NUCLEASE RESISTANT DOUBLE-STRANDED RIBONUCLEIC ACID

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ABSTRACT

This invention features modified iRNA agents having improved stability in cells and biological fluids, and methods of making and identifying iRNA agents having improved stability, and of using the iRNA agents to inhibit the expression or function of a target gene.
FIG. 1

(II): backbone attachment points

ligand/tethering attachment point

carrier

O
O

W

O

R = H, protecting group;

A: R = ( ),

R = ( ) + (LIGAND)

B:

carrier

O
O

PG

L

C:

carrier

R

R' O

P

N(R''2)
FIG. 3
FIG. 3 (Cont’d)
FIG. 3 (Cont’d)
5' conjugation of peptide into siRNA

1. Morpholine
2. Fmoc-peptide, HBTU

1. NH₃, aq
2. 80% AcOH

5'-Peptide-siRNA hybrid

FIG. 5
"C"-activation

\[ R_1 \]
\[ R - \text{CO-NH-NH} \]

\[ \text{Cl-CO}_2\text{Ar} \]

\[ R_1 \]
\[ R - \text{CO-NH-N-CO}_2\text{Ar} \]

"N"-activation

\[ R_2 \]
\[ \text{H}_2\text{N-X-CO-R}_3 \]

\[ \text{Cl-CO}_2\text{Ar} \]

\[ H_1 \]
\[ R_2 \]
\[ \text{Ar}_2\text{C-N-X-CO-R}_3 \]

\[ R - \text{CO-NH-N-CO}_2\text{NH-X-CO-R}_3 \]

FIG. 6
Synthesis of Ant and Tat N-methylpeptides

With respective sequences, synthesis of Ant and Tat peptides could be obtained.

FIG. 7
Synthesis of Ant and Tat peptides

With respective sequences, synthesis of Ant and Tat peptides could be obtained.

FIG. 8
FIG. 9
FIG. 10
Phosphorothioate Substitution Inhibits 3'-5' Exonuclease Cleavage
Effect of 3' End Modifications on 3'-5' Exonuclease Degradation in Human Serum
3' Abasic Pyrrolidine Cationic Modifications Provide Exonuclease Stability

PBS control time point at 4hr
90% human serum time points at 10', 15', 30', 1h, 2h, 4h
Terminal Cationic C6 Amino dT Modification Provides Exonucleolytic Stability

PBS control time point at 4hr
90% human serum time points at 10', 15', 30', 1h, 2h, 4h
3' Naproxen Conjugation Is Tolerated on Both the Sense and Antisense Strands
3' Abasic Pyrrolidine Cationic Modifications Are Tolerated on Both Sense and Antisense Strands
3' Terminal Cationic C6 Amino dT Modifications Are Tolerated on Both the Sense and Antisense Strands
Stabilization of siRNAs to Mouse Serum Using Conjugation Chemistry

Non-modified Luc siRNA

3'-Naproxen-modified Luc siRNA

Buffer time points: 0, 8h
Mouse serum time points: 0, 15', 30', 1h, 2h, 4h, 8h

5'-CUUACGCUGAGUCUUGCAdTdT
dTdTGAUGCGACUCGAAGCU-5'

5'-CUUACGCUGAGUCUUGCAdTdTTM3
M3dTdTGAUGCGACUCGAAGCU-5'

1s/1as
Stabilization of siRNAs in Liver Using Conjugation Chemistry

Non-modified Luc siRNA

3'-Naproxen-modified Luc siRNA

Buffer time points: 0, 4h, 8h
Liver homogenate time points: 0, 15', 30', 1h, 2h, 4h, 8h

1s/1as

5'-CUUACGCUGAGUACUUCGAdTdT
5'-CUUACGCUGAGUACUUCGAdTdTM3

dTdTGAAUGCGACUCAUGAAGCU-5'
m3dTdTGAUUCGACUCAUGAAGCU-5'
FIG. 23
FIG. 25
NUCLEASE RESISTANT DOUBLE-STRANDED RIBONUCLEIC ACID

CROSS-REFERENCE TO RELATED APPLICATIONS

[0001] This application is a continuation-in-part of International Application No. PCT/US.2004/011829, filed on Apr. 16, 2004, which claims the benefit of U.S. Provisional Application No. 60/493,986, filed on Aug. 8, 2003; U.S. Provisional Application No. 60/494,597, filed on Aug. 11, 2003; U.S. Provisional Application No. 60/506,341, filed on Sep. 26, 2003; U.S. Provisional Application No. 60/518,453, filed on Nov. 7, 2003; U.S. Provisional Application No. 60/463,772, filed on Apr. 17, 2003; U.S. Provisional Application No. 60/465,802, filed on Apr. 25, 2003; U.S. Provisional Application No. 60/469,612, filed on May 9, 2003; U.S. Provisional Application No. 60/510,246, filed on Oct. 9, 2003; U.S. Provisional Application No. 60/510,318, filed on Oct. 10, 2003; U.S. Provisional Application No. 60/503,414, filed on Sep. 15, 2003; U.S. Provisional Application No. 60/465,665, filed on Apr. 25, 2003; International Application No.: PCT/US.2004/07070, filed on Mar. 8, 2004; International Application No.: PCT/US.2004/10586, filed on Apr. 5, 2004; International Application No.: PCT/US.2004/11255, filed on Apr. 9, 2004; and International Application No.: PCT/US.2004/011822, filed on Apr. 16, 2004. The contents of all of these prior applications are hereby incorporated by reference in their entirety.

TECHNICAL FIELD

[0002] This invention features modified iRNA agents having improved stability in cells and biological fluids, and methods of making and identifying iRNA agents having improved stability, and of using the iRNA agents to inhibit the expression or function of a target gene.

BACKGROUND

[0003] Many diseases (e.g., cancers, hematopoietic disorders, endocrine disorders, and immune disorders) arise from the abnormal expression or activity of a particular gene or group of genes. Similarly, disease can result through expression of a mutant form of protein, as well as from expression of viral genes that have been integrated into the genome of their host. The therapeutic benefits of being able to selectively silence these abnormal or foreign genes are obvious.

[0004] Double-stranded RNA molecules (dsRNA) can block gene expression by virtue of a highly conserved regulatory mechanism known as RNA interference (RNAi). Briefly, the RNA III Dicer enzyme processes dsRNA into small interfering RNA (siRNA) of approximately 22 nucleotides. One strand of the siRNA (the “antisense strand”) then serves as a guide sequence to induce cleavage of messenger RNAs (mRNAs) including a nucleotide sequence which is at least partially complementary to the sequence of the antisense strand by an RNA-induced silencing complex RISC (Hammond, S. M., et al., Nature (2000) 404:293-296). The antisense strand is not cleaved or otherwise degraded in this process, and the RISC including the antisense strand can subsequently effect the cleavage of further mRNAs.

SUMMARY

[0005] The invention features iRNA agents having improved stability in cells and biological fluids, methods of making and identifying iRNA agents having improved stability, as well as compositions and methods for inhibiting the expression of a target gene in a cell using the iRNA agent. The invention also features compositions and methods for treating diseases caused by the expression or activity of the target gene. The invention features iRNA agents that contain modifications that increase resistance of the iRNA agents to exonucleolytic degradation, including 5’ exomucelloytic and 3’ exomucelloytic degradation. The modifications render the iRNA agents more resistant to enzymatic or chemical degradation, and thus more stable and bioavailable than an otherwise identical iRNA agent that does not include the modification.

[0006] In one aspect, the invention features iRNA agents having improved stability when administered to a subject. These agents can have, e.g., improved stability in cells and biological samples, particularly serum. The iRNA agent includes one or more of various modifications. For example, the iRNA agents can include a backbone modification to one or more of the terminal nucleotides on an iRNA strand. A terminal nucleotide can be the nucleotide at the last position, and can include the nucleotide(s) at the second, third, fourth, and fifth positions from the 5’ or 3’-most ends of the sense or antisense strand of an iRNA agent. In one embodiment, an iRNA agent includes a phosphorothioate linkage or P-alkyl modification in the linkages between one or more of the terminal nucleotides of an iRNA agent. In another embodiment, one or more terminal nucleotides of an iRNA agent include a sugar modification, e.g., a 2’ or 3’ sugar modification. Exemplary sugar modifications include, for example, a 2’-O-methylated nucleotide, a 2’-deoxy nucleotide, a 2’-deoxyfluoro nucleotide, a 2’-methoxyethenyl nucleotide, a 2’-O-NMA, a 2’-DMOAE, a 2’-AP, 2’-hydroxy, or a 2’-arfluoro or a locked nucleic acid (LNA), extended nucleic acid (ENA), hexasac nucleic acid (HNA), or cyclohexene nucleic acid (C6NA). A 2’ modification is preferably 2’OMe, more preferably, 2’ fluoro. In one embodiment the modification is an LNA and the LNA is on the 5’ or 3’ end of the sense strand, and not on the 5’ or 3’ end of the antisense strand.

[0007] In one embodiment, the iRNA agent includes a 3’ sugar modification, e.g., a 3’OMe modification. Preferably a 3’OMe modification is on the 3’ or 5’ end of the sense strand of the iRNA agent.

[0008] In some embodiments, an iRNA agent includes a 5’-methyl-pyrimidine (e.g., a 5’-methyl-uridine modification or a 5’-methyl-cytidine modification.

[0009] The modifications described herein can be combined onto a single iRNA agent. For example, in one embodiment, at least one terminal nucleotide of an iRNA agent has a phosphorothioate linkage and a 2’ sugar modification, e.g., a 2’OMe or 2’ F modification. In another embodiment, at least one terminal nucleotide of an iRNA agent has a 5’ Me-pyrimidine and a 2’ sugar modification, e.g., a 2’F or 2’OMe modification.

[0010] In one embodiment, an iRNA agent includes a nucleobase modification, such as a cationic modification, such as a 3’-basic cationic modification. The cationic modification can be, e.g., an alkylamino-dT (e.g., a C6 amino-dT), an alkylamino conjugate, a pyridoline conjugate, a pthalalimal or a hydroxyprolinol conjugate, on one or more of the terminal nucleotides of the iRNA agent. An alkylamino-dT...
conjugate is preferably attached to the 3’ end of the sense or antisense strand of an iRNA agent. A pyrrolidine linker is preferably attached to the 3’ or 5’ end of the sense strand, or the 3’ end of the antisense strand. An amidine uridine is preferably on the 3’ or 5’ end of the sense strand, and not on the 5’ end of the antisense strand.

[0011] In another embodiment, the iRNA agent includes a conjugate on one or more of the terminal nucleotides of the iRNA agent. The conjugate can be, for example, a lipophile, a terpene, a protein binding agent, a vitamin, a carbohydrate, or a peptide. For example, the conjugate can be naproxen, nitroindole (or another conjugate that contributes to stacking interactions), folate, ibuprofen, or a CS pyrimidine linker. In other embodiments, the conjugates are glycergic lipid conjugates (e.g., a dialkyl glyceric derivatives), vitamin E conjugates, or thio-cholesterol. Preferably, conjugates are on the 3’ end of the antisense strand, or on the 5’ or 3’ end of the sense strand, and preferably the conjugates are not on the 3’ end of the antisense strand and on the 3’ end of the sense strand.

[0012] In one embodiment, the conjugate is naproxen, and the conjugate is preferably on the 5’ or 3’ end of the sense or antisense strands. In one embodiment, the conjugate is cholesterol, and the conjugate is preferably on the 5’ or 3’ end of the sense strand and preferably not present on the antisense strand. In some embodiments, the cholesterol is conjugated to the iRNA agent by a pyrrolidine linker, or a serinol linker, or hydroxyprolinol linker. In other embodiments, the conjugate is a cholesterol, or cholesterol is conjugated to the iRNA agent by a disulfide linkage. In a particularly preferred embodiment, the conjugate is cholic acid, and the cholic acid is attached to the 5’ or 3’ end of the sense strand, or the 3’ end of the antisense strand. In one embodiment, the cholic acid is attached to the 3’ end of the sense strand and the 3’ end of the antisense strand.

[0013] In another embodiment, one or more terminal nucleotides has a 2’-5’ linkage. Preferably, a 2’-5’ linkage occurs on the sense strand, e.g., the 5’ end of the sense strand.

[0014] In one embodiment, the iRNA agent includes an L-sugar, preferably at the 5’ or 3’ end of the sense strand.

[0015] In one embodiment, the iRNA agent includes a methylphosphonate at one or more terminal nucleotides to enhance exonuclease resistance, e.g., at the 3’ end of the sense or antisense strands of the iRNA agent.

[0016] In one embodiment, an iRNA agent has been modified by replacing one or more ribonucleotides with deoxyribonucleotides. Preferably, adjacent deoxyribonucleotides are joined by phosphorothioate linkages, and the iRNA agent does not include more than four consecutive deoxyribonucleotides on the sense or the antisense strands.

[0017] In some embodiments, an iRNA agent having increased stability in cells and biological samples includes a difluorotolyl (DFT) modification, e.g., 2,4-difluorotolyl uracil, or a guanidine to inosine substitution.

[0018] In one embodiment the iRNA agent includes a terminal 5’-uridine-adenine-3’ (5’-UA-3’) dinucleotide wherein the uridine is a 2’-modified nucleotide, or a terminal 5’-uridine-guanine-3’ (5’-UG-3’) dinucleotide, wherein the 5’-uridine is a 2’-modified nucleotide, or a terminal 5’-cytidine-adenine-3’ (5’-CA-3’) dinucleotide, wherein the 5’-cytidine is a 2’-modified nucleotide, or a terminal 5’-uridine-3’ (5’-UU-3’) dinucleotide, wherein the 5’-uridine is a 2’-modified nucleotide, or a terminal 5’-cytidine-cytidine-3’ (5’-CC-3’) dinucleotide, wherein the 5’-cytidine is a 2’-modified nucleotide, or a terminal 5’-cytidine-uridine-3’ (5’-CU-3’) dinucleotide, wherein the 5’-cytidine is a 2’-modified nucleotide, or a terminal 5’-cytidine-uridine-3’ (5’-UC-3’) dinucleotide, wherein the 5’-uridine is a 2’-modified nucleotide. The chemically modified nucleotide in the iRNA agent may be a 2-O-methylated nucleotide. In some embodiments, the modified nucleotide can be a 2’-deoxy nucleotide, a 2’-deoxyfluoro nucleotide, a 2’-O-methoxyethyl nucleotide, a 2’-O-NMA, a 2’-DMAEVE, a 2’-AP, 2’-hydroxy, or a 2’-ara-fluoro, or a locked nucleic acid (LNA), extended nucleic acid (ENA), hexose nucleic acid (HNA), or cyclohexene nucleic acid (CeNA). The iRNA agents including the modifications described herein are particularly stabilized against exonuclease activity.

[0019] In one particularly preferred embodiment, the iRNA agent includes a cholesterol molecule on the 3’ end of an iRNA agent, and conjugated to the iRNA agent by a pyrrolidine linker, and at least one additional modification, e.g., a modification described herein, such as a phosphorothioate linkage, for inhibiting exonuclease degradation. In another particularly preferred embodiment, an iRNA agent includes a cholic acid modification on one or both of the 5’ ends of the sense or antisense strands, and at least one additional modification, e.g., a modification described herein, such as a phosphorothioate linkage, for inhibiting exonuclease degradation.

[0020] In one embodiment, the iRNA agent has a single overhang, e.g., one end of the iRNA agent has a 3’ or 5’ overhang and the other end of the iRNA agent is a blunt end. In another embodiment, the iRNA agent has a double overhang, e.g., both ends of the iRNA agent have a 3’ or 5’ overhang, such as a dinucleotide overhang. In another embodiment, both ends of the iRNA agent have blunt ends.

[0021] The iRNA agent may further include a sense RNA strand and an antisense RNA strand, wherein the antisense RNA strand is 25 or fewer nucleotides in length, and includes an antisense nucleotide sequence having 18-25 nucleotides in length. The iRNA agent may further include a nucleotide overhang having 1 to 4 unpaired nucleotides, which may be at the 3’-end of the antisense RNA strand, and the nucleotide overhang may have the nucleotide sequence 5’-GCG-3’ or 5’-CGC-3’. The unpaired nucleotides may have at least one phosphorothioate dinucleotide linkage, and at least one of the unpaired nucleotides may be chemically modified in the 2’-position. In one embodiment, the double-strand region of the iRNA agent includes phosphorothioate dinucleotide linkages on one or both of the sense and antisense strands. In one embodiment, the antisense RNA strand and the sense RNA strand are connected with a linker. The chemical linker may be a hexaethylene glycol linker, a poly-oxypolysaccharine-oxy-1,3-propanol linker, an allyl linker, or a polyethylene glycol linker.

[0022] In another aspect, the invention relates to a method of making an iRNA agent. The method includes preparing an antisense RNA strand and a sense RNA strand, wherein at least one of the RNA strands includes a modification described herein, e.g., a modification that confers an
enhanced resistance to exonucleolytic cleavage. The RNA strands may then be mixed to form a double stranded iRNA agent. The RNA strands may be prepared using solid-phase synthesis in a 3’ to 5’ direction.

[0023] In yet another aspect, the invention relates to a method for inhibiting the expression of a target gene in a cell. The method includes introducing an iRNA agent, as described above, into the cell, and maintaining the cell for a time sufficient to obtain iRNA agent-mediated downregulation, e.g., by degradation, of an mRNA transcript of the target gene. The cell may be a mammalian cell, such as a human cell.

[0024] In still another aspect, the invention relates to a pharmaceutical composition for inhibiting the expression of a target gene in an organism. The compositions include an iRNA agent, as described above, and a pharmaceutically acceptable carrier. The pharmaceutically acceptable carrier may be an aqueous solution, such as phosphate buffered saline, or it may include a micellar structure, such as a liposome, capsid, capsid polymer, polymeric nanocapsule, or polymeric microparticle.

[0025] In yet a further aspect, the invention relates to a method for treating a disease caused by the expression of a target gene in a subject. The method includes administering a pharmaceutical composition containing an iRNA agent, as described above, and a pharmaceutically acceptable carrier. The subject may be a mammal, such as a human.

[0026] In another aspect, the invention relates to methods of identifying an iRNA agent having increased stability in a biological sample. The method may include preparing a mixture of iRNA agent, incubating the mixture of iRNA agent in the biological sample, and identifying an iRNA agent exhibiting an increased stability as compared to other iRNA agent in the biological sample. The mixture of iRNA agent may be prepared by chemical synthesis or by extraction from a biological sample. Alternatively, the method may include introducing an iRNA agent into a cell, maintaining the cell under conditions suitable for expressing a protein encoded by a target gene, measuring an amount of the protein produced in the cell, comparing the amount of the protein produced in the cell to that in a control cell, and identifying an iRNA agent that causes a reduction in the amount of protein in the cell as compared to the control cell. The cell may be a mammalian cell.

[0027] In still another aspect, the invention relates to a method of increasing the nuclease resistance of an iRNA agent. The method includes forming an iRNA agent, as described above, wherein the nuclease resistance of the iRNA agent is increased compared to a control composition. Such method includes the steps of incorporating into the iRNA agent, a modification described herein. The modification can be added to one or more of the 5’ or 3’ terminal nucleotides of the sense and antisense strands of the iRNA agent.

[0028] While RNA interference using iRNA agent has been shown to be an effective means for selective gene silencing, RNA can have less than desired stability in some bodily fluids, particularly in serum. Thus, RNA, including iRNA agent, can be degraded between the time it is administered to a subject and the time it enters a target cell. Even within the cell, RNA can undergo rapid degradation by nucleases. Methods of the invention can provide more stable or nuclease resistant iRNA agents that can offer better bioavailability and hence improved effectiveness. This is an improvement over the current insufficient methods for stabilizing iRNA agent against degradation.

[0029] The details of one or more embodiments of the invention are set forth in the accompanying drawings and the description below. Other features, objects, and advantages of the invention will be apparent from the description and drawings, and from the claims.

DESCRIPTION OF DRAWINGS

[0030] FIG. 1 is a general synthetic scheme for incorporation of RRMS monomers into an oligonucleotide.

[0031] FIG. 2A is a list of substituents that may be present on silicon in OGF1.

[0032] FIG. 2B is a list of substituents that may be present on the C2'-orthoester group.

[0033] FIG. 3 is a list of representative RRMS cyclic carriers. Panel 1 shows pyrroline-based RRMSs; panel 2 shows 3-hydroxyproline-based RRMSs; panel 3 shows piperidine-based RRMSs; panel 4 shows morpholine and piperazine-based RRMSs; and panel 5 shows decalin-based RRMSs. R1 is succinate or phosphoramide and R2 is H or a conjugate ligand.

[0034] FIG. 4 is a general reaction scheme for 3’ conjugation of peptide into iRNA.

[0035] FIG. 5 is a general reaction scheme for 5’ conjugation of peptide into iRNA.

[0036] FIG. 6 is a general reaction scheme for the synthesis of aza-peptides.

[0037] FIG. 7 is a general reaction scheme for the synthesis of N-methyl amino acids and peptides.

[0038] FIG. 8 is a general reaction scheme for the synthesis of β-methyl amino acids and Ant and Tat peptides.

[0039] FIG. 9 is a general reaction scheme for the synthesis of Ant and Tat oligocarbamates.

[0040] FIG. 10 is a general reaction scheme for the synthesis of Ant and Tat oligouracils.

[0041] FIG. 11 is a schematic representation of peptide carriers.

[0042] FIG. 12 shows a gel electrophoretic separation of 3’ phosphorothiate modified dsRNAs following incubation in human serum.

[0043] FIG. 13 shows a gel electrophoretic separation of 3’ modified dsRNAs following incubation in human serum.
FIG. 14 shows a gel electrophoretic separation of 3' abasic pyrrolidine conjugated dsRNAs following incubation in human serum.

FIG. 15 shows a gel electrophoretic separation of 3' cationic C6 amino dT conjugated dsRNAs following incubation in human serum.

FIG. 16 is a graph showing luciferase gene expression activity in cells following transfection of 3'Naproxen modified dsRNAs.

FIG. 17 is a graph showing luciferase gene expression activity in cells following transfection of 3'abasic pyrrolidine modified dsRNAs.

FIG. 18 is a graph showing luciferase gene expression activity in cells following transfection of 3' cationic C6 amino dT modified dsRNAs.

FIG. 19 shows a gel electrophoretic separation of 3' naproxen conjugated dsRNAs following incubation in mouse serum.

FIG. 20 shows a gel electrophoretic separation of 3' naproxen conjugated dsRNAs following incubation in liver extract.

FIG. 21 is a denaturing gel analysis of the human serum stability assay for AL-DUP-1000. C is the 4 hour time point for siRNA duplex incubated in PBS buffer alone, OH— is the partial alkaline hydrolysis marker, *s/as represents siRNA duplex containing 5' end-labeled sense RNA and s/*as represents duplex containing 5' end-labeled anti-sense RNA. Samples were incubated in 90% human serum and time points were assayed at 10 seconds, 5 min, 15 min, 30 min, 1 hour, 2 hours and 4 hours. Black lines to the right of bands indicate exonucleolytic degradation fragments and the red lines highlight a few of the endonucleolytic degradation fragment.

FIG. 22A is a denaturing gel analysis of the human serum stability assay for AL-DUP-1393. C is the 4 hour time point for each siRNA duplex incubated in PBS buffer alone, *s/as represents siRNA duplex containing 5' end-labeled sense RNA and s/*as represents duplex containing 5' end-labeled antisense RNA. Samples were assayed at 10 seconds, 15 min, 30 min, 1 hour, 2 hours and 4 hours.

FIG. 22B is a denaturing gel analysis of the human serum stability assay for AL-DUP-1329. The lanes are labeled and the experiment was performed as described for FIG. 22A.

FIG. 23 is a denaturing gel analysis of AL-DUP-1036, AL-DUP-1363, and AL-DUP-1363 (see Table 2 for sequences). Black vertical lines highlight regions where exonucleolytic cleavage is suppressed, stars indicate sites of strong endonucleolytic cleavage in the antisense strand and weaker endonucleolytic cleavage in the sense strand. C is the 4 hour time point for each siRNA duplex incubated in PBS buffer alone, *s/as represents siRNA duplex containing 5' end-labeled sense RNA and s/*as represents duplex containing 5' end-labeled antisense RNA. Samples were assayed at 10 seconds, 15 min, 30 min, 1 hour, 2 hours and 4 hours.

FIG. 24. Human serum stability profile of siRNA duplexes containing cationic modifications. Denaturing gel analysis of AL-DUP-1000 (alkylamino-dT), AL-DUP-1403 (abasic pyrrolidine cationic), and AL-DUP-1403 (see Table 3 for sequences). Black line highlights region where exonucleolytic cleavage is suppressed and red star indicates site of strong endonucleolytic cleavage in the antisense strand. C is the 4 hour time point for each siRNA duplex incubated in PBS buffer alone, *s/as represents siRNA duplex containing 5' end-labeled sense RNA and s/*as represents duplex containing 5' end-labeled antisense RNA. Samples were assayed at 10 seconds, 15 min, 30 min, 1 hour, 2 hours and 4 hours.

FIG. 25 is a denaturing gel analysis of the human serum stability assay for AL-DUP-1069. The black vertical line highlights the region where exo nucleolytic cleavage is suppressed. C is the 4 hour time point for each siRNA duplex incubated in PBS buffer alone, *s/as represents siRNA duplex containing 5' end-labeled sense RNA and s/*as represents duplex containing 5' end-labeled antisense RNA. Samples were assayed at 10 seconds, 15 min, 30 min, 1 hour, 2 hours and 4 hours.

Like reference symbols in the various drawings indicate like elements.

DETAILED DESCRIPTION

The invention is based, at least in part, on the discovery that particular modifications to an iRNA agent are effective at increasing resistance of the iRNA agent to exonucleolytic degradation. Thus, the invention features iRNA agents that contain modifications that increase resistance of the iRNA agents to exonucleolytic degradation, including 5' exonucleolytic and 3' exonucleolytic degradation. These iRNA agents may have improved stability in cells and biological fluids.

The iRNA agents having improved stability can include one or more of various modifications. For example, the iRNA agent can include a backbone modification to one or more of the terminal nucleotides on an iRNA strand. Exemplary backbone modifications include phosphorothioate linkages or P-alkyl linkages between one or more of the terminal nucleotides of an iRNA agent. One or more terminal nucleotides of an iRNA agent can also include a sugar modification, e.g., a 2' or 3' sugar modification. Exemplary sugar modifications are described below. An iRNA agent can include multiple modifications that confer an enhanced resistance to exonucleolytic cleavage. For example, one or more terminal nucleotides of an iRNA agent can have a phosphorothioate linkage and a 2' sugar modification.

An iRNA agent having increased resistance to exonucleolytic degradation can include a nucleoside modification, a cationic modification on one or more of the terminal nucleotides of the iRNA agent.

Exemplary modifications for enhanced resistance to endonucleolytic cleavage are described below.
R₂ = H, OH, OMe, F, OCH₃, OCH₂F, OCH₂SCH₂S, OCH₂DNMe₂, OCH₃OCH₂H₂SMe₂, OCH₂CONH₂, OCH₃(OCH₃)₂SM, where X = O, S, NR₃, and R₃, R₄ = H, Me, Et, OCH₂CONH₂R₆, where R₃, R₄, where R₅ = H, Me, Et, and R₆ = H, Me, Et or a ligand.

X = OH, S, Me, 2-isopropyl, tert-butyl, phenyl, -NH-Alkyl, -N(formyl), -N alkyl (Ar, -CH₃)X or -NH(OCH₂)XR₃, where X = O, S, NR₃, R₇ = H, Me, Et, and R₆ = H, Me, Et, COMe and n = 1-12.

