL	Netherlands	YU	Yugoslavia
CH	Switzerland	KG	Kyrgyzstan	NO	Norway	ZW	Zimbabwe
CI	Côte d'Ivoire	KP	Democratic People's Republic of Korea	NZ	New Zealand		
CM	Cameroon			PL	Poland		
CN	China	KR	Republic of Korea	PT	Portugal		
CU	Cuba	KZ	Kazakstan	RO	Romania		
CZ	Czech Republic	LC	Saint Lucia	RU	Russian Federation		
DE	Germany	LI	Liechtenstein	SD	Sudan		
DK	Denmark	LK	Sri Lanka	SE	Sweden		
EE	Estonia	LR	Liberia	SG	Singapore		

RNA TARGETED 2'-MODIFIED OLIGONUCLEOTIDES THAT ARE
CONFORMATIONALLY PREORGANIZED

FIELD OF THE INVENTION

5 The present invention relates to nucleotides,
oligonucleotides incorporating such nucleotides and methods
of using such oligonucleotides. The oligonucleotides of the
invention are useful in therapeutic and investigative
purposes. More specifically, the present invention is
10 directed to the use of oligonucleotides having 2'-O-
modifications that will increase affinity and nuclease
resistance.

BACKGROUND OF THE INVENTION

 It is well known that most of the bodily states in
15 mammals, including most disease states, are affected by
proteins. Classical therapeutic modes have generally
focused on interactions with such proteins in an effort to
moderate their disease-causing or disease-potentiating
functions. However, recently, attempts have been made to
20 moderate the actual production of such proteins by
interactions with molecules that direct their synthesis,
such as intracellular RNA. By interfering with the
production of proteins, maximum therapeutic effect and
minimal side effects may be realized. It is the general

- 2 -

object of such therapeutic approaches to interfere with or otherwise modulate gene expression leading to undesired protein formation.

One method for inhibiting specific gene expression
5 is the use of oligonucleotides. Oligonucleotides are now accepted as therapeutic agents with great promise. Oligonucleotides are known to hybridize to single-stranded DNA or RNA molecules. Hybridization is the sequence-specific base pair hydrogen bonding of nucleobases of the
10 oligonucleotide to the nucleobases of the target DNA or RNA molecule. Such nucleobase pairs are said to be complementary to one another. The concept of inhibiting gene expression through the use of sequence-specific binding of oligonucleotides to target RNA sequences, also known as
15 antisense inhibition, has been demonstrated in a variety of systems, including living cells (for example see: Wagner et al., *Science* (1993) 260: 1510-1513; Milligan et al., *J. Med. Chem.*, (1993) 36:1923-37; Uhlmann et al., *Chem. Reviews*, (1990) 90:543-584; Stein et al., *Cancer Res.*, (1988)
20 48:2659-2668).

The events that provide the disruption of the nucleic acid function by antisense oligonucleotides (Cohen in *Oligonucleotides: Antisense Inhibitors of Gene Expression*, (1989) CRC Press, Inc., Boca Raton, FL) are
25 thought to be of two types. The first, hybridization arrest, denotes the terminating event in which the oligonucleotide inhibitor binds to the target nucleic acid and thus prevents, by simple steric hindrance, the binding of essential proteins, most often ribosomes, to the nucleic
30 acid. Methyl phosphonate oligonucleotides: Miller, P.S. and Ts'O, P.O.P. (1987) *Anti-Cancer Drug Design*, 2:117-128, and α -anomer oligonucleotides are the two most extensively studied antisense agents which are thought to disrupt nucleic acid function by hybridization arrest.

- 3 -

The second type of terminating event for antisense oligonucleotides involves the enzymatic cleavage of the targeted RNA by intracellular RNase H. A 2'-deoxyribofuranosyl oligonucleotide or oligonucleotide analog hybridizes with the targeted RNA and this duplex activates the RNase H enzyme to cleave the RNA strand, thus destroying the normal function of the RNA. Phosphorothioate oligonucleotides are the most prominent example of an antisense agent that operates by this type of antisense terminating event.

Oligonucleotides may also bind to duplex nucleic acids to form triplex complexes in a sequence specific manner via Hoogsteen base pairing (Beal et al., *Science*, (1991) 251:1360-1363; Young et al., *Proc. Natl. Acad. Sci.* (1991) 88:10023-10026). Both antisense and triple helix therapeutic strategies are directed towards nucleic acid sequences that are involved in or responsible for establishing or maintaining disease conditions. Such target nucleic acid sequences may be found in the genomes of pathogenic organisms including bacteria, yeasts, fungi, protozoa, parasites, viruses, or may be endogenous in nature. By hybridizing to and modifying the expression of a gene important for the establishment, maintenance or elimination of a disease condition, the corresponding condition may be cured, prevented or ameliorated.

In determining the extent of hybridization of an oligonucleotide to a complementary nucleic acid, the relative ability of an oligonucleotide to bind to the complementary nucleic acid may be compared by determining the melting temperature of a particular hybridization complex. The melting temperature (T_m), a characteristic physical property of double helices, denotes the temperature (in degrees centigrade) at which 50% helical (hybridized) versus coil (unhybridized) forms are present. T_m is measured by using the UV spectrum to determine the formation and

- 4 -

breakdown (melting) of the hybridization complex. Base stacking, which occurs during hybridization, is accompanied by a reduction in UV absorption (hypochromicity).

Consequently, a reduction in UV absorption indicates a
5 higher T_m . The higher the T_m , the greater the strength of the bonds between the strands.

Oligonucleotides may also be of therapeutic value when they bind to non-nucleic acid biomolecules such as intracellular or extracellular polypeptides, proteins, or
10 enzymes. Such oligonucleotides are often referred to as 'aptamers' and they typically bind to and interfere with the function of protein targets (Griffin, et al., *Blood*, (1993), 81:3271-3276; Bock, et al., *Nature*, (1992) 355: 564-566).

Oligonucleotides and their analogs have been
15 developed and used for diagnostic purposes, therapeutic applications and as research reagents. For use as therapeutics, oligonucleotides must be transported across cell membranes or be taken up by cells, and appropriately hybridize to target DNA or RNA. These critical functions
20 depend on the initial stability of the oligonucleotides toward nuclease degradation. A serious deficiency of unmodified oligonucleotides which affects their hybridization potential with target DNA or RNA for therapeutic purposes is the enzymatic degradation of
25 administered oligonucleotides by a variety of intracellular and extracellular ubiquitous nucleolytic enzymes referred to as nucleases. For oligonucleotides to be useful as therapeutics or diagnostics, the oligonucleotides should demonstrate enhanced binding affinity to complementary
30 target nucleic acids, and preferably be reasonably stable to nucleases and resist degradation. For a non-cellular use such as a research reagent, oligonucleotides need not necessarily possess nuclease stability.

A number of chemical modifications have been
35 introduced into oligonucleotides to increase their binding

- 5 -

affinity to target DNA or RNA and resist nuclease degradation.

Modifications have been made to the ribose phosphate backbone to increase the resistance to nucleases. 5 These modifications include use of linkages such as methyl phosphonates, phosphorothioates and phosphorodithioates, and the use of modified sugar moieties such as 2'-O-alkyl ribose. Other oligonucleotide modifications include those made to modulate uptake and cellular distribution. A number 10 of modifications that dramatically alter the nature of the internucleotide linkage have also been reported in the literature. These include non-phosphorus linkages, peptide nucleic acids (PNA's) and 2'-5' linkages. Another modification to oligonucleotides, usually for diagnostic and 15 research applications, is labeling with non-isotopic labels, e.g., fluorescein, biotin, digoxigenin, alkaline phosphatase, or other reporter molecules.

A variety of modified phosphorus-containing linkages have been studied as replacements for the natural, 20 readily cleaved phosphodiester linkage in oligonucleotides. In general, most of them, such as the phosphorothioate, phosphoramidates, phosphonates and phosphorodithioates all result in oligonucleotides with reduced binding to complementary targets and decreased hybrid stability. In 25 order to make effective therapeutics therefore this binding and hybrid stability of antisense oligonucleotides needs to be improved.

Of the large number of modifications made and studied, few have progressed far enough through discovery 30 and development to deserve clinical evaluation. Reasons underlying this include difficulty of synthesis, poor binding to target nucleic acids, lack of specificity for the target nucleic acid, poor *in vitro* and *in vivo* stability to nucleases, and poor pharmacokinetics. Several 35 phosphorothioate oligonucleotides and derivatives are

- 6 -

presently being used as antisense agents in human clinical trials for the treatment of various disease states. A submission for approval was recently made to both United States and European regulatory agencies for one antisense
5 drug, Fomivirsen, for use to treat cytomegalovirus (CMV) retinitis in humans.

The structure and stability of chemically modified nucleic acids is of great importance to the design of antisense oligonucleotides. Over the last ten years, a
10 variety of synthetic modifications have been proposed to increase nuclease resistance, or to enhance the affinity of the antisense strand for its target mRNA (Crooke *et al.*, *Med. Res. Rev.*, **1996**, 16, 319-344; De Mesmaeker *et al.*, *Acc. Chem. Res.*, **1995**, 28, 366-374). Although a great deal of
15 information has been collected about the types of modifications that improve duplex formation, little is known about the structural basis for the improved affinity observed.

RNA exists in what has been termed "A Form" geometry while DNA exists in "B Form" geometry. In general, RNA:RNA duplexes are more stable, or have higher melting temperatures (T_m) than DNA:DNA duplexes (Sanger *et al.*, *Principles of Nucleic Acid Structure*, **1984**, Springer-Verlag; New York, NY.; Lesnik *et al.*, *Biochemistry*, **1995**, 34, 10807-
25 10815; Conte *et al.*, *Nucleic Acids Res.*, **1997**, 25, 2627-2634). The increased stability of RNA has been attributed to several structural features, most notably the improved base stacking interactions that result from an A-form geometry (Searle *et al.*, *Nucleic Acids Res.*, **1993**, 21, 2051-
30 2056). The presence of the 2' hydroxyl in RNA biases the sugar toward a C3' *endo* pucker, i.e., also designated as Northern pucker, which causes the duplex to favor the A-form geometry. On the other hand, deoxy nucleic acids prefer a C2' *endo* sugar pucker, i.e., also known as Southern pucker,

- 7 -

which is thought to impart a less stable B-form geometry (Sanger, W. (1984) *Principles of Nucleic Acid Structure*, Springer-Verlag, New York, NY). In addition, the 2' hydroxyl groups of RNA can form a network of water mediated hydrogen bonds that help stabilize the RNA duplex (Egli et al., *Biochemistry*, **1996**, 35, 8489-8494).

DNA:RNA hybrid duplexes, however, are usually less stable than pure RNA:RNA duplexes, and depending on their sequence may be either more or less stable than DNA:DNA duplexes (Searle et al., *Nucleic Acids Res.*, **1993**, 21, 2051-2056). The structure of a hybrid duplex is intermediate between A- and B-form geometries, which may result in poor stacking interactions (Lane et al., *Eur. J. Biochem.*, **1993**, 215, 297-306; Fedoroff et al., *J. Mol. Biol.*, **1993**, 233, 509-523; Gonzalez et al., *Biochemistry*, **1995**, 34, 4969-4982; Horton et al., *J. Mol. Biol.*, **1996**, 264, 521-533). The stability of a DNA:RNA hybrid is central to antisense therapies as the mechanism requires the binding of a modified DNA strand to a mRNA strand. To effectively inhibit the mRNA, the antisense DNA should have a very high binding affinity with the mRNA. Otherwise the desired interaction between the DNA and target mRNA strand will occur infrequently, thereby decreasing the efficacy of the antisense oligonucleotide.

