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[Continued on next page]

(54) Title: CHEMICALLY MODIFIED OLIGONUCLEOTIDES AND SMALL MOLECULES FOR USE IN REDUCING MICRO RNA ACTIVITY LEVELS AND USES THEREOF

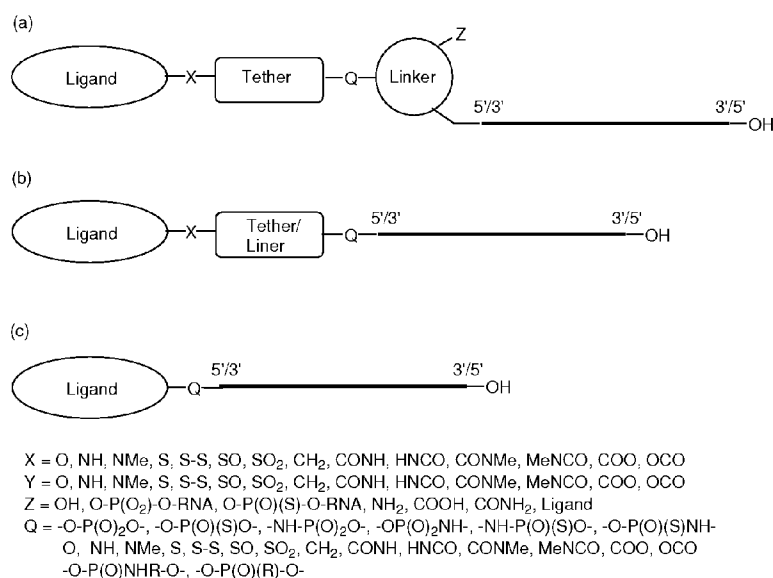


FIG. 5

(57) Abstract: This invention relates generally to chemically modified oligonucleotides useful for modulating activity of microRNAs and pre-microRNAs. More particularly, the invention relates to single stranded chemically modified oligonucleotides for inhibiting microRNA and pre-microRNA activity and to methods of making and using the modified oligonucleotides.

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CHEMICALLY MODIFIED OLIGONUCLEOTIDES AND SMALL MOLECULES FOR USE IN REDUCING MICRO RNA ACTIVITY LEVELS AND USES THEREOF

RELATED APPLICATIONS

5 This application claims the benefit of priority to United States Provisional Patent Application serial number 61/022,299, filed January 18, 2008.

TECHNICAL FIELD

 This invention relates generally to chemically modified oligonucleotides (antagomirs) and small molecules useful for minimizing the activity level of microRNAs. More particularly, the invention relates to single stranded, double stranded, partially double stranded and hairpin structured chemically modified oligonucleotides for minimizing the activity level of microRNAs and to methods of making and using the modified oligonucleotides and small molecules.

BACKGROUND

 A variety of nucleic acid species are capable of modifying gene expression. These include antisense RNA, siRNA, microRNA, RNA and DNA aptamers, and decoy RNAs. Each of these nucleic acid species can inhibit target nucleic acid activity, including gene expression.

20 MicroRNAs (miRNAs) are a class of 18-24 nt non-coding RNAs (ncRNAs) that exist in a variety of organisms, including mammals, and are conserved in evolution. miRNAs are processed from hairpin precursors of 70 nt (pre-miRNA) which are derived from primary transcripts (pri-miRNA) through sequential cleavage by the RNase III enzymes drosha and dicer. miRNAs can be encoded in intergenic regions, hosted within introns of pre-mRNAs or within ncRNA genes. Many microRNAs can be encoded in intergenic regions, hosted within introns of pre-mRNAs or within ncRNA genes. Many miRNAs also tend to be clustered and transcribed as polycistrons and often have similar spatial temporal expression patterns. MiRNAs have been found to have roles in a variety of biological processes including developmental timing, differentiation, apoptosis, cell proliferation, organ development, and metabolism.

30

SUMMARY

The present invention is based in part on the discovery that level of activity of endogenous microRNAs (miRNAs) or pre-microRNAs (pre-miRNAs) can be inhibited by an agent herein defined as an antagomir or by a small molecule, *e.g.*, through systemic administration of the antagomir or small molecule, as well as by parenteral administration of such agents. Embodiments of the invention provide specific compositions and methods that are useful in reducing miRNA and pre-miRNA activity levels, in *e.g.*, a mammal, such as a human. In particular, the present invention provides specific compositions and methods that are useful for reducing activity levels of the miRNAs miR-122, miR-16, miR-192, miR-194, miR-141, miR-143, miR-181, miR-181a, miR-181c, miR-192, miR-194, miR-200c, miR-206, miR-1, miR-205, miR-16, miR ebv-BHRF1-1, miR ebv-BHRF1-2, miR ebv-BHRF12-1, miR kshv-K3, miR kshv-K4-3p, miR kshv-mir-K2, miR kshv-mir-K5, miR kshv-mir-K6-3p, miR kshv-mir-K7, miR kshv-mir-K11, miR-31, miR-196, miR-215, miR-155, miR-142-5p, miR-142-3p, miR-143, Hsa-mir-146a, Hsa-mir-146b, mCMV-miR-01-1, mCMV-miR-01-2, mCMV-miR-23-1, mCMV-miR-23-2, mCMV-miR-44-1, miR-133, miR-133b, miR-124, miR-126, miR-126-3p, miR-126-5p, miR-21, miR-22, miR-122, or miR-33.

In one aspect, the invention features antagomirs. Antagomirs are single stranded, double stranded, partially double stranded and hairpin structured chemically modified oligonucleotides that target a microRNA. Figures 5-11 provides representative structures of antagomirs.

An antagomir consisting essentially of or including at least 12 or more contiguous nucleotides substantially complementary to an endogenous miRNA and more particularly agents that include 12 or more contiguous nucleotides substantially complementary to a target sequence of an miRNA or pre-miRNA nucleotide sequence. Preferably, an antagomir featured in the invention includes a nucleotide sequence sufficiently complementary to hybridize to a miRNA target sequence of about 12 to 25 nucleotides, preferably about 15 to 23 nucleotides. More preferably, the target sequence differs by no more than 1, 2, or 3 nucleotides from a sequence shown in Table 1, and in one embodiment, the antagomir is an agent shown in Table 2 or in Table 3. In one embodiment, the antagomir includes a non-nucleotide moiety, *e.g.*, a cholesterol moiety. The non-nucleotide moiety can be attached, *e.g.*, to the 3' or 5' end of the oligonucleotide agent. In a preferred embodiment, a cholesterol moiety is attached to the 3' end of the oligonucleotide agent.