Y = O or S.
[0062] Most Preferred Sugar with 2'-Substituents and Backbones
[0063] R=H, OH, OMe, F, O(CH₂)₂OCH₃, O(CH₂)₂SCH₃, O(CH₂)₂ONMe₂, O(CH₂)₂O(CH₂)₂NMMe₂, OCH₂CONH₂, O(CH₂)₂XR₂, where X=O, S, NR₃, and R₃, R₄=H, Me, Et, OCH₂CONR₂R₄, where R₃R₄, where R₃=H, Me, Et and R₄=H, Me, Et or a ligand

[0064] X=OH, —SH, —Me, —isopropyl, —tert-Butyl, —phenyl, —NH-Alkyl, —N(dialkyl), —N(alkyl)(aryl), —(CH₂)ₙXR₂, or —NH(CH₂)ₙXR₂, where X=O, S, NR₃, R₄=H, Me, Et, and R₅=H, Me, Et, COMe and n=1-12

[0065] Y=O, S or Se

[0066] Chiral Rp and Sp isomers, P=S preferably Sp

[0067] R₁, R₂=H, OH, OMe, F, O(CH₂)₂OCH₃, O(CH₂)₂SCH₃, O(CH₂)₂ONMe₂, O(CH₂)₂O(CH₂)₂NMMe₂, OCH₂CONH₂, O(CH₂)₂XR₂, where X=O, S, NR₃, and R₃, R₄=H, Me, Et, OCH₂CONR₂R₄, where R₃R₄, where R₃=H, Me, Et and R₄=H, Me, Et or a ligand

[0068] X=OH, —SH, —Me, —isopropyl, —tert-Butyl, —phenyl, —NH-Alkyl, —N(dialkyl), —N(alkyl)(aryl), —(CH₂)ₙXR₂, or —NH(CH₂)ₙXR₂, where X=O, S, NR₃, R₄=H, Me, Et, and R₅=H, Me, Et, COMe and n=1-12

[0069] Y=O, S or Se

[0070] Chiral Rp and Sp isomers, P=S preferably Sp
[0071] R₁, R₂=H, OH, OMe, F, O(CH₂)₂OCH₃, O(CH₂)₂SCH₂, O(CH₂)₂ONMe₂, O(CH₂)₂O(CH₂)₂NMMe₂, OCH₂CONH₂, O(CH₂)₂XR₂, where X=O, S, NR₄ and R₈, R₉=H, Me, Et; OCH₂CONR₂Rₘ, where R₉, R₈, where R₈=H, Me, Et and R₉=H, Me, Et or a ligand

[0072] X=OH, —SH, -Me, -isopropyl, -tert-Butyl, -phenyl, —NH-Alkyl, —N(dialkyl), —N(alkyl)(Aryl), —(CH₂)₂XR₂ or —NH(CH₂)₂XR₂, where X=O, S, NR₄, R₈=H, Me, Et, and R₉=H, Me, Et or a ligand

[0073] Y=O, S or Se

[0074] Chiral Rp and Sp isomers, P=S preferably Sp

[0077] Y=O, S or Se

[0078] Chiral Rp and Sp isomers, P=S preferably Sp

[0079] R=H, OH, OMe, F, O(CH₂)₂OCH₃, O(CH₂)₂SCH₂, O(CH₂)₂ONMe₂, O(CH₂)₂O(CH₂)₂NMMe₂, OCH₂CONH₂, O(CH₂)₂XR₂, where X=O, S, NR₄ and R₈, R₉=H, Me, Et; OCH₂CONR₂Rₘ, where R₉, R₈, where R₈=H, Me, Et, and R₉=H, Me, Et or a ligand

[0080] X=OH, —SH, -Me, -isopropyl, -tert-Butyl, -phenyl, —NH-Alkyl, —N(dialkyl), —N(alkyl)(Aryl), —(CH₂)₂XR₂ or —NH(CH₂)₂XR₂, where X=O, S, NR₄, R₈=H, Me, Et, and R₉=H, Me, Et or a ligand

[0081] Y=O, S or Se

[0082] Z=O, S, CH₂, CHF, CF₂

[0083] Chiral Rp and Sp isomers, P=S preferably Sp

[0084] R=H, OH, OMe, F, O(CH₂)₂OCH₃, O(CH₂)₂SCH₂, O(CH₂)₂ONMe₂, O(CH₂)₂O(CH₂)₂NMMe₂, OCH₂CONH₂, O(CH₂)₂XR₂, where X=O, S, NR₄ and R₈, R₉=H, Me, Et; OCH₂CONR₂Rₘ, where R₉, R₈, where R₈=H, Me, Et, and R₉=H, Me, Et or a ligand

[0085] X=OH, —SH, -Me, -isopropyl, -tert-Butyl, -phenyl, —NH-Alkyl, —N(dialkyl), —N(alkyl)(Aryl), —(CH₂)₂XR₂ or —NH(CH₂)₂XR₂, where X=O, S, NR₄, R₈=H, Me, Et, and R₉=H, Me, Et or a ligand
Y=O, S or Se

Chiral Rp and Sp isomers, P=S preferably Sp

Y=O, S or Se

Chiral Rp and Sp isomers, P=S preferably Sp

R=H, OH, OMe, F, O(CH_2)_2OCH_3, O(CH_2)_3SCH_3, O(CH_2)_2ONMe_2, O(CH_2)_2O(CH_2)_2NMe_2, OCH_2CONH_2, O(CH_2)_3XR_3, where X=O, S, NR_2, R_3, R_4=H, Me, Et, OCH_2CONR_2R_3, where R_2, R_3, where R_3=H, Me, Et and R_4=H, Me, Et or a ligand

X=OH, —SH, —Me, —isopropyl, —tert-Butyl, —phenyl, —NH-Alkyl, —N(dialkyl), —N(alkyl)(Aryl), —(CH_2)_3XR_3 or —NH(CH_2)_3XR_3, where X=O, S, NR_3, R_3=H, Me, Et, and R_4=H, Me, Et, COMe and n=1-12

Y=O, S or Se

Z_1, Z_2=H, OH, NR"R", SR"", OR"

Chiral Rp and Sp isomers, P=S preferably Sp

Z_1, Z_2=O, S, NR', CH_2, CHE, CF

Chiral Rp and Sp isomers, P=S preferably Sp

R=H, OH, OMe, F, O(CH_2)_2OCH_3, O(CH_2)_3SCH_3, O(CH_2)_2ONMe_2, O(CH_2)_2O(CH_2)_2NMe_2, OCH_2CONH_2, O(CH_2)_3XR_3, where X=O, S, NR_2, R_3, R_4=H, Me, Et, OCH_2CONR_2R_3, where R_2, R_3, where R_3=H, Me, Et and R_4=H, Me, Et or a ligand

X=OH, —SH, —Me, —isopropyl, —tert-Butyl, —phenyl, —NH-Alkyl, —N(dialkyl), —N(alkyl)(Aryl), —(CH_2)_3XR_3 or —NH(CH_2)_3XR_3, where X=O, S, NR_3, R_3=H, Me, Et, and R_4=H, Me, Et or a ligand

Y=O, S or Se

Z_1, Z_2=O, S, NR', CH_2, CHE, CF

Chiral Rp and Sp isomers, P=S preferably Sp

R=H, OH, OMe, F, O(CH_2)_2OCH_3, O(CH_2)_3SCH_3, O(CH_2)_2ONMe_2, O(CH_2)_2O(CH_2)_2NMe_2, OCH_2CONH_2, O(CH_2)_3XR_3, where X=O, S, NR_2, R_3, R_4=H, Me, Et, OCH_2CONR_2R_3, where R_2, R_3, where R_3=H, Me, Et and R_4=H, Me, Et or a ligand
where $X=O$, $S$, $NR_4$ and $R_4$, $R_4=H$, Me, Et; OCH$_2$CONR$_2$R$_6$ where $R_2$, R$_6$ where $R_2=H$, Me, Et and $R_6=H$, Me, Et or a ligand.

[0103] $X=OH$, $SH$, $Me$, isopropyl, -tert-Butyl, -phenyl, $NH$-Alkyl, $N$(dialkyl), $N$(alkyl)(Aryl), $-(CH_2)_nX_R$ and $-NH$(CH$_2$)$_nX_R$, where $X=O$, $S$, $NR_4$ and $R_4=H$, Me, Et; OCH$_2$CONR$_2$R$_6$ where $R_2$, R$_6$ where $R_2=H$, Me, Et and $R_6=H$, Me, Et or a ligand.

[0104] $Y=O$, $S$ or $Se$.

[0105] $Z=O$, $S$, $NR'$, $CH_2$, CHF, CF.


[0107] $R=H$, $OH$, $OME$, $F$, O(CH$_2$)$_2$OCH$_3$, O(CH$_2$)$_2$SMe, (CH$_2$)$_2$ONMe$_2$, O(CH$_2$)$_2$O(CH$_2$)$_2$NMe$_2$, OCH$_2$CONH$_2$, O(CH$_2$)$_2$XR$_2$, where $X=O$, $S$, $NR_4$ and $R_4$, $R_4=H$, Me, Et; OCH$_2$CONR$_2$R$_6$ where $R_2$, R$_6$ where $R_2=H$, Me, Et and $R_6=H$, Me, Et or a ligand.

[0108] $X=OH$, $SH$, $Me$, isopropyl, -tert-Butyl, -phenyl, $NH$-Alkyl, $N$(dialkyl), $N$(alkyl)(Aryl), $-(CH_2)_nX_R$ and $-NH$(CH$_2$)$_nX_R$, where $X=O$, $S$, $NR_4$ and $R_4=H$, Me, Et; OCH$_2$CONR$_2$R$_6$ where $R_2$, R$_6$ where $R_2=H$, Me, Et and $R_6=H$, Me, Et or a ligand.

[0109] $Y=O$, $S$ or $Se$.

[0110] $Z=O$, $S$, $NR'$, $CH_2$, CHF, CF.

[0111] $Z=H$, OR', $SR'$, $NRR'$.

[0112] Chiral $Rp$ and $Sp$ isomers, $P=S$ preferably $Sp$.

[0113] $R=H$, $OH$, $OME$, $F$, O(CH$_2$)$_2$OCH$_3$, O(CH$_2$)$_2$SCH$_3$, O(CH$_2$)$_2$ONMe$_2$, O(CH$_2$)$_2$O(CH$_2$)$_2$NMe$_2$, OCH$_2$CONH$_2$, O(CH$_2$)$_2$XR$_2$, where $X=O$, $S$, $NR_4$ and $R_4$, $R_4=H$, Me, Et; OCH$_2$CONR$_2$R$_6$ where $R_2$, R$_6$ where $R_2=H$, Me, Et and $R_6=H$, Me, Et or a ligand.

[0114] $Y=O$, $S$, $CMe_2$, CHF, CF$_2$.

[0115] $R=H$, $OH$, $OME$, $F$, O(CH$_2$)$_2$OCH$_3$, O(CH$_2$)$_2$SCH$_3$, O(CH$_2$)$_2$ONMe$_2$, O(CH$_2$)$_2$O(CH$_2$)$_2$NMe$_2$, OCH$_2$CONH$_2$, O(CH$_2$)$_2$XR$_2$, where $X=O$, $S$, $NR_4$ and $R_4$, $R_4=H$, Me, Et; OCH$_2$CONR$_2$R$_6$ where $R_2$, R$_6$ where $R_2=H$, Me, Et and $R_6=H$, Me, Et or a ligand.

[0116] $X=OH$, $SH$, $Me$, isopropyl, -tert-Butyl, -phenyl, $NH$-Alkyl, $N$(dialkyl), $N$(alkyl)(Aryl), $-(CH_2)_nX_R$ and $-NH$(CH$_2$)$_nX_R$, where $X=O$, $S$, $NR_4$ and $R_4=H$, Me, Et; OCH$_2$CONR$_2$R$_6$ where $R_2$, R$_6$ where $R_2=H$, Me, Et and $R_6=H$, Me, Et or a ligand.

[0117] $Y=O$, $S$ or $Se$.

[0118] $Z=O$, $S$, $NR'$, $CH_2$, CHF, CF$_2$.


[0120] $Z=H$, OR', $SR'$, $NRR'$.
[0120] R=H, OH, OMe, F, O(CH₂)OCH₃, O(CH₂)₂SCH₂, O(CH₂)₂ONMe₂, O(CH₂)₂O(CH₂)₂NMe₂, OCH₂CONH₂, O(CH₂)₂XR₃, where X=O, S, NR₄ and R₃, R₄=H, Me, Et, OCH₂CONR₃R₅, where R₅, R₆ where R₆=H, Me, Et and R₅=H, Me, Et or a ligand

[0121] X=OH, —SH, —Me, —isopropyl, —tert-Butyl, —phenyl, —NH-Alkyl, —N(dialkyl), —N(alkyl)(Aryl), —(CH₂)ₙXRₗ or —NH(CH₂)XRₗ, where X=O, S, NR₄, R₄=H, Me, Et, and R₅=H, Me, Et, COMe and n=1-12

[0122] Y=O, S or Se

[0123] Z=O, S, NR', CH₂, CHF, CF₂

[0124] R'=H, Me, Et, (CH₂)ₙOH; n=0-6

[0125] Chiral Rp and Sp isomers, P=S preferably Sp

[0126] L-Nucleoside Derived from 1-15

[0127] 2'-5' Linkage from 1-15

[0130] Y=O, S or Se

[0131] R=H, OH, OMe, F, O(CH₂)OCH₃, O(CH₂)₂SCH₂, O(CH₂)₂ONMe₂, O(CH₂)₂O(CH₂)₂NMe₂, OCH₂CONH₂, O(CH₂)₂XR₃, where X=O, S, NR₄ and R₃, R₄=H, Me, Et, OCH₂CONR₃R₅, where R₅, R₆ where R₆=H, Me, Et and R₅=H, Me, Et or a ligand

[0132] X=OH, —SH, —Me, —isopropyl, —tert-Butyl, —phenyl, —NH-Alkyl, —N(dialkyl), —N(alkyl)(Aryl), —(CH₂)ₙXRₗ or —NH(CH₂)XRₗ, where X=O, S, NR₄, R₄=H, Me, Et, and R₅=H, Me, Et, COMe and n=1-12
[0133] Y=O, S or Se

18A 3'-thiophosphate (3'-end)

19A 3'-thiophosphate (3'-end)

[0134] R=H, OH, OMe, F, O(CH$_2$)$_2$OCH$_3$, O(CH$_2$)$_2$SCH$_3$, O(CH$_2$)$_2$ONMe$_2$, O(CH$_2$)$_2$O(CH$_2$)$_2$NMe$_2$, OCH$_2$CONH$_2$, O(CH$_2$)$_2$XR$_3$, where X=O, S, NR$_4$ and R$_3$, R$_n$=H, Me, Et, etc.; OCH$_2$CONR$_2$R$_3$, where R$_4$, R$_5$, where R$_n$=H, Me, Et and R$_n$=H, Me, Et or a ligand

[0135] X=OH, —SH, -Me, -isopropyl, -tert-Butyl, -phenyl, —NH-Alkyl, —N(dialkyl), —N(alkyl)(Aryl), —(CH$_2$)$_n$XR$_7$, or —NH(CH$_2$)$_n$XR$_7$, where X=O, S, NR$_4$, R$_n$=H, Me, Et, and R$_n$=H, Me, Et, COMe and n=1-12

[0137] R=H, OH, OMe, F, O(CH$_2$)$_2$OCH$_3$, O(CH$_2$)$_2$SCH$_3$, O(CH$_2$)$_2$ONMe$_2$, O(CH$_2$)$_2$O(CH$_2$)$_2$NMe$_2$, OCH$_2$CONH$_2$, O(CH$_2$)$_2$XR$_3$, where X=O, S, NR$_4$ and R$_3$, R$_n$=H, Me, Et, etc.; OCH$_2$CONR$_2$R$_3$, where R$_4$, R$_5$, where R$_n$=H, Me, Et and R$_n$=H, Me, Et or a ligand

[0138] X=—OH, —SH, -Me, -isopropyl, -tert-Butyl, -phenyl, —NH-Alkyl, —N(dialkyl), —N(alkyl)(Aryl), —(CH$_2$)$_n$XR$_7$, or —NH(CH$_2$)$_n$XR$_7$, where X=O, S, NR$_4$, R$_n$=H, Me, Et, and R$_n$=H, Me, Et, COMe and n=1-12
[0139] Y=O, S or Se

20A S'-thiophosphate (S'-end)

20B S'-thiophosphate (S'-end)

[0140] R=H, OH, OMe, F, O(CH₂)₂OCH₃, O(CH₂)₂SCH₃, O(CH₂)₂ONMe₂, O(CH₂)₂O(CH₂)₂NMe₂, OCH₂CONH₂, O(CH₂)₂XR₂, where X=O, S, NR₃ and R₃, R₃=H, Me, Et, OCH₂CONR₃R₃, where R₃, R₃=H, Me, Et and R₃=H, Me, Et or a ligand

[0141] X=—OH, =—SH, =—Me, =—isopropyl, =—tert-Butyl, =—phenyl, =—NH-Alkyl, =—N(dialkyl), =—N(alkyl)(Aryl), =—(CH₂)ₙXR₂ or =—NH(CH₂)XR₃, where X=O, S, NR₃, R₃=H, Me, Et, and R₃=H, Me, Et, COMe and n=1-12

[0142] Y=O, S or Se

[0143] -continued

21B S'-thiophosphate (S'-end)

[0144] X=—OH, =—SH, =—Me, =—isopropyl, =—tert-Butyl, =—phenyl, =—NH-Alkyl, =—N(dialkyl), =—N(alkyl)(Aryl), =—(CH₂)ₙXR₂ or =—NH(CH₂)XR₃, where X=O, S, NR₃, R₃=H, Me, Et, and R₃=H, Me, Et, COMe and n=1-12

[0145] Y=O, S or Se

21A S'-thiophosphate (S'-end)

22A S'-deoxy (S'-end)
[0146] R=H, OH, OMe, F, O(CH₂)₂OCH₃, O(CH₂)₂SCH₃, O(CH₂)₂ONMe₂, O(CH₂)₂O(CH₂)₂NMe₂, OCH₂CONH₂, O(CH₂)₂XR₂, where X=O, S, NR₃ and R₂, R₃=H, Me, Et, etc.; OCH₂CONR₂R₃, where R₂, R₃=H, Me, Et and R₄=H, Me, Et or a ligand

[0147] X=—OH, —SH, -Me, -isopropyl, -tert-Butyl, -phenyl, —NH-Alkyl, —N(dialkyl), —N(alkyl)(aryl), —(CH₂)ₙXRₙ or —NH(CH₂)XRₙ, where X=O, S, NR₃, R₃=H, Me, Et, and R₄=H, Me, Et, COMe and n=1-12

[0148] Y=O, S or Se

[0149] Chiral Rp and Sp isomers, P=S preferably Sp

[0150] R=H, OH, OMe, F, O(CH₂)₂OCH₃, O(CH₂)₂SCH₃, O(CH₂)₂ONMe₂, O(CH₂)₂O(CH₂)₂NMe₂, OCH₂CONH₂, O(CH₂)₂XR₂, where X=O, S, NR₃ and R₂, R₃=H, Me, Et, etc.; OCH₂CONR₂R₃, where R₂, R₃=H, Me, Et and R₄=H, Me, Et or a ligand

[0151] X=—OH, —SH, -Me, -isopropyl, -tert-Butyl, -phenyl, —NH-Alkyl, —N(dialkyl), —N(alkyl)(aryl), —(CH₂)ₙXRₙ or —NH(CH₂)XRₙ, where X=O, S, NR₃, R₃=H, Me, Et, and R₄=H, Me, Et, COMe and n=1-12

[0152] Y=O, S or Se

[0153] Z=OH, OMe, SH, SMe, NH₂, NHMe, NMe₂, Aryl

[0154] Chiral Rp and Sp isomers, P=S preferably Sp
[0155] R=H, OH, OMe, F, O(CH2)2OCH3, O(CH2)2SCH3, O(CH2)2ONMe2, O(CH2)2O(CH2)2NMMe2, OCH2CONH2, O(CH2)2XR3, where X=O, S, NR4 and R3, R3=H, Me, Et, OCH2CONR5R6, where R5, R6, where R5=H, Me, Et and R6=H, Me, Et or a ligand

[0156] X=—OH, —SH, —Me, —isopropyl, —tert-Butyl, —phenyl, —NH-Alkyl, —N(dialkyl), —N(alkyl)(aryl), —(CH2)3XR7 or —NH(CH2)3XR7, where X=O, S, NR4, R3=H, Me, Et, and R6=H, Me, Et, COMe and n=1-12

[0157] Y=O, S or Se

[0158] Z=OH, OMe, SH, SMe, NH2, NHMe, NMe2, Aryl, alkyl

[0159] Chiral Rp and Sp isomers, P=S preferably Sp

[0160] R=H, OH, OMe, F, O(CH2)2OCH3, O(CH2)2SCH3, O(CH2)2ONMe2, O(CH2)2O(CH2)2NMMe2, OCH2CONH2, O(CH2)2XR3, where X=O, S, NR4 and R3, R3=H, Me, Et, OCH2CONR5R6, where R5, R6, where R5=H, Me, Et and R6=H, Me, Et or a ligand

[0161] X=—OH, —SH, —Me, —isopropyl, —tert-Butyl, —phenyl, —NH-Alkyl, —N(dialkyl), —N(alkyl)(aryl), —(CH2)3XR7 or —NH(CH2)3XR7, where X=O, S, NR4, R3=H, Me, Et, and R6=H, Me, Et, COMe and n=1-12

[0162] Y=O, S or Se

[0163] Chiral Rp and Sp isomers, P=S preferably Sp
[0164] R=H, OH or OMe, X=O, NH, NMe, S, CH₂, or CF₃; Y=O or S; Q', Q''=H; Q=H and Q'=Me or Et; or Q', Q''=Me or Et and n=0-5
[0166] R=H, OH or OMe; X=O, NH, NMe, S, CH₂, or CF₂; Y=O or S; Q', Q''=H Q'=H and Q''=Me or Et or Q', Q''=Me or Et; p=0-6 and q=0-6
R=H, OH or OMe; X=O, NH, NMe, S, CH₃, or CF₃; Y=O or S; Q', Q''=H; Q'=H and Q''=Me or Et or Q', Q''=Me or Et; p=0-6 and q=0-6
[0170] R=H, OH or OMe

-continued

[0171] R=H, OH or OMe
[0172] R=H, OH or OMe
[0174] R=H, OH or OMe -continued
[0175] R = H, OH or OMe
R=H, OH or OMe

For structures 27-81, R can also have the values for structures 1A-26.

The iRNA agent can include a conjugate (also called a ligand) on the 5' or 3' terminus of the sense or antisense strand. Exemplary ligands are shown below. The conjugate can be attached to the 3' or 5' end. Preferably, a cholesterol conjugate is on the 5' or 3' end of the sense strand. Preferably, a cholesterol conjugate is not on the antisense strand.

Preferred Ligands

Vitamins
Peptides
Carbohydrates
Terpenes
Steroids
Lipids
Amino acids
Naproxen
Ibuprofen
Amines (mono, di, tri, tetraalkyl or aryl)
Folate
Sugar (N-Acetylgalactosamine, galactosamine, galactose, Mannose)

Double-stranded (dsRNA) directs the sequence-specific silencing of mRNA through a process known as RNA interference (RNAi). The process occurs in a wide variety of organisms, including mammals and other vertebrates.

It has been demonstrated that 21-23 nt fragments of dsRNA are sequence-specific mediators of RNA silencing, e.g., by causing RNA degradation. While not wishing to be bound by theory, it may be that a molecular signal, which may be merely the specific length of the fragments, present in these 21-23 nt fragments recruits cellular factors that mediate RNAi. Described herein are methods for preparing and administering these 21-23 nt fragments, and other iRNAs agents, and their use for specifically inactivating gene function. The use of iRNAs agents (or recombinantly produced or chemically synthesized oligonucleotides of the same or similar nature) enables the targeting of specific mRNAs for silencing in mammalian cells. In addition, longer dsRNA agent fragments can also be used, e.g., as described below.
Although, in mammalian cells, long dsRNAs can induce the interferon response, which is frequently deleterious, sRNAs do not trigger the interferon response, at least not to an extent that is deleterious to the cell and host. In particular, the length of the RNA agent strands in an sRNA agent can be less than 31, 30, 28, 25, or 23 nt, e.g., sufficiently short to avoid inducing a deleterious interferon response. Thus, the administration of a composition of sRNA agent (e.g., formulated as described herein) to a mammalian cell can be used to silence expression of a target gene while circumventing the interferon response. Further, use of a discrete species of iRNA agent can be used to selectively target one allele of a target gene, e.g., in a subject heterozygous for the allele.

In a typical embodiment, the subject is a mammal such as a cow, horse, mouse, rat, dog, pig, goat, or a primate. The subject can be a dairy mammal (e.g., a cow, or goat) or other farmed animal (e.g., a chicken, turkey, sheep, pig, fish, shrimp). In a much preferred embodiment, the subject is a human, e.g., a normal individual or an individual that has, is diagnosed with, or is predicted to have a disease or disorder.

Ligand-Conjugated Monomer Subunits and Monomers for Oligonucleotide Synthesis

Definitions

The term “halo” refers to any radical of fluorine, chlorine, bromine or iodine.

The term “alkyl” refers to a hydrocarbon chain that may be a straight chain or branched chain, containing the indicated number of carbon atoms. For example, C<sub>3</sub>-C<sub>12</sub> alkyl indicates that the group may have from 1 to 12 (inclusive) carbon atoms in it. The term “haloalkyl” refers to an alkyl in which one or more hydrogen atoms are replaced by halo, and includes alkyl moieties in which all hydrogens have been replaced by halo (e.g., perfluoroalkyl). Alkyl and haloalkyl groups may be optionally inserted with O, N, or S. The terms “arylalkyl” refers to an alkyl moiety in which an alkyl hydrogen atom is replaced by an aryl group. Aralkyl includes groups in which more than one hydrogen atom has been replaced by an aryl group. Examples of “arylalkyl” include benzyl, 9-fluorenlyl, benzhydryl, and triyl groups.

The term “alkenyl” refers to a straight or branched hydrocarbon chain containing 2-8 carbon atoms and characterized in having one or more double bonds. Examples of a typical alkenyl include, but not limited to, alkenyl, propenyl, 2-butenyl, 3-hexenyl and 3-ocynyl groups. The term “alkynyl” refers to a straight or branched hydrocarbon chain containing 2-8 carbon atoms and characterized in having one or more triple bonds. Some examples of a typical alkynyl are ethynyl, 2-propynyl, and 3-methylbutynyl, and propargyl. The sp<sup>2</sup> and sp<sup>3</sup> carbons may optionally serve as the point of attachment of the alkenyl and alkynyl groups, respectively.

The terms “alkylamino” and “dialkylamino” refer to —NH(alkyl) and —N(alkyl), respectively. The term “arylalkylamino” refers to a —NH(arylalkyl) radical. The term “alkoxy” refers to an —O-alkyl radical, and the terms “cyclealkoxy” and “aralkoxy” refer to an —O-cyclealkyl and O-aralkyl radicals respectively. The term “siloxyl” refers to a R<sub>3</sub>SiO— radical. The term “mercapto” refers to an SH radical. The term “thioalkoxy” refers to an —S-alkyl radical.

The term “alkylene” refers to a divalent alkyl (i.e., —R—), e.g., —CH<sub>2</sub>—, —CH<sub>2</sub>CH<sub>2</sub>—, and —CH<sub>2</sub>CH<sub>3</sub>—. The term “alkyleneedioxo” refers to a divalent species of the structure —O—R—O—, in which R represents an alkylene.

The term “aryl” refers to an aromatic monocyclic, bicyclic, or tricyclic hydrocarbon ring system, wherein any ring atom can be substituted. Examples of aryl moieties include, but are not limited to, phenyl, naphthyl, anthracenyl, and pyrenyl.

The term “cycloalkyl” as employed herein includes saturated cyclic, bicyclic, tricyclic, or polycyclic hydrocarbon groups having 3 to 12 carbons, wherein any ring atom can be substituted. The cycloalkyl groups herein described may also contain fused rings. Fused rings are rings that share a common carbon-carbon bond or a common carbon atom (e.g., spiro-fused rings). Examples of cycloalkyl moieties include, but are not limited to, cyclohexyl, adamantyl, and norbornyl.