One synthetic 2'-modification that imparts increased nuclease resistance and a very high binding affinity to nucleotides is the 2'-methoxyethoxy (MOE, 2'-OCH₂CH₂OCH₃) side chain (Baker et al., *J. Biol. Chem.*, **1997**, 272, 11944-12000; Freier et al., *Nucleic Acids Res.*, **1997**, 25, 4429-4443). One of the immediate advantages of the MOE substitution is the improvement in binding affinity, which is greater than many similar 2' modifications such as O-methyl, O-propyl, and O-aminopropyl (Freier and Altmann, *Nucleic Acids Research*, (1997) 25:4429-4443). 2'-O-

- 8 -

Methoxyethyl-substituted also have been shown to be antisense inhibitors of gene expression with promising features for *in vivo* use (Martin, P., *Helv. Chim. Acta*, **1995**, 78, 486-504; Altmann *et al.*, *Chimia*, **1996**, 50, 168-
5 176; Altmann *et al.*, *Biochem. Soc. Trans.*, **1996**, 24, 630-637; and Altmann *et al.*, *Nucleosides Nucleotides*, 1997, **16**, 917-926). Relative to DNA, they display improved RNA affinity and higher nuclease resistance. Chimeric oligonucleotides with 2'-O-methoxyethyl-ribonucleoside wings
10 and a central DNA-phosphorothioate window also have been shown to effectively reduce the growth of tumors in animal models at low doses. MOE substituted oligonucleotides have shown outstanding promise as antisense agents in several disease states. One such MOE substituted oligonucleotide is
15 presently being investigated in clinical trials for the treatment of CMV retinitis.

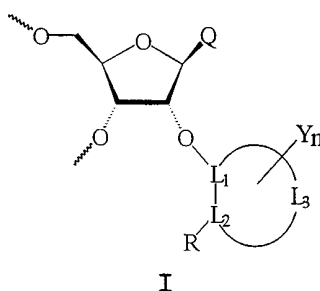
Although the known modification to oligonucleotides, including the use of the 2'-O-methoxyethyl modification, have contributed to the development of oligonucleotides for
20 various uses, for use in diagnostics, therapeutics and as research reagents, there still exists a need in the art for further modifications to oligonucleotides having enhanced hybrid binding affinity and/or increased nuclease resistance.

25

SUMMARY OF THE INVENTION

This invention is directed to nucleosides having novel 2'-O-modifications of formula I:

- 9 -



wherein

Q is a heterocyclic base;

L₁, L₂ and L₃ are atoms in a ring system, as shown,
 5 having from about 4 to about 7 carbon atoms or having from
 about 3 to about 6 carbon atoms and 1 or 2 hetero atoms
 wherein said hetero atoms are selected from oxygen, nitrogen
 and sulfur and wherein said ring system is aliphatic,
 unsaturated aliphatic, aromatic or heterocyclic;

10 R is OX, SX, N(H)X or NX₂;

X is H, C₁-C₈ alkyl, C₁-C₈ haloalkyl, C(=NH)N(H)Z,
 C(=O)N(H)Z and OC(=O)N(H)Z;

Y is alkyl or haloalkyl having 1 to about 10 carbon
 atoms, alkenyl having 2 to about 10 carbon atoms, alkynyl
 15 having 2 to about 10 carbon atoms, aryl having 6 to about 14
 carbon atoms, N(H)X, NX₂, OX, halo, SX or CN;

n is 0, 1 or 2 (i.e., the ring can bear 0, 1, or 2 Y
 groups); and

Z is H or C₁-C₈ alkyl.

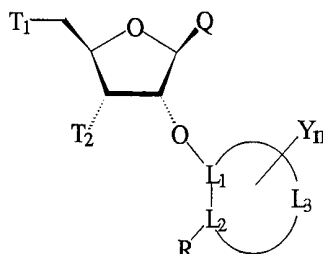
20 In preferred nucleosides of the invention: the ring
 system is phenyl, pyridyl, cyclopentyl, cyclobutyl,
 cyclohexyl, cycloheptyl, morpholino, piperidyl or
 piperazinyl; R is OH, SH, OCH₃, N(H)C(=NH)NH₂, NC(=O)NH₂, NH₂,
 or OC(=O)NH₂, preferably OCH₃, NH₂, OH, NC(=O)NH₂ or
 25 OC(=O)NH₂; L₁ and L₂ are each carbon atoms (with L₃ optionally
 being a heteroatom selected from O, S and N) or one L₁ and L₂
 is a carbon atom and the other of L₁ and L₂ is a heteroatom
 selected from O, S and N; Q is a purine or a pyrimidine,
 particularly adenine, cytosine, thymine, uracil, guanine or

- 10 -

2-aminoadenine; n is 0; and/or the ring system is phenyl or cyclohexyl.

The invention further includes compounds of the formula:

5



wherein

Q is a heterocyclic base;

T₁ and T₂, independently, are OH, a protected hydroxyl, an activated phosphate group, an activated phosphite group, a reactive group for forming an internucleotide linkage, a nucleotide, a nucleoside, or an oligonucleotide;

L₁, L₂ and L₃ comprise a ring system having from about 4 to about 7 carbon atoms or having from about 3 to about 6 carbon atoms and 1 or 2 hetero atoms wherein said hetero atoms are selected from oxygen, nitrogen and sulfur and wherein said ring system is aliphatic, unsaturated aliphatic, aromatic or heterocyclic;

R is OX, SX, N(H)X or NX₂;

X is H, C₁-C₈ alkyl, C₁-C₈ haloalkyl, C(=NH)N(H)Z, C(=O)N(H)Z and OC(=O)N(H)Z;

Y is alkyl or haloalkyl having 1 to about 10 carbon atoms, alkenyl having 2 to about 10 carbon atoms, alkynyl having 2 to about 10 carbon atoms, aryl having 6 to about 14 carbon atoms, N(H)X, NX₂, OX, halo, SX or CN;

n is 0, 1 or 2; and

Z is H or C₁-C₈ alkyl.

- 11 -

BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1 are computer model representations of nucleosides bearing novel 2'-modifications of the invention.

DESCRIPTION OF PREFERRED EMBODIMENTS

5 The present invention is directed to novel 2'-O-modified nucleotides and oligonucleotides incorporating these novel nucleotides. These novel 2'-O-modifications have certain desirable properties that contribute towards increases in binding affinity and/or nuclease resistance.

10 There are a number of potential items to consider when designing oligonucleotides that might have improvements in binding affinities. It appears that one effective approach to constructing modified oligonucleotides with very high RNA binding affinity is the combination of two or more different
15 types of modifications, each of which contributes favorably to various factors that might be important for binding affinity.

Freier and Altmann, *Nucleic Acids Research*, (1997) 25:4429-4443, recently published a study on the influence of
20 structural modifications of oligonucleotides on the stability of their duplexes with target RNA. In this study, the authors reviewed a series of oligonucleotides containing more than 200 different modifications that had been synthesized and assessed for their hybridization affinity
25 and T_m . Sugar modifications studied included substitutions on the 2'-position of the sugar, 3'-substitution, replacement of the 4'-oxygen, the use of bicyclic sugars, and four member ring replacements. Several nucleobase modifications were also studied including substitutions at
30 the 5, or 6 position of thymine, modifications of pyrimidine heterocycle and modifications of the purine heterocycle. Numerous backbone modifications were also investigated including backbones bearing phosphorus, backbones that did not bear a phosphorus atom, and backbones that were neutral.

- 12 -

Four general approaches might be used to improve hybridization of oligonucleotides to RNA targets. These include: preorganization of the sugars and phosphates of the oligodeoxynucleotide strand into conformations favorable for hybrid formation, improving stacking of nucleobases by the addition of polarizable groups to the heterocycle bases of the nucleotides of the oligonucleotide, increasing the number of H-bonds available for A-U pairing, and neutralization of backbone charge to facilitate removing undesirable repulsive interactions. This invention is predicated on the first of these, preorganization of the sugars and phosphates of the oligodeoxynucleotide strand into conformations favorable for hybrid formation, and can be used in combination with the other three approaches.

Sugars in DNA:RNA hybrid duplexes frequently adopt a C3' endo conformation. Thus modifications that shift the conformational equilibrium of the sugar moieties in the single strand toward this conformation should preorganize the antisense strand for binding to RNA. Of the several sugar modifications that have been reported and studied in the literature, the incorporation of electronegative substituents such as 2'-fluoro or 2'-alkoxy shift the sugar conformation towards the 3' endo (northern) pucker conformation. This pucker conformation further assisted in increasing the T_m of the oligonucleotide with its target. Large substituents at the 2'-position are, however, not well tolerated.

There is a clear correlation between substituent size at the 2'-position and duplex stability. Incorporation of alkyl substituents at the 2'-position typically leads to a significant decrease in binding affinity. Thus, small alkoxy groups generally are very favorable while larger alkoxy groups at the 2'-position generally are unfavorable. However, if the 2'-substituent contained an ethylene glycol

- 13 -

motif, then a strong improvement in binding affinity to the target RNA is observed.

The high binding affinity resulting from 2' substitution has been partially attributed to the 2' substitution causing a C3' *endo* sugar pucker which in turn may give the oligomer a favorable A-form like geometry. This is a reasonable hypothesis since substitution at the 2' position by a variety of electronegative groups (such as fluoro and *O*-alkyl chains) has been demonstrated to cause C3' *endo* sugar puckering (De Mesmaeker *et al.*, *Acc. Chem. Res.*, **1995**, 28, 366-374; Lesnik *et al.*, *Biochemistry*, **1993**, 32, 7832-7838).

In addition, for 2'-substituents containing an ethylene glycol motif, a *gauche* interaction between the oxygen atoms around the O-C-C-O torsion of the side chain may have a stabilizing effect on the duplex (Freier *et al.*, *Nucleic Acids Research*, (1997) 25:4429-4442). Such *gauche* interactions have been observed experimentally for a number of years (Wolfe *et al.*, *Acc. Chem. Res.*, **1972**, 5, 102; Abe *et al.*, *J. Am. Chem. Soc.*, **1976**, 98, 468). This *gauche* effect may result in a configuration of the side chain that is favorable for duplex formation. The exact nature of this stabilizing configuration has not yet been explained. While we do not want to be bound by theory, it may be that holding the O-C-C-O torsion in a single *gauche* configuration, rather than a more random distribution seen in an alkyl side chain, provides an entropic advantage for duplex formation.

To better understand the higher RNA affinity of 2'-*O*-methoxyethyl substituted RNA and to examine the conformational properties of the 2'-*O*-methoxyethyl substituent, a self-complementary dodecamer oligonucleotide

2'-*O*-MOE r(CGCGAAUUCGCG)

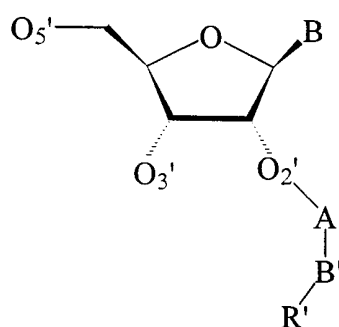
SEQ ID NO: 1

was synthesized, crystallized and its structure at a resolution of 1.7 Ångstrom was determined. This crystal

- 14 -

structure is the first crystal structure of a fully modified RNA oligonucleotide analogue. The duplex adopts an overall A-form conformation and all modified sugars display C3'-endo pucker. In most of the 2'-O-substituents, the torsion angle around the A'-B' bond, as depicted in Structure I below, of the ethylene glycol linker has a *gauche* conformation. For 2'-O-MOE, A' and B' of formula II below are methylene moieties of the ethyl portion of the MOE and R' is the methoxy portion.