An oligonucleotide agent featured herein, *e.g.*, an antagomir can be modified, for example, to provide increased stability against nucleolytic degradation. Exemplary

modifications include a modification of the nucleotide backbone such as modification of the phosphate linker or replacement of the phosphate linker; modification of the sugar moiety such as modification of the 2' hydroxyl on the ribose; replacement of the sugar moiety such as ribose or deoxyribose with a different chemical structure such as a PNA structure; or

5 modification of the nucleobase for example modification to a universal base or G-clamp. In some embodiments, the oligonucleotide agent includes a phosphorothioate in at least the first, second, or third internucleotide linkage at the 5' or 3' end of the nucleotide sequence. In one embodiment, the oligonucleotide agent includes a 2'-modified nucleotide, *e.g.*, a 2'-deoxy, 2'-deoxy-2'-fluoro, 2'-O-methyl, 2'-O-methoxyethyl (2'-O-MOE), 2'-O-aminopropyl (2'-O-AP),

10 2'-O-dimethylaminoethyl (2'-O-DMAOE), 2'-O-dimethylaminopropyl (2'-O-DMAP), 2'-O-dimethylaminoethoxyethyl (2'-O-DMAEOE), or 2'-O-N-methylacetamido (2'-O-NMA). In a particularly preferred embodiment, the oligonucleotide agent includes at least one 2'-O-methyl-modified nucleotide, and in some embodiments, all of the nucleotides of the oligonucleotide agent include a 2'-O-methyl modification.

15 The oligonucleotide agent can be further modified so as to be attached to a ligand, for example, a ligand selected to improve stability, distribution or cellular uptake of the agent, *e.g.*, cholesterol or folate. Exemplary lipophilic ligands include a cholesterol; a bile acid; and a fatty acid (*e.g.*, lithocholic-oleyl, lauroyl, docosyl, stearyl, palmitoyl, myristoyl, oleoyl, linoleoyl). In some preferred embodiments, the oligonucleotide agent is combined with a

20 targeting agent such as a folate moiety.

The oligonucleotide agent can further be in isolated form or can be part of a pharmaceutical composition used for the methods described herein, particularly as a pharmaceutical composition formulated for parental administration. The pharmaceutical compositions can contain one or more oligonucleotide agents, and in some embodiments, will

25 contain two or more oligonucleotide agents, each one directed to a different miRNA.

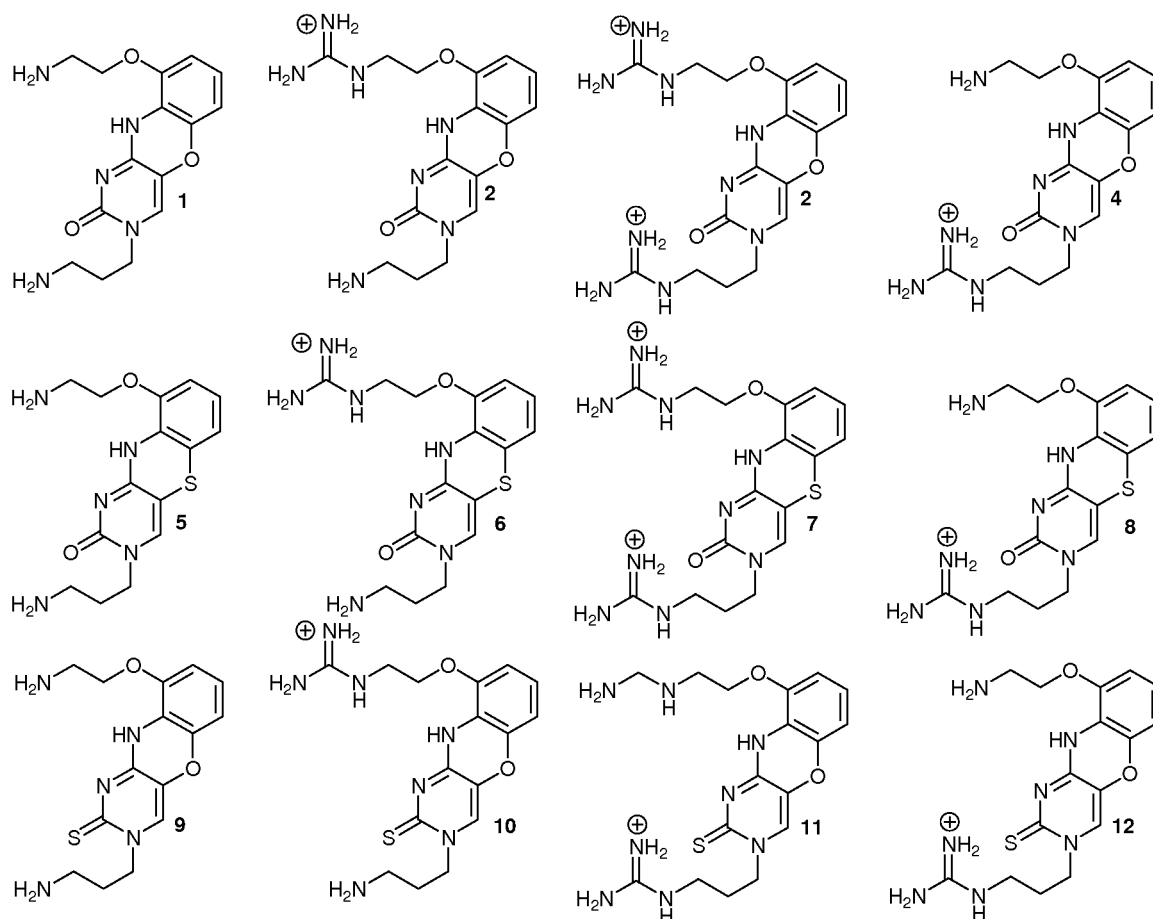
An antagomir that is substantially complementary to a nucleotide sequence of an miRNA can be delivered to a cell or a human to inhibit or reduce the activity level of an endogenous miRNA, such as when aberrant or undesired miRNA activity, or insufficient activity of a target mRNA that hybridizes to the endogenous miRNA, is linked to a disease or