The term “heterocyclic” refers to a nonaromatic 3-10 membered monocyclic, 8-12 membered bicyclic, or 11-14 membered tricyclic ring system having 1-3 heteroatoms if monocyclic, 1-6 heteroatoms if bicyclic, or 1-9 heteroatoms if tricyclic, said heteroatoms selected from O, N, or S (e.g., carbon atoms and 1-3, 1-6, or 1-9 heteroatoms of N, O, or S if monocyclic, bicyclic, or tricyclic, respectively), wherein any ring atom can be substituted. The heterocyclic groups herein described may also contain fused rings. Fused rings are rings that share a common carbon-carbon bond or a common carbon atom (e.g., spiro-fused rings). Examples of heterocyclic include, but are not limited to tetrahydrofuranyl, tetrahydropyran, piperdiny, morpholin, pyrrolinyl and pyrrolidinyl.

The term “cycloalkenyl” as employed herein includes partially unsaturated, nonaromatic, cyclic, bicyclic, tricyclic, or polycyclic hydrocarbon groups having 5 to 12 carbons, preferably 5 to 8 carbons, wherein any ring atom can be substituted. The cycloalkenyl groups herein described may also contain fused rings. Fused rings are rings that share a common carbon-carbon bond or a common carbon atom (e.g., spiro-fused rings). Examples of cycloalkenyl moieties include, but are not limited to cyclohexenyl, cyclohexadienyl, or norbornenyl.

The term “heterocycloalkenyl” refers to a partially saturated, nonaromatic 5-10 membered monocyclic, 8-12 membered bicyclic, or 11-14 membered tricyclic ring system having 1-3 heteroatoms if monocyclic, 1-6 heteroatoms if bicyclic, or 1-9 heteroatoms if tricyclic, said heteroatoms selected from O, N, or S (e.g., carbon atoms and 1-3, 1-6, or 1-9 heteroatoms of N, O, or S if monocyclic, bicyclic, or tricyclic, respectively), wherein any ring atom can be substituted. The heterocycloalkenyl groups herein described may also contain fused rings. Fused rings are rings that share a common carbon-carbon bond or a common carbon atom (e.g., spiro-fused rings). Examples of heterocycloalkenyl include, but are not limited to tetrahydrofuranyl and dihydrofuran.

The term “heterynaryl” refers to an aromatic 5-8 membered monocyclic, 8-12 membered bicyclic, or 11-14 membered tricyclic ring system having 1-3 heteroatoms if monocyclic, 1-6 heteroatoms if bicyclic, or 1-9 heteroatoms if tricyclic, said heteroatoms selected from O, N, or S (e.g., carbon atoms and 1-3, 1-6, or 1-9 heteroatoms of N, O, or
S if monocyclic, bicyclic, or tricyclic, respectively), wherein any ring atom can be substituted. The heteroaryl groups herein described may also contain fused rings that share a common carbon-carbon bond.

[0221] The term “oxo” refers to an oxygen atom, which forms a carbonyl when attached to carbon, an N-oxide when attached to nitrogen, and a sulfoxide or sulfone when attached to sulfur.

[0222] The term “acyl” refers to an alkylcarboxylic, cyanoalkylicarboxylic, arylcarboxylic, heterocyclicarboxylic, or heteroarylcycarboxyl substituent, any of which may be further substituted by substituents.

[0223] The term “substituents” refers to a group “substituted” on an alkyl, cycloalkyl, alkenyl, alkynyl, heterocyclic, heterocycloalkenyl, cycloalkenyl, aryl, or heteroaryl group at any atom of that group. Suitable substituents include, without limitation, alkyl, alkenyl, alkynyl, halo, hydroxy, cyano, nitro, amino, SO₂H, sulfate, phosphate, phosphonate, sulfonate, ester, amine, amide, anilide, esteramide, esteramine, esteramide, esteramine, esteramide, esteramine, or esteramide, respectively, alkyl, aralkyl, heteroaryl, and combinations thereof, amine (mono-, di-, alkyl, cycloalkyl, aralkyl, heteroaryl, and combinations thereof), ester (alkyl, aralkyl, heteroaryl), amide (mono-, di-, alkyl, aralkyl, heteroaryl, and combinations thereof), sulfonamide (mono-, di-, alkyl, aralkyl, heteroaryl, and combinations thereof), unsubstituted aryl, unsubstituted heteroaryl, unsubstituted heterocyclic, and unsubstituted cycloalkyl. In one aspect, the substituents on a group are independently any one or any subset of the aforementioned substituents.

[0224] The terms “adeninyl, cytosinyl, guaninyl, thyminyl, and uracilly” and the like refer to radicals of adenine, cytosine, guanine, thymine, and uracil.

[0225] A “protected” moiety refers to a reactive functional group, e.g., a hydroxyl group or an amino group, or a class of molecules, e.g., sugars, having one or more functional groups, in which the reactivity of the functional group is temporarily blocked by the presence of an attached protecting group. Protecting groups useful for the monomers and methods described herein can be found, e.g., in Greene, T. W., Protective Groups in Organic Synthesis (John Wiley and Sons: New York, 1981), which is hereby incorporated by reference.

[0226] General

[0227] An RNA agent, e.g., an RNA agent, containing a preferred, but nonlimiting ligand-conjugated monomer subunit is presented as formula (II) below and in the scheme in FIG. 1. The carrier (also referred to in some embodiments as a “linker”) can be a cyclic or acyclic moiety and includes two “backbone attachment points” (e.g., hydroxyl groups) and a ligand. The ligand can be directly attached (e.g., conjugated to the carrier or indirectly attached (e.g., conjugated) to the carrier by an intervening tether (e.g., an acyclic chain of one or more atoms; or a nucleobase, e.g., a naturally occurring nucleobase optionally having one or more chemical modifications, e.g., an unusual base; or a universal base). The carrier therefore also includes a “ligand or tethering attachment point” for the ligand and tether/tethered ligand, respectively.

[0228] The ligand-conjugated monomer subunit may be the 5’ or 3’ terminal subunit of the RNA molecule, i.e., one of the two “W” groups may be a hydroxyl group, and the other “W” group may be a chain of two or more unmodified or modified ribonucleotides. Alternatively, the ligand-conjugated monomer subunit may occupy an internal position, and both “W” groups may be one or more unmodified or modified ribonucleotides. More than one ligand-conjugated monomer subunit may be present in a RNA molecule, e.g., an RNA agent. Preferred positions for inclusion of tethered ligand-conjugated monomer subunits, e.g., one in which a lipophilic moiety, e.g., cholesterol, is tethered to the carrier are at the 3’ terminus, the 5’ terminus, or an internal position of the sense strand.

[0229] The modified RNA molecule of formula (II) can be obtained using oligonucleotide synthetic methods known in the art. In a preferred embodiment, the modified RNA molecule of formula (II) can be prepared by incorporating one or more of the corresponding monomer compounds (see, e.g., A, B, and C below and in the scheme in FIG. 1) into a growing sense or antisense strand, utilizing, e.g., phosphoramidite or H-phosphonate coupling strategies.

[0230] The monomers, e.g., a ligand-conjugated monomer, generally include two different functionalized hydroxyl groups (OFG¹ and OFG²), which are linked to the carrier molecule (see A below and in FIG. 1, and a ligand/tethering attachment point. As used herein, the term “functionalized hydroxyl group” means that the hydroxyl proton has been replaced by another substituent. As shown in representative structures B and C below and in FIG. 1, one hydroxyl group (OFG¹) on the carrier is functionalized with a protecting group (PG). The other hydroxyl group (OFG²) can be functionalized with either (1) a liquid or solid phase synthesis support reagent (solid circle) directly or indirectly through a linker, L, as in B, or (2) a phosphorus-containing moiety, e.g., a phosphoramidite as in C. The tethering attachment point may be connected to a hydrogen atom, a suitable protecting group, a tether, or a tethered ligand at the time that the monomer is incorporated into the growing sense or antisense strand (see variable “R” in A below). Thus, the tethered ligand can be, but need not be attached to the monomer at the time that the monomer is incorporated into the growing strand. In certain embodiments, the tether, the ligand or the tethered ligand may be linked to a “precursor” ligand-conjugated monomer subunit after a “precursor” ligand-conjugated monomer subunit has
been incorporated into the strand. The wavy line used below (and elsewhere herein) refers to a connection, and can represent a direct bond between the moiety and the attachment point or a tethering molecule which is interposed between the moiety and the attachment point. Directly tethered means the moiety is bound directly to the attachment point. Indirectly tethered means that there is a tether molecule interposed between the attachment point and the moiety.

![Diagram](Image)

[0231] The (OFG) protecting group may be selected as desired, e.g., from T. W. Greene and P. G. M. Wuts, Protective Groups in Organic Synthesis, 2d Ed., John Wiley and Sons (1991). The protecting group is preferably stable under amidite synthesis conditions, storage conditions, and oligonucleotide synthesis conditions. Hydroxyl groups, —OH, are nucleophilic groups (i.e., Lewis bases), which react through the oxygen with electrophiles (i.e., Lewis acids). Hydroxyl groups in which the hydrogen has been replaced with a protecting group, e.g., a triarylmethyl group or a trialkylsilylethyl group, are essentially unreactive as nucleophiles in displacement reactions. Thus, the protected hydroxyl group is useful in preventing e.g., homocoupling of compounds exemplified by structure C during oligonucleotide synthesis. In some embodiments, a preferred protecting group is the dimethoxytrityl group. In other embodiments, a preferred protecting group is a silicon-based protecting group having the formula below:

![Diagram](Image)

[0232] X5', X5", and X5''' can be selected from substituted or unsubstituted alkyl, cycloalkyl, aryl, aralkyl, heteroaryl, alkoxy, cycloalkoxy, aralkoxy, aryloxy, heteroaryloxy, or siloxy (i.e., R,SiO—, the three “R” groups can be any combination of the above listed groups). X5', X5", and X5''' may all be the same or different; also contemplated is a combination in which two of X5', X5", and X5''' are identical and the third is different. In certain embodiments X5', X5", and X5''' include at least one alkoxy or siloxy groups and may be any one of the groups listed in FIG. 2A, a preferred combination includes X5', X5"-trimethylsilyloxy and X5'''=1,3-tris(hydroxymethyl)-2-propoxy or cyclododecyleoxy.

[0233] Other preferred combinations of X5', X5", and X5''' include those that result in OFG groups that meet the deprotection and stability criteria delineated below. The group is preferably stable under amidite synthesis conditions, storage conditions, and oligonucleotide synthesis conditions. Rapid removal, i.e., less than one minute, of the silyl group from e.g., a support-bound oligonucleotide is desirable because it can reduce synthesis times and thereby reduce exposure time of the growing oligonucleotide chain to the reagents. Oligonucleotide synthesis can be improved if the silyl protecting group is visible during deprotection, e.g., from the addition of a chromophore silyl substituent.

[0234] Selection of silyl protecting groups can be complicated by the competing demands of the essential characteristics of stability and facile removal, and the need to balance these competitive goals. Most substituents that increase stability can also increase the reaction time required for removal of the silyl group, potentially increasing the level of difficulty in removal of the group.

[0235] The addition of alkoxy and siloxy substituents to OFG silicon-containing protecting groups increases the susceptibility of the protecting groups to fluoride cleavage of the silyl ether bonds. Increasing the steric bulk of the substituents preserves stability while not decreasing fluoride lability to an equal extent. An appropriate balance of substituents on the silyl group makes a silyl ether a viable nucleoside protecting group.

[0236] Candidate OFG silicon-containing protecting groups may be tested by exposing a tetrahydrofuran solution of a preferred carrier bearing the candidate OFG group to five molar equivalents of tetrahydrofuran at room temperature. The reaction time may be determined by monitoring the disappearance of the starting material by thin layer chromatography.

[0237] When the OFG in B includes a linker, e.g., a relatively long organic linker, connected to a soluble or
insoluble support reagent, solution or solid phase synthesis techniques can be employed to build up a chain of natural and/or modified ribonucleotides once OFG is deprotected and free to react as a nucleophile with another nucleoside or monomer containing an electrophilic group (e.g., an amide group). Alternatively, a natural or modified ribonucleotide or oligoribonucleotide chain can be coupled to monomer C via an amide group or H-phosphonate group at OFG. Subsequent to this operation, OFG is deblocked, and the restored nucleophilic hydroxyl group can react with another nucleoside or monomer containing an electrophilic group. R' can be substituted or unsubstituted alkyl or aralkyl. In preferred embodiments, R' is methyl, allyl or 2-cyanomethyl. R' may a C₃-C₁₀ alkyl group, preferably it is a branched group containing three or more carbons, e.g., isopropyl.

[0238] OFG in B can be hydroxyl functionalized with a linker, which in turn contains a liquid or solid phase synthesis support reagent at the other linker terminus. The support reagent can be any support medium that can support the monomers described herein. The monomer can be attached to an insoluble support via a linker, L₁, which allows the monomer (and the growing chain) to be solubilized in the solvent in which the support is placed. The solubilized, yet immobilized, monomer can react with reagents in the surrounding solvent; unreacted reagents and soluble by-products can be readily washed away from the solid support to which the monomer or monomer-derived products is attached. Alternatively, the monomer can be attached to a soluble support moiety, e.g., polyethylene glycol (PEG) and liquid phase synthesis techniques can be used to build up the chain. Linker and support medium selection is within skill of the art. Generally the linkers may be =C(O)(CH₂)₆COO−, or =C(O)(CH₂)₆S−, in which q can be 0, 1, 2, 3, or 4; preferably, it is oxlyl, succinyl or thioglycolyl. Standard control pore glass solid phase synthesis supports can not be used in conjunction with fluoride labile 5′ silyl protecting groups because the glass is degraded by fluoride with a significant reduction in the amount of full-length product. Fluoride-stable polystyrene based supports or PEG are preferred.

[0239] The ligand/tethering attachment point can be any divalent, trivalent, tetravalent, pentavalent or hexavalent atom. In some embodiments, ligand/tethering attachment point can be a carbon, oxygen, nitrogen or sulfur atom. For example, a ligand/tethering attachment point precursor functional group can have a nucleophilic heteroatom, e.g., —SH, —NH₂, secondary amino, ONH₂, or NH₂NH₂. As another example, the ligand/tethering attachment point precursor functional group can be an olefin, e.g., —CH=CH₂, and the precursor functional group can be attached to a ligand, a tether, or tethered ligand using, e.g., transition metal catalyzed carbon-carbon (for example olefin metathesis) processes or cycloaditions (e.g., Diels-Alder). As a further example, the ligand/tethering attachment point precursor functional group can be an electrophilic moiety, e.g., an aldehyde. When the carrier is a cyclic carrier, the ligand/tethering attachment point can be an endocyclic atom (i.e., a constituent atom in the cyclic moiety, e.g., a nitrogen atom) or an exocyclic atom (i.e., an atom or group of atoms attached to a constituent atom in the cyclic moiety).

[0240] The carrier can be any organic molecule containing attachment points for OFG, OFG, and the ligand. In certain embodiments, carrier is a cyclic molecule and may contain heteroatoms (e.g., O, N or S). E.g., carrier molecules may include aryl (e.g., benzene, biphenyl, etc.), cyclosilky (e.g., cyclohexane, cis or trans decalin, etc.), cycloalkenyl (e.g., cyclohexenyl), or heterocyclic (tetrahydropyran, piperazine, pyrrolidine, etc.). In other embodiments, the carrier can be an acyclic moiety, e.g., based on serinol. Any of the above cyclic systems may include substituents in addition to OFG, OFG, and the ligand.

[0241] Sugar-Based Monomers

[0242] In some embodiments, the carrier molecule is an oxygen containing heterocycle. Preferably the carrier is a ribose sugar as shown in structure LCM-I. In this embodiment, the monomer, e.g., a ligand-conjugated monomer is a nucleoside.

[0243] “B” represents a nucleobase, e.g., a naturally occurring nucleobase optionally having one or more chemical modifications, e.g., and unnatural base; or a universal base.

[0244] As used herein, an “unusual” nucleobase can include any one of the following:

[0245] 2-methyladeninyl,
[0246] N6-methyladeninyl,
[0247] 2-methylthio-N-6-methyladeninyl,
[0248] N6-isopentenyladeninyl,
[0249] 2-methylthio-N-6-isopentenyladeninyl,
[0250] N6-(cis-hydroxyisopentenyl)adeninyl,
[0251] 2-methylthio-N-6-(cis-hydroxyisopentenyl)adeninyl,
[0252] N6-glycinylcarbamoyladeninyl,
[0253] N6-threonylcarbamoyladeninyl,
[0254] 2-methylthio-N-6-threonyl carbamoyladeninyl,
[0255] N6-methyl-N-6-threonylcarbamoyladeninyl,
[0256] N6-hydroxynorvalylcarbamoyladeninyl,
[0257] 2-methylthio-N-6-hydroxynorvalyl carbamoyladeninyl,
[0258] N6,N6-dimethyladeninyl,
[0259] 3-methylcytosinyl,
[0260] 5-methylcytosinyl,
[0261] 2-thiocytosinyl,

[0262] 5-formylcytosinyl,

[0263] N4-methylcytosinyl,

[0264] 5-hydroxymethylcytosinyl,

[0265] 1-methylguaninyl,

[0266] N2-methylguaninyl,

[0267] 7-methylguaninyl,

[0268] N2,N2-dimethylguaninyl,
A universal base can form base pairs with each of the natural DNA/RNA bases, exhibiting relatively little discrimination between them. In general, the universal bases are non-hydrogen bonding, hydrophobic, aromatic moieties which can stabilize e.g., duplex RNA or RNA-like molecules, via stacking interactions. A universal base can also include hydrogen bonding substituents. As used herein, a “universal base” can include anthracenes, pyrenes or any one of the following:

- 5-(carboxyhydroxymethyl)uracilyl methyl ester,
- 5-methoxycarbonylmethyluracilyl,
- 5-methoxy carbonylmethyl-2-thiouracilyl,
- 5-aminomethyl-2-thiouracilyl,
- 5-methylaminomethyluracilyl,
- 5-methylaminomethyl-2-thiouracilyl,
- 5-methylaminomethyl-2-selenouracilyl,
- 5-carbamoylmethyluracilyl,
- 5-carboxymethylaminomethyluracilyl,
- 5-carboxymethylaminomethyl-2-thiouracilyl,
- 3-methyluracilyl,
- 1-methyl-3-(3-amino-3-carboxypropyl) pseudouracilyl,
- 5-carboxymethyluracilyl,
- 5-methylhydouracilyl, or
- 3-methyl pseudouracilyl.

A universal base can form base pairs with each of the natural DNA/RNA bases, exhibiting relatively little discrimination between them. In general, the universal bases are non-hydrogen bonding, hydrophobic, aromatic moieties which can stabilize e.g., duplex RNA or RNA-like molecules, via stacking interactions. A universal base can also include hydrogen bonding substituents. As used herein, a “universal base” can include anthracenes, pyrenes or any one of the following:
In some embodiments, B can form part of a tether that connects a ligand to the carrier. For example, the tether can be $B = \text{CH} = \text{CH} = \text{C(O)} \text{NH} = (\text{CH}_2)_n = \text{NH} \text{C(O)}\text{-LIGAND}$. In a preferred embodiment, the double bond is trans, and the ligand is a substituted or unsubstituted cholesterolyl radical (e.g., attached through the D-ring side chain or the C-3 hydroxyl); an aralkyl moiety having at least one stereogenic center and at least one substituent on the aryl portion of the aralkyl group; or a nucleobase. In certain embodiments, B, in the tether described above, is uracil or a universal base, e.g., an aryl moiety, e.g., phenyl, optionally having additional substituents, e.g., one or more fluoro groups. B can be substituted at any atom with the remainder of the tether.

$X^2$ can include "oxy" or "deoxy" substituents in place of the 2'-OH, or be a ligand or a tethered ligand.

Examples of "oxy"-substituents include alkoxy or aryloxy (OR, e.g., R = H, alkyl, cycloalkyl, aryl, aralkyl, heteroaryl, sugar, or protecting group); polyethylene glycols (PEG), $O(\text{CH}_2\text{CH}_2\text{O})_n\text{CH}_2\text{OR}$; "locked" nucleic acids (LNA) in which the 2' hydroxyl is connected, e.g., by a methylene bridge, to the 4' carbon of the same ribose sugar;

O—PROTECTED AMINE (AMINE=NH$_2$; alkylamino, dialkylamino, heterocyclyl, arylamino, diaryl amino, heteroaryl amino, or diheteroaryl amino, ethylene diamine, polyamino) and aminoalkoxy, $O(\text{CH}_2)_n$PROTECTED AMINE, (e.g., AMINE=NH$_2$; alkylamino, dialkylamino, heterocyclyl, arylamino, diaryl amino, heteroaryl amino, or diheteroaryl amino, ethylene diamine, polyamino), and orthoester. Amines protecting groups can include formyl, amido, benzyl, allyl, etc.

Preferred orthoesters have the general formula $J$. The groups $R^{37}$ and $R^{38}$ may be the same or different and can be any combination of the groups listed in FIG. 2B. A preferred orthoester is the "ACE" group, shown below as structure K.
“Deoxy” substituents include hydrogen (i.e. deoxyribose sugars); halo (e.g., fluoro); protected amino (e.g., \( \text{NH}_2 \); alkylmino, dialkylmino, heterocyclyl, arylmino, diaryl amino, heteroaryl amino, diheteroaryl amino, or amino acid in which all amino are protected); fully protected polyamino (e.g., \( \text{NH}(-\text{CH}_2\text{CH}_2\text{NH}_2)\text{CH}_2\text{CH}_2\text{AMINE} \), wherein AMINE=\( \text{NH}_2 \); alkylmino, dialkylmino, heterocyclyl, arylmino, diaryl amino, heteroaryl amino, or diheteroaryl amino and all amino groups are protected), ---NH-C(O)R (R=alkyl, cycloalkyl, aryl, aralkyl, heteroaryl or sugar), cyano; alkyl-thio-alkyl; thioalkoxy; and alkyl, cycloalkyl, aryl, alkenyl and alkynyl, which may be optionally substituted with e.g., a protected amino functionality. Preferred substituents are 2'-methoxyethyl, 2'-OCH\(_3\), O-allyl, 2'-C—allyl, and 2'-fluoro.

\[ X^3 \] is as described for OFG\(^2 \) above.

PG can be a triarylmethyl group (e.g., a dimethoxytrityl group) or Si(X\(^3\))(X\(^3\)) in which (X\(^3\)), (X\(^3\)), and (X\(^3\)) are as described elsewhere.

Sugar Replacement-Based Monomers, e.g., Ligand-Conjugated Monomers (Cyclic)

Cyclic sugar replacement-based monomers, e.g., sugar replacement-based ligand-conjugated monomers, are also referred to herein as ribose replacement monomer subunit (RRMS) monomer compounds. Preferred carriers have the general formula (LCM-2) provided below (In that structure preferred backbone attachment points can be chosen from R\(^1\) or R\(^2\); R\(^3\) or R\(^4\); or R\(^5\) and R\(^10\) if Y is CR\(^R\) (two positions are chosen to give two backbone attachment points, e.g., R\(^3\) and R\(^4\), or R\(^3\) and R\(^5\)). Preferred tethering attachment points include R\(^2\); R\(^3\) or R\(^5\) when X is CH\(_2\). The carriers are described below as an entity, which can be incorporated into a strand. Thus, it is understood that the structures also encompass the situations wherein one (in the case of a terminal position) or two (in the case of an internal position) of the attachment points, e.g., R\(^1\) or R\(^2\); R\(^3\) or R\(^4\); or R\(^9\) or R\(^10\) (when Y is CR\(^R\)X), is connected to the phosphate, or modified phosphate, e.g., sulfur containing, backbone. E.g., one of the above-named R groups can be —CH\(_2\)OH, wherein one bond is connected to the carrier and one to a backbone atom, e.g., a linking oxygen or a central phosphorus atom.)

[0317] in which,

[0314] X is N(CO)R\(^7\), NR\(^7\) or CH\(_2\);

[0315] Y is NR\(^8\), O, S, CR\(^R\)R\(^10\);

[0316] Z is CR\(^R\)R\(^12\) or absent;

[0317] Each of R\(^1\), R\(^2\), R\(^3\), R\(^4\), R\(^5\), and R\(^10\) is, independently, H, OR\(^6\), or (CH\(_2\))\(_n\)OR\(^6\) provided that at least two of R\(^1\), R\(^2\), R\(^3\), R\(^4\), R\(^5\), and R\(^10\) are OR\(^6\) and/or (CH\(_2\))\(_n\)OR\(^6\);

[0318] Each of R\(^5\), R\(^6\), R\(^7\), and R\(^8\) is, independently, a ligand, H, C\(_1\)-C\(_2\) alkyl optionally substituted with 1-3 R\(^13\), or C(O)NH\(^R\); or R\(^5\) and R\(^7\) together are C\(_1\)-C\(_8\) cycloalkyl optionally substituted with R\(^14\);

[0319] R\(^7\) can be a ligand, e.g., R\(^7\) can be R\(^2\), or R\(^7\) can be a ligand tethered indirectly to the carrier, e.g., through a tethering moiety, e.g., C\(_1\)-C\(_2\) alkyl substituted with NR\(^R\);, or C\(_1\)-C\(_2\) alkyl substituted with NHC(O)R\(^8\);

[0320] R\(^8\) is H or C\(_1\)-C\(_6\) alkyl;

[0321] R\(^13\) is hydroxy, C\(_1\)-C\(_4\) alkoxyl, or halo;

[0322] R\(^14\) is NR\(^R\)R\(^12\);

[0323] R\(^15\) is C\(_1\)-C\(_6\) alkyl optionally substituted with cyano, or C\(_2\)-C\(_6\) alkkenyl;

[0324] R\(^16\) is C\(_1\)-C\(_{10}\) alkyl;

[0325] R\(^17\) is a liquid or solid phase support reagent;

[0326] L is \( \text{OC}(-\text{CH}_2)\text{N}(-\text{CH}_2)\text{S} \) or \( \text{OC}(-\text{CH}_2)\text{N}(-\text{CH}_2)\text{S} \);

[0327] R\(^\star\) is a protecting group, e.g., CA\(_{R}\); (e.g., a dimethoxytrityl group) or Si(X\(^3\))(X\(^3\))(X\(^3\)) in which (X\(^3\)), (X\(^3\)), and (X\(^3\)) are as described elsewhere.

[0328] R\(^8\) is P(O)(O\(^\star\))H, P(O\(^\star\))N(R\(^\star\)); or L-R\(^\star\);

[0329] R\(^\star\) is H or C\(_1\)-C\(_6\) alkyl;

[0330] R\(^\star\) is H or a ligand;

[0331] Each Ar is, independently, C\(_6\)-C\(_{10}\) aryl optionally substituted with C\(_1\)-C\(_4\) alkenyl;

[0332] n is 1-4; and q is 0-4.

Exemplary carriers include those in which, e.g., X is N(CO)R\(^7\) or NR\(^7\), Y is CR\(^R\) or CH\(_2\), and Z is absent; or X is N(CO)R\(^7\) or NR\(^7\), Y is CR\(^R\) or CH\(_2\), Y is OR\(^6\), and Z is CR\(^R\)R\(^12\); or X is N(CO)R\(^7\) or NR\(^7\), Y is OR\(^6\), and Z is CR\(^R\)R\(^12\); or X is CH\(_2\); Y is CR\(^R\)R\(^10\); Z is CR\(^R\)R\(^12\); and R\(^5\) and R\(^10\) together form C\(_6\).
cycloalkyl \((H, z=2)\), or the indane ring system, e.g., \(X\) is \(\text{CH}_2\); \(Y\) is \(\text{CR}^3\text{R}^5\); \(Z\) is \(\text{CR}^{11}\text{R}^{14}\), and \(R^2\) and \(R^{14}\) together form \(C_8\) cycloalkyl \((H, z=1)\).