10



II

The crystallization conditions used were 2 mM oligonucleotide, 50 mM Na Hepes pH 6.2-7.5, 10.50 mM MgCl₂, 15% PEG 400. The crystal data showed: space group C2, cell constants $a=41.2$ Å, $b=34.4$ Å, $c=46.6$ Å, $\beta=92.4^\circ$. The resolution was 1.7 Å at -170°C . The current R -factor was 20% (R_{free} 26%).

In the crystal, the 2'-O-MOE RNA duplex adopts a general orientation such that the crystallographic 2-fold rotation axis does not coincide with the molecular 2-fold rotation axis. The duplex adopts the expected A-type geometry and all of the 24 2'-O-MOE substituents were visible in the electron density maps at full resolution. The electron density maps as well as the temperature factors of substituent atoms indicate flexibility of the 2'-O-MOE substituent in some cases.

Most of the 2'-O-MOE substituents display a *gauche* conformation around the C-C bond of the ethyl linker.

- 15 -

However, in two cases, a *trans* conformation around the C-C bond is observed. The lattice interactions in the crystal include packing of duplexes against each other via their minor grooves. Therefore, for some residues, the
5 conformation of the 2'-O-substituent is affected by contacts to an adjacent duplex. In general, variations in the conformation of the substituents (e.g. g^+ or g^- around the C-C bonds) create a range of interactions between substituents, both inter-strand, across the minor groove,
10 and intra-strand. At one location, atoms of substituents from two residues are in van der Waals contact across the minor groove. Similarly, a close contact occurs between atoms of substituents from two adjacent intra-strand residues.

15 Previously determined crystal structures of A-DNA duplexes were for those that incorporated isolated 2'-O-methyl T residues. In the crystal structure above for the 2'-O-MOE substituents, a conserved hydration pattern has been observed for the 2'-O-MOE residues. A single water
20 molecule is seen located between O2', O3' and the methoxy oxygen atom of the substituent, forming contacts to all three of between 2.9 and 3.4 Å. In addition, oxygen atoms of substituents are involved in several other hydrogen bonding contacts. For example, the methoxy oxygen atom of
25 a particular 2'-O-substituent forms a hydrogen bond to N3 of an adenosine from the opposite strand via a bridging water molecule.

In several cases a water molecule is trapped between the oxygen atoms O2', O3' and OC' of modified nucleosides.
30 2'-O-MOE substituents with *trans* conformation around the C-C bond of the ethylene glycol linker are associated with close contacts between OC' and N2 of a guanosine from the opposite strand, and, water-mediated, between OC' and N3(G). When combined with the available thermodynamic data for
35 duplexes containing 2'-O-MOE modified strands, this crystal

- 16 -

structure allows for further detailed structure-stability analysis of other antisense modifications.

In extending the crystallographic structure studies, molecular modeling experiments were performed to study further enhanced binding affinity of oligonucleotides having 2'-O-modifications of the invention. The computer simulations were conducted on compounds of SEQ ID NO: 1, above, having 2'-O-modifications of the invention located at each of the nucleoside of the oligonucleotide. The simulations were effect with the oligonucleotide in aqueous solution and were done using the AMBER force field method (Cornell et al., *J. Am. Chem. Soc.*, **1995**, 117, 5179-5197). All the calculations were performed on an Indigo2 SGI machine (Silicon Graphics, Mountain View, CA).

New 2'-O-modifications of the inventions include, but are not limited to, those having a ring structure that incorporates a two atom portion identical to the A' and B' atoms of formula II. The ring structure is attached at the 2' position of a sugar moiety of one or more nucleoside that are incorporated into an oligonucleotide. The 2'-oxygen of the nucleoside links to a carbon atom (corresponding to the A' atom of formula II and position L₁ of formula I as described above) of the modification. These ring structures can be aliphatic, unsaturated aliphatic, aromatic or heterocyclic. A further atom of the ring, the L₂ position of formula I as described above (corresponding to the B' atom of formula II), bears a further oxygen atom or an sulfur or nitrogen atom thereon. This oxygen, sulfur or nitrogen atom is bonded to one or more hydrogen atoms, alkyl or haloalkyl moieties or it can be part of a further chemical moiety such as ureido, carbamate, amide or amidine moiety. The remainder of the ring structure restricts rotation about the bond joining these two ring atoms. This assists in positioning the "further oxygen, sulfur or nitrogen atom" (part of the R position as described above) such that the

- 17 -

further atom can be located juxtaposed to the 3'-oxygen atom (O3') of the nucleoside.

The ring structure can be further modified with a Y group of formula I above. This Y group is useful for
5 modifying the hydrophilic and hydrophobic properties of the ring to which it is attached and thus the properties of an oligonucleotide that includes the 2'-O-modifications of the invention. Further Y can be selected to be a group capable of assuming a charged structure, e.g. an amine. This is
10 particularly useful in modifying the overall charge of an oligonucleotide that includes a 2'-O-modifications of the invention. When an oligonucleotide is linked by charged phosphate groups, e.g. phosphorothioate or phosphodiester linkages, location of a counter ion on the 2'-O-
15 modification, e.g. Y is an amine functionality, locally naturalizes the charge in the local environment of the nucleotide bearing the 2'-O-modification. Such neutralization of charge will modulate uptake, cell localization and other pharmacokinetic and pharmacodynamic
20 effects of the oligonucleotide.

Preferred ring structures of the invention for inclusion as a 2'-O modification include cyclohexyl, cyclopentyl and phenyl rings as well as heterocyclic rings having spacial footprints similar to cyclohexyl, cyclopentyl
25 and phenyl rings. Particularly preferred 2'-O-substituent groups of the invention are listed below including an abbreviation for each:

	2'-O-(trans 2-methoxy cyclohexyl)	--	2'-O-(TMCHL)
30	2'-O-(trans 2-methoxy cyclopentyl)	--	2'-O-(TMCPL)
	2'-O-(trans 2-ureido cyclohexyl)	--	2'-O-(TUCHL)
	2'-O-(trans 2-methoxyphenyl)	--	2'-O-(2MP)

Structural details for duplexes incorporating such 2'-O-substituents were analyzed using the described AMBER force

- 18 -

field program on the Indigo2 SGI machine. The simulated structure maintained a stable A-form geometry throughout the duration of the simulation. The presence of the 2' substitutions locked the sugars in the C3'-endo

5 conformation.

The simulation for the TMCHL modification revealed that the 2'-O-(TMCHL) side chains have a direct interaction with water molecules solvating the duplex. The oxygen atoms in the 2'-O-(TMCHL) side chain are capable of forming a

10 water-mediated interaction with the 3' oxygen of the phosphate backbone as shown in Figure 1. The presence of the two oxygen atoms in the 2'-O-(TMCHL) side chain gives rise to favorable gauche interactions. The barrier for rotation around the O-C-C-O torsion is made even larger by

15 this novel modification. The preferential preorganization in an A-type geometry increases the binding affinity of the 2'-O-(TMCHL) to the target RNA. The locked side chain conformation in the 2'-O-(TMCHL) group created a more favorable pocket for binding water molecules. The presence

20 of these water molecules played a key role in holding the side chains in the preferable gauche conformation. While not wishing to be bound by theory, the bulk of the substituent, the diequatorial orientation of the substituents in the cyclohexane ring, the water of hydration

25 and the potential for trapping of metal ions in the conformation generated will additionally contribute to improved binding affinity and nuclease resistance of oligonucleotides incorporating nucleosides having this 2'-O-modification.

30 As described for the TMCHL modification above, identical computer simulations of the 2'-O-(TMCPL), the 2'-O-(2MP) and 2'-O-(TUCHL) modified oligonucleotides in aqueous solution also illustrate that stable A-form geometry will be maintained throughout the duration of the

35 simulation. The presence of the 2' substitution will lock

- 19 -

the sugars in the C3'-endo conformation and the side chains will have direct interaction with water molecules solvating the duplex. The oxygen atoms in the respective side chains are capable of forming a water-mediated interaction with the 3' oxygen of the phosphate backbone. The presence of the two oxygen atoms in the respective side chains give rise to the favorable gauche interactions. The barrier for rotation around the respective O-C-C-O torsions will be made even larger by respective modification. The preferential preorganization in A-type geometry will increase the binding affinity of the respective 2'-O-modified oligonucleotides to the target RNA. The locked side chain conformation in the respective modifications will create a more favorable pocket for binding water molecules. The presence of these water molecules plays a key role in holding the side chains in the preferable gauche conformation. The bulk of the substituent, the diequatorial orientation of the substituents in their respective rings, the water of hydration and the potential trapping of metal ions in the conformation generated will all contribute to improved binding affinity and nuclease resistance of oligonucleotides incorporating nucleosides having these respective 2'-O-modification.

The modifications of the invention can be used on a variety of nucleosides that are incorporated into oligonucleotides. These sugars can be normal sugars or they might be modified sugars. Representative modified sugars include carbocyclic or acyclic sugars, sugars having substituent groups at their 2' position, sugars having substituent groups at their 3' position, and sugars having substituents in place of one or more hydrogen atoms of the sugar. Other altered base moieties and altered sugar moieties are disclosed in United States Patent 3,687,808 and PCT application PCT/US89/02323.

- 20 -

Altered base moieties or altered sugar moieties also include other modifications consistent with the spirit of this invention. Such oligonucleotides are best described as being structurally distinguishable from, yet functionally
5 interchangeable with, naturally occurring or synthetic wild type oligonucleotides. All such oligonucleotides are comprehended by this invention so long as they function effectively to mimic the structure of a desired RNA or DNA strand.

10 Additional modifications may also be made at other positions on the oligonucleotide, particularly the 3' position of the sugar on the 3' terminal nucleotide and the 5' position of 5' terminal nucleotide. For example, one
15 additional modification of the oligonucleotides of the invention involves chemically linking to the oligonucleotide one or more moieties or conjugates which enhance the activity, cellular distribution or cellular uptake of the oligonucleotide. Such moieties include but are not limited to lipid moieties such as a cholesterol moiety (Letsinger et
20 al., *Proc. Natl. Acad. Sci. USA*, 1989, 86, 6553), cholic acid (Manoharan et al., *Bioorg. Med. Chem. Lett.*, 1994, 4, 1053), a thioether, e.g., hexyl-S-tritylthiol (Manoharan et al., *Ann. N.Y. Acad. Sci.*, 1992, 660, 306; Manoharan et al., *Bioorg. Med. Chem. Lett.*, 1993, 3, 2765), a thiocholesterol
25 (Oberhauser et al., *Nucl. Acids Res.*, 1992, 20, 533), an aliphatic chain, e.g., dodecandiol or undecyl residues (Saison-Behmoaras et al., *EMBO J.*, 1991, 10, 111; Kabanov et al., *FEBS Lett.*, 1990, 259, 327; Svinarchuk et al., *Biochimie*, 1993, 75, 49), a phospholipid, e.g., di-
30 hexadecyl-rac-glycerol or triethylammonium 1,2-di-O-hexadecyl-rac-glycero-3-H-phosphonate (Manoharan et al., *Tetrahedron Lett.*, 1995, 36, 3651; Shea et al., *Nucl. Acids Res.*, 1990, 18, 3777), a polyamine or a polyethylene glycol chain (Manoharan et al., *Nucleosides & Nucleotides*, 1995,

- 21 -

14, 969), or adamantane acetic acid (Manoharan et al.,
Tetrahedron Lett., 1995, 36, 3651), a palmityl moiety
(Mishra et al., *Biochim. Biophys. Acta*, 1995, 1264, 229), or
an octadecylamine or hexylamino-carbonyl-oxycholesterol
5 moiety (Crooke et al., *J. Pharmacol. Exp. Ther.*, 1996, 277,
923).