30 disorder. In one embodiment, an antagomir featured in the invention has a nucleotide sequence that is substantially complementary to miR-122 (see Table 1), which hybridizes to numerous RNAs, including aldolase A mRNA, N-myc downstream regulated gene (Ndr3) mRNA, IQ motif containing GTPase activating protein-1 (Iqgap1) mRNA, HMG-CoA-reductase (Hmgcr) mRNA, and citrate synthase mRNA and others. In a preferred

embodiment, the antagomir that is substantially complementary to miR-122 is antagomir-122 (Table 2). Aldolase A deficiencies have been found to be associated with a variety of disorders, including hemolytic anemia, arthrogryposis complex congenita, pituitary ectopia, rhabdomyolysis, hyperkalemia. Humans suffering from aldolase A deficiencies also
5 experience symptoms that include growth and developmental retardation, midfacial hypoplasia, hepatomegaly, as well as myopathic symptoms. Thus a human who has or who is diagnosed as having any of these disorders or symptoms is a candidate to receive treatment with an antagomir that hybridizes to miR-122 and can receive such treatment.

In some embodiments, an antagomir featured in the invention has a nucleotide
10 sequence that is substantially complementary to miR-16, miR-192, miR-194, or other miRNA described herein.

In one aspect, the invention features a small molecule antagonist of an miRNA or pre-miRNA, e.g., miR-122. In certain embodiments, the small molecule antagonists is a molecule shown in exemplification 1.



Exemplification 1. RNA binding molecules proposed as microRNA antagonists

5

In one aspect, the invention features a method of reducing the levels of miRNA or pre-miRNA activity in a cell of a subject, *e.g.*, a human subject. The method includes the step of administering an antagomir to the subject, where the antagomir is substantially single-stranded and includes a sequence that is substantially complementary to 12 to 23 contiguous nucleotides, and preferably 15 to 23 contiguous nucleotides, of a target sequence of an miRNA or pre-miRNA nucleotide sequence. Preferably, the target sequence differs by no more than 1, 2, or 3 nucleotides from a microRNA or pre-microRNA sequence, such as a microRNA sequence shown in Table 1.

10

In one embodiment, the methods featured in the invention are useful for reducing the level of an endogenous miRNA (*e.g.*, miR-122, miR-16, miR-192 or miR-194) or pre-miRNA in a cell, *e.g.*, in a cell of a subject, such as a human subject. Such methods include contacting the cell with an antagomir described herein for a time sufficient to allow uptake of the antagomir into the cell.

15

In another aspect, the invention features a pharmaceutical composition including an antagomir described herein, and a pharmaceutically acceptable carrier. In a preferred embodiment, the antagomir included in the pharmaceutical composition hybridizes to miR-122, miR-16, miR-192, or miR-194.

5 In another aspect the invention features a method of inhibiting miRNA activity (*e.g.*, miR-122, miR-16, miR-192, or miR-194 activity) or pre-miRNA activity in a cell, *e.g.*, a cell of a subject. The method includes contacting the cell with an effective amount of an antagomir described herein, which is substantially complementary to the nucleotide sequence of the target miRNA or the target pre-miRNA. Such methods can be performed on a
10 mammalian subject by administering to a subject one of the oligonucleotide agents/pharmaceutical compositions described herein.

In another aspect the invention features a method of increasing levels of an RNA or protein that are encoded by a gene whose expression is down-regulated by an miRNA, *e.g.*, an endogenous miRNA, such as miR-122, miR-16, miR-192 or mir-194. The method
15 includes contacting the cell with an effective amount of an antagomir described herein, which is substantially complementary to the nucleotide sequence of the miRNA. While not wishing to be bound by theory it is believed that the antogmir binds to and effectively inhibits translation of the RNA transcribed from the gene. For example, the invention features a method of increasing aldolase A protein levels in a cell. Similarly, the invention features a
20 method of increasing Ndr3, Iqgap1, Hmgcr, and/or citrate synthase protein levels in a cell. The methods include contacting the cell with an effective amount of an antagomir described herein (*e.g.*, in Table 2 and Table 3), which is substantially complementary to the nucleotide sequence of miR-122 (see Table 1).

In another aspect, the invention provides methods of increasing expression of a target
25 gene by providing an antagomir to which a lipophilic moiety is conjugated, *e.g.*, a lipophilic conjugated antagomir described herein, to a cell. The antagomir preferably hybridizes to an miRNA (*e.g.*, miR-122, miR-16, miR-192, or miR-194) or a pre-miRNA. In a preferred embodiment the conjugated antagomir can be used to increase expression of a target gene in an organism, *e.g.*, a mammal, *e.g.*, a human, or to increase expression of a target gene in a
30 cell line or in cells which are outside an organism. An mRNA transcribed from the target gene hybridizes to an endogenous miRNA, which consequently results in downregulation of mRNA expression. An antagomir featured in the invention hybridizes to the endogenous miRNA and consequently causes an increase in mRNA expression. In the case of a whole organism, the method can be used to increase expression of a gene and treat a condition

associated with a low level of expression of the gene. For example, an antagomir that targets miR-122 (e.g., antagomir-122) can be used to increase expression of an aldolase A gene to treat a subject having, or at risk for developing, hemolytic anemia, arthrogryposis complex congenita, pituitary ectopia, rhabdomyolysis, hyperkalemia, or any other disorder associated with aldolase A deficiency. Administration of an antagomir that targets miR-122 (e.g., antagomir-122) can be also be used to increase expression of an Ndr3, Iqgap1, Hmgcr, or citrate synthase gene to treat a subject having, or at risk for developing, a disorder associated with a decreased expression of any one of these genes.

DESCRIPTION OF DRAWINGS

FIG 5. Ligand conjugated oligonucleotide to modulate activity of miRNA: (a) ligand of interest is conjugated to the oligonucleotide via a tether and linker; (b) ligand of interest is conjugated to the oligonucleotide via a linker without a tether or tether without an additional linker and (c) a ligand of interest is attached directly to the oligonucleotide.