[0334] In certain embodiments, the carrier may be based on the pyrrole ring system or the 4-hydroxyproline ring system, e.g., \(X\) is \(\text{N(CO)}\text{R}^7\) or \(\text{NR}^7\), \(Y\) is \(\text{CR}^9\text{R}^{10}\), and \(Z\) is absent \((D)\). \(\text{OFG}^2\) is preferably attached directly to one of the carbons in the five-membered ring \((-\text{OFG}^2\text{ in } D)\). For the pyrrole-based carriers, \(-\text{CH}_2\text{OFG}^1\) may be attached to \(C-2\) and \(\text{OFG}^2\) may be attached to \(C-3\); or \(-\text{CH}_2\text{OFG}^1\) may be attached to \(C-3\) and \(\text{OFG}^2\) may be attached to \(C-4\). In certain embodiments, \(-\text{CH}_2\text{OFG}^1\) and \(\text{OFG}^2\) may be geminally substituted to one of the above-referenced carbons. For the 3-hydroxyproline-based carriers, \(-\text{CH}_2\text{OFG}^1\) may be attached to \(C-2\) and \(\text{OFG}^2\) may be attached to \(C-4\). The pyrrole- and 4-hydroxyproline-based monomers may therefore contain linkages (e.g., carbon-carbon bonds) wherein bond rotation is restricted about that particular linkage, e.g., restriction resulting from the presence of a ring. Thus, \(-\text{CH}_2\text{OFG}^1\) and \(\text{OFG}^2\) may be cis or trans with respect to one another in any of the pairings delineated above. Accordingly, all cis/trans isomers are expressly included.

The monomers may also contain one or more asymmetric centers and thus occur as racemates and racemic mixtures, single enantiomers, individual diastereomers and diastereomeric mixtures. All such isomeric forms of the monomers are expressly included (e.g., the centers bearing \(\text{CH}_2\text{OFG}^1\) and \(\text{OFG}^2\) can both have the \(R\) configuration; or both have the \(S\) configuration; or one center can have the \(R\) configuration and the other center can have the \(S\) configuration and vice versa). The tethering attachment point is preferably nitrogen. Preferred examples of carrier \(D\) include the following:

[0335] A tether-ligand group, e.g., a methylene group, connected to one of the carbons in the five-membered ring \((-\text{CH}_2\text{OFG}^1\text{ in } D)\). \(\text{OFG}^2\) is preferably attached directly to one of the carbons in the five-membered ring \((-\text{OFG}^2\text{ in } D)\). For the pyrrole-based carriers, \(-\text{CH}_2\text{OFG}^1\) may be attached to \(C-2\) and \(\text{OFG}^2\) may be attached to \(C-3\); or \(-\text{CH}_2\text{OFG}^1\) may be attached to \(C-3\) and \(\text{OFG}^2\) may be attached to \(C-4\). In certain embodiments, \(-\text{CH}_2\text{OFG}^1\) and \(\text{OFG}^2\) may be geminally substituted to one of the above-referenced carbons. For the 3-hydroxyproline-based carriers, \(-\text{CH}_2\text{OFG}^1\) may be attached to \(C-2\) and \(\text{OFG}^2\) may be attached to \(C-4\). The pyrrole- and 4-hydroxyproline-based monomers may therefore contain linkages (e.g., carbon-carbon bonds) wherein bond rotation is restricted about that particular linkage, e.g., restriction resulting from the presence of a ring. Thus, \(-\text{CH}_2\text{OFG}^1\) and \(\text{OFG}^2\) may be cis or trans with respect to one another in any of the pairings delineated above. Accordingly, all cis/trans isomers are expressly included.

[0336] In certain embodiments, the carrier may be based on the piperidine ring system \((E)\), e.g., \(X\) is \(\text{N(CO)}\text{R}^7\) or \(\text{NR}^7\), \(Y\) is \(\text{CR}^9\text{R}^{10}\), and \(Z\) is \(\text{CR}^{11}\text{R}^{14}\). \(\text{OFG}^1\) is preferably attached to a primary carbon, e.g., an exocyclic alkyne group, e.g., a methylene group \((n=1)\) or ethylene group \((n=2)\), connected to one of the carbons in the six-membered ring \([-\text{CH}_2\text{OFG}^1\text{ in } E]\). \(\text{OFG}^2\) is preferably attached directly to one of the carbons in the six-membered ring \((-\text{OFG}^2\text{ in } E)\). \(-\text{CH}_2\text{OFG}^1\) and \(\text{OFG}^2\) may be disposed in a geminal manner on the ring, i.e., both groups may be attached to the same carbon, e.g., at \(C-2, C-3,\) or \(C-4\).
Alternatively, \((\text{CH}_2)_n\text{OFG}^1\) and \(\text{OFG}^2\) may be disposed in a vicinal manner on the ring, i.e., both groups may be attached to adjacent ring carbon atoms, e.g.,

\[\text{OFG}^1\] may be attached to C-2 and \(\text{OFG}^2\) may be attached to C-3; \((\text{CH}_2)_n\text{OFG}^1\) may be attached to C-3 and \(\text{OFG}^2\) may be attached to C-2; \((\text{CH}_2)_n\text{OFG}^1\) may be attached to C-3 and \(\text{OFG}^2\) may be attached to C-4; \((\text{CH}_2)_n\text{OFG}^1\) may be attached to C-4 and \(\text{OFG}^2\) may be attached to C-3. The piperidine-based monomers may therefore contain linkages (e.g., carbon-carbon bonds) wherein bond rotation is restricted about that particular linkage, e.g., restriction resulting from the presence of a ring. Thus, \(\text{CH}_2\text{OFG}^1\) and \(\text{OFG}^2\) may be cis or trans with respect to one another in any of the pairings delineated above. Accordingly, all cis/trans isomers are expressly included. The monomers may also contain one or more asymmetric centers and thus occur as racemates and racemic mixtures, single enantiomers, individual diastereomers and diastereomeric mixtures. All such isomeric forms of the monomers are expressly included (e.g., the centers bearing \(\text{CH}_2\text{OFG}^1\) and \(\text{OFG}^2\) can both have the R configuration; or both have the S configuration; or one center can have the R configuration and the other center can have the S configuration and vice versa). The tethering attachment point is preferably nitrogen.

In certain embodiments, the carrier may be based on the piperazine ring system (F), e.g., X is N(CO)R\(^7\) or NR\(^7\), Y is NR\(^7\), and Z is CR\(^7\)R\(^{12}\); or the morpholine ring system (G), e.g., X is N(CO)R\(^7\) or NR\(^7\), Y is O, and Z is CR\(^1\)R\(^{12}\). \(\text{OFG}^1\) is preferably attached to a primary carbon, e.g., an exocyclic methylene group (n=1) or ethylene group (n=2) connected to one of C-2, C-3, C-4, or C-5. \((\text{CH}_2)_n\text{OFG}^1\) in H) \(\text{OFG}^2\) is preferably attached directly to one of C-2, C-3, C-4, or C-5 ( \(\text{OFG}^2\) in H), \((\text{CH}_2)_n\text{OFG}^1\) and \(\text{OFG}^2\) may be disposed in a geminal manner on the ring, i.e., both groups may be attached to the same carbon, e.g., at C-2, C-3, C-4, or C-5. Alternatively, \((\text{CH}_2)_n\text{OFG}^1\) and \(\text{OFG}^2\) may be disposed in a vicinal manner on the ring, i.e., both groups may be attached to adjacent ring carbon atoms, e.g., \((\text{CH}_2)_n\text{OFG}^1\) may be attached to C-2 and \(\text{OFG}^2\) may be attached to C-3; \((\text{CH}_2)_n\text{OFG}^1\) may be attached to C-3 and \(\text{OFG}^2\) may be attached to C-2; \((\text{CH}_2)_n\text{OFG}^1\) may be attached to C-3 and \(\text{OFG}^2\) may be attached to C-4; \((\text{CH}_2)_n\text{OFG}^1\) may be attached to C-4 and \(\text{OFG}^2\) may be attached to C-3; \((\text{CH}_2)_n\text{OFG}^1\) may be attached to C-4 and \(\text{OFG}^2\) may be attached to C-5; or \((\text{CH}_2)_n\text{OFG}^1\) may be attached to C-5 and \(\text{OFG}^2\) may be attached to C-4. The decalin or indane-based monomers may therefore contain linkages (e.g., carbon-carbon bonds) wherein bond rotation is restricted about that particular linkage, e.g., restriction resulting from the presence of a ring. Thus, \((\text{CH}_2)_n\text{OFG}^1\) and \(\text{OFG}^2\) may be cis or trans with respect to one another in any of the pairings delineated above. Accordingly, all cis/trans isomers are expressly included. The monomers may also contain one or more asymmetric centers and thus occur as racemates and racemic mixtures, single enantiomers, individual diastereomers and diastereomeric mixtures. All such isomeric forms of the monomers are expressly included (e.g., the centers bearing \(\text{CH}_2\text{OFG}^1\) and \(\text{OFG}^2\) can both have the R configuration; or both have the S configuration; or one center can have the R configuration and the other center can have the S configuration and vice versa). The tethering attachment point is preferably nitrogen in both F and G.
r Racemic mixtures, single enantiomers, individual diastereomers and diastereomeric mixtures. All such isometric forms of the monomers are expressly included (e.g., the centers bearing CH₂OFG¹ and OFG² may both have the R configuration; or both have the S configuration; or one center can have the R configuration and the other center can have the S configuration and vice versa). In a preferred embodiment, the substituents at C-1 and C-6 are trans with respect to one another. The tethering attachment point is preferably C-6 or C-7.

Other carriers may include those based on 3-hydroxyprone (J). Thus, -(CH₂)₃OFG² and OFG² may be cis or trans with respect to one another. Accordingly, all cis/trans isomers are expressly included. The monomers may also contain one or more asymmetric centers.

Thus occur as racemates and racemic mixtures, single enantiomers, individual diastereomers and diastereomeric mixtures. All such isometric forms of the monomers are expressly included (e.g., the centers bearing CH₂OFG¹ and OFG² may both have the R configuration; or both have the S configuration; or one center can have the R configuration and the other center can have the S configuration and vice versa). The tethering attachment point is preferably nitrogen.

Representative cyclic, sugar replacement-based carriers are shown in FIG. 3.

Sugar Replacement-Based Monomers (Acyclic)

Acyclic sugar replacement-based monomers, e.g., sugar replacement-based ligand-conjugated monomers, are also referred to herein as ribose replacement monomer subunit (RRMS) monomer compounds. Preferred acyclic carriers can have formula LCM-3 or LCM-4 below.

In some embodiments, each of x, y, and z can be independently of one another, 0, 1, 2, or 3. In formula LCM-3, when y and z are different, then the tertiary carbon can have either the R or S configuration. In preferred embodiments, x is zero and y and z are each 1 in formula LCM-3 (e.g., based on serinol), and y and z are each 1 in formula LCM-3. Each of formula LCM-3 or LCM-4 below can optionally be substituted, e.g., with hydroxy, alkoxy, perhaloalkyl.
with hydroxy, alkoxy, perhaloalkyl, and/or optionally inserted with one or more additional heteroatoms, e.g., N, O, or S. The double bond can be cis or trans or E or Z.

[0352] In other embodiments the tether may include an electrophilic moiety, preferably at the terminal position of the tether. Preferred electrophilic moieties include, e.g., an aldehyde, alkyl halide, mesylate, tosylate, nosylate, or brosylate, or an activated carboxylic acid ester, e.g., an NHS ester, or a pentfluorophenyl ester. Preferred tethers (underlined) include TAP-(CH₃)₂CHO; TAP-C(O)(CH₃)₂CHO; or TAP-NR'"(CH₃)₂CHO, in which n is 1-6 and R"" is C₁-C₆ alkyl; or TAP-(CH₃)₂C(O)ONHS; TAP-C(O)(CH₃)₂C(O)ONHS; or TAP-NR""(CH₃)₂C(O)ONHS, in which n is 1-6 and R"" is C₁-C₆ alkyl; TAP-(CH₃)₂C(O)OC₂F₅; TAP-C(O)(CH₃)₂C(O)OC₂F₅; or TAP-NR""(CH₃)₂C(O)OC₂F₅, in which n is 1-11 and R"" is C₁-C₆ alkyl; or —(CH₂)n—CH₃—G; TAP-C(O)(CH₃)₂CH₃—G; or TAP-NR""(CH₃)₂CH₃—G, in which n can be as described elsewhere and R"" is C₁-C₆ alkyl (LG can be a leaving group, e.g., halide, mesylate, tosylate, nosylate, brosylate). Tethering can be carried out by coupling a nucleophilic group of a ligand, e.g., a thiol or amino group with an electrophilic group on the tether.

[0353] In other embodiments, it can be desirable for the monomer to include a phthalimido group (K) at the terminal position of the tether.

As discussed above, the ligand or tethered ligand may be present on the ligand-conjugated monomer when the ligand-conjugated monomer is incorporated into the growing strand. In some embodiments, the ligand may be incorporated into a “precursor” ligand-conjugated monomer subunit after a “precursor” ligand-conjugated monomer subunit has been incorporated into the growing strand. For example, a monomer having, e.g., an amino-terminated tether, e.g., TAP-(CH₃)NH₂ may be incorporated into a growing sense or antisense strand. In a subsequent operation, i.e., after incorporation of the precursor monomer subunit into the strand, a ligand having an electrophilic group, e.g., a pentfluorophenyl ester or aldehyde group, can subsequently be attached to the precursor ligand-conjugated monomer by coupling the electrophilic group of the ligand with the terminal nucleophilic group of the precursor ligand-conjugated monomer subunit tether.

[0359] In preferred embodiments, a ligand alters the distribution, targeting or lifetime of an RNA agent into which it is incorporated. In preferred embodiments a ligand provides an enhanced affinity for a selected target, e.g., molecule, cell or cell type, compartment, e.g., a cellular or organ compartment, tissue, organ or region of the body, as, e.g., compared to a species absent such a ligand.

[0360] Preferred ligands can improve transport, hybridization, and specificity properties and may also improve nuclease resistance of the resultant natural or modified oligoribonucleotide, or a polymeric molecule comprising any combination of monomers described herein and/or natural or modified ribonucleotides.

[0361] Ligands in general can include therapeutic modifiers, e.g., for enhancing uptake; diagnostic compounds or reporter groups e.g., for monitoring distribution; crosslinking agents; nuclease-resistance conferring moieties; and natural or unusual nucleobases. General examples include lipophiles, lipids, steroids (e.g., cholesterol, uvaol, becogenin, diosgenin), terpenes (e.g., triterpenes, e.g., sarsasapogenin, Friedelin, epifriedelanol derivatized lithocholic acid), vitamins (e.g., folic acid, vitamin A, vitamin E, biotin, pyridoxal), carbohydrates, proteins, protein binding agents, integrin targeting molecules, polycations, peptides, polyamines, and peptide mimics.

[0362] The ligand can be a peptide or peptidomimetic. A peptidomimetic (also referred to herein as an oligopeptidomimetic) is a molecule capable of folding into a defined three-dimensional structure similar to a natural peptide. The attachment of peptide and peptidomimetics to an RNA agent can affect pharmacokinetic distribution of the RNA, such as by enhancing cellular recognition and absorption. The peptide or peptidomimetic moiety can be about 5-50 amino acids long, e.g., about 5, 10, 15, 20, 25, 30, 35, 40, 45, or 50 amino acids long.

[0354] In other embodiments, other protected amino groups can be at the terminal position of the tether, e.g., alloc, monomethoxy triyl (MNT), trifluoro acetyl, Fmoc, or aryl sulfonyl (e.g., the aryl portion can be ortho-nitrophenyl or ortho, para-dinitrophenyl).

[0355] Any of the tethers described herein may further include one or more additional linking groups, e.g., —O—(CH₂)n—, —(CH₂)n—SS—, —(CH₂)n—, or —(CH=CH) —.

[0356] Tethered Ligands

[0357] A wide variety of entities, e.g., ligands, can be tethered to an RNA agent, e.g., to the carrier of a ligand-conjugated monomer subunit. Examples are described below in the context of a ligand-conjugated monomer subunit that is only preferred, entities can be coupled at other points to an RNA agent.

[0358] Preferred moieties are ligands, which are coupled, preferably covalently, either directly or indirectly via an intervening tether, to the carrier. In preferred embodiments, the ligand is attached to the carrier via an intervening tether.
[0363] In some embodiments, the ligand can be one of the following triterpenes:
In some embodiments, the ligand can be substituted or unsubstituted cholesterol, or a stereoisomer thereof or one of the following steroids:

- Diosgenin
- Heconigen
- Uvulol

iRNA Agent Structure

The monomers described herein can be used to make oligonucleotides which are useful as iRNA agents, e.g., RNA molecules, (double-stranded; single-stranded) that mediate RNAi, e.g., with respect to an endogenous gene of a subject or to a gene of a pathogen. In most cases the iRNA agent will incorporate monomers described herein together with naturally occurring nucleosides or nucleotides or with other modified nucleosides or nucleotides. The modified monomers can be present at any position in the iRNA agent, e.g., at the termini or in the middle region of an iRNA agent or in a duplex region or in an unpaired region. In a preferred embodiment iRNA agent can have any architecture, e.g., architecture described herein. E.g., it can be incorporated into an iRNA agent having an overhang structure, a hairpin or other single strand structure or a two-strand structure, as described herein.

An “RNA agent” as used herein, is an unmodified RNA, modified RNA, or nucleoside surrogate, all of which are defined herein (see, e.g., the section below entitled RNA Agents). While numerous modified RNAs and nucleoside surrogates are described, preferred examples include those which have greater resistance to nuclease degradation than do unmodified RNAs. Preferred examples include those which have a 2' sugar modification, a modification in a single strand overhang, preferably a 3' single strand overhang, or, particularly if single stranded, a 5' modification which includes one or more phosphate groups or one or more analogs of a phosphate group.

An “iRNA agent” as used herein, is an RNA agent which can, or which can be cleaved into an RNA agent which can, down regulate the expression of a target gene, preferably an endogenous or pathogen target RNA. While not wishing to be bound by theory, an iRNA agent may act by one or more of a number of mechanisms, including post-transcriptional cleavage of a target mRNA sometimes referred to in the art as RNAi, or pre-transcriptional or pre-translational mechanisms. An iRNA agent can include a single strand or can include more than one strands, e.g., it can be a double stranded iRNA agent. If the iRNA agent is a single strand it is particularly preferred that it includes a 5' modification which includes one or more phosphate groups or one or more analogs of a phosphate group.

The RRMS-containing iRNA agent should include a region of sufficient homology to the target gene, and be of sufficient length in terms of nucleotides, such that the iRNA agent, or a fragment thereof, can mediate down regulation of the target gene. (For ease of exposition the term nucleotide or ribonucleotide is sometimes used herein in reference to one or more monomeric subunits of an RNA agent. It will be understood herein that the usage of the term “ribonucleotide” or “nucleotide”, herein can, in the case of a modified RNA or nucleotide surrogate, also refer to a modified nucleotide, or surrogate replacement moiety at one or more positions.) Thus, the iRNA agent is or includes a region which is at least partially, and in some embodiments fully, complementary to the target RNA. It is not necessary that there be perfect complementarity between the iRNA agent and the target, but the correspondence must be sufficient to enable the iRNA agent, or a cleavage product thereof, to direct sequence specific silencing, e.g., by RNAi cleavage of the target RNA, e.g., mRNA.

As discussed elsewhere herein, an iRNA agent will often be modified or include nucleoside surrogates in addition to the ribose replacement modification subunit (RRMS). Single stranded regions of an iRNA agent will often be modified or include nucleoside surrogates, e.g., the unpaired region or regions of a hairpin structure, e.g., a region which links two complementary regions, can have modifications or nucleoside surrogates. Modification to stabilize one or more 3'- or 5'-terminus of an RNA agent, e.g., against exonucleases, or to favor the antisense sRNA agent to enter into RISC are also favored. Modifications can include C3 (or C6, C7, C12) amino linkers, thiol linkers, carboxyl linkers, non-nucleotide spacers (C3, C6, C9, C12, abasic, triethylene glycol, hexaethylene glycol), special biotin or fluorescein reagents that come as phosphoramidites and that have another DMT-protected hydroxyl group, allowing multiple couplings during RNA synthesis.

In addition to homology to target RNA and the ability to down regulate a target gene, an iRNA agent will preferably have one or more of the following properties:

- It will be of the Formula 1, 2, 3, or 4 set out in the RNA Agent section below;
- If single stranded it will have a 5' modification which includes one or more phosphate groups or one or more analogs of a phosphate group;
(0374) (3) it will, despite modifications, even to a very large number, or all of the nucleosides, have an antisense strand that can present bases (or modified bases) in the proper three dimensional framework so as to be able to form correct base pairing and form a duplex structure with a homologous target RNA which is sufficient to allow down regulation of the target, e.g., by cleavage of the target RNA;

(0375) (4) it will, despite modifications, even to a very large number, or all of the nucleosides, still have “RNA-like” properties, i.e., it will possess the overall structural, chemical and physical properties of an RNA molecule, even though not exclusively, or even partly, of ribonucleotide-based content. For example, an RNA agent can contain, e.g., a sense and/or an antisense strand in which all of the nucleotide sugars contain, e.g., 2’ fluoros in place of 2’ hydroxyl. This deoxyribonucleotide-containing agent can still be expected to exhibit RNA-like properties. While not wishing to be bound by theory, the electronegative fluoride may serve an axial orientation when attached to the C2’ position of ribose. This spatial preference of fluoride can, in turn, force the sugars to adopt a C3’-endo puck. This is the same puckering mode as observed in RNA molecules and gives rise to the RNA-characteristic A-family-type helix. Further, since fluoride is a good hydrogen bond acceptor, it may participate in the same hydrogen bonding interactions with water molecules that are known to stabilize RNA structures. (Generally, it is preferred that a modified moiety at the 2’ sugar position will be able to enter into H-bonding which is more characteristic of the OH moiety of a ribonucleotide than the H moiety of a deoxyribonucleotide. A preferred RNA agent will: exhibit a C3’-endo puck in all, or at least 50, 75, 80, 85, 90, or 95% of its sugars; exhibit a C2’-endo puck in a sufficient amount of its sugars that it can give rise to a the RNA-characteristic A-family-type helix; will have no more than 20, 10, 5, 4, 3, 2, or 1 sugar which is not a C2’-endo puck structure. These limitations are particularly preferably in the antisense strand;

(0376) (4) regardless of the nature of the modification, and even though the RNA agent can contain deoxynucleotides or modified deoxynucleotides, particularly in overhang or other single strand regions, it is preferred that DNA molecules, or any molecule in which more than 50, 60, or 70% of the nucleotides in the molecule, or more than 50, 60, or 70% of the nucleotides in a duplexed region are deoxyribonucleotides, or modified deoxyribonucleotides which are deoxy at the 2’ position, are excluded from the definition of RNA agent.

(0377) A “single strand RNA agent” as used herein, is an RNA agent which is made up of a single molecule. It may include a duplexed region, formed by intra-strand pairing, e.g., it may be, or include, a hairpin or pan-handle structure. Single strand RNA agents are preferably antisense with regard to the target molecule. In preferred embodiments single strand RNA agents are 5’ phosphorylated or include a phosphoryl analog at the 5’ prime terminus. 5’-phosphate modifications include those which are compatible with RISC mediated gene silencing. Suitable modifications include: 5’-monophosphate ([(OH)2(O)P-O-5’); 5’-diphosphate ([(OH)2(O)P-O-P(OH)(O)-O-5’); 5’-triphosphate ([(OH)2(O)P-O-P(OH)(O)-O-5’); 5’-guanosine cap (7-methylated or non-methylated) (7m-G-O-5’(HO)(O)P-O—(HO)(O)P—O—P(HO)(O)—O-5’); 5’-adenosine cap (Appp), and any modified or unmodified nucleotide cap structure (N—O—[HO](O)P—O—(HO)(O)P—O—P(HO)(O)—O-5’); 5’-monothiophosphate (phosphorothionate, (HO)(OH)(S)P-O-P(HO)(O)—O-5’); 5’-monothiophosphate (phosphorothionate, (HO)(OH)(HS)P-O-P(HO)(O)—O-5’); 5’-phosphorothiolate ([(HO)2(O)P-O-S-5’); any additional combination of oxygen/sulfur replaced monophosphate, diphosphate and triphosphates (e.g. 5’-alpha-thiotriphosphate, 5’-gamma-thiotriphosphate, etc.), 5’-phosphoramidates ((HO)2(O)P—NH-O—, (HO)(NH)2(O)P—O—5’), 5’-alkylphosphonates (R=alkyl=methyl, ethyl, isopropyl, propyl, etc., e.g. RP(OH)(O)—O—5’, (OH)2(O)P—5’—CH—), 5’-alkylether-phosphorothiates (R=alkylether=methoxymethyl, (MeOCH2—), ethoxymethyl, etc., e.g. RP(OH)(O)—O—5’). (These modifications can also be used with the antisense strand of a double stranded RNA.)

(0378) It may be desirable to modify one or both of the antisense and sense strands of a double strand RNA agent. In some cases they will have the same modification or the same class of modification but in other cases the sense and antisense strand will have different modifications, e.g., in some cases it is desirable to modify only the sense strand. It may be desirable to modify only the sense strand, e.g., to activate it, e.g., the sense strand can be modified in order to inactivate the sense strand and prevent formation of an active sRNA:protein or RISC. This can be accomplished by a modification which prevents 5’-phosphorylation of the sense strand, e.g., by modification with a 5’-O-methyl ribonucleotide (see Nykanen et al., 2001 ATP requirements and small interfering RNA structure in the RNA interference pathway. Cell 107, 309-321.) Other modifications which prevent phosphorylation can also be used, e.g., simply substituting the 5’OH by H rather than O-Me. Alternatively, a large bulky group may be added to the 5-phosphate turning it into a phosphodiester linkage, though this may be less desirable as phosphorylases can cleave such a linkage and release a functional sRNA 5’-end. Antisense strand modifications include 5’ phosphorylation as well as any of the other 5’ modifications discussed herein, particularly the 5’ modifications discussed above in the section on single stranded sRNA molecules.

(0379) It is preferred that the sense and antisense strands be chosen such that the ds sRNA agent includes a single strand or unpaired region at one or both ends of the molecule. Thus, a ds sRNA agent contains sense and antisense strands, preferable paired to contain an overhang, e.g., one or two 5’ or 3’ overhangs but preferably a 3’ overhang of 2-3 nucleotides. Most embodiments will have a 3’ overhang. Preferred sRNA agents will have single-stranded overhangs, preferably 3’ overhangs, of 1 or preferably 2 or 3 nucleotides in length at each end. The overhangs can be the result of one strand being longer than the other, or the result of two strands of the same length being staggered. 5’ ends are preferably phosphorylated.

(0380) Preferred lengths for the duplexed region is between 15 and 30, most preferably 18, 19, 20, 21, 22, and 23 nucleotides in length, e.g., in the sRNA agent range discussed above. sRNA agents can resemble in length and structure the natural Dicer processed products from long dsRNAs. Embodiments in which the two strands of the sRNA agent are linked, e.g., covalently linked are also included. Hairpin, or other single strand structures which provide the required double stranded region, and preferably a 3’ overhang are also within the invention.
As nucleic acids are polymers of subunits or monomers, many of the modifications described below occur at a position which is repeated within a nucleic acid, e.g., a modification of a base, or a phosphate moiety, or the a non-linking O of a phosphate moiety. In some cases the modification will occur at all of the subject positions in the nucleic acid but in many, and in fact in most cases it will not. By way of example, a modification may only occur at a 3’ or 5’ terminal position, may only occur in a terminal regions, e.g., at a position on a terminal nucleotide or in the last 2, 3, 4, 5, or 10 nucleotides of a strand. A modification may occur in a double strand region, a single strand region, or in both. A modification may occur only in the double strand region of an RNA or may only occur in a single strand region of an RNA. E.g., a phosphorothioate modification at a non-linking O position may only occur at one or both termini, may only occur in a terminal regions, e.g., at a position on a terminal nucleotide or in the last 2, 3, 4, 5, or 10 nucleotides of a strand, or may occur in double strand and single strand regions, particularly at termini. The 5’ end or ends can be phosphorylated.