As used herein, the term "alkyl" includes but is not
limited to straight chain, branch chain, and cyclic
unsaturated hydrocarbon groups including but not limited to
10 methyl, ethyl, and isopropyl groups. Alkyl groups of the
present invention may be substituted. Representative alkyl
substituents are disclosed in United States Patent No.
5,212,295, at column 12, lines 41-50, hereby incorporated by
reference in its entirety.

15 Alkenyl groups according to the invention are to
straight chain, branch chain, and cyclic hydrocarbon groups
containing at least one carbon-carbon double bond, and
alkynyl groups are to straight chain, branch chain, and
cyclic hydrocarbon groups containing at least one carbon-
20 carbon triply bond. Alkenyl and alkynyl groups of the
present invention can be substituted.

Aryl groups are substituted and unsubstituted aromatic
cyclic moieties including but not limited to phenyl,
naphthyl, anthracyl, phenanthryl, pyrenyl, and xylyl groups.

25 Alkaryl groups are those in which an aryl moiety links an
alkyl moiety to a core structure, and aralkyl groups are
those in which an alkyl moiety links an aryl moiety to a
core structure.

In general, the term "hetero" denotes an atom other
30 than carbon, preferably but not exclusively N, O, or S.
Accordingly, the term "heterocyclic ring" denotes an alkyl
ring system having one or more heteroatoms (i.e., non-carbon
atoms). Preferred heterocyclic rings include, for example
but not limited to imidazole, pyrrolidine, 1,3-dioxane,
35 piperazine, morpholine rings. As used herein, the term

- 22 -

"heterocyclic ring" also denotes a ring system having one or more double bonds, and one or more heteroatoms. Preferred heterocyclic rings include, for example but not limited to the pyrrolidino ring.

5 Oligonucleotides according to the present invention that are hybridizable to a target nucleic acid preferably comprise from about 5 to about 50 nucleosides. It is more preferred that such compounds comprise from about 8 to about 30 nucleosides, with 15 to 25 nucleosides being particularly
10 preferred. As used herein, a target nucleic acid is any nucleic acid that can hybridize with a complementary nucleic acid-like compound. Further in the context of this invention, "hybridization" shall mean hydrogen bonding, which may be Watson-Crick, Hoogsteen or reversed Hoogsteen
15 hydrogen bonding between complementary nucleobases.

"Complementary" as used herein, refers to the capacity for precise pairing between two nucleobases. For example, adenine and thymine are complementary nucleobases which pair through the formation of hydrogen bonds. "Complementary"
20 and "specifically hybridizable," as used herein, refer to precise pairing or sequence complementarity between a first and a second nucleic acid-like oligomers containing nucleoside subunits. For example, if a nucleobase at a certain position of the first nucleic acid is capable of
25 hydrogen bonding with a nucleobase at the same position of the second nucleic acid, then the first nucleic acid and the second nucleic acid are considered to be complementary to each other at that position. The first and second nucleic acids are complementary to each other when a sufficient
30 number of corresponding positions in each molecule are occupied by nucleobases which can hydrogen bond with each other. Thus, "specifically hybridizable" and "complementary" are terms which are used to indicate a sufficient degree of complementarity such that stable and
35 specific binding occurs between a compound of the invention

- 23 -

and a target RNA molecule. It is understood that an oligomeric compound of the invention need not be 100% complementary to its target RNA sequence to be specifically hybridizable. An oligomeric compound is specifically
5 hybridizable when binding of the oligomeric compound to the target RNA molecule interferes with the normal function of the target RNA to cause a loss of utility, and there is a sufficient degree of complementarity to avoid non-specific binding of the oligomeric compound to non-target sequences
10 under conditions in which specific binding is desired, *i.e.* under physiological conditions in the case of *in vivo* assays or therapeutic treatment, or in the case of *in vitro* assays, under conditions in which the assays are performed.

The oligonucleotides of the present invention can be
15 used in diagnostics, therapeutics and as research reagents. They can be used in pharmaceutical compositions by including a suitable pharmaceutically acceptable diluent or carrier. They further can be used for treating organisms having a disease characterized by the undesired production of a
20 protein. The organism should be contacted with an oligonucleotide having a sequence that is capable of specifically hybridizing with a strand of nucleic acid coding for the undesirable protein. Treatments of this type can be practiced on a variety of organisms ranging from
25 unicellular prokaryotic and eukaryotic organisms to multicellular eukaryotic organisms. Any organism that utilizes RNA-DNA transcription or RNA-protein translation as a fundamental part of its hereditary, metabolic or cellular control is susceptible to therapeutic and/or prophylactic
30 treatment in accordance with this invention. Seemingly diverse organisms such as bacteria, yeast, protozoa, algae, all plants and all higher animal forms including warm-blooded animals, can be treated. Further each cell of multicellular eukaryotes can be treated since they include
35 both DNA-RNA transcription and RNA-protein translation as

- 24 -

integral parts of their cellular activity. Many of the organelles (e.g., mitochondria and chloroplasts) of eukaryotic cells also include transcription and translation mechanisms. Thus, single cells, cellular populations or
5 organelles can also be included within the definition of organisms that can be treated with therapeutic or diagnostic oligonucleotides. As used herein, therapeutics is meant to include the eradication of a disease state, by killing an organism or by control of erratic or harmful cellular growth
10 or expression.

Oligonucleotides according to the invention can be assembled in solution or through solid-phase reactions, for example, on a suitable DNA synthesizer utilizing nucleosides, phosphoramidites and derivatized controlled
15 pore glass (CPG) according to the invention and/or standard nucleotide precursors. In addition to nucleosides that include a novel modification of the inventions other nucleoside within an oligonucleotide may be further modified with other modifications at the 2' position. Precursor
20 nucleoside and nucleotide precursors used to form such additional modification may carry substituents either at the 2' or 3' positions. Such precursors may be synthesized according to the present invention by reacting appropriately protected nucleosides bearing at least one free 2' or 3'
25 hydroxyl group with an appropriate alkylating agent such as , but not limited to, alkoxyalkyl halides, alkoxyalkylsulfonates, hydroxyalkyl halides, hydroxyalkyl sulfonates, aminoalkyl halides, aminoalkyl sulfonates, phthalimidoalkyl halides, phthalimidoalkyl sulfonates,
30 alkylaminoalkyl halides, alkylaminoalkyl sulfonates, dialkylaminoalkyl halides, dialkylaminoalkylsulfonates, dialkylaminooxyalkyl halides, dialkylaminooxyalkyl sulfonates and suitably protected versions of the same. Preferred halides used for alkylating reactions include
35 chloride, bromide, fluoride and iodide. Preferred sulfonate

- 25 -

leaving groups used for alkylating reactions include, but are not limited to, benzenesulfonate, methylsulfonate, tosylate, p-bromobenzenesulfonate, triflate, trifluoroethylsulfonate, and (2,4-
5 dinitroanilino)benzenesulfonate.

Suitably protected nucleosides can be assembled into oligonucleotides according to known techniques. See, for example, Beaucage *et al.*, *Tetrahedron*, **1992**, 48, 2223.

The ability of oligonucleotides to bind to their
10 complementary target strands is compared by determining the melting temperature (T_m) of the hybridization complex of the oligonucleotide and its complementary strand. The melting temperature (T_m), a characteristic physical property of double helices, denotes the temperature (in degrees
15 centigrade) at which 50% helical (hybridized) versus coil (unhybridized) forms are present. T_m is measured by using the UV spectrum to determine the formation and breakdown (melting) of the hybridization complex. Base stacking, which occurs during hybridization, is accompanied by a
20 reduction in UV absorption (hypochromicity). Consequently, a reduction in UV absorption indicates a higher T_m . The higher the T_m , the greater the strength of the bonds between the strands. The structure-stability relationships of a large number of nucleic acid modifications have been
25 reviewed (Freier and Altmann, *Nucl. Acids Research*, **1997**, 25, 4429-443).

The relative binding ability of the oligonucleotides of the present invention was determined using protocols as described in the literature (Freier and Altmann, *Nucl. Acids
30 Research*, **1997**, 25, 4429-443). Typically absorbance versus temperature curves were determined using samples containing 4 μ M oligonucleotide in 100 mM Na⁺, 10 mM phosphate, 0.1 mM EDTA, and 4 μ M complementary, length matched RNA.

The *in vivo* stability of oligonucleotides is an
35 important factor to consider in the development of

- 26 -

oligonucleotide therapeutics. Resistance of oligonucleotides to degradation by nucleases, phosphodiesterases and other enzymes is therefore determined. Typical *in vivo* assessment of stability of the
5 oligonucleotides of the present invention is performed by administering a single dose of 5 mg/kg of oligonucleotide in phosphate buffered saline to BALB/c mice. Blood collected at specific time intervals post-administration is analyzed by HPLC or capillary gel electrophoresis (CGE) to determine
10 the amount of oligonucleotide remaining intact in circulation and the nature the of the degradation products.

Heterocyclic bases amenable to the present invention include both naturally and non-naturally occurring nucleobases and heterocycles. A representative list
15 includes adenine, guanine, cytosine, uridine, and thymine, as well as other synthetic and natural nucleobases such as xanthine, hypoxanthine, 2-aminoadenine, 6-methyl and other alkyl derivatives of adenine and guanine, 2-propyl and other alkyl derivatives of adenine and guanine, 5-halo uracil and
20 cytosine, 6-azo uracil, cytosine and thymine, 5-uracil (pseudo uracil), 4-thiouracil, 8-halo, oxa, amino, thiol, thioalkyl, hydroxyl and other 8-substituted adenines and guanines, 5-trifluoromethyl and other 5-substituted uracils and cytosines, 7-methylguanine. Further heterocyclic bases
25 include those disclosed in United States Patent No. 3,687,808, those disclosed in the *Concise Encyclopedia Of Polymer Science And Engineering*, pages 858-859, Kroschwitz, J.I., ed. John Wiley & Sons, 1990, and those disclosed by Englisch, et al., *Angewandte Chemie, International Edition*
30 1991, 30, 613.

Additional objects, advantages, and novel features of this invention will become apparent to those skilled in the art upon examination of the following examples, which are not intended to be limiting. All oligonucleotide sequences
35 are listed in a standard 5' to 3' order from left to right.

- 27 -

EXAMPLE 1**2'-O-(guaiacolyl)-5-methyluridine**

2-Methoxyphenol (6.2g, 50 mmol) was slowly added to a solution of borane in tetrahydrofuran (1 M, 10 mL, 10 mmol) with stirring in a 100 mL bomb. Hydrogen gas evolved as the solid dissolved O-2,2'-anhydro-5-methyluridine (1.2g, 5 mmol), and sodium bicarbonate (2.5 mg) were added and the bomb was sealed, placed in an oil bath and heated to 155 °C for 36 hours. The bomb was cooled to room temperature and opened. The crude solution was concentrated and the residue partitioned between water (200 mL) and hexanes (200 mL). The excess phenol was extracted into hexanes. The aqueous layer was extracted with ethyl acetate (3x200 mL) and the combined organic layer was washed once with water, dried over anhydrous sodium sulfate and concentrated. The residue was purified by silica gel flash column chromatography using methanol:methylene chloride (1/10, v/v) as the eluent. Fractions were collected and the target fractions were concentrated to give 490 mg of pure product as a white solid. $R_f = 0.545$ in $\text{CH}_2\text{Cl}_2/\text{CH}_3\text{OH}$ (10:1). MS/ES for $\text{C}_{17}\text{H}_{20}\text{N}_2\text{O}_7$, 364.4; Observed 364.9.