FIG 6. Ligand conjugated double stranded oligonucleotide to modulate activity of miRNA: (a) ligand of interest is conjugated to the oligonucleotide via a tether and linker; (b) ligand of interest is conjugated to the oligonucleotide via a linker without a tether or tether without an additional linker and (c) a ligand of interest is attached directly to the oligonucleotide.

FIG 7. Ligand conjugated antisense strand comprising partially double stranded oligonucleotides to modulate activity of miRNA. (a-c) ligand of interest is conjugated to the oligonucleotide via a tether and linker; (d-f) ligand of interest is conjugated to the oligonucleotide via a linker without a tether or tether without an additional linker and (g-i) a ligand of interest is attached directly to the oligonucleotide.

FIG 8. Ligand conjugated partial sense strand comprising partially double stranded oligonucleotides to modulate activity of miRNA. (a-c) ligand of interest is conjugated to the oligonucleotide via a tether and linker; (d-f) ligand of interest is conjugated to the oligonucleotide via a linker without a tether or tether without an additional linker and (g-i) a ligand of interest is attached directly to the oligonucleotide.

FIG 9. Ligand conjugated partial hairpin oligonucleotides to modulate activity of miRNA. (a-b) ligand of interest is conjugated to either 3' or 5' end of the hairpin via a tether and linker; (c-d) ligand of interest is conjugated to the hairpin via a linker without a tether or

tether without an additional linker and (e-f) a ligand of interest is attached directly to the oligonucleotide. The hairpin is comprised of nucleotides or non-nucleotide linkages.

FIG 10. Ligand conjugated hairpin oligonucleotides to modulate activity of miRNA.

(a) ligand of interest is conjugated to either 3' or 5' end of the hairpin via a tether and linker;
 5 (b) ligand of interest is conjugated to the hairpin via a linker without a tether or tether without an additional linker and (c) a ligand of interest is attached directly to the oligonucleotide. The hairpin is comprised of nucleotides or non-nucleotide linkages.

FIG. 11. Cholesterol conjugated oligonucleotides to modulate activity of miRNA. (a)

5' cholesterol conjugate; (b) 3' cholesterol conjugate and (c) cholesterol conjugate building
 10 blocks for oligonucleotide synthesis. The oligonucleotide can be miRNA, anti-miRNA, chemically modified RNA or DNA; DNA or DNA analogues for antisense application.

DETAILED DESCRIPTION

15 The present invention is based in part on the discovery that activity levels of endogenous microRNAs (miRNAs) or pre-microRNAs (pre-miRNAs) can be inhibited by an antagomir, *e.g.*, through systemic administration of an antagomir, as well as by parenteral administration of such agents. Based on these findings, the present invention provides specific compositions and methods that are useful in reducing miRNA and pre-miRNA
 20 activity levels, in *e.g.*, a mammal, such as a human. In particular, the present invention provides specific compositions and methods that are useful for reducing levels of the miRNAs miR-122, miR-16, miR-192, and miR-194, herein defined as antagomirs.

In one aspect, the invention features antagomirs. An antagomir is a single-stranded, double stranded, partially double stranded or hairpin structured chemically modified
 25 oligonucleotide agents that consisting of, consisting essentially of or comprising at least 12 or more contiguous nucleotides substantially complementary to an endogenous miRNA and more particularly agents that include 12 or more contiguous nucleotides substantially complementary to a target sequence of an miRNA or pre-miRNA nucleotide sequence. As used herein partially double stranded refers to double stranded structures that contain less
 30 nucleotides than the complementary strand. In general, such partial double stranded agents will have less than 75% double stranded structure, preferably less than 50%, and more

preferably less than 25%, 20 % or 15% double stranded structure. Figures 5-11 provides representative structures of antagomirs.

Preferably, an antagomir featured in the invention includes a nucleotide sequence sufficiently complementary to hybridize to an miRNA target sequence of about 12 to 25 nucleotides, preferably about 15 to 23 nucleotides. More preferably, the target sequence differs by no more than 1, 2, or 3 nucleotides from a sequence shown in Table 1, and in one embodiment, the antagomir is an agent shown in Table 2 or Table 3. In one embodiment, the antagomir includes a non-nucleotide moiety, *e.g.*, a cholesterol moiety. The non-nucleotide moiety can be attached, *e.g.*, to the 3' or 5' end of the oligonucleotide agent. In a preferred embodiment, a cholesterol moiety is attached to the 3' end of the oligonucleotide agent.

In some embodiments, the oligonucleotide agent is modified, for example, to further stabilize against nucleolytic degradation. Exemplary modifications include a nucleotide base or modification of a sugar moiety. The oligonucleotide agent can include modified linker agent such as a phosphorothioate in at least the first, second, or third internucleotide linkage at the 5' or 3' end of the nucleotide sequence. In one embodiment, the oligonucleotide agent includes a 2'-modified nucleotide, *e.g.*, a 2'-deoxy, 2'-deoxy-2'-fluoro, 2'-O-methyl, 2'-O-methoxyethyl (2'-O-MOE), 2'-O-aminopropyl (2'-O-AP), 2'-O-dimethylaminoethyl (2'-O-DMAOE), 2'-O-dimethylaminopropyl (2'-O-DMAP), 2'-O-dimethylaminoethoxyethyl (2'-O-DMAEOE), or 2'-O-N-methylacetamido (2'-O-NMA). In a particularly preferred embodiment, the oligonucleotide agent includes at least one 2'-O-methyl-modified nucleotide, and in some embodiments, all of the nucleotides of the oligonucleotide agent include a 2'-O-methyl modification. In some embodiments, the sugar moiety of the nucleotide can be replaced, for example, with a non-sugar moiety such as a PNA.

The oligonucleotide agent can be further modified so as to be attached to a ligand. The ligand can be selected, for example, to improve stability, distribution or cellular uptake of the agent, *e.g.*, cholesterol or folate.

The oligonucleotide agent can further be in isolated form or can be part of a pharmaceutical composition used for the methods described herein, particularly as a pharmaceutical composition formulated for parental administration. The pharmaceutical compositions can contain one or more oligonucleotide agents, and in some embodiments, will contain two or more oligonucleotide agents, each one directed to a different miRNA.