In some embodiments it is particularly preferred, e.g., to enhance stability, to include particular bases in overhangs, or to include modified nucleotides or nucleotide surrogates, in single strand overhangs, e.g., in a 5’ or 3’ overhang, or in both. E.g., it can be desirable to include purine nucleotides in overhangs. In some embodiments all or some of the bases in a 3’ or 5’ overhang will be modified, e.g., with a modification described herein. Modifications can include, e.g., the use of modifications at the 2’ OH group of the ribose sugar, e.g., the use of deoxyribonucleotides, e.g., deoxythymidine, instead of ribonucleotides, and modifications in the phosphate group, e.g., phosphothioate modifications. Overhangs need not be homologous with the target sequence.

Modifications and nucleotide surrogates are discussed below.

The scaffold presented above in Formula 1 represents a portion of a ribonucleic acid. The basic components are the ribose sugar, the base, the terminal phosphates, and phosphate internucleotide linkers. Where the bases are naturally occurring bases, e.g., adenine, uracil, guanine or cytosine, the sugars are the unmodified 2’ hydroxyl ribose sugar (as depicted) and W, X, Y, and Z are all O, Formula 1 represents a naturally occurring unmodified oligoribonucleotide.

Unmodified oligoribonucleotides may be less than optimal in some applications, e.g., unmodified oligoribonucleotides can be prone to degradation by e.g., cellular nucleases. Nucleases can hydrolyze nucleic acid phosphodiester bonds. However, chemical modifications to one or more of the above RNA components can confer improved properties, and, e.g., can render oligoribonucleotides more stable to nucleases. Unmodified oligoribonucleotides may also be less than optimal in terms of offering tethering points for attaching ligands or other moieties to an RNA agent.

Modified nucleic acids and nucleotide surrogates can include one or more of:

(i) alteration, e.g., replacement, of one or both of the non-linking (X and Y) phosphate oxygens and/or one or more of the linking (W and Z) phosphate oxygens (When the phosphate is in the terminal position, one of the positions W or Z will not link the phosphate to an additional element in a naturally occurring ribonucleic acid. However, for simplicity of terminology, except where otherwise noted, the W position at the 5’ end of a nucleic acid and the terminal Z position at the 3’ end of a nucleic acid, are within the term “linking phosphate oxygens” as used herein);

(ii) alteration, e.g., replacement, of a constituent of the ribose sugar, e.g., of the 2’ hydroxyl on the ribose sugar, or wholesale replacement of the ribose sugar with a structure other than ribose, e.g., as described herein;

(iii) wholesale replacement of the phosphate moiety (bracket I) with “dephospho” linkers;

(iv) modification or replacement of a naturally occurring base;

(v) replacement or modification of the ribose-phosphate backbone (bracket II);

(vi) modification of the 3’ end or 5’ end of the RNA, e.g., removal, modification or replacement of a terminal phosphate group or conjugation of a moiety, e.g. a fluorescently labeled moiety, to either the 3’ or 5’ end of RNA.

The terms replacement, modification, alteration, and the like, as used in this context, do not imply any process limitation, e.g., modification does not mean that one must start with a reference or naturally occurring ribonucleic acid and modify it to produce a modified ribonucleic acid but rather modified simply indicates a difference from a naturally occurring molecule.

It is understood that the actual electronic structure of some chemical entities cannot be adequately represented by only one canonical form (i.e. Lewis structure). While not wishing to be bound by theory, the actual structure can instead be some hybrid or weighted average of two or more canonical forms, known collectively as resonance forms or structures. Resonance structures are not discrete chemical entities and exist only on paper. They differ from one another only in the placement of or “localization” of the bonding and nonbonding electrons for a particular chemical entity. It can
be possible for one resonance structure to contribute to a greater extent to the hybrid than the others. Thus, the written and graphical descriptions of the embodiments of the present invention are made in terms of what the art recognizes as the predominant resonance form for a particular species. For example, any phosphoramidate (replacement of a nonlinking oxygen with nitrogen) would be represented by X=O and Y=N in the above figure.

[0395]  Specific modifications are discussed in more detail below.

[0396]  The Phosphate Group

[0397]  The phosphate group is a negatively charged species. The charge is distributed equally over the two nonlinking oxygen atoms (i.e., X and Y in formula I above). However, the phosphate group can be modified by replacing one of the oxygens with a different substituent. One result of this modification to RNA phosphate backbones can be increased resistance of the oligoribonucleotide to nucleolytic breakdown. Thus while not wishing to be bound by theory, it can be desirable in some embodiments to introduce alterations which result in either an uncharged linker or a charged linker with unsymmetrical charge distribution.

[0398]  Examples of modified phosphate groups include phosphorothioate, phosphoro- selenoates, boronate phosphates, boronate phosphate esters, hydrogen phosphonates, phosphoroamidates, alkyl or aryl phosphonates and phosphoristeres. Phosphorothioates have both non-linking oxygens replaced by sulfur. Unlike the situation where only one of X or Y is altered, the phosphorus center in the phosphorothioates is achiral which precludes the formation of oligoribonucleotides diastereomers. Diastereomer formation can result in a preparation in which the individual diastereomers exhibit varying resistance to nucleases. Further, the hybridization affinity of RNA containing chiral phosphate groups can be lower relative to the corresponding unmodified RNA species. Thus, while not wishing to be bound by theory, modifications to both X and Y which eliminate the chiral center, e.g., phosphorothioate formation, may be desirable in that they cannot produce diastereomer mixtures. Thus, X can be any one of S, Se, B, C, H, N, or OR (R is alkyl or aryl). Thus Y can be any one of S, Se, B, C, H, N, or OR (R is alkyl or aryl). Replacement of X and/or Y with sulfur is preferred.

[0399]  The phosphate linker can also be modified by replacement of a linking oxygen (i.e., W or Z in formula I) with nitrogen (bridged phosphoramidates), sulfur (bridged phosphorothioates) and carbon (bridged methylene phosphonates). The replacement can occur at a terminal oxygen position (W (3) or position Z (5)). Replacement of W with carbon or Z with nitrogen is preferred.

[0400]  Candidate agents can be evaluated for suitability as described below.

[0401]  The Sugar Group

[0402]  A modified RNA can include modification of all or some of the sugar groups of the ribonucleic acid. E.g., the 2'-hydroxyl group (OH) can be modified or replaced with a number of different “oxy” or “deoxy” substituents. While not being bound by theory, enhanced stability is expected since the hydroxyl can no longer be deprotonated to form a 2'-alkoxide ion. The 2'-alkoxide can catalyze degradation by intramolecular nucleophilic attack on the linker phosphorus atom. Again, while not wishing to be bound by theory, it can be desirable to some embodiments to introduce alterations in which alkoxide formation at the 2' position is not possible.

[0403]  Examples of “oxy”-2' hydroxyl group modifications include alkoxyl or aryloxyl (OR, e.g., R=H, alkyl, cycloalkyl, aryl, aralkyl, heteroaryl or sugar); polyethyleneglycols (PEG), O(CH₂CH₂O)₉CH₂CH₂OR; “locked” nucleic acids (LNA) in which the 2' hydroxyl is connected, e.g., by a methylene bridge, to the 4' carbon of the same ribose sugar; O-AMINE (AMINE=NH₂; alkylamino, dialkylamino, heterocyclic, aryldiamino, diaryl amino, heteroaryl amino, or diheteroaryl amino, ethylene diamine, polyamino) and aminoalcohols, O(CH₂)₉AMINE, (e.g., AMINE=NH₂; alkylamino, dialkylamino, heterocyclic, arylamino, diaryl amino, heteroaryl amino, or diheteroaryl amino, ethylene diamine, polyamino). It is noteworthy that oligonucleotides containing only the methoxethyl group (MOE), O(CH₂CH₂OCH₃, a PEG derivative), exhibit nuclease stabilities comparable to those modified with the robust phosphorothioate modification.

[0404]  “Deoxy” modifications include hydrogen (i.e. deoxyribose sugars, which are of particular relevance to the overhang portions of partially ds RNA), halo (e.g., fluoro); amino (e.g. NH₂; alkylamino, dialkylamino, heterocyclic, aryldiamino, diaryl amino, heteroaryl amino, or diheteroaryl amino, amido, or amino acid); NH(CH₂CH₂NH₂)CH₂CH₂AMINE (AMINE=NH₂; alkylamino, dialkylamino, heterocyclic, aryldiamino, diaryl amino, heteroaryl amino, or diheteroaryl amino) —NH(R(OR=alkyl, cycloalkyl, aryl, aralkyl, heteroaryl or sugar), cyano; mercapto; alkyl-thio-alkyl; thio-alkoxy; and alkyl, cycloalkyl, aryl, alkenyl and alkynyl, which may be optionally substituted with e.g., an amino functionality. Preferred substituents are 2'-methoxethyl, 2'-OCH₃; 2'-O-alkyl, 2'-O-C— alkyl, and 2'-fluoro.

[0405]  The sugar group can also contain one or more carbons that possess the opposite stereochemical configuration than that of the corresponding carbon in ribose. Thus, modified RNA can include nucleotides containing, e.g., arabinose, as the sugar.

[0406]  Modified RNAs can also include “abasic” sugars, which lack a nucleobase at C-1'. These abasic sugars can also be further contain modifications at one or more of the constituent sugar atoms.

[0407]  To maximize nuclease resistance, the 2' modifications can be used in combination with one or more phosphate linker modifications (e.g., phosphorothioate). The so-called “chimeric” oligonucleotides are those that contain two or more different modifications.

[0408]  The modification can also entail the wholesale replacement of a ribose structure with another entity at one or more sites in the RNA agent.

[0409]  Candidate modifications can be evaluated as described below.

[0410]  Replacement of the Phosphate Group

[0411]  The phosphate group can be replaced by nonphosphorus containing connectors (cf. Bracket I in formula I above). While not wishing to be bound by theory, it is believed that since the charged phosphodiester group is the reaction center in nucleolytic degradation, its replacement
with neutral structural mimics should impart enhanced nuclease stability. Again, while not wishing to be bound by theory, it may be desirable to introduce alterations that improve nuclease resistance. Other examples of terminal modifications include dyes, intercalating agents (e.g., acridines), cross-linkers (e.g., psoralene, mitomycin C), porphyrins (TPPC4, tetrathiaporphyrin, Sapphyrin), polycyclic aromatic hydrocarbons (e.g., phenazine, dihydrophenazine), artificial endonucleases (e.g., EDTA), lipophilic carriers (e.g., cholesterol, cholic acid, adamantane acetic acid, 1-pyrene butyric acid, dihydrotestosterone, 1,3-Bis(hexadecl)glycerol, geranylgeranyl pyrophosphate, borneol, menthol, 1,3-propanediol, heptadecyl glycerol, palmic acid, myristic acid, O3-(alcohol)thiophthalic acid, O3-(alcohol)thiophthalic acid, dimethoxytrityl, or phenoxazine) and peptide conjugates (e.g., antennapedia peptide, Tat peptide), alkylating agents, phosphate, amino, mercapto, PEG (e.g., PEG-40K), MPEG, polyamine, alkyl, substituted alkyl, radiolabeled markers, enzymes, hapten (e.g., biotin), transport/absorption facilitators (e.g., aspirin, vitamin E, folic acid), synthetic ribonucleases (e.g.,imidazole, bisimidazole, histamine, imidazole clusters, acridine-imidazole conjugates, Eu3+ complexes of tetraaza macrocycles).

[0412] Examples of moieties which can replace the phosphate group include siloxane, carbonate, carboxymethyl, carbamate, amide, thioether, ethylene oxide linker, sulfonate, sulfonamide, thioformaldehyde, formicaldehyde, oxime, methyleneimino, methylenemethylimino, methylidencyrazo, methylenedimethylidrazo and methylenecycloheximino. Preferred replacements include the methylenecarbonylalamin and methylenemethylimino groups.

[0413] Candidate modifications can be evaluated as described below.

[0414] Replacement of Ribophosphate Backbone

[0415] Oligonucleotide-mimicking scaffolds can also be constructed wherein the phosphate linker and ribose sugar are replaced by nuclease resistant nucleoside or nucleotide surrogates (see Bracket II of Formula 1 above). While not wishing to be bound by theory, it is believed that the absence of a repetitively charged backbone diminishes binding to proteins that recognize polynucleotides (e.g., nuclease). Again, while not wishing to be bound by theory, it may be desirable to introduce alterations in which the bases are tethered by a neutral surrogate backbone.

[0416] Examples include the mophilino, cyclobutyl, pyrrolidine and peptide nucleic acid (PNA) nucleoside surrogates. A preferred surrogate is a PNA surrogate.

[0417] Candidate modifications can be evaluated as described below.

[0418] Terminal Modifications

[0419] The 3' and 5' ends of an oligonucleotide can be modified. Such modifications can be at the 3' end, 5' end or both ends of the molecule. They can include modification or replacement of an entire terminal phosphate or of one or more of the atoms of the phosphate group. E.g., the 3' and 5' ends of an oligonucleotide can be conjugated to other functional molecular entities such as labeling moieties, e.g., fluorophores (e.g., pyrene, TAMRA, fluorescein, Cy3 or Cy5 dyes) or protecting groups (based e.g., on sulfur, silicon, boron or ester). The functional molecular entities can be attached to the sugar through a phosphate group and/or a spacer. The terminal atom of the spacer can connect to or replace the linking atom of the phosphate group or the C-3' or C-5' O, N, S or C group of the sugar. Alternatively, the spacer can connect to or replace the terminal atom of a nucleotide surrogate (e.g., PNA). These spacers or linkers can include e.g., -(CH$_2$)$_n$-, -(CH$_2$)$_n$N-, -(CH$_2$)$_n$O-, -(CH$_2$)$_n$S-, O(CH$_2$CH$_2$)$_n$CH$_2$OH (e.g., $n=3$ or 6), abasic sugars, amide, carboxy, amine, oxime, oximine, thioether, disulfide, thiourea, sulfonamide, or morpholino, or biotin and fluorescein reagents. When a spacer/phosphate-functional molecular entity-spacer/phosphate array is interconnected between two strands of RNA, this array can substitute for a hairpin RNA loop in a hairpin-type RNA agent. The 3' end can be an -OH group. While not wishing to be bound by theory, it is believed that conjugation of certain moieties can improve transport, hybridization, and specificity properties. Again, while not wishing to be bound by theory, it may be desirable to introduce terminal alterations that improve nuclease resistance. Other examples of terminal modifications include dyes, intercalating agents (e.g., acridines), cross-linkers (e.g., psoralene, mitomycin C), porphyrins (TPPC4, tetrathiaporphyrin, Sapphyrin), polycyclic aromatic hydrocarbons (e.g., phenazine, dihydrophenazine), artificial endonucleases (e.g., EDTA), lipophilic carriers (e.g., cholesterol, cholic acid, adamantane acetic acid, 1-pyrene butyric acid, dihydrotestosterone, 1,3-Bis(hexadecl)glycerol, geranylgeranyl pyrophosphate, borneol, menthol, 1,3-propanediol, heptadecyl glycerol, palmic acid, myristic acid, O3-(alcohol)thiophthalic acid, O3-(alcohol)thiophthalic acid, dimethoxytrityl, or phenoxazine) and peptide conjugates (e.g., antennapedia peptide, Tat peptide), alkylating agents, phosphate, amino, mercapto, PEG (e.g., PEG-40K), MPEG, polyamine, alkyl, substituted alkyl, radiolabeled markers, enzymes, hapten (e.g., biotin), transport/absorption facilitators (e.g., aspirin, vitamin E, folic acid), synthetic ribonucleases (e.g., imidazole, bisimidazole, histamine, imidazole clusters, acridine-imidazole conjugates, Eu3+ complexes of tetraaza macrocycles).

[0420] Terminal modifications can be added for a number of reasons, including as discussed elsewhere herein to modulate activity or to modulate resistance to degradation. Terminal modifications useful for modulating activity include modification of the 5' end with phosphate or phosphate analogs. E.g., in preferred embodiments IRNA agents, especially antisense strands, are 5' phosphorylated or include a phosphoryl analog at the 5' prime terminus. 5'-phosphate modifications include those which are compatible with RISC mediated gene silencing. Suitable modifications include: 5'-monophosphate ([(H)]O(2)O(5)&mdash;O(5)); 5'-diphosphate ([(H)]O(2)O(5)&mdash;O(5)&mdash;P(H)O(5)&mdash;O(5)); 5'-triphosphate ([(H)]O(2)O(5)&mdash;O(5)&mdash;P(H)O(5)&mdash;O(5)&mdash;P(H)O(5)&mdash;O(5)); 5'-guanosine cap (7-methylated or non-methylated) (7&mdash;G-O(5)&mdash;[(H)]O(2)O(5)&mdash;O(5)&mdash;P(H)O(5)&mdash;O(5)); 5'-adenosine cap (Acpp), and any modified or unmethylated nucleotide cap structure (N&mdash;[(H)]O(2)O(5)&mdash;P(H)O(5)&mdash;O(5)&mdash;P(H)O(5)&mdash;O(5)); 5'-monothiophosphate (phosphorothioate; [(H)]O(2)S(5)&mdash;O(5)); 5'-monothiophosphate (phosphorothioate; [(H)]O(2)S(5)&mdash;O(5)); 5'-monothiophosphate ([(H)]O(2)O(5)&mdash;S&mdash;); any additional combination of oxygen/sulfur replaced monophosphate, diphosphate and triphosphates (e.g. 5'-alpha-thiophosphite, 5'-gamma-thiophosphite, etc.), 5'-phosphoramidates ([(H)]O(2)O(5)&mdash;N&mdash;H; [(H)]O(2)O(5)&mdash;N&mdash;H; 5'-alkylphosphonates (R=alkyl)methyl, ethyl, isopropyl, propyl, etc., e.g., RP(OH)(O)&mdash;O(5) &mdash; (O)(2)P(5)&mdash;CH$_2$&mdash;); 5'-alkylethyl phosphonates (R=alkylether-methoxymethyl (MeOCH$_2$&mdash;), ethoxymethyl, etc., e.g., RP(OH)(O)&mdash;O(5) &mdash;).

[0421] Terminal modifications can also be useful for monitoring distribution, and in such cases the preferred groups to be added include fluorophores, e.g., fluorescein or an Alexa dye, e.g., Alexa 488. Terminal modifications can also be useful for enhancing uptake, useful modifications for this include cholesterol. Terminal modifications can also be useful for cross-linking an RNA agent to another moiety; modifications useful for this include mitomycin C.

[0422] Candidate modifications can be evaluated as described below.

[0423] The Bases

[0424] Adenine, guanine, cytosine and uracil are the most common bases found in RNA. These bases can be modified
or replaced to provide RNA's having improved properties. E.g., nuclease resistant oligoribonucleotides can be prepared with these bases or with synthetic and natural nucleobases (e.g., inosine, thymine, xanthine, hypoxanthine, nodularine, isoguanosine, or tubercidine) and any one of the above modifications. Alternatively, substituted or modified analogs of any of the above bases, e.g., “usual bases” and “universal bases" described herein, can be employed. Examples include without limitation 2-aminoadenine, 6-methyl and other alkyl derivatives of adenine and guanine, 2-propyl and other alkyl derivatives of adenine and guanine, 5-haloaracil and cytosine, 5-propynyl uracil and cytosine, 6-azo uracil, cytosine and thymine, 5-uracil (pseudouracil), 4-thiouracil, 5-haloaracil, 5-(2-aminopropyl)uracil, 5-amino allyl uracil, 8-halo, amino, thio, thioalkyl, hydroxyl and other 8-substituted adenines and guanines, 5-trifluoromethyl and other 5-substituted uracils and cytosines, 7-methylguanine, 5-substituted pyrimidines, 6-azapyrimidines and N-2, N-6 and O-6 substituted purines, including 2-aminopropyladenine, propynyluracil and 5-propynylcytosine, dihydrouracil, 3-deaza-5-azacytosine, 2-aminopurine, 5-alkyluracil, 7-alkylguanine, 5-alkyl cytosine, 7-deazaadenine, N6, N6-dimethyladenine, 2,6-diaminopurine, 5-amino allyl uracil, N3-methyluracil, substituted 1,2,4-triazoles, 2-pyridine, 5-nitrodinole, 5-nitropyrrrole, 5-methoxyuracil, uracil-5-oxoacetylic acid, 5-methoxycarbonylmethyluracil, 5-methyl-2-thiouracil, 5-methoxycarbonylmethyl-2-thiouracil, 3-(3-amino-3caryboxypropynyl)uracil, 3-methylcytosine, 5-methylcytosine, N' acetyl cytosine, 2-thiocytosine, N6-methyladenine, N6-isopentyladenine, 2-methylthio-N6-isopentenyladenine, N6-methylguainines, or O-alkylated bases. Further purines and pyrimidines include those disclosed in U.S. Pat. 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 Engelsch et al., Angewandte Chemie, Internationale Edition, 1991, 30, 613.

[0425] Generally, base changes are less preferred for promoting stability, but they can be useful for other reasons, e.g., some, e.g., 2,6-diaminopurine and 2 amino purine, are fluorescent. Modified bases can reduce target specificity. This should be taken into consideration in the design of RNA agents.

[0426] Candidate modifications can be evaluated as described below.

[0427] Evaluation of Candidate RNA's

[0428] One can evaluate a candidate RNA agent, e.g., a modified RNA, for a selected property by exposing the agent or modified molecule and a control molecule to the appropriate conditions and evaluating for the presence of the selected property. For example, resistance to a degrader can be evaluated as follows. A candidate modified RNA (and preferably a control molecule, usually the unmodified version) can be exposed to degradative conditions, e.g., exposed to a milieu, which includes a degradative agent, e.g., a nuclease. E.g., one can use a biological sample, e.g., one that is similar to a milieu, which might be encountered, in therapeutic use, e.g., blood or a cellular fraction, e.g., a cell-free homogenate or disrupted cells. The candidate and control could then be evaluated for resistance to degradation by any of a number of approaches. For example, the candidate and control could be labeled, preferably prior to exposure, with, e.g., a radioactive or enzymatic label, or a fluorescent label, such as Cy3 or Cy5. Control and modified RNA's can be incubated with the degradative agent, and optionally a control, e.g., an inactivated, e.g., heat inactivated, degradative agent. A physical parameter, e.g., size, of the modified and control molecules are then determined. They can be determined by a physical method, e.g., by polyacrylamide gel electrophoresis or a sizing column, to assess whether the molecule has maintained its original length, or assessed functionally. Alternatively, Northern blot analysis can be used to assay the length of an unlabeled modified molecule.

[0429] A functional assay can also be used to evaluate the candidate agent. A functional assay can be applied initially or after an earlier non-functional assay, e.g., assay for resistance to degradation) to determine if the modification alters the ability of the molecule to silence gene expression. For example, a cell, e.g., a mammalian cell, such as a mouse or human cell, can be co-transfected with a plasmid expressing a fluorescent protein, e.g., GFP, and a candidate RNA agent homologous to the transcript encoding the fluorescent protein (see, e.g., WO 00/44914). For example, a modified dsRNA homologous to the GFP mRNA can be assayed for the ability to inhibit GFP expression by monitoring for a decrease in cell fluorescence, as compared to a control cell, and in which the transfection did not include the candidate dsRNA, e.g., controls with no agent added and/or controls with a non-modified RNA added. Efficacy of the candidate agent on gene expression can be assessed by comparing cell fluorescence in the presence of the modified and unmodified dsRNA agents.

[0430] In an alternative functional assay, a candidate dsRNA agent homologous to an endogenous mouse gene, preferably a maternal expressed gene, such as c-mos, can be injected into an immature mouse oocyte to assess the ability of the agent to inhibit gene expression in vivo (see, e.g., WO 01/36646). A phenotype of the oocyte, e.g., the ability to maintain arrest in metaphase II, can be monitored as an indicator that the agent is inhibiting expression. For example, cleavage of c-mos mRNA by a dsRNA agent would cause the oocyte to exit metaphase arrest and initiate parthenogenetic development (Colledge et al. Nature 370: 65-68, 1994; Hashimoto et al. Nature, 370:68-71, 1994). The effect of the modified agent on target RNA levels can be verified by Northern blot to assay for a decrease in the level of target mRNA, or by Western blot to assay for a decrease in the level of target protein, as compared to a negative control. Controls can include cells in which no agent is added and/or cells in which a non-modified RNA is added.

[0431] References

[0432] General References

[0433] The oligoribonucleotides and oligoribonucleosides used in accordance with this invention may be with solid phase synthesis, see for example “Oligonucleotide synthesis, a practical approach”, Ed. M. J. Gait, IRL Press, 1984; “Oligonucleotides and Analogues, A Practical Approach”, Ed. F. Eckstein, IRL Press, 1991 (especially Chapter 1, Modern machine-aided methods of oligodeoxynucleotide synthesis, Chapter 2, Oligoribonucleotide synthesis, Chapter 3, 3'-O-Methyloligonucleotide synthesis and applications, Chapter 4, Phosphorothioate oligonucleotides, Chapter 5, Synthesis of oligonucleotide phospho-

[0434] Modification described in WO 00/44895, WO01/75164, or WO02/44321 can be used herein.

[0435] The disclosure of all publications, patents, and published patent applications listed herein are hereby incorporated by reference.

[0436] Phosphate Group References


[0438] Sugar Group References


[0440] Replacement of the Phosphate Group References

[0441] Methyleneethylmimid linked oligoribonucleosides, also identified herein as MMI linked oligoribonucleosides, methyleneethylhydrazo linked oligonucleosides, also identified herein as MDH linked oligoribonucleosides, and methylene carbamoyl amino linked oligonucleosides, also identified herein as amide-3 linked oligoribonucleosides, and methylene carbamoyl linked oligonucleosides, also identified herein as amide-4 linked oligoribonucleosides as well as mixed backbone compounds having, as for instance, alternating MMI and PO or PS linkages can be prepared as is described in U.S. Pat. Nos. 5,378,825, 5,386,023, 5,489,677 and in published PCT applications PCT/US92/04294 and PCT/US92/04305 (published as WO 92/20822 WO and 92/20823, respectively). Formacetal and thioformacetal linked oligoribonucleosides can be prepared as is described in U.S. Pat. Nos. 5,264,562 and 5,264,564. Ethylene oxide linked oligoribonucleosides can be prepared as is described in U.S. Pat. No. 5,223,618. Siloxane replacements are described in Cormier, J. F. et al. Nucleic Acids Res. 1988, 16, 4583. Carbonate replacements are described in Tittensor, J. R. J. Chem. Soc. C 1971, 1933. Carboxymethyl replacements are described in Edge, M. D. et al. J. Chem. Soc. Perkin Trans. 1972, 1991. Carbamate replacements are described in Stirchak, E. P. Nucleic Acids Res. 1989, 17, 6129.

[0442] Replacement of the Phosphate-Ribose Backbone Reference

[0443] Cyclobutyl sugar surrogate compounds can be prepared as is described in U.S. Pat. No. 5,359,044. Pyrrolidine sugar surrogate can be prepared as is described in U.S. Pat. No. 5,519,134. Morpholine sugar surrogates can be prepared as is described in U.S. Pat. Nos. 5,142,047 and 5,235,033, and other related patent disclosures. Peptide Nucleic Acids (PNAs) are known per se and can be prepared in accordance with any of the various procedures referred to in Peptide Nucleic Acids (PNA): Synthesis, Properties and Potential Applications, Bioorganic & Medicinal Chemistry, 1996, 4, 5-23. They may also be prepared in accordance with U.S. Pat. No. 5,539,083.

[0444] Terminal Modification References


[0446] Bases References

[0447] N-2 substituted pyrimidine nucleoside amides can be prepared as is described in U.S. Pat. No. 5,459,255. 3-Deaza pyrimidine nucleoside amides can be prepared as is described in U.S. Pat. No. 5,457,191. 5,6-Substituted pyrimidine nucleoside amides can be prepared as is described in U.S. Pat. No. 5,614,617. 5-Propynyl pyrimidine nucleoside amides can be prepared as is described in U.S. Pat. No. 5,484,908. Additional references can be disclosed in the above section on base modifications.