Example 2**5'-Dimethoxytrityl-2'-O-(2-methoxyphenyl)-5-methyluridine-3'-O-(2-cyanoethyl-N,N-diisopropylamino) phosphoramidite**

2'-O-(guaiacolyl)-5-methyl-uridine is treated with 1.2 equivalents of dimethoxytrityl chloride (DMT-Cl) in pyridine to yield the 5'-O-dimethoxy tritylated nucleoside. After evaporation of the pyridine and work up (CH_2Cl_2 /saturated NaHCO_3 solution) the compound is purified in a silica gel column. The 5'-protected nucleoside is dissolved in anhydrous methylene chloride and under argon atmosphere, N,N-diisopropylaminohydro-tetrazolide (0.5 equivalents) and bis-N,N-diisopropylamino-2-cyanoethyl-phosphoramidite (2 equivalents) are added via syringe over 1 min. The reaction

- 28 -

mixture is stirred under argon at room temperature for 16 hours and then applied to a silica column. Elution with hexane:ethylacetate (25:75) yielded the title compound.

Example 3

5 5'-Dimethoxytrityl-2'-O-(2-methoxyphenyl)-5-methyluridine-3'-O-succinate

The 5'-protected nucleoside from Example 2 is treated with 2 equivalents of succinic anhydride and 0.2 equivalents of 4-N,N-dimethylaminopyridine in pyridine. After 2 hours
10 the pyridine is evaporated, the residue is dissolved in CH₂Cl₂ and washed three times with 100 mL of 10% citric acid solution. The organic layer is dried over anhydrous MgSO₄ to give the desired succinate. The succinate is then attached to controlled pore glass (CPG) using established procedures
15 (Pon, R.T., Solid phase supports for oligonucleotide synthesis, in *Protocols for Oligonucleotides and Analogs*, S. Agrawal (Ed.), Humana Press: Totawa, NJ, 1993, 465-496).

Example 4

5'-Dimethoxytrityl-2'-O-(trans-2-methoxycyclohexyl)-5-methyl 20 uridine

2'-3'-O-dibutylstannyl-5-methyl uridine (Wagner et al., *J. Org. Chem.*, 1974, 39, 24) is alkylated with trans-2-methoxycyclohexyl tosylate at 70°C in DMF. A 1:1 mixture of 2'-O- and 3'-O-(trans-2-methoxycyclohexyl)-5-methyluridine
25 is obtained in this reaction. After evaporation of the DMF solvent, the crude mixture is dissolved in pyridine and treated with dimethoxytritylchloride (DMT-cl) (1.5 equivalents). The resultant mixture is purified by silica gel flash column chromatography to give the title compound.

- 29 -

Example 5

5'-Dimethoxytrityl-2'-O-(*trans*-2-methoxycyclohexyl)-5-methyluridine-3'-O-(2-cyanoethyl-N,N-diisopropylamino) phosphoramidite

- 5 5'-Dimethoxytrityl-2'-O-(*trans*-2-methoxycyclohexyl)-5-methyl uridine is phosphitylated according to the procedure described in Example 2 to give the required phosphoramidite.

Example 6

5'-Dimethoxytrityl-2'-O-(*trans*-2-methoxycyclohexyl)-5-methyluridine-3'-O-(succinyl-amino) CPG

5'-Dimethoxytrityl-2'-O-(*trans*-2-methoxycyclohexyl)-5-methyl uridine is succinylated and attached to the controlled pore glass to give the solid support bound nucleoside.

Example 7

trans-2-ureido-cyclohexanol

- Trans*-2-amino-cyclohexanol (Aldrich) is treated with triphosgene in methylene chloride (1/3 equivalent). To the resulting solution, excess ammonium hydroxide is added to give after work up the title compound.

Example 8

2'-O-(*trans*-2-uriedo-cyclohexyl)-5-methyl uridine

- Trans*-2-uriedo-cyclohexanol (50 mmol) is added to a solution of borane in tetrahydrofuran (1 M, 10mL, 10 mmol) while stirring in a 10 mL bomb. Hydrogen gas evolves as the reactant dissolves. O2,2'-anhydro-5-methyluridine (5 mmol) and sodium bicarbonate (2.5 mg) are added to the bomb and sealed. Then it is heated to 140° for 72 hrs. The bomb is cooled to room temperature and opened. The crude material was worked up as illustrated for Example 1 followed by

- 30 -

purification by silica gel flash column chromatography to give the title compound.

Example 9

5'-O-(Dimethoxytrityl)-2'-O-(*trans*-2-uriedo-cyclohexyl) 3'-
5 O-(2-cyanoethyl, N,N,-diisopropyl) uridine phosphoramidite
2'-O-(*trans*-2-uriedo-cyclohexyl)-5-methyl uridine
tritylated at the 5'-OH and phosphitylated at the 3'-OH
following the procedures illustrated in example 2 to give
the title compound.

10 Example 10

5'-O-dimethoxytrityl-2'-O-(*trans*-2-uriedo-cyclohexyl)-5-
methyl-3'-O-(succinyl)-amino CPG uridine
5'-O-dimethoxytrityl-2'-O-(*trans*-2-uriedo-cyclohexyl)-
5-methyl uridine is succinylated and attached to CPG as
15 illustrated in Example 6 above.

Example 11

2'-O-(*trans*-2-methoxy-cyclohexyl) adenosine

Trans-2-methoxycyclopentanol, *trans*-2-methoxy-
cylcohexanol, *trans*-2-methoxy-cyclopentyl tosylate and
20 *trans*-2-methoxy-cyclohexyl tosylate are prepared according
to reported procedures (Roberts, D.D., Hendrickson, W., *J.*
Org. Chem., **1967**, 34, 2415-2417; *J. Org. Chem.*, **1997**, 62,
1857-1859). A solution of adenosine (42.74 g, 0.16 mol) in
dry dimethylformamide (800 mL) at 5°C is treated with sodium
25 hydride (8.24 g, 60% in oil prewashed thrice with hexanes,
0.21 mol). After stirring for 30 min, *trans*-2-methoxy-
cyclohexyl tosylate (0.16 mol) is added over 20 minutes at
5°C. The reaction is stirred at room temperature for 48
hours, then filtered through Celite. The filtrate is
30 concentrated under reduced pressure followed by

- 31 -

coevaporation with toluene (2x100 mL) to give the title compound.

Example 12

N⁶-Benzoyl-2'-O-(*trans*-2-methoxycyclohexyl) adenosine

5 A solution of 2'-O-(*trans*-2-methoxy-cyclohexyl) adenosine (0.056 mol) in pyridine (100 mL) is evaporated under reduced pressure to dryness. The residue is redissolved in pyridine (560 mL) and cooled in an ice water bath. Trimethylsilyl chloride (36.4 mL, 0.291 mol) is added
10 and the reaction is stirred at 5°C for 30 minutes. Benzoyl chloride (33.6 mL, 0.291 mol) is added and the reaction is allowed to warm to 25°C for 2 hours and then cooled to 5°C. The reaction is diluted with cold water (112 mL) and after stirring for 15 min, concentrated ammonium hydroxide (112
15 mL). After 30 min, the reaction is concentrated under reduced pressure (below 30°C) followed by coevaporation with toluene (2x100 mL). The residue is dissolved in ethyl acetate-methanol (400 mL, 9:1) and the undesired silyl by-products are removed by filtration. The filtrate is
20 concentrated under reduced pressure and purified by silica gel flash column chromatography (800 g, chloroform-methanol 9:1). Selected fractions are combined, concentrated under reduced pressure and dried at 25°C/0.2 mmHg for 2 h to give the title compound.

25 Example 13

N⁶-Benzoyl-5'-O-(dimethoxytrityl)-2'-O-(*trans*-2-methoxycyclohexyl) adenosine

 A solution of N⁶-benzoyl-2'-O-(*trans*-2-methoxy-cyclohexyl) adenosine (0.285 mol) in pyridine (100 mL) is
30 evaporated under reduced pressure to an oil. The residue is redissolved in dry pyridine (300 mL) and 4,4'-dimethoxy-triphenylmethyl chloride (DMT-Cl, 10.9 g, 95%, 0.31 mol) added. The mixture is stirred at 25°C for 16 h and then

- 32 -

poured onto a solution of sodium bicarbonate (20 g) in ice water (500 mL). The product is extracted with ethyl acetate (2x150 mL). The organic layer is washed with brine (50 mL), dried over sodium sulfate (powdered) and evaporated under
5 reduced pressure (below 40°C). The residue is chromatographed on silica gel (400 g, ethyl acetate-hexane 1:1. Selected fractions were combined, concentrated under reduced pressure and dried at 25°C/0.2 mmHg to give the title compound.

10 Example 14

[N⁶-Benzoyl-5'-O-(4,4'-dimethoxytrityl)-2'-O-(*trans*-2-methoxycyclohexyl) adenosine-3'-O-yl]-N,N-diisopropylamino-cyanoethoxy phosphoramidite

Phosphitylation of N⁶-benzoyl-5'-O-(dimethoxytrityl)-2'-
15 O-(*trans*-2-methoxycyclohexyl) adenosine was performed as illustrated in Example 2 to give the title compound.

EXAMPLE 15

General procedures for oligonucleotide synthesis

Oligonucleotides are synthesized on a PerSeptive
20 Biosystems Expedite 8901 Nucleic Acid Synthesis System. Multiple 1-mmol syntheses are performed for each oligonucleotide. The 3'-end nucleoside containing solid support is loaded into the column. Trityl groups are removed with trichloroacetic acid (975 mL over one minute)
25 followed by an acetonitrile wash. The oligonucleotide is built using a modified diester or thioate protocol.

Phosphodiester protocol

All standard amidites (0.1 M) are coupled over a 1.5 minute time frame, delivering 105 µL material. All novel
30 amidites are dissolved in dry acetonitrile (100 mg of amidite/1 mL acetonitrile) to give approximately 0.08-0.1 M solutions. The 2'-modified amidite is double coupled using 210 µL over a total of 5 minutes. Total coupling time is

- 33 -

approximately 5 minutes (210 mL of amidite delivered).
1-H-tetrazole in acetonitrile is used as the activating
agent. Excess amidite is washed away with acetonitrile.
(1S)-(+)-(10-camphorsulfonyl) oxaziridine (CSO, 1.0 g
5 CSO/8.72 mL dry acetonitrile) is used to oxidize (3 minute
wait step) delivering approximately 375 μ L of oxidizer.
Standard amidites are delivered (210 μ L) over a 3-minute
period.

Phosphorothioate protocol

10 The 2'-modified amidite is double coupled using 210 μ L
over a total of 5 minutes. The amount of oxidizer,
3H-1,2-benzodithiole-3-one-1,1-dioxide (Beaucage reagent,
3.4 g Beaucage reagent/200 mL acetonitrile), is 225 μ L (one
minute wait step). The unreacted nucleoside is capped with
15 a 50:50 mixture of tetrahydrofuran/acetic anhydride and
tetrahydrofuran/pyridine/1-methyl imidazole. Trityl yields
are followed by the trityl monitor during the duration of
the synthesis. The final DMT group is left intact. After
the synthesis, the contents of the synthesis cartridge (1
20 mmole) is transferred to a Pyrex vial and the
oligonucleotide is cleaved from the controlled pore glass
(CPG) using 30% ammonium hydroxide (NH_4OH , 5 mL) for
approximately 16 hours at 55°C.