An antagomir, *e.g.*, one that is substantially complementary to a nucleotide sequence of an miRNA, can be delivered to a cell or a human to inhibit or reduce the activity of an endogenous miRNA, such as when aberrant or undesired miRNA activity, or insufficient

activity of a target mRNA that hybridizes to the endogenous miRNA, is linked to a disease or disorder. In one embodiment, an antagomir featured in the invention has a nucleotide sequence that is substantially complementary to miR-122 (see Table 1), which hybridizes to numerous RNAs, including aldolase A mRNA, N-myc downstream regulated gene (Ndr3) mRNA, IQ motif containing GTPase activating protein-1 (Iqgap1) mRNA, HMG-CoA-reductase (Hmgcr) mRNA, and citrate synthase mRNA and others. In a preferred embodiment, the antagomir that is substantially complementary to miR-122 is antagomir-122 (Table 2). Aldolase A deficiencies have been found to be associated with a variety of disorders, including hemolytic anemia, arthrogryposis complex congenita, pituitary ectopia, rhabdomyolysis, hyperkalemia. Humans suffering from aldolase A deficiencies also experience symptoms that include growth and developmental retardation, midfacial hypoplasia, hepatomegaly, as well as myopathic symptoms. Thus a human who has or who is diagnosed as having any of these disorders or symptoms is a candidate to receive treatment with an antagomir, such as a single-stranded oligonucleotide agent, that hybridizes to miR-122 and can receive such treatment.

In some embodiments, an antagomir featured in the invention has a nucleotide sequence that is substantially complementary to miR-16, miR-192, or miR-194 (see Table 1).

In one embodiment, the antagomir is selected from those shown in Table 2 and Table 3. The single-stranded oligonucleotide agents of Table 2 are complementary to miR-122,.

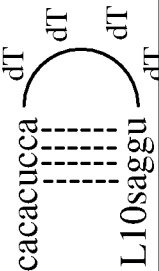
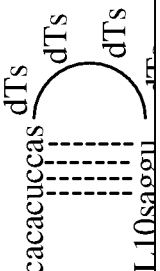
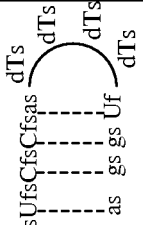
Table 1. Exemplary miRNAs identified in *mus musculus*

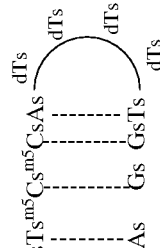
miRNA	Sequence	SEQ ID NO:
miR-122	5'-UGGAGUGUGACAAUGGUGUUUGU-3'	1
miR-16	5'-UAGCAGCACGUAAAUAUUGGCG-3'	2
miR-192	5'-CUGACCUAUGAAUUGACAGCC-3'	3
miR-194	5'-UGUAAACAGCAACUCCAUGUGGA-3'	4

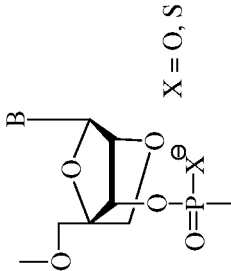
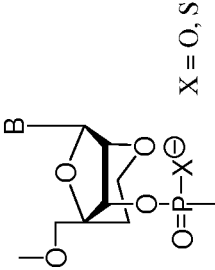
Table 2. Antagomir-122 and analogs

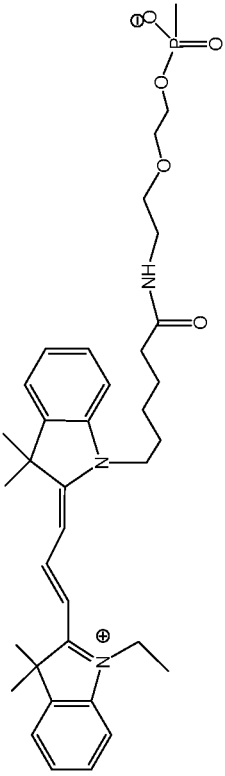
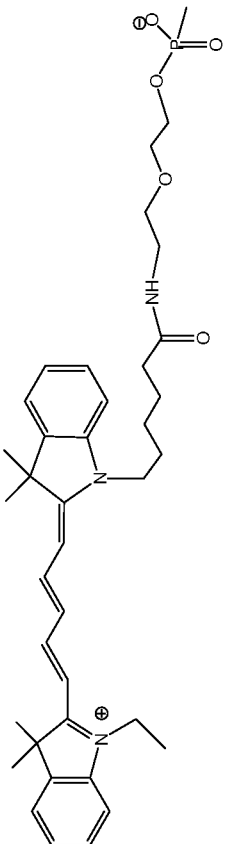
Anti-122					
3038	ascsaacacacauugucacacuscscasL10	Parent antagomir all 2'-OMe, 2 + 4 PS, Cholesterol		8341.315	
Mismatches					
3040	ascsaacacacacugucacauuscscasL10	Parent: 3038, 4 mismatches(a to c, c to a, u to c, c to u), 2+4 PS and cholesterol	15	8341.315	
3355	ascsaacacacacugucacauuscscasL10	3 mismatches, 2+4 PS and cholesterol	165	8365.3397	
3356	ascsaacacacacugucacauuscscasL10	2 mismatches, 2+4 PS and cholesterol	70	8341.315	
3357	ascsaacacacacauugucacauuscscasL10	1 mismatch, 2+4 PS and cholesterol	95	8342.2998	
3542	ascsaacacacacacugucacacuscscasL10	one mismatch (u to c), 2+4 PS and cholesterol	40	8340.3302	
3543	cscsaacacacacauugucacacuscscasL10	one mismatch (a to c), 2+4 PS and cholesterol	38	8317.2903	
3034	ACACACAACACUGUCACAUUCCA	Parent 3040 with 4 mm, all ribo, no PS, no cholesterol		7217.3932	
3039	ascsascsasasascscsuscscsascscsascscasL10	Four mm, all PS, cholesterol	35	8614.4302	
Ribo, PS and Cholesterol modifications					
3033	ACAAACACCAUUGUCACACUCCA	All ribo, no PS, no cholesterol	25	7217.3932	
3631	AcAAAcACcAUuGUcAcACUCscsa	Only PyPu are 2'-OMe, 2 PS, no cholesterol	28	7361.7372	