[0448] Preferred iRNA Agents

Preferred RNA agents have the following structure (see Formula 2 below):
Referring to Formula 2 above, \( R', R, \) and \( R^3 \) are each, independently, \( H, \) (i.e. abasic nucleotides), adenine, guanine, cytosine and uracil, inosine, thymine, xanthine, hypoxanthine, isobuturidine, isosuganisine, 2-amino-adenine, 6-methyl and other alkyl derivatives of adenine and guanine, 2-propyl and other alkyl derivatives of adenine and guanine, 6-haloaracil and cytosine, 5-propynyl uracil and cytosine, 6-azo uracil, cytosine and thymine, 5-uracil (pseudouracil), 4-thiouracil, 5-haloaracil, 5-(2-amino-propyl)uracil, 5-amino allyl uracil, 8-halo, amino, thiol, thioalkyl, hydroxyl and other 8-substituted adenines and guanines, 5-trifluoromethyl and other 5-substituted uracils and cytosines, 7-methylguanine, 5-substituted pyrimidines, 6-aza-pyrimidines and N-2, N-6 and O-6 substituted purines, including 2-amino-4-propyladenine, 5-propynyluracil and 5-propynylcytosine, dihydroaracil, 3-deaza-5-azacytosine, 2-aminopurine, 5-alkylcytosine, 7-alkylguanine, 5-alkylcytosine, 7-deaza-cytosine, N6, N6-dimethyladenine, 2,6-diaminopurine, 5-alkyllyruracil, N3-methyluracil, substituted 1,2,4-triazole, 2-pyridinone, 5-nitroindole, 3-nitropyrole, 5-methoxyuracil, uracil-5-oxoacetic acid, 5-methoxycarbonylmethyluracil, 5-methyl-2-thiouracil, 5-methoxy-2-carboxymethyl-2-thiouracil, 3-(3-amino-3-carboxypropyl)uracil, 3-methylcytosine, 5-methylcytosine, N4-acetyl cytosine, 2-thiocytosine, N6-methyladenine, N6-isopentylcytosine, 2-methylthio-N-6-isopentyladenine, N-methylguanines, or O-alkylated bases.

\[ R^1, R^2, \text{and } R^3 \text{ are each, independently, } H, \text{ OR, } O(CH_2CH_2O)_nCH_2CH_2OR, O(CH_2)_nR, O(CH_2)_nOR, H; \text{ halo; NH}_3; \text{ NHR}^6; \text{ NH(CH_2CH_NH)_nCH_2CH_NHR}^6; \text{ NH(O)R}^8; \text{ cyano; mercapto, SR}^2; \text{ alkyl-thio-alkyl; alkyl, aralkyl, cycloalkyl, aroyl, heteroaryl, alkenyl, alkynyl, each of which may be optionally substituted with halo, hydroxy, oxo, nitro, haloalkyl, alkyl, aryl, alkyl, alkoxy, arlyoxy, amino, alkylamino, dialkylamino, heterocyclyl, arylamino, diaryl amino, heteroaryl amino, diheteroaryl amino, acylamino, alky carbamoyl, ary carbamoyl, aminocarboxyl, alkoxy carbonyl, carboxy, hydroxyl, alkylsulfonyl, alkylsulfonamido, aminesulfanilamido, aralkylsulfonamido, alkylcarbonyl, arlyoxy, cyano, or ureido; or } R^1, R^2, \text{ or } R^3 \text{ together with } R^7 \text{ to form an } [--O--CH_2--] \text{ covalently bound bridge between the sugar 2' and 4' carbons.}

\[ A^2 \text{ is:}

\[ A^3 \text{ is:}

and

\[ A' \text{ is:} \]

\[ \begin{align*}
  \text{W}_1 & \quad \text{Z}_1 \\
  \text{X}_1 & \quad \text{Y}_1 \\
  \text{Z}_2 & \\
  \text{X}_2 & \\
  \text{Z}_3 & \\
  \text{X}_3 & \\
  \text{Z}_4 & \\
  \text{X}_4 
\end{align*} \]

\[ H; \text{Z}; \text{an inverted nucleotide}; \text{a basic nucleotide}; \text{or absent.} \]

\[ W^1 \text{ is } \text{OH}, (\text{CH}_2)^{10} \text{R}^{10}, (\text{CH}_2)\text{NHR}^{10}, (\text{CH}_2)\text{OR}^{10}, (\text{CH}_2)\text{SR}^{10}, (\text{CH}_2)\text{R}^{10}, (\text{CH}_2)\text{OR}^{10}, (\text{CH}_2)\text{NR}^{10}, (\text{CH}_2)\text{R}^{10}, (\text{CH}_2)\text{SS(CH}_2)^{10}, (\text{CH}_2)\text{R}^{10}, (\text{CH}_2)\text{NR}^{10}, (\text{CH}_2)\text{R}^{10}, (\text{CH}_2)\text{SR}^{10}, (\text{CH}_2)\text{R}^{10}, (\text{CH}_2)\text{NR}^{10}, (\text{CH}_2)\text{R}^{10}, (\text{CH}_2)\text{SR}^{10}, (\text{CH}_2)\text{R}^{10}, (\text{CH}_2)\text{NR}^{10}, (\text{CH}_2)\text{R}^{10}, (\text{CH}_2)\text{SR}^{10}, (\text{CH}_2)\text{R}^{10}, (\text{CH}_2)\text{NR}^{10}, (\text{CH}_2)\text{R}^{10}, (\text{CH}_2)\text{SR}^{10}. \]

Referring to Formula 3, \( A'-A' \) is \( L - G - L \); \( A' \) and/or \( A'^0 \) may be absent, in which \( L \) is a linker, wherein one or both \( L \) may be present or absent and is selected from the group consisting of \( \text{CH}_2(\text{CH}_2)_n \); \( n \text{NH}_2; \text{O}(\text{CH}_2)_m; S(\text{CH}_2)_n \). \( G \) is a functional group selected from the group consisting of siloxane, carbonate, carbonylmethyl, carboxylic acid, amide, thioether, ethylene oxide linker, sulfonate, sulfonamide, thiomethyl, formic acid, oxime, methylene-
imino, methylenemethylimino, methylenedihydrazone, methylenedimethylhydrazone and methylenoxymethylimino.

[R0468] R^10, R^20, and R^30 are each, independently, H, (i.e., basic nucleotides), adenine, guanine, cytosine and uracil, inosine, thymine, xanthine, hypoxanthine, xubulanine, tubercidine, isoquinouline, 2-aminoadenine, 6-methyl and other alkyl derivatives of adenine and guanine, 2-propyl and other alkyl derivatives of adenine and guanine, 5-haloaracil and cytosine, 5-propyl uracil and cytosine, 6-azo uracil, cytosine and thymine, 5-uracil (pseudouracil), 4-thiouracil, 5-haloaracil, 5-(2-amino propyl)uracil, 5-amino allyl uracil, 8-halo, amino, thiol, thioalkyl, hydroxyl and other 8-substituted adenines and guanines, 5-trifluoromethyl and other 5-substituted uracils and cytosines, 7-methylguanine, 5-substituted pyrimidines, 6-azapyrimidines and N-2, N-6 and O-6 substituted purines, including 2-amino propyl adenine, 5-propynyl uracil and 5-propynylcytosine, dicytrouracil, 3-deaza-5-azacytosine, 2-amino purine, 5-alkyluracil, 7-alkylguanine, 5-alkyl cytosine, 7-deazaadenine, 7-deazaguanine, N6, N6-dimethyl adenine, 2,6-diaminopurine, 5-amino-allyl-uracil, N3-methyluracil substituted 1,2,4-triazoles, 2-pyridinone, 5-nitroindole, 3-nitropyrrrole, 5-methoxy uracil, uracil-5-oxyacetic acid, 5-methoxy ammonium methyl uracil, 5-methyl-2-thiouracil, 5-methoxy carbonylbromethyluracil, 5-methylaminomethyl-2-thiouracil, 5-methylaminomethyl-2-thiouracil, 3-(3-amino-3-carboxypropyl)uracil, 3-methylcytosine, N4-acyl cytosine, 2-thiocytesine, N6-methyladenine, N6-isopentenyladenine, 2-methylthio-6-isopentenyladenine, N-methyl guanines, or O-alkylated bases.

[R0469] R^10, R^20, and R^30 are each, independently, OR, OR', OR", O(CH2)OR', CH2OR, CH2OR', CH2OR", or OR'.

[R0470] x is 5-100 or chosen to comply with a length for an RNA agent described herein; and g is 0-2.

[R0471] Preferred nucleoside surrogates have the following structure (see Formula 4 below): S is a nucleoside surrogate selected from the group consisting of mophilino, cyclobutyl, pyrrolidine, and peptide nucleic acid. L is a linker and is selected from the group consisting of CH2(CH2)n; N(CH2)m; O(CH2)p; S(CH2)q; —(O(CH2))r— or may be absent. M is an amide bond; sulfonamide; sulfinate; phosphate group; modified phosphate group as described herein; or may be absent.

[R0472] Preferred surrogates have the following structure (see Formula 4 below):

[R0473] [SLR^{2000}(M-SLR^{2000}), M-SLR^{2000}] FORMULA 4

[R0474] While not wishing to be bound by theory, it is believed that modifications of the sugar, base, and/or phosphate backbone in an iRNA agent can enhance endonuclease and exonuclease resistance, and can enhance interactions with transporter proteins and one or more of the functional components of the RISC complex. Preferred modifications
are those that increase exonuclease and endonuclease resistance and thus prolong the half-life of the iRNA agent prior to interaction with the RISC complex, but at the same time do not render the iRNA agent resistant to endonuclease activity in the RISC complex. Again, while not wishing to be bound by any theory, it is believed that placement of the modifications at or near the 3' and/or 5' end of antisense strands can result in iRNA agents that meet the preferred nuclease resistance criteria delineated above. Again, still while not wishing to be bound by any theory, it is believed that placement of the modifications at e.g., the middle of a sense strand can result in iRNA agents that are relatively less likely to undergo off-targeting.

[0481] Modifications described herein can be incorporated into any double-stranded RNA and RNA-like molecule described herein, e.g., an iRNA agent. An iRNA agent may include a duplex comprising a hybridized sense and antisense strand, in which the antisense strand and/or the sense strand may include one or more of the modifications described herein. The anti sense strand may include modifications at the 3' end and/or the 5' end and/or at one or more positions that occur 1-6 (e.g., 1-5,1-4, 1-3,1-2) nucleotides from either end of the strand. The sense strand may include modifications at the 3' end and/or the 5' end and/or at any one of the intervening positions between the two ends of the strand. The iRNA agent may also include a duplex comprising two hybridized antisense strands. The first and/or the second antisense strand may include one or more of the modifications described herein. Thus, one and/or both antisense strands may include modifications at the 3' end and/or the 5' end and/or at one or more positions that occur 1-6 (e.g., 1-5,1-4, 1-3,1-2) nucleotides from either end of the strand. Particular configurations are discussed below.

[0482] Modifications that can be useful for producing iRNA agents that meet the preferred nucleic resistance criteria delineated above can include one or more of the following chemical and/or stereochemical modifications of the sugar, base, and/or phosphate backbone:

[0483] (i) chiral (S)-thiotates. Thus, preferred NRM's include nucleotide dimers with an enriched or pure for a particular chiral form of a modified phosphate group containing a heteroatom at the nonbridging position, e.g., Sp or Rp, at the position X, where this is the position normally occupied by the oxygen. The atom at X can also be S, Se, Ne, or Br. When X is S, enriched or chirally pure Sp linkage is preferred. Enriched means at least 70, 80, 90, 95, or 99% of the preferred form. Such NRM's are discussed in more detail below;

[0484] (ii) attachment of one or more cationic groups to the sugar, base, and/or the phosphorus atom of a phosphate or modified phosphate backbone moiety. Thus, preferred NRM's include monomers at the terminal position derivatized at a cationic group. As the 5' end of an antisense sequence should have a terminal —OH or phosphate group this NRM is preferably not used at the 5' end of an anti-sense sequence. The group should be attached at a position on the base which minimizes interference with H bond formation and hybridization, e.g., away from the face which interacts with the complementary base on the other strand, e.g., at the 5' position of a pyrimidine or a 7-position of a purine. These are discussed in more detail below;

[0485] (iii) nonphosphate linkages at the termini. Thus, preferred NRM's include Non-phosphate linkages, e.g., a linkage of 4 atoms which confers greater resistance to cleavage than does a phosphate bond. Examples include 3' CH2NCH2O—CH25' and 3' CH2NH(O—)—CH25';

[0486] (iv) 3'-bridging thiophosphates and 5'-bridging thiophosphates. Thus, preferred NRM's can include these structures;

[0487] (v) L-RNA, 2'-5' linkages, inverted linkages, a-nucleosides. Thus, other preferred NRM's include: L-nucleosides and dimeric nucleotides derived from L-nucleosides; 2'-5' phosphate, non-phosphate and modified phosphate linkages (e.g., thio phosphates, phosphorimidates and boronophosphates); dimers having inverted linkages, e.g., 3'-3' or 5'5' linkages; monomers having an alpha linkage at the 1' site on the sugar, e.g., the structures described herein having an alpha linkage;

[0488] (vi) conjugate groups. Thus, preferred NRM's can include e.g., a targeting moiety or a conjugated ligand described herein conjugated with the monomer, e.g., through the sugar, base, or backbone;

[0489] (vi) abasic linkages. Thus, preferred NRM's can include an abasic monomer, e.g., an abasic monomer as described herein (e.g., a nucleobaseless monomer); an aromatic or heterocyclic or polyheterocyclic aromatic monomer as described herein; and

[0490] (vii) 5'-phosphonates and 5'-phosphate prodrugs. Thus, preferred NRM's include monomers, preferably at the terminal position, e.g., the 5' position, in which one or more atoms of the phosphate group is derivatized with a protecting group, which protecting group or groups, are removed as a result of the action of a component in the subject's body, e.g., a carboxyesterase or an enzyme present in the subject's body. E.g., a phosphate prodrug in which a carboxy esterase cleaves the protected molecule resulting in the production of a thioate anion which attacks a carbon adjacent to the 0 of a phosphate and resulting in the production of an unprotected phosphate.

[0491] One or more different NRM modifications can be introduced into an iRNA agent or into a sequence of an iRNA agent. An NRM modification can be used more than once in a sequence or in an iRNA agent. As some NRM's interfere with hybridization the total number incorporated, should be such that acceptable levels of iRNA agent duplex formation are maintained.

[0492] In some embodiments NRM modifications are introduced into the terminal the cleavage site or in the cleavage region of a sequence (a sense strand or sequence) which does not target a desired sequence or gene in the subject. This can reduce off-target silencing.

[0493] Chiral (S)-Thiotates

[0494] A modification can include the alteration, e.g., replacement, of one or both of the non-linking (X and Y)
phosphate oxygens and/or of one or more of the linking (W and Z) phosphate oxygens. Formula X below depicts a phosphate moiety linking two sugar/sugar surrogate-base moieties, SB₁ and SB₂.

\[
\text{FORMULA X}
\]

[0495] In certain embodiments, one of the non-linking phosphate oxygens in the phosphate backbone moiety (X and Y) can be replaced by any one of the following: S, Se, BR₃ (R is hydrogen, alkyl, aryl, etc.), C (i.e., an alkyl group, an aryl group, etc.), H, NR₂ (R is hydrogen, alkyl, aryl, etc.), or OR (R is alkyl or aryl). The phosphorus atom in an unmodified phosphate group is achiral. However, replacement of one of the non-linking oxygens with one of the above atoms or groups of atoms renders the phosphorus atom chiral; in other words a phosphorus atom in a phosphate group modified in this way is a stereogenic center. The stereogenic phosphorus atom can possess either the “R” configuration (herein Rₚ) or the “S” configuration (herein Sₚ). Thus if 60% of a population of stereogenic phosphorus atoms have the Rₚ configuration, then the remaining 40% of the population of stereogenic phosphorus atoms have the Sₚ configuration.

[0496] In some embodiments, iRNA agents, having phosphate groups in which a phosphate non-linking oxygen has been replaced by another atom or group of atoms, may contain a population of stereogenic phosphorus atoms in which at least about 50% of these atoms (e.g., at least about 60% of these atoms, at least about 70% of these atoms, at least about 80% of these atoms, at least about 90% of these atoms, at least about 95% of these atoms, at least about 98% of these atoms, at least about 99% of these atoms) have the Sₚ configuration. Alternatively, iRNA agents having phosphate groups in which a phosphate non-linking oxygen has been replaced by another atom or group of atoms may contain a population of stereogenic phosphorus atoms in which at least about 50% of these atoms (e.g., at least about 60% of these atoms, at least about 70% of these atoms, at least about 80% of these atoms, at least about 90% of these atoms, at least about 95% of these atoms, at least about 98% of these atoms, at least about 99% of these atoms) have the Rₚ configuration. In other embodiments, the population of stereogenic phosphorus atoms may have the Sₚ configuration and may be substantially free of stereogenic phosphorus atoms having the Rₚ configuration. In still other embodiments, the population of stereogenic phosphorus atoms may have the Rₚ configuration and may be substantially free of stereogenic phosphorus atoms having the Sₚ configuration.

“Substantially free of stereogenic phosphorus atoms having the Sₚ configuration” means that moieties containing stereogenic phosphorus atoms having the Rₚ configuration cannot be detected by conventional methods known in the art (chiral HPLC, "H NMR analysis using chiral shift reagents, etc.).

[0497] In a preferred embodiment, modified iRNA agents contain a phosphorothioate group, i.e., a phosphate group in which a phosphate non-linking oxygen has been replaced by a sulfur atom. In an especially preferred embodiment, the population of phosphorothioate stereogenic phosphorus atoms may have the Sₚ configuration and be substantially free of stereogenic phosphorus atoms having the Rₚ configuration.

[0498] Phosphorothioates may be incorporated into iRNA agents using dimers e.g., formulas X-1 and X-2. The former can be used to introduce phosphorothioate at the 3’ end of a strand, while the latter can be used to introduce this modification at the 5’ end or at a position that occurs e.g., 1, 2, 3, 4, 5, or 6 nucleotides from either end of the strand. In the above formulas, Y can be 2-cyanoethoxy, W and Z can be 0, R₂ can be, e.g., a substituent that can impart the C-3 endo configuration to the sugar (e.g., OH, F, OCH₃), DMT is dimethoxytrityl, and “BASE” can be a natural, unusual, or a universal base.
[0500] X-1 and X-2 can be prepared using chiral reagents or directing groups that result in phosphorothioate-containing dimers having a population of stereogenic phosphorus atoms having essentially only the R<sub>5</sub> configuration (i.e., being substantially free of the S<sub>5</sub> configuration) or only the S<sub>5</sub> configuration (i.e., being substantially free of the R<sub>5</sub> configuration). Alternatively, dimers can be prepared having a population of stereogenic phosphorus atoms in which about 50% of the atoms have the R<sub>5</sub> configuration and about 50% of the atoms have the S<sub>5</sub> configuration. Dimers having stereogenic phosphorus atoms with the R<sub>5</sub> configuration can be identified and separated from dimers having stereogenic phosphorus atoms with the S<sub>5</sub> configuration using e.g., enzymatic degradation and/or conventional chromatography techniques.

[0501] Cationic Groups

[0502] Modifications can also include attachment of one or more cationic groups to the sugar, base, and/or the phosphorus atom of a phosphate or modified phosphate backbone moiety. A cationic group can be attached to any atom capable of substitution on a natural, unusual or universal base. A preferred position is one that does not interfere with hybridization, i.e., does not interfere with the hydrogen bonding interactions needed for base pairing. A cationic group can be attached e.g., through the C'2 position of a sugar or analogous position in a cyclic or acyclic sugar surrogate. Cationic groups can include e.g., protonated amino groups, derived from e.g., O-AMINE (AMINE=NH<sub>2</sub>); alkylaminio, dialkylaminio, heterocyclyl, aryamino, diaryl amino, heteroaryl amino, or diheteroaryl amino, ethylene diamine, polyamines); aminoalkoxy, e.g., O(CH<sub>2</sub>)<sub>n</sub>AMINE, (e.g., AMINE=NH<sub>2</sub>; alkylaminio, dialkylaminio, heterocyclyl, aminocyclyl, diaryl amino, heteroaryl amino, or diheteroaryl amino, ethylene diamine, polyamines); aminoalkoxy, e.g., NH<sub>2</sub>-alkylaminio, dialkylaminio, heterocyclyl, aryamino, diaryl amino, heteroaryl amino, or diheteroaryl amino, ethylene diamine, polyamines); aminoalkoxy, e.g., NH<sub>2</sub>-alkylaminio, dialkylaminio, heterocyclyl, aryamino, diaryl amino, heteroaryl amino, or diheteroaryl amino, ethylene diamine, polyamines); aminoalkoxy, e.g., NH<sub>2</sub>-alkylaminio, dialkylaminio, heterocyclyl, aryamino, diaryl amino, heteroaryl amino, or diheteroaryl amino, ethylene diamine, polyamines).

[0503] Nonphosphate Linkages

[0504] Modifications can also include the incorporation of nonphosphate linkages at the 5' and/or 3' end of a strand. Examples of nonphosphate linkages which can replace the phosphate group include methyl phosphonate, hydroxylamino, siloxane, carbonate, carboxymethyl, carbamate, amide, thioether, ethylene oxide linker, sulfonate, sulfonamide, thiocarbamoyl, formacetal, oxime, methylenemino, methylenemethylamino, methylenedimethylamino, ethyldihydrazo and methyleneoxymethylimino. Preferred replacements include the methyl phosphonate and hydroxylamino groups.

[0505] 3'-Bridging Thiophosphates and 5'-Bridging Thiophosphates; Locked-RNA, 2'-5' Linkages, Inverted Linkages, α-Nucleosides; Conjugate Groups; Abasic Linkages; and 5'-Phosphate and 5'-Phosphate Prodrugs

[0506] Referring to formula X above, modifications can include replacement of one of the bridging or linking phosphate oxygens in the phosphate backbone moiety (W and Z). Unlike the situation where only one of X or Y is altered, the phosphorus center in the phosphorodithioates is achiral which precludes the formation of iRNA agents containing a stereogenic phosphorus atom.

[0507] Modifications can also include linking two sugars via a phosphate or modified phosphate group through the 2' position of a first sugar and the 5' position of a second sugar. Also contemplated are inverted linkages in which both a first and second sugar are each linked through the respective 3' positions. Modified RNA's can also include "abasic" sugars, which lack a nucleobase at C-1'. The sugar group can also contain one or more carbons that possess the opposite stereochemical configuration than that of the corresponding carbon in ribose. Thus, a modified iRNA agent can include nucleotides containing e.g., arabinose, as the sugar. In another subset of this modification, the natural, unusual, or universal base may have the α-configuration. Modifications can also include L-RNA.

[0508] Modifications can also include 5'-phosphonates, e.g., P(O(OH))<sub>2</sub>-X-CH(=O)-sugar (X=CH<sub>2</sub> or CHF<sub>2</sub>) in 5'-phosphate prodrugs, e.g., P(O)(CH<sub>2</sub>)<sub>2</sub>CH(=O)-sugar. In the latter case, the prodrug groups may be decomposed via reaction first with carboxy esterases. The remaining ethyl thiolate group via intramolecular S<sub>2</sub> displacement can depurate as epissulfide to afford the underivatized phosphate group.

[0509] Modification can also include the addition of conjugating groups described elsewhere herein, which are preferably attached to an iRNA agent through any amino group available for conjugation.

[0510] Nucleoside modifications include some which can be placed only at the terminus and others which can go at any position. Generally the modifications that can inhibit hybridization so it is preferably to use them only in terminal regions, and preferable to not use them at the cleavage site or in the cleavage region of an sequence which target a subject sequence or gene. The can be used anywhere in a sense sequence, provided that sufficient hybridization between the two sequences of the iRNA agent is maintained. In some embodiments it is desirably to put the NRM at the cleavage site or in the cleavage region of a sequence which does not target a subject sequence or gene, as it can minimize off-target silencing.

[0511] In addition, an iRNA agent described herein can have an overhang which does not form a duplex structure with the other sequence of the iRNA agent—it is an overhang, but it does hybridize, either with itself, or with another nucleic acid, other than the other sequence of the iRNA agent.

[0512] In most cases, the nuclease-resistance promoting modifications will be distributed differently depending on whether the sequence will target a sequence in the subject (often referred to as an anti-sense sequence) or will not target a sequence in the subject (often referred to as a sense sequence). If a sequence is to target a sequence in the subject, modifications which inhibit or inhibit endonuclease cleavage should not be inserted in the region which is subject to RISC mediated cleavage, e.g., the cleavage site or the cleavage region (As described in Ebishir et al., 2001, Genes and Dev. 15: 188, hereby incorporated by reference, cleavage of the target occurs about in the middle of a 20 or 21 nt guide RNA, or about 10 or 11 nucleotides upstream of the first nucleotide which is complementary to the guide
sequence. As used herein cleavage site refers to the nucleotide on either side of the cleavage site, on the target or on the RNA agent strand which hybridizes to it. Cleavage region means an nucleotide with 1, 2, or 3 nucleotides of the cleavage site, in either direction.)

[0513] Such modifications can be introduced into the terminal regions, e.g., at the terminal position or with 2, 3, 4, or 5 positions of the terminus, of a sequence which targets or a sequence which does not target a sequence in the subject.

[0514] An RNA agent can have a first and a second strand chosen from the following:

[0515] a first strand which does not target a sequence and which has an NRM modification at or within 1, 2, 3, 4, 5, or 6 positions from the 3' end;

[0516] a first strand which does not target a sequence and which has an NRM modification at or within 1, 2, 3, 4, 5, or 6 positions from the 5' end;

[0517] a first strand which does not target a sequence and which has an NRM modification at or within 1, 2, 3, 4, 5, or 6 positions from the 3' end and which has an NRM modification at or within 1, 2, 3, 4, 5, or 6 positions from the 5' end;

[0518] a first strand which does not target a sequence and which has an NRM modification at the cleavage site or in the cleavage region;

[0519] a first strand which does not target a sequence and which has an NRM modification at the cleavage site or in the cleavage region and one or more of an NRM modification at or within 1, 2, 3, 4, 5, or 6 positions from the 3' end, a NRM modification at or within 1, 2, 3, 4, 5, or 6 positions from the 5' end, or NRM modifications at or within 1, 2, 3, 4, 5, or 6 positions from both the 3' and the 5' end (5' end NRM modifications are preferentially not at the terminus but rather at a position 1, 2, 3, 4, 5, or 6 away from the 5' terminus of an antisense strand);

[0520] a second strand which targets a sequence and which has an NRM modification at or within 1, 2, 3, 4, 5, or 6 positions from the 3' end;

[0521] a second strand which targets a sequence and which has an NRM modification at or within 1, 2, 3, 4, 5, or 6 positions from the 5' end (5' end NRM modifications are preferentially not at the terminus but rather at a position 1, 2, 3, 4, 5, or 6 away from the 5' terminus of an antisense strand);

[0522] a second strand which targets a sequence and which has an NRM modification at or within 1, 2, 3, 4, 5, or 6 positions from the 3' end and which has a NRM modification at or within 1, 2, 3, 4, 5, or 6 positions from the 5' end;

[0523] a second strand which targets a sequence and which preferably does not have an NRM modification at the cleavage site or in the cleavage region;

[0524] a second strand which targets a sequence and which does not have an NRM modification at the cleavage site or in the cleavage region and one or more of an NRM modification at or within 1, 2, 3, 4, 5, or 6 positions from the 3' end, a NRM modification at or within 1, 2, 3, 4, 5, or 6 positions from the 5' end, or NRM modifications at or within 1, 2, 3, 4, 5, or 6 positions from both the 3' and the 5' end (5' end NRM modifications are preferentially not at the terminus but rather at a position 1, 2, 3, 4, 5, or 6 away from the 5' terminus of an antisense strand).