Oligonucleotide Purification

25 After the deprotection step, the samples are filtered
from CPG using Gelman 0.45 μ m nylon acrodisc syringe
filters. Excess NH_4OH is evaporated away in a Savant AS160
automatic speed vac. The crude yield is measured on a
Hewlett Packard 8452A Diode Array Spectrophotometer at 260
30 nm. Crude samples are then analyzed by mass spectrometry
(MS) on a Hewlett Packard electrospray mass spectrometer.
Trityl-on oligonucleotides are purified by reverse phase
preparative high performance liquid chromatography (HPLC).
HPLC conditions are as follows: Waters 600E with 991
35 detector; Waters Delta Pak C4 column (7.8X300mm); Solvent A:

- 34 -

50 mM triethylammonium acetate (TEA-Ac), pH 7.0; Solvent B: 100% acetonitrile; 2.5 mL/min flow rate; Gradient: 5% B for first five minutes with linear increase in B to 60% during the next 55 minutes. Fractions containing the desired product/s (retention time = 41 minutes for DMT-ON-16314; retention time = 42.5 minutes for DMT-ON-16315) are collected and the solvent is dried off in the speed vac. Oligonucleotides are detritylated in 80% acetic acid for approximately 60 minutes and lyophilized again. Free trityl and excess salt are removed by passing detritylated oligonucleotides through Sephadex G-25 (size exclusion chromatography) and collecting appropriate samples through a Pharmacia fraction collector. The solvent is again evaporated away in a speed vac. Purified oligonucleotides are then analyzed for purity by CGE, HPLC (flow rate: 1.5 mL/min; Waters Delta Pak C4 column, 3.9X300mm), and MS. The final yield is determined by spectrophotometer at 260 nm.+

PROCEDURES

PROCEDURE 1

20 ICAM-1 Expression

Oligonucleotide Treatment of HUVECs: Cells were washed three times with Opti-MEM (Life Technologies, Inc.) prewarmed to 37°C. Oligonucleotides were premixed with 10µg/mL Lipofectin (Life Technologies, Inc.) in Opti-MEM, serially diluted to the desired concentrations, and applied to washed cells. Basal and untreated (no oligonucleotide) control cells were also treated with Lipofectin. Cells were incubated for 4 h at 37°C, at which time the medium was removed and replaced with standard growth medium with or without 5 mg/mL TNF-α ® & D Systems). Incubation at 37°C was continued until the indicated times.

Quantitation of ICAM-1 Protein Expression by

Fluorescence-activated Cell Sorter: Cells were removed from plate surfaces by brief trypsinization with 0.25% trypsin in

- 35 -

PBS. Trypsin activity was quenched with a solution of 2% bovine serum albumin and 0.2% sodium azide in PBS (+Mg/Ca). Cells were pelleted by centrifugation (1000 rpm, Beckman GPR centrifuge), resuspended in PBS, and stained with 3 μ l/10⁵ cells of the ICAM-1 specific antibody, CD54-PE (Pharmingen). Antibodies were incubated with the cells for 30 min at 4°C in the dark, under gently agitation. Cells were washed by centrifugation procedures and then resuspended in 0.3 mL of FACSFlow buffer (Becton Dickinson) with 0.5% formaldehyde (Polysciences). Expression of cell surface ICAM-1 was then determined by flow cytometry using a Becton Dickinson FACSscan. Percentage of the control ICAM-1 expression was calculated as follows: [(oligonucleotide-treated ICAM-1 value) - (basal ICAM-1 value)/(non-treated ICAM-1 value) - (basal ICAM-1 value)]. (Baker, Brenda, et. al. 2'-O-(2-Methoxy)ethyl-modified Anti-intercellular Adhesion Molecule 1 (ICAM-1) Oligonucleotides Selectively Increase the ICAM-1 mRNA Level and Inhibit Formation of the ICAM-1 Translation Initiation Complex in Human Umbilical Vein Endothelial Cells, *The Journal of Biological Chemistry*, 272, 11994-12000, 1997.)

ICAM-1 expression of 2'-O-(trans-2-methoxycyclohexyl) modified oligonucleotides of the invention is measured by the reduction of ICAM-1 levels in treated HUVEC cells. The oligonucleotides are believed to work by a direct binding RNase H independent mechanism. Appropriate scrambled control oligonucleotides are used as controls. They have the same base composition as the test sequence.

Sequences that contain the 2'-O-(trans-2-methoxycyclohexyl) modification as listed in Table 1 below are prepared and tested in the above assay. SEQ ID NO: 4, a C-raf targeted oligonucleotide, was used as a control.

- 36 -

Table 1
Oligonucleotides Containing
2'-O-(trans-2-methoxycyclohexyl) modification

5	SEQ ID	Sequence (5'-3')	Target
	NO:		
	2	5'-T _s C ^m _s T _s G _s A _s G _s T _s A _s G _s C ^m _s A _s G _s A _s G _s G _s A _s G _s C ^m _s T _s C-3'	Human ICAM -1
	3	5'-T _o C ^m _o T _o G _o A _o G _o T _o A _o G _o C ^m _o A _o G _o A _o G _o G _o A _o G _o C ^m _o T _o C-3'	Human ICAM -1
	4	5'-A _s T _s G _s C ^m _s A _s T _s T _s C ^m _s T _s G _s C ^m _s C ^m _s C ^m _s C ^m _s C ^m _s A _s A _s G _s G _s A-3'	mouse C-raf
	5	5'-G _s C ^m _s C ^m _s C ^m _s A _s A _s G _s C ^m _s T _s G _s G _s C ^m _s A _s T _s C ^m _s C ^m _s G _s T _s C ^m _s A-3'	Human ICAM-1

- 10 All nucleosides in bold are 2'-O-(trans-2-methoxycyclohexyl); subscript S indicates a phosphorothioate linkage; subscript O indicates a phosphodiester linkage; and superscript m on C (C^m) indicates a 5-methyl-C.

PROCEDURE 2

15 Enzymatic Degradation of 2'-O-modified oligonucleotides

Three oligonucleotides are synthesized incorporating the modifications shown in Table 2 below at the 3'-end. These modified oligonucleotides are subjected to snake venom phosphodiesterase action.

- 20 Oligonucleotides (30 nanomoles) are dissolved in 20 mL of buffer containing 50 mM Tris-HCl pH 8.5, 14 mM MgCl₂, and 72 mM NaCl. To this solution 0.1 units of snake-venom phosphodiesterase (Pharmacia, Piscataway, NJ), 23 units of nuclease P1 (Gibco LBRL, Gaithersburg, MD), and 24 units of
- 25 calf intestinal phosphatase (Boehringer Mannheim, Indianapolis, IN) are added and the reaction mixture is incubated at 37°C for 100 hours. HPLC analysis is carried out using a Waters model 715 automatic injector, model 600E

- 37 -

pump, model 991 detector, and an Alltech (Alltech Associates, Inc., Deerfield, IL) nucleoside/nucleotide column (4.6 x 250 mm). All analyses are performed at room temperature. The solvents used are A: water and B: acetonitrile. Analysis of the nucleoside composition is accomplished with the following gradient: 0-5 min., 2% B (isocratic); 5-20 min., 2% B to 10% B (linear); 20-40 min., 10% B to 50% B. The integrated area per nanomole is determined using nucleoside standards. Relative nucleoside ratios are calculated by converting integrated areas to molar values and comparing all values to thymidine, which is set at its expected value for each oligomer.

Table 2

15	Relative Nuclease Resistance of 2'-Modified Chimeric Oligonucleotides	
	5'-TTT TTT TTT TTT TTT T*T*T*T*-3' SEQ ID NO 6	
	(Uniform phosphodiester)	
	T* = 2'-modified T	Modification
20	-O-CH ₂ -CH ₂ -CH ₃	Pr
	-O-CH ₂ -CH ₂ -O-CH ₃	MOE
	-O-(TMCHL)	TMCHYL

PROCEDURE 3

General procedure for the evaluation of gapped 2'-O-TMCHYL modified oligonucleotides targeted to *Ha-ras*

Different types of human tumors, including sarcomas, neuroblastomas, leukemias and lymphomas, contain active oncogenes of the *ras* gene family. *Ha-ras* is a family of small molecular weight GTPases whose function is to regulate cellular proliferation and differentiation by transmitting signals resulting in constitutive activation of *ras* are associated with a high percentage of diverse human cancers.

- 38 -

Thus, *ras* represents an attractive target for anticancer therapeutic strategies.

SEQ ID NO: 7 is a 20-base phosphorothioate oligodeoxynucleotide targeting the initiation of translation region of human *Ha-ras* and it is a potent isotype-specific inhibitor of *Ha-ras* in cell culture based on screening assays ($IC_{50}=45$ nm). Treatment of cells *in vitro* with SEQ ID NO: 7 results in a rapid reduction of *Ha-ras* mRNA and protein synthesis and inhibition of proliferation of cells containing an activating *Ha-ras* mutation. When administered at doses of 25 mg/kg or lower by daily intraperitoneal injection (IP), SEQ ID NO: 7 exhibits potent antitumor activity in a variety of tumor xenograft models, whereas mismatch controls do not display antitumor activity. SEQ ID NO: 7 has been shown to be active against a variety of tumor types, including lung, breast, bladder, and pancreas in mouse xenograft studies (Cowser, L.M. *Anti-cancer drug design*, 1997, 12, 359-371). A second-generation analog of SEQ ID NO: 7, where the 5' and 3' termini ("wings") of the sequence are modified with 2'-methoxyethyl (MOE) modification and the backbone is kept as phosphorothioate (Table 2, SEQ ID NO: 13), exhibits IC_{50} of 15 nm in cell culture assays. thus, a 3-fold improvement in efficacy is observed from this chimeric analog. Because of the improved nuclease resistance of the 2'-MOE phosphorothioate, SEQ ID NO: 13 increases the duration of antisense effect *in vitro*. This will relate to frequency of administration of this drug to cancer patients. SEQ ID NO: 13 is currently under evaluation in *ras* dependent tumor models (Cowser, L.M. *Anti-cancer drug design*, 1997, 12, 359-371). The parent compound, SEQ ID NO: 7, is in Phase I clinical trials against solid tumors by systemic infusion. Antisense oligonucleotides having the 2'-O-TMCHL modification are prepared and tested in the aforementioned assays in the manner described to determine activity.

- 39 -

Table 3
Ha-ras Antisense Oligonucleotides With 2'-O-TMCHYL
Modifications and Their Controls

5

SEQ ID NO:	Sequence	Backbone	2'-Modif.	Comments
7	5'-TsCsCs GsTsCs AsTsCs Gs CsTs CsCsTs CsAsGs GsG-3'	P=S	2'-H	parent
8	5'-TsCsAs GsTsAs AsTsAs Gs GsCs CsCsAs CsAsTs GsG-3'	P=S	2'-H	mismatch control
9	5'-ToToCo <u>GsTsCs AsTsCs Gs CsTs</u> CoCoTo CoAoGo GoG-3'	P=O/P=S/ P=O	2'-O-TMCHL in the wings	Gapmer (mixed backbone)
10	5'-TsCsCs <u>GsTsCs AsTsCs Gs CsTs</u> CsCsTs CsAsGs GsG-3'	P=S	2'-O-TMCHL in the wings	Gapmer as uniform thioate
11	5'-ToCoAo <u>GsTsAs AsTsAs GsCsCs GsCsCs Gs</u> Co Co CoCoAo CoAoTo GoG-3'	P=O/P=S/ P=O	2'-O-TMCHL in the wings	Gapmer (mixed backbone)
12	5'-TsCsAs <u>GsTsAs AsTs As GsCsCs GsCsCs CsCsAs CsAsTs GsC-3'</u>	P=S	2'-O-TMCHL in the wings	Gapmer as uniform thioate
13	5'-TsCsCs <u>GsTsCs AsTsCs Gs CsTs</u> CsCsTs CsAsGs GsG-3'	P=S	2'-MOE in the wings	Gapmer with MOE as control
14	5'-TsCsAs <u>GsTsAs AsTsAsGsCs CsGsCsCsCsCsAsCsAsTs</u> GsC-3'	P=S	2'-MOE in the wings	Gapmer with MOE as control

10

15 underlined portions of sequences are 2'-deoxy

- 40 -

PROCEDURE 4

General procedure for the evaluation of 2'-O-TMCHL
oligonucleotides targeted to HCV

Uniformly modified 2'-O-TMCHYL phosphodiester
5 oligonucleotides are evaluated for antisense inhibition of
HCV gene via a translation arrest mechanism.