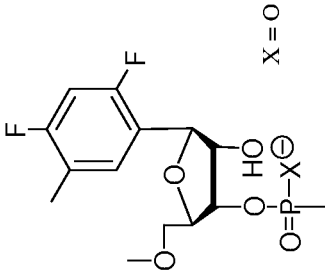
3633	AcAAAcAccAuuGucAcAcucscsa	All Pu are ribo, 2 PS, no cholesterol	50	7445.8968	
3632	AcAAAcAccAuuGucAcAcuccsAsL10	All Pu are ribo, 2 PS, cholesterol	70	8136.7866	
3630	AcAAAcACcAUuGUcAcACUCcsAsL10	Only PyPu are ribo, 2 PS, cholesterol	60	8052.627	
PS and Cholesterol modifications					
3037	ascsasasascscsasusugscsascsascscsasL10	Parent: 3038, all PS, cholesterol	127	8614.4302	
3225	ascsasasascscscsasusugscsascsascscscsa	Parent 3038, all PS, no cholesterol	386	7893.4482	
3226	ascsaacaccauugucacucscsa	Partial PS, no cholesterol	168	7620.333	
3350	acaaacaccauugucacuccaL10	No PS, cholesterol	30	8244.9214	
3383	acaaacaccauugucacuccasL10	1 PS, cholesterol	45	8260.987	
3811	ascacacaaacacugucacauscscsa	2 + 4 PS, no cholesterol	189	7636.3986	
Different lengths					
3351	csasaaacaccauugucacucscsasL10	25mer, 2 + 4 PS, cholesterol	20	8979.7306	
3352	csasaacaccauugucacuscscsL10	21 mer, 2 + 4 PS, cholesterol	28	7654.85	
3353	asacaccauugucacascscsL10	19 mer, 2 + 4 PS, cholesterol	32	7016.4344	
3354	asascaccauugucacscscsL10	17 mer, 2 + 4 PS, cholesterol	120	6353.9941	

Hairpin					
3384	Acaaacaccauugucaca cuccadTdTdTdTuggasL10	dT loop, cholesterol			10858.6361
					
3385	acaaacaccauugucacacuccasdTsdTs dTsuggasL10	dTs loop, cholesterol	45		10938.9641
					
2'-F modifications					
3803	asCfsasasasCfsasCfsasUfsUfsgsUfsCfsasCfsasCfsUfs sCfsCfsa	All PS, all Py 2'-F, no cholesterol			
3804	asCfsasasasCfsasCfsasUfsUfsgsUfsCfsasCfsasCfsUfs sCfsCfsasdTsdTsUfsggsa 	All PS, all Py 2'-F, dTs hairpin loop, no cholesterol	100		10452.1377
3805	AfsCfsAfsAfsAfsCfsAfsCfsCfsAfsUfsUfsgsUfsCfsAfsCfsAfs fsCfsUfsCfsCfsAf	All 2'F, all PS, No cholesterol	45		7616.6317

LNA and cholesterol modifications					
3806	As^{m5}CsAsAsAscsascscsasusugscscsas^{m5}CsTs^{m5}CsA	5 X LNA, 13 X 2'-OMe, 5 X LNA, All PS, no cholesterol	60	7943.4222	
3807	As(m5Cs)AsAsAsdCsdAsdCsdAsdTsdGsdTsdCsdAsdTsdAs^{m5}CsTs^{m5}CsA	5 X LNA, DNA, 5 X LNA, no cholesterol		7595.164	
3810	As^{m5}CsAsAsAscsascscsasusugscscsas^{m5}CsTs^{m5}CsA s^{m5}CsAs dTsdTsdTsdTsTsGsGsA	5 X LNA, DNA, 5 X LNA, dTs loop, no cholesterol	70	10676.5717	
					
3808	As^{m5}CsAsAsAscsascscsasusugscscsas^{m5}CsTs^{m5}CsA	5 X ethylene LNA, 13 X 2'-OMe, 5 X ethylene LNA, All PS, no cholesterol		8083.6882	
3809	As^{m5}CsAsAsAsdCsdAsdCsdAsdTsdGsdTsdCsdAsdTsdAs^{m5}CsTs^{m5}CsA	5 X ethylene LNA, DNA, 5 X ethylene LNA, All PS, no cholesterol		7735.43	

Methylene LNA		Ethylene LNA			
					
2'-OMOE modifications					
876	As ^{m5} <u>Cs</u> AAA ^{m5} <u>CA</u> ^{m5} <u>C</u> ^{m5} <u>CATTGT</u> ^{m5} <u>CA</u> ^{m5} <u>CA</u> ^{m5} <u>Cs</u> Is ^{m5} <u>Cs</u> ^{m5}	All 2'-O-Methoxyethyl, 2 + 4 PS	50	8831.9538	
877	As ^{m5} <u>Cs</u> AsAsAs ^{m5} <u>Cs</u> As ^{m5} <u>Cs</u> ^{m5} <u>Cs</u> AsIsIsGsIs ^{m5} <u>Cs</u> As ^{m5} <u>Cs</u> As ^{m5} <u>Cs</u> Is ^{m5} <u>Cs</u> ^{m5} <u>Cs</u> A	All 2'-O-Methoxyethyl, all PS	100	9089.0034	
550	As ^{m5} <u>Cs</u> AAA ^{m5} <u>CA</u> ^{m5} <u>C</u> ^{m5} <u>CATTGT</u> ^{m5} <u>CA</u> ^{m5} <u>CA</u> ^{m5} <u>CI</u> s ^{m5} <u>Cs</u> ^{m5} <u>C</u> sAsL10	cholesterol, all 2'-OMethoxyethyl, 2 + 4 PS	220	9536.8702	
551	As ^{m5} <u>Cs</u> AsAsAs ^{m5} <u>Cs</u> As ^{m5} <u>Cs</u> ^{m5} <u>Cs</u> AsIsIsGsIs ^{m5} <u>Cs</u> As ^{m5} <u>Cs</u> As ^{m5} <u>Cs</u> Is ^{m5} <u>Cs</u> ^{m5} <u>Cs</u> AsL10	cholesterol, all 2'-O-Methoxyethyl, all PS	40	9809.9854	
Partial duplexes					
400	uggagug 5'-ascsaaacaccaugucacacuscscasL10-3' gugaggu	(7mer, 2'-OMe) Complementary to parent antagomir-3038	23	2358.5853	