[0525] An RNA agent can also target two sequences and can have a first and second strand chosen from:

[0526] a first strand which targets a sequence and which has an NRM modification at or within 1, 2, 3, 4, 5, or 6 positions from the 3' end;

[0527] a first strand which targets a sequence and which has an NRM modification at or within 1, 2, 3, 4, 5, or 6 positions from the 5' end (5' end NRM modifications are preferentially not at the terminus but rather at a position 1, 2, 3, 4, 5, or 6 away from the 5' terminus of an antisense strand);

[0528] a first strand which targets a sequence and which has an NRM modification at or within 1, 2, 3, 4, 5, or 6 positions from the 3' end and which has a NRM modification at or within 1, 2, 3, 4, 5, or 6 positions from the 5' end;

[0529] a first strand which targets a sequence and which preferably does not have an NRM modification at the cleavage site or in the cleavage region;

[0530] a first strand which targets a sequence and which does not have an NRM modification at the cleavage site or in the cleavage region and one or more of an NRM modification at or within 1, 2, 3, 4, 5, or 6 positions from the 3' end, a NRM modification at or within 1, 2, 3, 4, 5, or 6 positions from the 5' end, or NRM modifications at or within 1, 2, 3, 4, 5, or 6 positions from both the 3' and the 5' end (5' end NRM modifications are preferentially not at the terminus but rather at a position 1, 2, 3, 4, 5, or 6 away from the 5' terminus of an antisense strand) and a second strand which targets a sequence and which has an NRM modification at or within 1, 2, 3, 4, 5, or 6 positions from the 3' end;

[0531] a second strand which targets a sequence and which has an NRM modification at or within 1, 2, 3, 4, 5, or 6 positions from the 3' end (5' end NRM modifications are preferentially not at the terminus but rather at a position 1, 2, 3, 4, 5, or 6 away from the 5' terminus of an antisense strand);

[0532] a second strand which targets a sequence and which has an NRM modification at or within 1, 2, 3, 4, 5, or 6 positions from the 3' end and which has a NRM modification at or within 1, 2, 3, 4, 5, or 6 positions from the 5' end;

[0533] a second strand which targets a sequence and which preferably does not have an NRM modification at the cleavage site or in the cleavage region;
a second strand which targets a sequence and which does not have an NRM modification at the cleavage site or in the cleavage region and one or more of an NRM modification at or within 1, 2, 3, 4, 5, or 6 positions from the 3' end, a NRM modification at or within 1, 2, 3, 4, 5, or 6 positions from the 5' end, or NRM modifications at or within 1, 2, 3, 4, 5, or 6 positions from both the 3' and the 5' end (5' end NRM modifications are preferentially not at the terminus but rather at a position 1, 2, 3, 4, 5, or 6 away from the 5' terminus of an antisense strand).

Ribose Mimics

The monomers and methods described herein can be used to prepare an RNA, e.g., an iRNA agent, that incorporates a ribose mimic, such as those described herein and those described in co-pending U.S. Provisional Application Ser. No. 60/454,962, filed on Mar. 13, 2003, and International Application No. PCT/US04/07070, both of which are hereby incorporated by reference.

Thus, an aspect of the invention features an iRNA agent that includes a secondary hydroxyl group, which can increase efficacy and/or confer nuclease resistance to the agent. Nucleases, e.g., cellular nucleases, can hydrolyze nucleic acid phosphodiester bonds, resulting in partial or complete degradation of the nucleic acid. The secondary hydroxy group confers nuclease resistance to an iRNA agent by rendering the iRNA agent less prone to nucleosome degradation relative to an iRNA which lacks the modification. While not wishing to be bound by theory, it is believed that the presence of a secondary hydroxy group on the iRNA agent can act as a structural mimic of a 3' ribose hydroxyl group, thereby causing it to be less susceptible to degradation.

The secondary hydroxy group refers to an "OH" radical that is attached to a carbon atom substituted by two other carbons and a hydrogen. The secondary hydroxy group that confers nuclease resistance as described above can be part of any acyclic carbon-containing group. The hydroxyl may also be part of any cyclic carbon-containing group, and preferably one or more of the following conditions is met (1) there is no ribose moiety between the hydroxyl group and the terminal phosphate group or (2) the hydroxyl group is not on a sugar moiety which is coupled to a base. The hydroxyl group is located at least two bonds (e.g., at least three bonds away, at least four bonds away, at least five bonds away, at least six bonds away, at least seven bonds away, at least eight bonds away, at least nine bonds away, at least ten bonds away, etc.) from the terminal phosphate group phosphorus of the iRNA agent. In preferred embodiments, there are five intervening bonds between the terminal phosphate group phosphorus and the secondary hydroxy group.

Preferred iRNA agent delivery modules with five intervening bonds between the terminal phosphate group phosphorus and the secondary hydroxy group have the following structure (see formula Y below):

Referring to formula Y, A is an iRNA agent, including any iRNA agent described herein. The iRNA agent may be connected directly or indirectly (e.g., through a spacer or linker) to "W" of the phosphate group. These spacers or linkers can include e.g., —(CH₂)ₙ—, —(CH₂)ₙN—, —(CH₂)ₙO—, —(CH₂)ₙS—, O(CH₂CH₂O)ₙCH₂CH₂OH (e.g., n=3 or 6), abasic sugars, amide, carboxy, amine, oximate, oxamime, theoether, disulfide, thioeurea, sulfonamide, or morpholinio, or biotin and fluorescein reagents.

The iRNA agents can have a terminal phosphate group that is unmodified (e.g., W, X, Y, and Z are O) or modified. In a modified phosphate group, W and Z can be independently NH, O, or S, and X and Y can be independently S, Se, BH₄⁻, C₁-C₆ alkyl, C₆-C₁₀ aryl, H, O, O', alkoxy or amino (including alkylamino, aminolino, etc.). Preferably, W, X, Y and Z are 0 and Y is S.

R₄ and R₅ are each, independently, hydrogen; or C₁-C₁₀₀ alkyl, optionally substituted with hydroxy, amino, halo, phosphate or sulfate and/or may be optionally inserted with O, N, O, alkyl or alkynyl.

R₅ is hydrogen; C₁-C₆ alkyl, optionally substituted with hydroxy, amino, halo, phosphate or sulfate and/or may be optionally inserted with O, N, O, alkyl or alkynyl; or, when n is 1, R₂ may be taken together with with R₃ or R₆ to form a ring of 5-12 atoms.

R₄ is hydrogen; C₁-C₁₀₀ alkyl, optionally substituted with hydroxy, amino, halo, phosphate or sulfate and/or may be optionally inserted with O, N, O, alkyl or alkynyl; or, when n is 1, R₅ may be taken together with with R₂ or R₆ to form a ring of 5-12 atoms.

R₅ is hydrogen, C₁-C₁₀₀ alkyl optionally substituted with hydroxy, amino, halo, phosphate or sulfate and/or may be optionally inserted with O, N, O, alkyl or alkynyl; or, when n is 1, R₅ may be taken together with with R₄ to form a ring of 5-12 atoms.

R₆ is hydrogen, C₁-C₁₀₀ alkyl, optionally substituted with hydroxy, amino, halo, phosphate or sulfate and/or may be optionally inserted with O, N, O, alkyl or
alkynyl, or, when \( n = 1 \), \( R_6 \) may be taken together with with \( R_6 \) to form a ring of 6-10 atoms;

\[ R_6 \] is hydrogen, \( C_2-C_{30} \) alkyl, or \( OC(O)CH_2C(O)NHRO \); \( T \) is hydrogen or a functional group; \( n \) and \( q \) are each independently 1-100; \( R_6 \) is \( C_2-C_{16} \) alkyl or \( C_2-C_{40} \) aryl; and \( R_6 \) is hydrogen, \( C_1-C_{10} \) alkyl, \( C_6-C_{10} \) aryl or a solid support agent.

Preferred embodiments may include one of more of the following subsets of RNAi agent delivery modules:

In one subset of RNAi agent delivery modules, \( A \) can be connected directly or indirectly through a terminal 3' or 5' ribose sugar carbon of the RNA agent.

In another subset of RNAi agent delivery modules, \( X, W, \) and \( Z \) are 0 and \( Y \) is S.

In still yet another subset of RNAi agent delivery modules, \( n = 1 \), \( R_6 \) and \( R_7 \) are taken together to form a ring containing six atoms and \( R_8 \) and \( R_8 \) are taken together to form a ring containing six atoms. Preferably, the ring system is a trans-decalin. For example, the RNAi agent delivery module of this subset can include a compound of Formula (Y-1):

\[ \text{A} \]

\[ \text{O} \]

\[ \text{CO} \]

\[ \text{S} \]

\[ \text{NHT} \]

\[ \text{HO} \]

The functional group can be, for example, a targeting group (e.g., a steroid or a carbohydrate), a reporter group (e.g., a fluorophore), or a label (an isotopically labelled moiety). The targeting group can further include protein binding agents, endothelial cell targeting groups (e.g., RGD peptides and mimetics), cancer cell targeting groups (e.g., folate Vitamin B12, Biotin), bone cell targeting groups (e.g., bisphosphonates, polyglycamat, polyaspartates), multivalent mannose (for e.g., macrophage testing), lactose, galactose, N-acetyl-galactosamine, monoclonal antibodies, glycoproteins, lectins, melanotropin, or thyrotropin.

As can be appreciated by the skilled artisan, methods of synthesizing the compounds of the formulae herein will be evident to those of ordinary skill in the art. The synthesized compounds can be separated from a reaction mixture and further purified by a method such as column chromatography, high pressure liquid chromatography, or recrystallization. Additionally, the various synthetic steps may be performed in an alternate sequence or order to give the desired compounds. Synthetic chemistry transformations and protecting group methodologies (protection and deprotection) useful in synthesizing the compounds described herein are known in the art and include, for example, those such as described in R. Larock, *Comprehensive Organic Transformations*, VCH Publishers (1989); T. W. Greene and P. G. M. Wuts, *Protective Groups in Organic Synthesis*, 2d. Ed., John Wiley and Sons (1991); L. Fieser and M. Fieser, *Fieser and Fieser's Reagents for Organic Synthesis*, John Wiley and Sons (1994); and L. Paquette, ed., *Encyclopedia of Reagents for Organic Synthesis*, John Wiley and Sons (1995), and subsequent editions thereof.

**Pharmaceutical Compositions**

In one embodiment, the invention relates to a pharmaceutical composition containing a modified RNAi agent, as described in the preceding sections, and a pharmaceutically acceptable carrier, as described below. A pharmaceutical composition including the modified RNAi agent is useful for treating a disease caused by expression of a target gene. In this aspect of the invention, the RNAi agent of the invention is formulated as described below. The pharmaceutical composition is administered in a dosage sufficient to inhibit expression of the target gene.

The pharmaceutical compositions of the present invention are administered in dosages sufficient to inhibit the expression or activity of the target gene. Compositions containing the RNAi agent of the invention can be administered at surprisingly low dosages. A maximum dosage of 5 mg RNAi agent per kilogram body weight per day may be sufficient to inhibit or completely suppress the expression or activity of the target gene.

In general, a suitable dose of modified RNAi agent will be in the range of 0.001 to 500 milligrams per kilogram body weight of the recipient per day (e.g., about 1 microgram per kilogram to about 500 milligrams per kilogram, about 100 micrograms per kilogram to about 100 milligrams per kilogram, about 1 milligrams per kilogram to about 75 milligrams per kilogram, about 10 micrograms per kilogram to about 50 milligrams per kilogram, or about 1 microgram per kilogram to about 50 micrograms per kilogram). The pharmaceutical composition may be administered once per day, or the RNAi agent may be administered as two, three, four, five, six or more sub-doses at appropriate intervals throughout the day. In that case, the RNAi agent contained in each sub-dose must be correspondingly smaller in order to achieve the total daily dosage. The dosage unit can also be compounded for delivery over several days, e.g., using a conventional sustained release formulation which provides sustained release of the RNAi agent over a several day period. Sustained release formulations are well known in the art. In this embodiment, the dosage unit contains a corresponding multiple of the daily dose.

The skilled artisan will appreciate that certain factors may influence the dosage and timing required to effectively treat a subject, including but not limited to the severity of the infection or disease, previous treatments, the general health and/or age of the subject, and other diseases present. Moreover, treatment of a subject with a therapeutically effective amount of a composition can include a single treatment or a series of treatments. Estimates of effective dosages and in vivo half-lives for the individual RNAi agent encompassed by the invention can be made using conventional methodologies or on the basis of in vivo testing using an appropriate animal model, as described elsewhere herein.

Advances in mouse genetics have generated a number of mouse models for the study of various human diseases. For example, mouse repositories can be found at The Jackson Laboratory, Charles River Laboratories, Taconic, Harlan, Mutant Mouse Regional Resource Centers (MMRRC) National Network and at the European Mouse...
Mutant Archive. Such models may be used for in vivo testing of iRNA agent, as well as for determining a therapeutically effective dose.

[0559] The pharmaceutical compositions encompassed by the invention may be administered by any means known in the art including, but not limited to oral or parenteral routes, including intravenous, intramuscular, intraperitoneal, subcutaneous, transdermal, airway (aerosol), ocular, rectal, vaginal and topical (including buccal and sublingual) administration. In preferred embodiments, the pharmaceutical compositions are administered by intravenous or intraparenchymal infusion or injection. The pharmaceutical compositions can also be administered intraparenchymally, intrathecally, and/or by stereotactic injection.

[0560] For oral administration, the iRNA agent useful in the invention will generally be provided in the form of tablets or capsules, as a powder or granules, or as an aqueous solution or suspension.

[0561] Tablets for oral use may include the active ingredients mixed with pharmaceutically acceptable excipients such as inert diluents, disintegrating agents, binding agents, lubricating agents, sweetening agents, flavoring agents, coloring agents and preservatives. Suitable inert diluents include sodium and calcium carbonate, sodium and calcium phosphate, and lactose, while corn starch and alginic acid are suitable disintegrating agents. Binding agents may include starch and gelatin, while the lubricating agent, if present, will generally be magnesium stearate, stearic acid or talc. If desired, the tablets may be coated with a material such as glyceryl monostearate or glyceryl distearate, to delay absorption in the gastrointestinal tract.

[0562] Capsules for oral use include hard gelatin capsules in which the active ingredient is mixed with a solid diluent, and soft gelatin capsules wherein the active ingredient is mixed with water or an oil such as peanut oil, liquid paraffin or olive oil.

[0563] For intramuscular, intraperitoneal, subcutaneous and intravenous use, the pharmaceutical compositions of the invention will generally be provided in sterile aqueous solutions or suspensions, buffered to an appropriate pH and isotonicity. Suitable aqueous vehicles include Ringer’s solution and isotonic sodium chloride. In a preferred embodiment, the carrier consists exclusively of an aqueous buffer. In this context, “exclusively” means no auxiliary agents or encapsulating substances are present which might affect or mediate uptake of the iRNA agent in the cells that harbor the target gene or virus. Such substances include, for example, micellar structures, such as liposomes or capsids, as described above. Although microinjection, lipofection, viruses, viroids, capsids, capsoids, or other auxiliary agents are required to introduce iRNA agent into cell cultures, surprisingly these methods and agents are not necessary for uptake of the iRNA agent in vivo. The iRNA agent of the present invention are particularly advantageous in that they do not require the use of an auxiliary agent to mediate uptake of the iRNA agent into the cells, many of which agents are toxic or associated with deleterious side effects. Aqueous suspensions according to the invention may include suspending agents such as cellulose derivatives, sodium alginate, polyvinyl-pyrrolidone and gum tragacanth, and a wetting agent such as lecithin. Suitable preservatives for aqueous suspensions include ethyl and n-propyl p-hydroxybenzoate.

[0564] The pharmaceutical compositions can also include encapsulated formulations to protect the iRNA agent against rapid elimination from the body, such as a controlled release formulation, including implants and microencapsulated delivery systems. Biodegradable, biocompatible polymers can be used, such as ethylene vinyl acetate, polyanhydrides, polyglycolic acid, collagen, polyethylene glycols, and polyactic acid. Methods for preparation of such formulations will be apparent to those skilled in the art. The materials can also be obtained commercially from Alza Corporation and Nova Pharmaceuticals, Inc. Liposomal suspensions (including liposomes targeted to infected cells with monoclonal antibodies to viral antigens) can also be used as pharmaceutically acceptable carriers. These can be prepared according to methods known to those skilled in the art, for example, as described in U.S. Pat. No. 4,522,811; PCT publication WO 91/6309; and European patent publication EP-A-43075, which are incorporated by reference herein.

[0565] Toxicity and therapeutic efficacy of the iRNA agent can be determined by standard pharmaceutical procedures in cell cultures or experimental animals, e.g., for determining the LD50 (the dose lethal to 50% of the population) and the ED50 (the dose therapeutically effective in 50% of the population). The dose ratio between toxic and therapeutic effects is the therapeutic index and it can be expressed as the ratio LD50/ED50. iRNA agents that exhibit high therapeutic indices are preferred.

[0566] The data obtained from cell culture assays and animal studies can be used in formulating a range of dosage for use in humans. The dosages of compositions of the invention are preferably within a range of circulating concentrations that include the ED50 with little or no toxicity. The dosage may vary within this range depending upon the dosage form employed and the route of administration utilized. For any iRNA agent used in the method of the invention, the therapeutically effective dose can be estimated initially from cell culture assays. A dose may be formulated in animal models to achieve a circulating plasma concentration range of the iRNA agent or, when appropriate, of the polypeptide product of a target sequence (e.g., achieving a decreased concentration of the polypeptide) that includes the IC50 (i.e., the concentration of the test iRNA agent which achieves a half-maximal inhibition of symptoms) as determined in cell culture. Such information can be used to more accurately determine useful doses in humans. Levels in plasma may be measured, for example, by high performance liquid chromatography.

[0567] In addition to their administration individually or as a plurality, as discussed above, iRNA agents relating to the invention can be administered in combination with other known agents effective in treating viral infections and diseases. In any event, the administering physician can adjust the amount and timing of the iRNA agent administration on the basis of results observed using standard measures of efficacy known in the art or described herein.

[0568] For oral administration, the iRNA agent useful in the invention will generally be provided in the form of tablets or capsules, as a powder or granules, or as an aqueous solution or suspension.


[0570] In one embodiment, the invention relates to a method for treating a subject having a disease or at risk of
developing a disease caused by the expression of a target gene. In this embodiment, iRNA agents can act as novel therapeutic agents for controlling one or more of cellular proliferative and/or differentiative disorders, disorders associated with bone metabolism, immune disorders, hematopoietic disorders, cardiovascular disorders, liver disorders, viral diseases, or metabolic disorders. The method includes administering a pharmaceutical composition of the invention to the patient (e.g., a human), such that expression of the target gene is silenced. Because of their high efficiency and specificity, the iRNA agent of the present invention specifically targets mRNA of target genes of diseased cells and tissues, as described below, and at surprisingly low dosages. The pharmaceutical compositions are formulated as described in the preceding section, which is hereby incorporated by reference herein.


[0572] In the prevention of disease, the target gene may be one which is required for initiation or maintenance of the disease, or which has been identified as being associated with a higher risk of contracting the disease. In the treatment of disease, the iRNA agent can be brought into contact with the cells or tissue exhibiting the disease. For example, iRNA agent substantially identical to all or part of a mutated gene associated with cancer, or one expressed at high levels in tumor cells, may be brought into contact with or introduced into a cancerous cell or tumor gene.

[0573] Examples of cellular proliferative and/or differentiative disorders include cancer, e.g., a carcinoma, sarcoma, metastatic disorder or hematopoietic neoplastic disorder, such as a leukemia. A metastatic tumor can arise from a multitude of primary tumor types, including but not limited to those of prostate, colon, lung, breast and liver origin. As used herein, the terms “cancer,” “hyperproliferative,” and “neoplastic” refer to cells having the capacity for autonomous growth, i.e., an abnormal state or condition characterized by rapidly proliferating cell growth. These terms are meant to include all types of cancerous growths or oncogenic processes, metastatic tissues or malignantly transformed cells, tissues, or organs, irrespective of histopathologic type or stage of invasiveness. Proliferative disorders also include hematopoietic neoplastic disorders, including diseases involving hyperplastic/neoplastic cells of hematopoietic origin, e.g., arising from myeloid, lymphoid or erythroid lineages, or precursor cells thereof.

[0574] The pharmaceutical compositions of the present invention can also be used to treat a variety of immune disorders, in particular those associated with overexpression or aberrant expression of a gene or expression of a mutant gene. Examples of hematopoietic disorders or diseases include, without limitation, autoimmune diseases (including, for example, diabetes mellitus, arthritis (including rheumatoid arthritis, juvenile rheumatoid arthritis, osteoarthritis, psoriatic arthritis), multiple sclerosis, encephalomyelitis, myasthenia gravis, systemic lupus erythematosus, autoimmune thyroiditis, dermatitis (including atopic dermatitis and eczematous dermatitis), psoriasis, Sjogren’s Syndrome, Crohn’s disease, aphtous ulcer, iritis, conjunctivitis, keratoconjunctivitis, ulcerative colitis, asthma, allergic asthma, cutaneous lupus erythematosus, scleroderma, vaginitis, pruritis, drug eruptions, leprosy reversal reactions, erythema nodosum leprosum, autoimmune uveitis, allergic encephalomyelitis, acute necrotizing hemorrhagic encephalopathy, idiopathic bilateral progressive sensorineural hearing loss, aplastic anemia, pure red cell anemia, idiopathic thrombocytopenia, polyendocrinitis, Wegener’s granulomatosis, chronic active hepatitis, Steven-Johnson syndrome, idiopathic sprue, ichnus planus, Graves’ disease, sarcoidosis, primary biliary cirrhosis, uveitis posterior, and interstitial lung fibrosis), graft-versus-host disease, cases of transplantation, and allergy.

[0575] In another embodiment, the invention relates to methods for treating viral diseases, including but not limited to hepatitis C, hepatitis B, herpes simplex virus (HSV), HIV-AIDS, poliovirus, and smallpox virus. iRNA agent of the invention are prepared as described herein to target expressed sequences of a virus, thus ameliorating viral activity and replication. The iRNA agents can be used in the treatment and/or diagnosis of viral infected tissue, both animal and plant. Also, such iRNA agent can be used in the treatment of virus-associated carcinoma, such as hepatocellular cancer.

[0576] For example, the iRNA agent of the present invention are useful for treating a subject having an infection or a disease associated with the replication or activity of a (+) strand RNA virus having a 3’-UTR, such as HCV. In this embodiment, the iRNA agent can act as novel therapeutic
agents for inhibiting replication of the virus. The method includes administering a pharmaceutical composition of the invention to the patient (e.g., a human), such that viral replication is inhibited. Examples of (+) strand RNA viruses which can be targeted for inhibition include, without limitation, picornaviruses, caliciviruses, nodaviruses, coronaviruses, arteriviruses, flaviviruses, and togaviruses. Examples of picornaviruses include enterovirus (poliovirus 1), rhinovirus (human rhinovirus 1A), hepatitis A virus, cardiovirus (encephalomyocarditis virus), alphavirus (foot-and-mouth disease virus 0), and parainfluenza (human coho

[0581] The methods for inhibiting the expression of a target gene can be applied to any gene one wishes to silence, thereby specifically inhibiting its expression, provided the cell or organism in which the target gene is expressed includes the cellular machinery which effects RNA interference. Examples of genes which can be targeted for silencing include, without limitation, developmental genes including but not limited to adhesion molecules, cyclin kinase inhibitors, Wnt family members, Pax family members, Winged helix family members, Hox family members, cytokines/lymphokines and their receptors, growth/differentiation factors and their receptors, and neurotransmitters and their receptors; (2) oncogenes including but not limited to ABL1, BCL1, BCL2, BCL6, BCLAF1, CBL, CSFIR, ERBB, ERBB2, ETS 1, ETS 1, ETVE, FGR, FOS, FYN, HCR, HRAS, JUN, KRAS, LCK, LYN, MDM2, MLL, MYB, MYC, MYC1, MYCN, NRAS, PIM1, PML, RET, SRC, TAL1, TCL3 and YES; (3) tumor suppressor genes including but not limited to APC, BRCA1, BRCA2, MADH4, MCC, NFI, NF2, RB1, TP53 and WT1; and (4) enzymes including but not limited to ACP desaturases and hydroxylases, ADP-glucose pyrophosphorylases, ATPases, alcohol dehydrogenases, amylases, amyloglucosidases, catalases, cellulases, cyclooxygenases, decarboxylases, dextrinases, DNA and RNA polymerases, galactosidases, glucanases, glucose oxidases, GTPases, helicases, hemicellulases, integrases, invertases, isomerases, kinases, lactases, lipases, lipoxygenases, lyszymes, pectinesterases, peroxidases, phosphatas, phospholipases, phosphorylases, polygalacturonases, proteinases and peptidases, pullulanases, recombinases, reverse transcriptases, topoisomerases, and xylanases.

[0582] In addition to in vivo gene inhibition, the skilled artisan will appreciate that the iRNA agent of the present invention is useful in a wide variety of in vitro applications. Such in vitro applications, include, for example, scientific and commercial research (e.g., elucidation of physiological pathways, drug discovery and development), and medical and veterinary diagnostics. In general, the method involves the introduction of the iRNA agent into a cell using known techniques (e.g., absorption through cellular processes, or by auxiliary agents or devices, such as electroporation and lipofection), then maintaining the cell for a time sufficient to obtain degradation of an mRNA transcript of the target gene.


[0584] In yet another aspect, the invention relates to methods for identifying iRNA agent having increased stability in biological tissues and fluids such as serum. iRNA agent having increased stability have enhanced resistance to degradation, e.g., by chemicals or nucleases (particularly endonucleases) which normally degrade RNA molecules. Methods for detecting increases in nucleic acid stability are well known in the art. Any assay capable of measuring or detecting differences between a test iRNA agent and a
control iRNA agent in any measurable physical parameter may be suitable for use in the methods of the present invention. In general, because the inhibitory effect of an iRNA agent on a target gene activity or expression requires that the molecule remain intact, the stability of a particular iRNA agent can be evaluated indirectly by observing or measuring a property associated with the expression of the gene. Thus, the relative stability of an iRNA agent can be determined by observing or detecting (1) an absence or observable decrease in the level of the protein encoded by the target gene, (2) an absence or observable decrease in the level of mRNA product from the target gene, and (3) a change or loss in phenotype associated with expression of the target gene. In the context of a medical treatment, the stability of an iRNA agent may be evaluated based on the degree of the inhibition of expression or function of the target gene, which in turn may be assessed based on a change in the disease condition of the patient, such as reduction in symptoms, remission, or a change in disease state.

[0585] In one embodiment, the method includes preparing an iRNA agent as described above (e.g., through chemical synthesis), incubating the iRNA agent with a biological sample, then analyzing and identifying those iRNA agent that exhibit an increased stability as compared to a control iRNA agent.

[0586] In an exemplified embodiment, iRNA agent is produced in vitro by mixing/annealing complementary single-stranded RNA strands, preferably in a molar ratio of at least about 3:7, more preferably in a molar ratio of about 4:6, and most preferably in essentially equal molar amounts (e.g., a molar ratio of about 5:5). Preferably, the single-stranded RNA strands are denatured prior to mixing/annealing, and the buffer in which the mixing/annealing reaction takes place contains a salt, preferably potassium chloride. Single-stranded RNA strands may be synthesized by solid phase synthesis using, for example, an Expedite 8090 synthesizer (Applied Biosystems, Applied Deutschland GmbH, Darmstadt, Germany), as described above.

[0587] iRNA agent are incubated with a biological sample under the conditions sufficient or optimal for enzymatic function. After incubating with a biological sample, the stability of the iRNA agent is analyzed by means conventional in the art, for example using RNA gel electrophoresis as exemplified herein. For example, when the sample is serum, the iRNA agent may be incubated at a concentration of 1-10 μM, preferably 2-8 μM, more preferably 3-6 μM, and most preferably 4-5 μM. The incubation temperature is preferably between 25° C. and 45° C., more preferably between 35° C. and 40° C., and most preferably about 37° C.