Hepatitis C virus (HCV) is known to be responsible for
liver disease in many millions of people throughout the
world. HCV is an enveloped, positive-strand RNA virus of
10 the flavivirus family. Initial infections in humans are
typically asymptomatic, but chronic infection often ensues
in which liver cirrhosis and hepatocellular carcinoma are
long-term sequelae. Interferon- α (IFN- α) therapy is widely
used in attempts to eradicate the virus from chronically
15 infected individuals, but long-term remissions are achieved
in only about 20% of patients, even after 6 months of
therapy. So far, there is no antiviral drug available for
the treatment of HCV. (Blair et al., 1998). Drug discovery
and development efforts have been hampered by the lack of
20 suitable cell culture replication assays for HCV, and
vaccine production has been hampered by genetic variability
of the virus' envelope genes. Specific inhibitors of cloned
viral enzymes such as proteases and the viral polymerase
have not yet been reported.

25 Antisense oligonucleotide therapy represents a novel
approach to the control of HCV infection. Several antisense
oligonucleotides complementary to HCV RNA sequences adjacent
to the polyprotein initiation codon of HCV have been
designed at Isis (Hanecak et al., *J. Virol.*, 1996, 70, 5203-
30 5212). The target genome is highly conserved among
independent HCV isolates.

It was shown that an RNase H-independent antisense
oligonucleotide had greater activity than its parent
phosphorothioate (which will work by RNase H mechanism)
35 which was targeted to the AUG site of a core protein

- 41 -

sequence of HCV in a human hepatocyte cell line employing a uniformly modified 2'-O-(methoxyethyl) phosphodiester (P=O 20 mer) Hanecak et al., *J. Virol.*, **1996**, 70, 5203-5212

Hanecak et al., *J. Virol.*, **1996**, 70, 5203-5212. Hepatitis C virus core protein levels were reduced as efficiently as the corresponding 2'-deoxyphosphorothioate with an IC₅₀ of 100 nm. SEQ ID NO: 16 was a potent inhibitor of core protein expression without affecting HCV RNA levels. This suggested the inhibition of HCV translation. The parent compound (SEQ ID NO: 15) had T_m of 50.8°C while the 2'-MOE compound (SEQ ID NO: 16) had a T_m of 83.8°C. Thus, SEQ ID NO: 16 had a better affinity for HCV RNA. The replicative cycle of HCV takes place in the cytoplasm of infected cells, in which RNase H levels have been reported to reduce relative to those of the nucleus. For this reason, it is better to utilize an antisense oligonucleotide which will work by non-RNase H mechanism to inhibit HCV. Oligonucleotide SEQ ID NO: 16 is an attractive lead since it contains a P=O linkage with a 2'-MOE modification. SEQ ID NO: 17 will be tested in accordance with the testing of SEQ ID NO: 15 and 16.

Table 4

5'-TTT AGG ATT CGT GCT CAT GG-3'

Antisense Oligonucleotide Targeting HCVC 5'-NCR

Nucleotide Numbers 340-359

SEQ ID NO:	Backbone	2'-modification	T _m (°C)
15	P=S	2'-deoxy	50.8
16	P=O	2'-MOE	83.8
17	P=O	2'-2'-O-TMCHL	

PROCEDURE 5

In vitro Assays

Isis antisense oligonucleotides complementary to the HCV polyprotein initiation codon sequence are known to inhibit expression of the viral core protein in immortalized cell lines engineered to express HCV RNA from recombinant DNA

- 42 -

integrated into the host cell genome Hanecak et al., *J. Virol.*, **1996**, *70*, 5203-5212. Non-complementary control oligonucleotides have no effect on HCV RNA or protein levels in this system. H8Ad17C cells will be treated with a range
5 of concentration of oligonucleotides shown in Table 5 above (0-200 nm) in the presence of cationic lipids and total protein levels will be evaluated 20 hours later by western blot analysis.

PROCEDURE 6

10 *In vivo* model for HCV

Animal models of HCV infection are not readily available. An alternative approach has been developed to evaluate antisense oligonucleotides to inhibit HCV gene expression in livers of mice. For these experiments, HCV sequences,
15 including SEQ ID NO: 16 target sequence, were fused to a luciferase reporter gene and inserted into a Vaccinia virus. Infection of mice with this recombinant vaccination virus results in quantifiable levels of luciferase in liver tissue. Potent phosphorothioate antisense oligonucleotides
20 have been shown to work in this model. SEQ ID NO: 17 (the 2'-O-TMCHL RNA analog of SEQ ID NO: 16) will be evaluated for inhibition of expression of the HCV-luciferase construct in livers of mice infected with the recombinant vaccinia virus. Inhibition will be evaluated for sequence-dependency
25 and dose response. HCV-luciferase expression in livers of mice infected with a control vaccinia virus vector lacking HCV target sequences will be used as control and the effect of antisense drug in these control systems will be evaluated. (Antisense oligonucleotide-mediated inhibition
30 of hepatitis C virus gene expression in mouse liver (Anderson et al., Meeting Abstracts, International Hepatitis Meeting, Hawaii, 1997)).

- 43 -

PROCEDURE 7

In vivo nuclease resistance

The *in vivo* Nuclease Resistance of gapmers having the 2'-O-TMCHL is studied in mouse plasma and tissues (kidney and liver). For this purpose, the C-raf oligonucleotide series SEQ ID NO: 18 will be used and the following five oligonucleotides listed in Table 5 below will be evaluated for their relative nuclease resistance.

Table 5

SEQ ID	Sequence	Backbone	Description
18	5'-ATG CAT TCT GCC CCA AGGA-3'	P=S, 2'-H	(control) rodent C-raf antisense oligo
19	<u>AoToGoCoAsTsTsCsTsGsCsC</u> sCsCs <u>AoAoGoGoA</u>	P=O/P=S/ P=O	(control) <u>2'-MOE/2'-H/</u> <u>2'-MOE</u>
20	<u>AsTsGsCsAsTsTsCsTsGsCsC</u> sCsCs <u>AsAsGsGsA</u>	P=S	(control) 2'-MOE/2'-H/2'- MOE
21	<u>AoToGoCoAsTsTsCsTsGsCsC</u> sCsCs <u>AoAoGoGoA</u>	P=O/P=S/ P=O	<u>2'-2'-O-TMCHL/</u> 2'-H/ <u>2'-2'-O-</u> <u>TMCHL</u>
22	<u>AsTsGsCsAsTsTsCsTsGsCsC</u> sCsCs <u>AsAsGsGsA</u>	P=S	<u>2'-2'-O-TMCHL/</u> 2'-H/ <u>2'-2'-O-</u> <u>TMCHL</u>

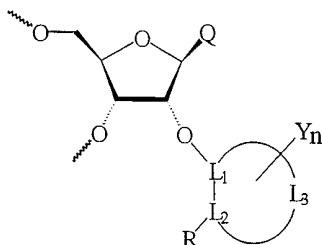
- 44 -

PROCEDURE 8**Animal studies**

For each oligonucleotide to be studied, 9 male BALB/c mice (Charles River, Wilmington, MA), weighing about 25 g are used (Crooke et al., *J. Pharmacol. Exp. Ther.*, 1996, 277, 923). Following a 1-week acclimation, the mice receive a single tail vein injection of oligonucleotide (5 mg/kg) administered in phosphate buffered saline (PBS), pH 7.0. The final concentration of oligonucleotide in the dosing solution is (5 mg/kg) for the PBS formulations. One retro-orbital bleed (either 0.25, 9.05, 2 or 4 post dose) and a terminal bleed (either 1, 3, 8 or 24 h post dose) is collected from each group. The terminal bleed (approximately 0.6-0.8 mL) is collected by cardiac puncture following ketamine/xylazine anesthesia. The blood is transferred to an EDTA-coated collection tube and centrifuged to obtain plasma. At termination, the liver and kidneys will be collected from each mouse. Plasma and tissues homogenates will be used for analysis for determination of intact oligonucleotide content by CGE. All samples will be immediately frozen on dry ice after collection and stored at -80°C until analysis.

WHAT IS CLAIMED IS:

1. An oligonucleotide having at least one nucleoside of formula:



5 wherein

Q is a heterocyclic base;

L₁, L₂ and L₃ comprise a ring system having from about 4 to about 7 carbon atoms or having from about 3 to about 6 carbon atoms and 1 or 2 hetero atoms wherein said hetero atoms are selected from oxygen, nitrogen and sulfur and wherein said ring system is aliphatic, unsaturated aliphatic, aromatic or heterocyclic;

R is OX , SX , N(H)X or NX_2 ;

X is H, C₁-C₈ alkyl, C₁-C₈ haloalkyl, C(=NH)N(H)Z,
 15 C(=O)N(H)Z and OC(=O)N(H)Z;

Y is alkyl or haloalkyl having 1 to about 10 carbon atoms, alkenyl having 2 to about 10 carbon atoms, alkynyl having 2 to about 10 carbon atoms, aryl having 6 to about 14 carbon atoms, N(H)X, NX₂, OX, halo, SX or CN;

20 n is 0, 1 or 2; and

Z is H or C₁-C₈ alkyl.

2. The oligonucleotide of claim 1 wherein said ring system is phenyl, pyridyl, cyclopentyl, cyclobutyl, cyclohexyl, cycloheptyl, morpholino, piperidyl or
25 piperazinyl.

3. The oligonucleotide of claim 1 wherein said R is OH, SH, OCH₃, N(H)C(=NH)NH₂, NC(=O)NH₂, NH₂, or OC(=O)NH₂.

- 46 -

4. The oligonucleotide of claim 1 wherein L_1 and L_2 are each carbon atoms.

5. The oligonucleotide of claim 1 wherein one of L_1 and L_2 is a carbon atom and the other of L_1 and L_2 is a
5 heteroatom selected from O, S and N.

6. The oligonucleotide of claim 1 wherein each of L_1 and L_2 is a carbon atom and L_3 is a heteroatom selected from O, S and N.

7. The oligonucleotide of claim 1 wherein Q is a
10 purine or a pyrimidine.

8. The oligonucleotide of claim 1 wherein Q is adenine, cytosine, thymine, uracil, guanine or 2-aminoadenine.

9. The oligonucleotide of claim 3 wherein R is OCH_3 .
15

10. The oligonucleotide of claim 3 wherein R is NH_2 or OH.

11. The oligonucleotide of claim 3 wherein R is $\text{NC}(=\text{O})\text{NH}_2$ or $\text{OC}(=\text{O})\text{NH}_2$.

20 12. The oligonucleotide of claim 1 wherein n is 0.

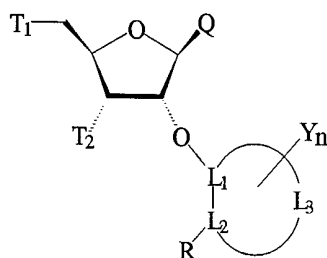
13. The oligonucleotide of claim 1 wherein said ring system is phenyl.