401	gacaaug		(7mer, 2'-OMe) Complementary to parent antagomir-3038	20	2325.6017	
402	5'-ascsaaacaccauugucacacuscscsasL10-3' uggaaug		(7mer, 2'-OMe) Complementary to mm antagomir (3040)	24	2342.5859	
403	ascacacaacacugucacauuscscsasL10 gacagug		(7mer, 2'-OMe) Complementary to mm antagomir (3040)	20	2341.6011	
Dye conjugates						
3358	Q37sascaaacaccauugucacacuscscsasL10		Parent 3038, Cy5 dye		8988.0903	
3359	Q38sascacacaacacugucacauuscscsasL10		Parent: 3034 with 4mm, 5'-Cy3-dye		8962.053	
3474	Q38sascaaacaccauugucacacuscscsasL10		Parent 3038, 5'-Cy3-dye		8962.053	
Q38	Q37					
 						
Difluorotoluoyl modification						

3552	ascsaacaccacauY1gucacacuscscsasL10	one DFT		8343.321	
DFT 					
G-clamp					
3625	ascsaacaccacauY5scsasL10	Y5=2'-O-methyl-(9-aminoethoxy) phenoxazine ribonucleotide-3'-phosphate	22	8490.	
3626	ascsasascscscsasusgscscscscscsasY5scsasL10	All PS, Y5, cholesterol	57	8763.	

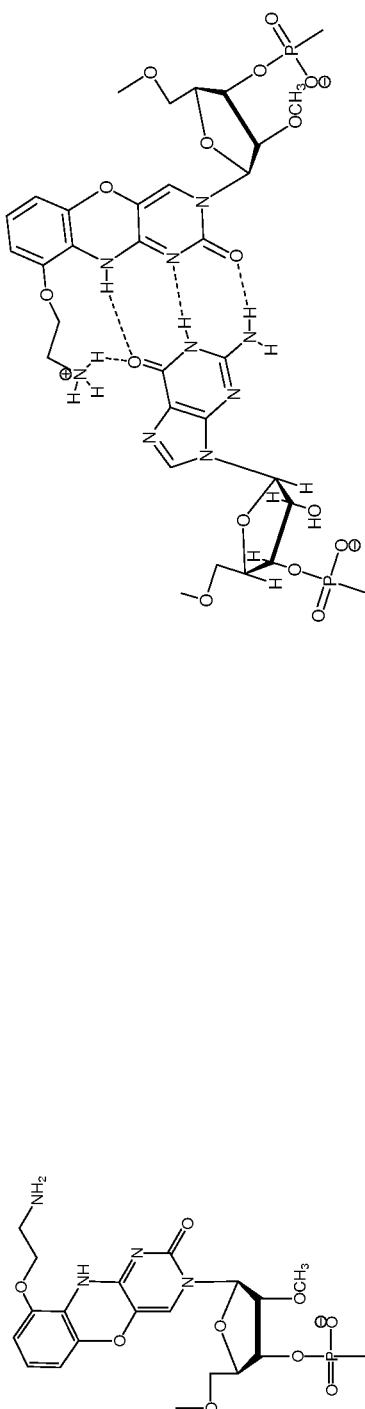
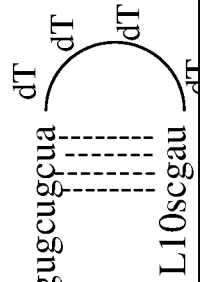
2'-O-Methyl-(9-aminoethoxy) phenoxazine ribonucleotide-3'-phosphate

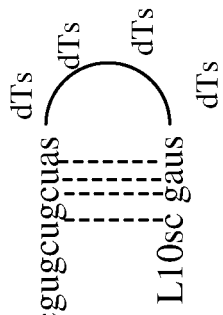
Table 3. Other Antagomirs

Antagomir-141					
30061	cscsaucuuuaccagacagugsusL10	All 2'OMe, 2 +4 PS, cholesterol	34	8033.0357	
Antagomir-143					
30064	usgsagcuacagucucuauscsasL10	All 2'OMe, 2 +4 PS, cholesterol	18	8073.0598	
Antagomir-181					
3537	asascuaccgacagcguugaugsusL10	All 2'OMe, 2 +4 PS, cholesterol	75	8774.5394	
3538	asascuaaccgaccgcuugaagsusL10	Parent: 3537, 4 mismatches; c to a, a to c, g to u, u to g	75	8774.5394	
3697	ascsucaccgacagcguugaugsusL10	Parent 3537, one 5' a deletion, cholesterol		8431.3069	
3698	Q38sascucaccgacagcguugaugsusL10	3697 with Cy3 dye, cholesterol	13	9052.0449	
3869	Q37sascucaccgacagcguugaugsusL10	3697 with Cy5 dye, cholesterol	30	9078.0822	
3870	Q37sascucaccgacagcguugaugsusL10	3869, cg switched to gu, cholesterol	17	8758.8744	
MM-antagomir-181a					
3874	Q38sascucaccgacagcguuuuasusL10	2 + 4 PS, cholesterol, Cy3, cholesterol	35	8973.9663	
30048	Q38sascgacgagagccuugaugsusL10	Parent 3874; two mismatches (c to g, c to g), cholesterol		9109.0532	