[0588] The biological sample used in the incubation step may be derived from tissues, cells, biological fluids or isolates thereof. For example, the biological sample may be isolated from a subject, such as a whole organism or a subset of its tissues or cells. The biological sample may also be a component part of the subject, such as a body fluid, including but not limited to blood, serum, plasma, mucus, lymphatic fluid, synovial fluid, cerebrospinal fluid, saliva, amniotic fluid, amniotic cord blood, urine, vaginal fluid and semen. Preferably, the biological sample is a serum derived from a blood sample of a subject. The subject is preferably a mammal, more preferably a human or a mouse.

[0589] In another embodiment, the method includes selecting an iRNA agent having increased stability by measuring the mRNA and/or protein expression levels of a target gene in a cell following introduction of the iRNA agent. In this embodiment, an iRNA agent of the invention inhibits expression of a target gene in a cell, and thus the method includes selecting an iRNA agent that induces a measurable reduction in expression of a target gene as compared to a control iRNA agent. Assays that measure gene expression by monitoring RNA and/or protein levels can be performed within about 24 hours following uptake of the iRNA agent by the cell. For example, RNA levels can be measured by Northern blot techniques, RNase Protection Assays, or Quality Control-PCR (QC-PCR) (including quantitative reverse transcription coupled PCR (RT-PCR) and analogous methods known in the art). Protein levels can be assayed, for example, by Western blot techniques, flow cytometry, or reporter gene expression (e.g., expression of a fluorescent reporter protein, such as green fluorescent protein (GFP)). RNA and/or protein levels resulting from target gene expression can be measured at regular time intervals following introduction of the test iRNA agent, and the levels are compared to those following introduction of a control iRNA agent into cells. A control iRNA agent can be a nonsensical iRNA agent (i.e., an iRNA agent having a scrambled sequence that does not target any nucleotide sequence in the subject), an iRNA agent that can target a gene not present in the subject (e.g., a luciferase gene, when the iRNA agent is tested in human cells), or an iRNA agent otherwise previously shown to be ineffective at silencing the target gene. The mRNA and protein levels of the test sample and the control sample can be compared. The test iRNA agent is selected as having increased stability when there is a measurable reduction in expression levels following absorption of the test iRNA agent as compared to the control iRNA agent. mRNA and protein measurements can be made using any art-recognized technique (see, e.g., Chiang, M. Y., et al., J. Biol. Chem. (1991) 266:18162-71; Fisher, T. et al., Nucl. Acids Res. (1993) 21:3857; and Chen et al., J. Biol. Chem. (1996)271:28259).

[0590] The ability of an iRNA agent composition of the invention to inhibit gene expression can be measured using a variety of techniques known in the art. For example, Northern blot analysis can be used to measure the presence of RNA encoding a target protein. The level of the specific mRNA produced by the target gene can be measured, e.g., using RT-PCR. Because iRNA agent directs the sequence-specific degradation of endogenous mRNA through RNAs, the selection methods of the invention encompass any technique that is capable of detecting a measurable reduction in the target RNA. In yet another example, Western blots can be used to measure the amount of target protein present. In still another embodiment, a phenotype influenced by the amount of the protein can be detected. Techniques for performing Western blots are well known in the art (see, e.g., Chen, et al., J. Biol. Chem. (1996) 271:28259).

[0591] When the target gene is to be silenced by an iRNA agent that targets a promoter sequence of the target gene, the target gene can be fused to a reporter gene, and reporter gene expression (e.g., transcription and/or translation) can be monitored. Similarly, when the target gene is to be silenced by an iRNA agent that targets a sequence other than a promoter, a portion of the target gene (e.g., a portion including the target sequence) can be fused with a reporter
gene so that the reporter gene is transcribed. By monitoring a change in the expression of the reporter gene in the presence of the siRNA agent, it is possible to determine the effectiveness of the siRNA agent in inhibiting the expression of the reporter gene. The expression levels of the reporter gene in the presence of the test siRNA agent versus a control siRNA agent are then compared. The test siRNA agent is selected as having increased stability when there is a measurable reduction in expression levels of the reporter gene as compared to the control siRNA agent. Examples of reporter genes useful for use in the present invention include, without limitation, those coding for luciferase, GFP, chloramphenicol acetyl transferase (CAT), β-galactosidase, and alkaline phosphatase. Suitable reporter genes are described, for example, in Current Protocols in Molecular Biology, John Wiley & Sons, New York (Ausubel, F. A., et al. eds., 1989); Gould, S. J., and S. Subramani, Anal. Biochem. (1988) 7:404-408; Gorman, C. M., et al., Mol. Cell. Biol. (1982) 2:1044-1051; and Selden, R., et al., Mol. Cell. Biol. (1986) 6:3173-3179; each of which is hereby incorporated by reference.

EXAMPLES

Example 1

Terminal Modifications Protect Sites of Exonuclease Cleavage

Serum Incubation Assay

Blood of 8 human volunteers (270 mL) was collected and kept at room temperature for 3 hours. The blood pool was then centrifuged at 20°C and 3000 x g using MegaFreeze 1.0 (Heraeus Instruments, Kendro Laboratory Products GmbH, Langenselbold) to separate the serum from the cellular fraction. The supernatant was stored in aliquots at -20°C and used as needed. Human serum obtained from Sigma (Sigma-Aldrich Chemie GmbH, Taufkirchen, Germany) was employed in control assays. Assay results reported herein were consistent among the different serum sources tested.

Double stranded RNAs (300 pmol, ca. 4.2 μg) were incubated at 37°C in 60 μl 90% human serum/10% phosphate buffered saline for 0, 15, 30, 60, 120 or 240 minutes, or 8 hours, 16 hours or 24 hours. A 10 μl sample was taken from the incubation mixture, frozen in liquid nitrogen and stored at -80°C until analysis.

Analysis by Electrophoresis and “Stains all” Detection

For analysis, samples were thawed, their constituents were isolated by phenol-extraction and ethanol-precipitation, separated on denaturing 14% polyacrylamide gels (6M Urea, 20% formamide, Carl Roth GmbH & Co KG Karlsruhe, Germany) and detected by staining with the “stains-all” reagent (Sigma-Aldrich Chemie GmbH, Steinheim, Germany). Reaction mixtures from incubation of selected dsRNAs were further analysed covering the time points 8 hours, 16 hours and 24 hours.

Cell Culture

HeLa SS6 cells were grown at 37° C. in Dulbecco’s modified Eagle medium (DMEM) supplemented with 10% fetal bovine serum (FBS), 100 units/mL penicillin, and 100 μg/mL streptomycin (Invitrogen, Carlsbad, Calif.). Cells were passaged regularly to maintain exponential growth. Twenty-four hours prior to siRNA transfection, cells were seeded on opaque, white 96-well plates (Costar, Corning, N.Y.) at a concentration of 15,000 cells/well in 150 μL antibiotic-free, phenol red-free DMEM (Invitrogen).

Dual Luciferase Gene Silencing Assays

In vitro activity of siRNAs was determined using a high-throughput 96-well plate format luciferase silencing assay. Cells were first transiently transfected with plasmids encoding firefly (target) and renilla (control) luciferase. DNA transfections were performed using Lipofectamine 2000 (Invitrogen) (0.5 μL/μg total DNA) and the plasmids gWiz-Luc (Aldevron, Fargo, N.Dak.) (200 ng/well) and pRL-CMV (Promega, Madison, Wis.) (200 ng/well). After 2 h, the plasmid transfection medium was removed, and the firefly luciferase targeting siRNAs were added to the cells at 100 nM concentration. siRNA transfections were performed using TransIT-TKO (Mirus, Madison, Wis.) (0.3 mL/well). After 24 h, cells were analyzed for both firefly and renilla luciferase expression using a plate luminometer (VICTOR2, PerkinElmer, Boston, Mass.) and the Dual-Glo Luciferase Assay Kit (Promega). Firefly/renilla luciferase expression ratios were used to determine percent gene silencing relative to mock-treated (no siRNA) controls.

RESULTS

dsRNAs including phosphorothioate modifications were incubated in 90% human serum at 37° C. as described above. Gel electrophoresis of the RNA products revealed that the phosphorothioate modifications enhanced stability (Fig. 1). Similar results were observed when 3’-Naproxen, 5-Nitroindole, alkyaminod-T, abasic pyrroldine, and cationic C6 amino dT were conjugated to the 3’ end of dsRNAs (Fig. 8, 2, 3, and 4).

The dual luciferase assay revealed that modifications than enhanced resistance to exonuclease activity retained silencing activity. FIGS. 5, 6, and 7 show the silencing activity of 3’ conjugated Naproxen, abasic pyrroldine, and cationic C6 amino dT, respectively.

FIGS. 8 and 9 show that the 3’Naproxen conjugated dsRNAs had enhanced exonuclease resistance in mouse serum and liver tissue, as well as in human serum (see FIG. 2).

Example 2

siRNA Modifications Enhanced Duplex Stability

Radiolabel method for monitoring serum stability of siRNA duplexes: siRNA duplexes were prepared at a
stock concentration of 1 μM in which either the sense (S) or antisense strand (AS) contained a trace amount of 5'-32P labeled material (e.g. 32P-S/AS and S/32P-AS). The presence of the end-labeled sense or antisense strand allowed for monitoring of the individual strand within the context of the siRNA duplex. Therefore, two duplex preparations were made for each siRNA sequence tested. siRNA duplexes were incubated in 90% human serum at a final concentration of 100 nM duplex. Samples were removed and quenched in a stop mix at appropriate times. For a typical time course, 10 seconds, 15 minutes, 30 minutes, 1 hour, 2 hours and 4 hours points were taken. Samples were analyzed by denaturing polyacrylamide gel electrophoresis along with a control sample (4 hour buffer-alone incubation) and a partial alkaline hydrolysis ladder of the labeled sense or antisense strand as a marker. The gel was imaged using a Fuji phosphorimager to detect the full length sense and antisense strands along with any degradation fragments that were generated by serum nucleases during incubation.

Since there is the possibility of losing the 5' phosphate label due to phosphatase activity in the serum, an alternative to 5' end labeling is to place an internal 32P or 32P label within either the sense or antisense strand. This labeling method is much more laborious than 5' end labeling and currently we have no evidence that dephosphorylation occurs during serum incubation.

A series of chemical modifications that fall into the following categories; backbone modification, sugar modification, nucleobase modification and 3' conjugate, were tested and showed enhanced serum stability as compared to a unmodified siRNA duplex. A description of each modification, its location within the siRNA duplex, and the serum stability data follows.

Serum stability of unmodified parent duplex: The unmodified parent duplex, AL-DUP-1000, was used to establish the serum stability baseline for evaluating the effect of chemical modifications on nuclease resistance.

AL-DUP-1000
5'-CUUACUGAGUCUCUGAATdTcT-3'  ALN-SEQ-1000  SEQ ID NO: 3
3'dToTGAAUCUGACUCUGAAGCU-5'  ALN-SEQ-1001  SEQ ID NO:

AL-DUP-1000 was subjected to the serum stability assay to evaluate its inherent nuclease resistance and to define its degradation pattern (FIG. 21). Denaturing gel electrophoresis was use analyze AL-DUP-1000 in a human serum stability assay. An siRNA duplex containing 5' end-labeled sense RNA (s/s) and a duplex containing 5' end-labeled antisense RNA (as/s) were each incubated in 90% human serum and time points were assayed at 10 seconds, 5 min, 15 min, 30 min, 1 hour, 2 hours and 4 hours. The control was a 4 hour time point for siRNA duplex incubated in PBS buffer alone, OH-was the partial alkaline hydrolysis marker. This unmodified duplex was observed to be degraded by both 3'-5' exonucleases and endonucleases (FIG. 21).

Cleavage of the 3' end of both the sense and antisense strands by 3'-5' exonucleases occurs within the first 5 minutes of incubation resulting in the loss of the 3' terminal dT residues (top vertical lines in s/s and s/as panels of FIG. 21). In addition to exonuclease degradation, both strands were cleaved by endonucleases. There was a major endonuclease site at position sixteen of the antisense strand (bottom vertical lines in s/as and s/as* panels of FIG. 21) that appears as early as 10 seconds. Very little full length sense or antisense strand was remaining after 1 hour in human serum. Chemical modifications were introduced within the context of the parent duplex to evaluate their effect on nuclease resistance. These chemical modifications fall within one of the following classes: backbone modification, sugar modification, nucleobase modification, cationic modification and conjugate.

Backbone modifications enhanced nuclease resistance: Specific phosphodiester linkages of the siRNA duplex were replaced by either phosphorothioate or methyolphosphonate and their stability was evaluated in the human serum stability assay. Table 1 contains the sequences of the duplexes tested. Substitution of the phosphodiester linkage at the 3' end of both the sense and antisense strands inhibited exonucleolytic degradation of the 3' overhangs (FIGS. 22A and 22B) as compared to the unmodified parent duplex (refer to FIG. 21). Full length starting material was present for four hours for both the sense and antisense strands. The endonucleolytic cleavage pattern seen in the unmodified duplex was unchanged. Similar results were obtained for duplexes that contained additional phosphorothioates at their 3' ends (data not shown). The placement of phosphorothioates at the endonucleolytic cleavage sites (duplexes 1419, 1420 and 1421) did not inhibit endonucleolytic cleavage at these sites (data not shown). In summary, a single phosphorothioate or methylyphosphonate between the two 3' terminal nucleotides was sufficient to protect the 3' ends from exonuclease degradation. Additional phosphorothioates at the 3' ends appear to enhance this effect, which may be necessary for long term exposure to serum nucleases.

Table 1

<table>
<thead>
<tr>
<th>siRNA duplexes containing backbone modifications.</th>
<th>(s = phosphorothioate, mp = methylyphosphonate)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Alynylam Duplex Duplex Sequence</td>
<td>Alynylam Sequence</td>
</tr>
<tr>
<td>AL-DUP-1393 5'-CUUACCGUGAGUCUCUGAATdTcT-3'</td>
<td>ALN-SEQ-1026</td>
</tr>
<tr>
<td>3'-dTcTGAUUGGACUCUAAGGCU-5'</td>
<td>ALN-SEQ-1027</td>
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</table>

Jun. 2, 2005
**TABLE 1-continued**

<table>
<thead>
<tr>
<th>Alnylam Duplex Sequence</th>
<th>Alnylam Duplex Sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>AL-DUP-1394 5'-CUUACCUAGACUCUGAAUACU*G+*dT+*dT-3' 3'-*dT-*dT+GAUCCGACUCAUGAAGCU-5'</td>
<td>AL-SEQ-1028</td>
</tr>
<tr>
<td>AL-DUP-1395 5'-CUUACCUAGACUCUGAAUACU*G+*dT+*dT-3' 3'-*dT-*dT+GAUCCGACUCAUGAAGCU-5'</td>
<td>AL-SEQ-1030</td>
</tr>
<tr>
<td>AL-DUP-1396 5'-CUUACCUAGACUCUGAAUACU*G+*dT+*dT-3' 3'-*dT-*dT+GAUCCGACUCAUGAAGCU-5'</td>
<td>AL-SEQ-1031</td>
</tr>
<tr>
<td>AL-DUP-1419 5'-CUUACCUAGACUCUGAAUACU*G+*dT+*dT-3' 3'-*dT-*dT+GAUCCGACUCAUGAAGCU-5'</td>
<td>AL-SEQ-1032</td>
</tr>
<tr>
<td>AL-DUP-1420 5'-CUUACCUAGACUCUGAAUACU*G+*dT+*dT-3' 3'-*dT-*dT+GAUCCGACUCAUGAAGCU-5'</td>
<td>AL-SEQ-1033</td>
</tr>
<tr>
<td>AL-DUP-1421 5'-CUUACCUAGACUCUGAAUACU*G+*dT+*dT-3' 3'-*dT-*dT+GAUCCGACUCAUGAAGCU-5'</td>
<td>AL-SEQ-1034</td>
</tr>
<tr>
<td>AL-DUP-1329 5'-CUUACCUAGACUCUGAAUACU*G+*dT+*dT-3' 3'-*dT-*dT+GAUCCGACUCAUGAAGCU-5'</td>
<td>AL-SEQ-1035</td>
</tr>
</tbody>
</table>

**TABLE 2**

<table>
<thead>
<tr>
<th>Alnylam Duplex Sequence</th>
<th>Alnylam Duplex Sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>AL-DUP-1027 5'-CUUACCUAGACUCUGAAUACU*G+*dT+*dT-3' 3'-*dT-*dT+GAUCCGACUCAUGAAGCU-5'</td>
<td>AL-SEQ-1006</td>
</tr>
<tr>
<td>AL-DUP-1036 5'-CUUACCUAGACUCUGAAUACU*G+*dT+*dT-3' 3'-*dT-*dT+GAUCCGACUCAUGAAGCU-5'</td>
<td>AL-SEQ-1008</td>
</tr>
<tr>
<td>AL-DUP-13ff 5'-CUUACCUAGACUCUGAAUACU*G+*dT+*dT-3' 3'-*dT-*dT+GAUCCGACUCAUGAAGCU-5'</td>
<td>AL-SEQ-gggg</td>
</tr>
<tr>
<td>AL-DUP-1363 5'-CUUACCUAGACUCUGAAUACU*G+*dT+*dT-3' 3'-*dT-*dT+GAUCCGACUCAUGAAGCU-5'</td>
<td>AL-SEQ-1162</td>
</tr>
</tbody>
</table>

[0613] Sugar modifications enhanced nuclease resistance: The effect of replacing the 2'OH with 2'OMe was evaluated at the sites of endonucleolytic cleavage as well as at the 3' ends of the siRNA duplex. The duplexes tested in the human serum stability assay are shown in Table 2. Some of these duplexes also contained phosphorothioate linkages to evaluate whether the combination of the two modifications enhance nuclease resistance more significantly. Substitution of the terminal dT residues with 2'OMe-U (AL-DUP-1027) reduced 3'-5' exonuclease degradation slightly over the unmodified parent duplex (data not shown); however, the extent of exocleavase protection by 2'OMe-U was far less than that achieved by placing a phosphorothioate between the two terminal dT residues (see FIG. 22A). Addition of a single phosphorothioate between the two terminal 2'OMe-uridine residues effectively inhibited 3'-5' exonucleolytic cleavage as seen in FIG. 23 for duplexes AL-DUP-1036, AL-DUP-13ff, and AL-DUP-1363. 2'OMe substitution on its own was much more effective at protecting from endonucleolytic cleavage when placed at the internal cleavage sites. The parent duplex was cleaved 3' of U at two UpA sites within the duplex. Both strand cleavage sites are due to the symmetry of this dinucleotide repeat and mapping data was used to confirm the sites of cleavage (data not shown). Placement of 2'OMe at the strong endonucleolytic site (FIG. 23, star in s' as gel, AL-DUP-13ff) resulted in inhibition of cleavage at this site. The second, weaker endonucleolytic site (FIG. 23, black star in s' as gel), however, was slightly enhanced when the strong site was protected with 2'OMe (FIG. 23, compare AL-DUP-13ff to AL-DUP-1036). Protection of both sites with 2'OMe (AL-DUP-1363) resulted in reduced endonucleolytic cleavage at both sites (FIG. 23). The inhibitory effect of the 2'OMe substitution is consistent with the mechanism of endonucleolytic cleavage, which requires the 2'OH as a nucleophile in the cleavage reaction. 2'OMe modification will also be an effective means to protect the 3' overhang of single overhang siRNA duplexes where the 3' overhang is composed of ribonucleotides. In this situation, 2'OMe substitution can be used to block the possible loss of the
terminal two nucleotides by endonucleolytic cleavage and phosphorothioate can be used to protect from exonuclease degradation.

[0615] Cationic modifications enhanced nuclease resistance: The effect of three different cationic chemical modifications on nuclease resistance was tested and compared to the parent unmodified duplex. The structures of the three cationic modifications tested are shown below.

![Diagram of cationic modifications](image)

**TABLE 3** siRNA duplexes containing cationic substitutions.

<table>
<thead>
<tr>
<th>Alkylamino Duplex</th>
<th>Duplex Sequence</th>
<th>Alkylam Sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>AL-DUP-10aa</td>
<td>5'-CUAUGCUGAGUACUCUUCGAdTaaT-3'</td>
<td>AL-SEQ-1017</td>
</tr>
<tr>
<td></td>
<td>3'-aadTdTGAUGCCUCUGCAGACCU-5'</td>
<td>AL-SEQ-1018</td>
</tr>
<tr>
<td>AL-DUP-10bb</td>
<td>5'-CUAUGCUGAGUACUCUUCGAdTaaT-3'</td>
<td>AL-SEQ-1015</td>
</tr>
<tr>
<td></td>
<td>3'-aadTdTGAUGCCUCUGCAGACCU-5'</td>
<td>AL-SEQ-1016</td>
</tr>
<tr>
<td>AL-DUP-1ccc</td>
<td>5'-CUAUGCUGAGUACUCUUCGAdTaaT-3'</td>
<td>AL-SEQ-dkkk</td>
</tr>
<tr>
<td></td>
<td>3'-aadTdTGAUGCCUCUGCAGACCU-5'</td>
<td>AL-SEQ-dkdd</td>
</tr>
<tr>
<td>AL-DUP-1403</td>
<td>5'-C<em>ValUCCCGAGUACUCUUCGAAU</em>U-3'</td>
<td>AL-SEQ-2080</td>
</tr>
<tr>
<td></td>
<td>3'-U<em>GAGaaUCCCGAGUACUCUUCGAAU</em>U-5'</td>
<td>AL-SEQ-2081</td>
</tr>
<tr>
<td>AL-DUP-1406</td>
<td>5'-C<em>ValUCCCGAGUACUCUUCGAAU</em>U-3'</td>
<td>AL-SEQ-2082</td>
</tr>
<tr>
<td></td>
<td>3'-U<em>GAGaaUCCCGAGUACUCUUCGAAU</em>U-5'</td>
<td>AL-SEQ-2083</td>
</tr>
</tbody>
</table>

[0616] The sequences of the duplexes assayed in the human serum stability assay are shown in Table 3. Both alkylamino-dT and abasic pyrroline cationic modifications were placed at the 3' terminal overhang to evaluate their effect on 3'-5' exonuclease degradation. Alkylamino-uridines were placed at the internal endonucleolytic cleavage sites to evaluate their ability to inhibit endonucleolytic cleavage. As seen in FIG. 24, replacing the 3' terminal dT residue with a single alkylamino-dT efficiently inhibited 3'-5' exonucleolytic degradation (FIG. 24, AL-DUP-10aa, left gel image). Replacement of both dT residues in the overhang with alkylamino-dT resulted in a similar extent of inhibition (data not shown). Addition of an abasic pyrroline cationic modification at the 3' terminus of each strand also protected against exonuclease degradation (FIG. 24, middle gel image). Both the alkylamino-dT and abasic pyrroline modifications protected from 3'-5' exonucleolytic cleavage up to 24 hours (data not shown). Placement of alkylamino-U at the internal cleavage sites inhibited endonucleolytic cleavage as shown in FIG. 24 for duplex AL-DUP-1403.

The ends of this duplex were stabilized from exonuclease degradation by 2'OMe-U and phosphorothioate substitutions in order to separate the two different cleavage events. Endonucleolytic cleavage was inhibited at both internal cleavage sites by alkylamino-U substitution for AL-DUP-1406 (data not shown).

[0617] 3' conjugates enhanced nuclease resistance: Conjugation of naproxen and ibuprofen to the 3' end of the
siRNA were tested for their ability to inhibit 3'-5' exonucleolytic degradation. The structure of naproxen is shown in below:

[0618] Table 4 lists the siRNAs that were tested in the human serum stability assay. Conjugation of either naproxen or ibuprofen to the 3' end inhibited exonucleolytic degradation. FIG. 18 shows the serum stability data for the naproxen modified duplex (AL-DUP-1069) and similar results were obtained for AL-DUP1413. Presumably the conjugates inhibit exonuclease cleavage by sterically blocking the exonuclease from binding to the 3' end of the siRNA duplex. Similar data was also obtained for AL-DUP-1069 in pooled mouse serum.

<table>
<thead>
<tr>
<th>Table 4</th>
</tr>
</thead>
<tbody>
<tr>
<td>siRNA duplexes containing 3 conjugates.</td>
</tr>
<tr>
<td>(Nap = Naproxen, Ibu = Ibuprofen)</td>
</tr>
<tr>
<td>AL-DUP-1069</td>
</tr>
<tr>
<td></td>
</tr>
<tr>
<td>AL-DUP-1413</td>
</tr>
<tr>
<td></td>
</tr>
</tbody>
</table>

[0619] A number of embodiments of the invention have been described. Nevertheless, it will be understood that various modifications may be made without departing from the spirit and scope of the invention. Other embodiments are in the claims.

What is claimed is:

1. An iRNA agent comprising at least one ribonucleotide modification, wherein the modification confers improved exonuclease stability to the iRNA agent when the iRNA agent is contacted with a biological sample.
2. The iRNA agent of claim 1, wherein the iRNA agent comprises at least two ribonucleotide modifications.
3. The iRNA agent of claim 1, wherein the iRNA agent comprises at least three ribonucleotide modifications.
4. The iRNA agent of claim 1, wherein the iRNA agent comprises at least four ribonucleotide modifications.
5. The iRNA agent of claim 1, wherein the iRNA agent comprises at least five ribonucleotide modifications.
6. The iRNA agent of claim 1, wherein biological sample comprises serum.
7. The iRNA agent of claim 1, wherein the modification comprises a backbone modification between one or more of the terminal nucleotides on an iRNA strand of the iRNA agent.
8. The iRNA agent of claim 7, wherein the backbone modification comprises a phosphorothioate linkage between one or more of the terminal nucleotides of an iRNA agent.
9. The iRNA agent of claim 1, wherein the modification comprises a modification of a nucleotide sugar.
10. The iRNA agent of claim 9, wherein the nucleotide is a terminal nucleotide.
11. The iRNA agent of claim 10, wherein the terminal nucleotide comprises a 2' or 3' sugar modification.
12. The iRNA agent of claim 10, wherein the iRNA agent comprises a 2'-O-methylated nucleotide, a 2'-deoxygenated nucleotide, a 2'-deoxyfluoro nucleotide, a 2'-methoxyethyl nucleotide, a 2'-MOE nucleotide, a 2'-DMAEOE nucleotide, a 2'-AP nucleotide, a 2'-hydroxy nucleotide, a 2'-arabino nucleotide, a locked nucleic acid, an extended nucleic acid, hexose nucleic acid, or cyclohexene nucleic acid.
13. The iRNA agent of claim 12, wherein the iRNA agent comprises a 2'-O-methylated nucleotide.
14. The iRNA agent of claim 12, wherein the iRNA agent comprises a 2'-deoxyfluoro nucleotide.
15. The iRNA agent of claim 12, wherein the iRNA agent comprises a locked nucleic acid and the locked nucleic acid is on a 5' or 3' end of a sense strand of the iRNA agent, and not on a 5' or 3' end of an antisense strand of the iRNA agent.
26. The iRNA agent of claim 1, wherein the iRNA agent comprises a terminal nucleotide having a nucleobase modification and a terminal nucleotide having a 2' or 3' sugar modification.

27. The iRNA agent of claim 1, wherein the iRNA agent comprises at least one conjugate.

28. The iRNA agent of claim 27, wherein the conjugate is attached to one or more terminal nucleotides of the iRNA agent.

29. The iRNA agent of claim 27, wherein the conjugate is selected from a lipophile, a terpen, a protein binding agent, a vitamin, a carbohydrate, or a peptide.

30. The iRNA agent of claim 27, wherein the conjugate is on a 3' end of an antisense strand of the iRNA agent, or on a 5' or 3' end of a sense strand on the iRNA agent.

31. The iRNA agent of claim 30, the conjugate is not on the 3' end of the antisense strand and on the 3' end of the sense strand.

32. The iRNA agent of claim 27, wherein conjugate is naproxen.

33. The iRNA agent of claim 27, wherein the conjugate is cholesterol.

34. The iRNA agent of claim 27, wherein the conjugate is cholic acid.