14. The oligonucleotide of claim 1 wherein said ring system is cyclohexyl.

25

- 47 -

15. A compound having the formula:



wherein

Q is a heterocyclic base;

5 T₁ and T₂, independently, are OH, a protected hydroxyl, an activated phosphate group, an activated phosphite group, a reactive group for forming an internucleotide linkage, a nucleotide, a nucleoside, or an oligonucleotide;

10 L₁, L₂ and L₃ comprise a ring system having from about 4 to about 7 carbon atoms or having from about 3 to about 6 carbon atoms and 1 or 2 hetero atoms wherein said hetero atoms are selected from oxygen, nitrogen and sulfur and wherein said ring system is aliphatic, unsaturated aliphatic, aromatic or heterocyclic;

15 R is OX, SX, N(H)X or NX₂;

X is H, C₁-C₈ alkyl, C₁-C₈ haloalkyl, C(=NH)N(H)Z, C(=O)N(H)Z and OC(=O)N(H)Z;

20 Y is alkyl or haloalkyl having 1 to about 10 carbon atoms, alkenyl having 2 to about 10 carbon atoms, alkynyl having 2 to about 10 carbon atoms, aryl having 6 to about 14 carbon atoms, N(H)X, NX₂, OX, halo, SX or CN;

n is 0, 1 or 2; and

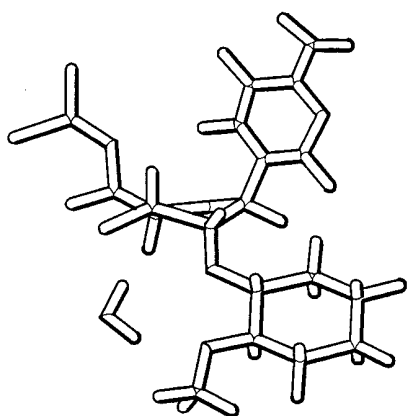
Z is H or C₁-C₈ alkyl.

25 16. The compound of claim 15 wherein said ring system is phenyl, pyridyl, cyclopentyl, cyclobutyl, cyclohexyl, cycloheptyl, morpholino, piperidyl or piperazinyl.

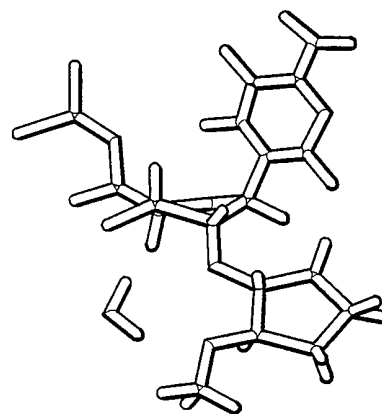
17. The compound of claim 15 wherein said R is OH, SH, OCH₃, N(H)C(=NH)NH₂, NC(=O)NH₂, NH₂, or OC(=O)NH₂.

- 48 -

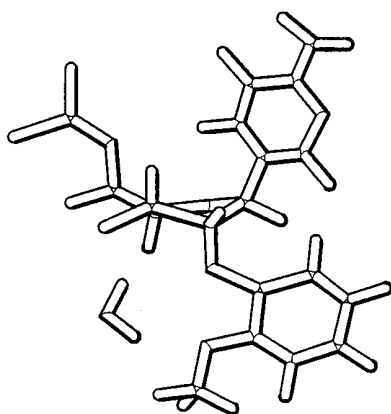
18. The compound of claim 15 wherein L_1 and L_2 are each carbon atoms.
19. The compound of claim 15 wherein one of L_1 and L_2 is a carbon atom and the other of L_1 and L_2 is a heteroatom
5 selected from O, S and N.
20. The compound of claim 15 wherein each of L_1 and L_2 is a carbon atom and L_3 is a heteroatom selected from O, S and N.
21. The compound of claim 15 wherein Q is a purine or
10 a pyrimidine.
22. The compound of claim 15 wherein Q is adenine, cytosine, thymine, uracil, guanine or 2-aminoadenine.
23. The compound of claim 17 wherein R is OCH_3 .
- 15 24. The compound of claim 17 wherein R is NH_2 or OH.
25. The compound of claim 17 wherein R is $NC(=O)NH_2$ or $OC(=O)NH_2$.
26. The compound of claim 15 wherein n is 0.
27. The compound of claim 15 wherein said ring system
20 is phenyl.
28. The compound of claim 15 wherein said ring system is cyclohexyl.



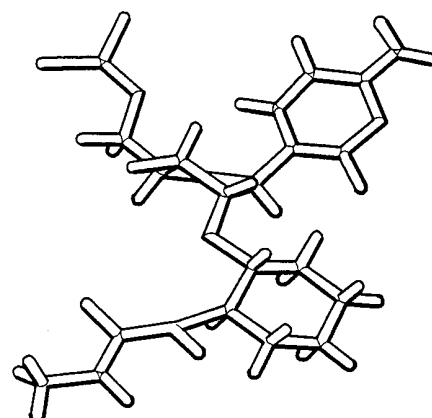
2'-O-(TMCHL)



2'-O-(TMCPL)



2'-O-(2MP)



2'-O-(TUCHL)

TMCHL - trans 2-methoxy cyclohexyl
TMCPL - trans 2-methoxy cyclopentyl
TUCHL - 2-ureido cyclopentyl
2MP - trans 2-methoxyphenyl

FIG. 1

SEQUENCE LISTING

<110> ISIS Pharmaceuticals, Inc. et al

<120> RNA Targeted 2'-Modified Oligonucleotides That Are
Conformationally Preorganized

<130> ISIS3968

<140>

<141>

<150> 09/123,108

<151> 1998-07-27

<160> 22

<170> PatentIn Ver. 2.0

<210> 1

<211> 12

<212> RNA

<213> Artificial Sequence

<220>

<223> Description of Artificial Sequence: Novel Sequence

<400> 1

cgcgaaauucg cg

12

<210> 2

<211> 20

<212> DNA

<213> Artificial Sequence

<220>

<223> Description of Artificial Sequence: Novel Sequence

<400> 2

tctgagtagc agaggagctc

20

<210> 3

<211> 20

<212> DNA

<213> Artificial Sequence

<220>

<223> Description of Artificial Sequence: Novel Sequence

<400> 3
tctgagtagc agaggagctc 20

<210> 4
<211> 20
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence: Novel Sequence

<400> 4
atgcattctg cccccaagga 20

<210> 5
<211> 20
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence: Novel Sequence

<400> 5
gcccaagctg gcatccgtca 20

<210> 6
<211> 19
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence: Novel Sequence

<400> 6
tttttttttt ttttttttt 19

<210> 7
<211> 20
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence: Novel Sequence

<400> 7
tccgtcatcg ctcttcaggg 20

<210> 8
<211> 20
<212> DNA
<213> Artificial Sequence

<220>

<223> Description of Artificial Sequence: Novel Sequence

<400> 8
tcagtaatag gccacatgg

20

<210> 9
<211> 20
<212> DNA
<213> Artificial Sequence

<220>

<223> Description of Artificial Sequence: Novel Sequence

<400> 9
ttcgtcatcg ctctcaggg

20

<210> 10
<211> 20
<212> DNA
<213> Artificial Sequence

<220>

<223> Description of Artificial Sequence: Novel Sequence

<400> 10
tccgtcatcg ctctcaggg

20

<210> 11
<211> 26
<212> DNA
<213> Artificial Sequence

<220>

<223> Description of Artificial Sequence: Novel Sequence

<400> 11
tcagtaatag ccgccgcccc acatgg

26

<210> 12
<211> 23
<212> DNA
<213> Artificial Sequence

<220>

<223> Description of Artificial Sequence: Novel Sequence

<400> 12

tcagtaatag ccgccccaca tgc

23

<210> 13

<211> 20

<212> DNA

<213> Artificial Sequence

<220>

<223> Description of Artificial Sequence: Novel Sequence

<400> 13

tccgtcatcg ctcctcaggg

20

<210> 14

<211> 23

<212> DNA

<213> Artificial Sequence

<220>

<223> Description of Artificial Sequence: Novel Sequence

<400> 14

tcagtaatag ccgccccaca tgc

23

<210> 15

<211> 20

<212> DNA

<213> Artificial Sequence

<220>

<223> Description of Artificial Sequence: Novel Sequence

<400> 15

tttaggattc gtgctcatgg

20

<210> 16

<211> 20

<212> DNA

<213> Artificial Sequence

<220>

<223> Description of Artificial Sequence: Novel Sequence

<400> 16
tttaggattc gtgctcatgg 20

<210> 17
<211> 20
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence: Novel Sequence

<400> 17
tttaggattc gtgctcatgg 20

<210> 18
<211> 19
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence: Novel Sequence

<400> 18
atgcattctg cccaagga 19

<210> 19
<211> 19
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence: Novel Sequence

<400> 19
atgcattctg cccaagga 19

<210> 20
<211> 19
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence: Novel Sequence

<400> 20
atgcattctg cccaagga 19

<210> 21

<211> 19
<212> DNA
<213> Artificial Sequence

<220>

<223> Description of Artificial Sequence: Novel Sequence

<400> 21
atgcattctg cccaagga

19

<210> 22
<211> 19
<212> DNA
<213> Artificial Sequence

<220>

<223> Description of Artificial Sequence: Novel Sequence

<400> 22
atgcattctg cccaagga

19

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US99/16541

A. CLASSIFICATION OF SUBJECT MATTER

IPC(6) : Please See Extra Sheet.

US CL : 536/23.1, 26.23, 26.26, 26.8, 27.6, 27.8, 27.81, 28.5, 28.53

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 536/23.1, 26.23, 26.26, 26.8, 27.6, 27.8, 27.81, 28.5, 28.53

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched
NONE

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

File CA structure search

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	US 5,436,331 A (REESE et al.) 25 July 1995, see abstract, third chemical structure.	15-28
X	US 5,496,546 A (WANG et al.) 05 March 1996, see abstract.	1-28
X	US 5,625,046 A (REESE et al.) 29 April 1997, see abstract.	1-28
X,P	US 5,858,988 A (WANG) 12 January 1999, see abstract.	1-28
X	ROZNER et al. Evaluation of 2'-Hydroxyl Protection in RNA-Synthesis Using the H-Phosphonate Approach. Nucleic Acids Research. 11 January 1994, Vol. 22, No. 1, pages 94-99, see abstract.	1-28

☒ Further documents are listed in the continuation of Box C. ☐ See patent family annex.

* Special categories of cited documents:	*T* later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
A document defining the general state of the art which is not considered to be of particular relevance	*X* document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
E earlier document published on or after the international filing date	*Y* document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
L document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)	*Z* document member of the same patent family
O document referring to an oral disclosure, use, exhibition or other means	
P document published prior to the international filing date but later than the priority date claimed	

Date of the actual completion of the international search

20 AUGUST 1999

Date of mailing of the international search report

14 OCT 1999

Name and mailing address of the ISA/US
Commissioner of Patents and Trademarks
Box PCT
Washington, D.C. 20231

Facsimile No. (703) 305-3230

Authorized officer

D. Lawrence Fox
LAWRENCE ERIC CRANE

Telephone No. (703) 308-1235

INTERNATIONAL SEARCH REPORT

International application No.

PCT/US99/16541

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	VIRGINIO et al. Trinitrophenyl-Substituted Nucleotides Are Potent Antagonists Selective for P2X1, P2X2, and Heteromeric P2X2/3 Receptors. Molecular Pharmacology. June 1998, Vol. 53, No. 6, pages 969-973, see abstract.	15-28
X	REESE et al. 4-Methoxytetrahydropyran-4-yl - A Symmetrical Alternative to the Tetrahydropyranyl Protecting Group. Tetrahedron. February 1970, Vol. 26, No. 4, pages 1023-1030, see abstract.	15-28
X	MCGREGOR et al. Preparation of Oligoribonucleotides Containing 4-Thiouridine Using Fpmp Chemistry. Photocrosslinking to RNA Binding Proteins Using 350 nm Irradiation. Nucleic Acids Research. 15 August 1996, Vol. 24, No. 16, pages 3173-3180, see abstract.	15-28

INTERNATIONAL SEARCH REPORT

International application No.

PCT/US99/16541

A. CLASSIFICATION OF SUBJECT MATTER:

IPC (6):

C07H 21/02, 21/04, 19/04, 19/20, 19/048, 19/10, 19/167, 19/173, 19/06, 19/09