MM-antagomir-181c					
3875	Q38sascucaccgacagguuagaagsusL10	2 + 4 PS, cholesterol, Cy3	9		8732.8371
30049	Q38sascugacggacugguucaaacsusL10	Parent 3875 with five mismatches; 2x c to g, a to u, a to u, 2 x g to c	47		8709.7972
Antagomir-192					
3548	gsgccguccauuaauagaucscasL10	All 2'-Ome, cholesterol, 2 +4 PS	42		7791.9065
3228	gsgscugucaauuagguucscasL10 (Trityl ON)		200		7808.8907
Antagomir-194					
3549	uscscuccauagagcugcugcusascasL10	All 2'-Ome, 2 +4 PS, cholesterol	14		8047.0655
3229	uscscacauggagugcuguuascsasL10	All 2'-Ome, 2 +4 PS, cholesterol	150		8089.0592
Antagomir-200c					
30062	cscsaucauuaccgcagcuasusL10	All 2'-Ome, 2 +4 PS, cholesterol	35		7992.0268
Antagomir-206					
30063	cscsacacauccuuacauucscscasL10	All 2'-Ome, 2 +4 PS, cholesterol	40		7911.9786
Antagomir-1					
3595	usascauacuucuuuacauuscsca	All 2'-Ome, 2 +4 PS	15		6890.8088

3596	usascsasusascsususcscsasusascscsasL10	All 2'-Ome, all PS	42	7115.7272	
3597	usascacuucuuuacauuscscsasL10	cholesterol, all 2'-Ome, 2 + 4 PS	140	7595.7252	
3598	usascsasusascsususcscsasusascscsasL10	cholesterol, all 2'-Ome, all PS	85	7836.7092	
Antagomir-GFP					
3600	usgscgucuccgacguagcscsusL10	3'-cholesterol, all 2'-Ome, 2 + 4 PS	37	7736.8166	
Antagomir-205					
3674	UCCUUAUCCACCGGAGUCUG			6891.0905	
3675	csasgacuccgguggaagaasgsgsasL10	Complementary to 3674, cholesterol, all 2'-Ome, 2 + 4 PS		8253.2417	
3676	csasgsascsuscsccsgsgsusgsgsasasasgsgsasL10	Complementary to 3674, cholesterol, all 2'-Ome, all PS	30	8510.2913	
3677	cscsgacuacgguggaagaasgsusL10	4 mismatches in 3675; a to c, c to a, u to g, g to u, cholesterol		8253.2417	
3678	cscsgsascsusascsgsgsusgsgsasasgsgsasusL10	3637 with all PS, cholesterol		8510.2913	
Antagomir-16					
3700	cgccaaauuuuacgucgcuadTdTdT dTuagcsL10	dT loop hairpin	80	10526.3321	



3701	cgccaauuuuacgugcugcuasdTsdTs dTsuagcsL10 	dTs loop hairpin	90	10606.6601	
3812	csgsccaauuuuacgugcugscsusa		156	7344.1187	
3227	csgsccaauuuuacgugcugscsusaL10	All 2'OMe, 2 +4 PS, cholesterol	120	7255.8942	
Anti-16					
3687	acguaaa	7 mer, all 2'-OMe	44	2309.6023	
3688	uasgcagc	7mer, all 2'-OMe, 1PS	18	2317.6426	
Antagomir-ebv-BHRF1-1					
3689	asascuccgggucgucaggsusasL10	All 2'-OMe, 3'-cholesterol, 2 + 4 PS		8167.1378	
Antagomir-ebv-BHRF1-2					
3690	ususcaauuucgcccgaaggsusasL10	All 2'OMe, 2 +4 PS, cholesterol	9	8400.2929	
Antagomir-ebv-BHRF12-1					
3691	gscsuuacaccaguuuccugusasasL10	All 2'OMe, 2 +4 PS, cholesterol	18	8329.2036	

Antagomir-kshv-K3				
3692	uscsgcugccgcuccagaaugsgsasL10	All 2'OMe, 2 +4 PS, cholesterol	9	8423.2816
Antagomir-kshv-K4-3p				
3693	uscsgcuaggccucagauuscusasL10	All 2'OMe, 2 +4 PS, cholesterol	9	8049.0351
Antagomir-kshv-mir-K2				
30031	csasgaucgacccggacucacagsgsusL10	All 2'OMe, 2 +4 PS, cholesterol	32	8110.1295
Antagomir-kshv-mir-K5				
30032	cscsggcaaguuccaggcaucscusasL10	All 2'OMe, 2 +4 PS, cholesterol	30	8086.1048
Antagomir-kshv-mir-K6-3p				
30033	csuscaacagcccgaaaaccasuscsasL10	All 2'OMe, 2 +4 PS, cholesterol	28	8060.1617
Antagomir-kshv-mir-K7				
30034	usgsagcgcagcaacaugggasuscsasL10	All 2'OMe, 2 +4 PS, cholesterol	38	8532.4254
Antagomir-kshv-mir-K11				
30035	uscsggacacaggcuagcaususasL10	All 2'OMe, 2 +4 PS, cholesterol	15	8158.1789
Antagomir-kshv-mir-mm-K11				

30036	uscsgcagacuggguaagcaususasL10	All 2'OMe, 2 +4 PS, cholesterol	48	8175.1631	
Antagomir-luc-1309					
3694	ascscgccugaagucucugaususasL10	All 2'OMe, 2 +4 PS, cholesterol		8072.075	
Antagomir-31					
3695	csasgcuaugccagcaucuugscscsusL10	All 2'OMe, 2 +4 PS, cholesterol		8024.0256	
Antagomir-196					
3699	cscsaacaacaugaaacuacsusL10	All 2'OMe, 2 +4 PS, cholesterol	70	7725.9393	
Antagomir-215					
3753	gsuscuguaaaucagguuscsasL10	All 2'OMe, 2 +4 PS, cholesterol	100	7753.852	
Antagomir-155					
3754	cscscucaucaaaauagcaususasL10	All 2'OMe, 2 +4 PS, cholesterol		8000.0521	
3871	Q37scscuccuacacaaauagcaususasL10	3754 with Cy5 dye			
30050	Q38scscgcuaacagaaauagcaususasL10	3754 with Cy3 dye	15	8769.958	
Antagomir-142-5p					
3755	gsusagucuuucuaucuuusagsL10	All 2'OMe, 2 +4 PS, cholesterol			

3872	Q37sgsuagugcuuuuacuauusasusgsL10	3755 with Cy5 dye	18		
30051	Q38sgsaacugguuuguaguuuusasusgsL10	3755 with Cy3 dye	10	8089.3506	
Antagomir-142-3p					
3756	cscsaauaaguaggaacacacuscasL10	All 2'OMe, 2 +4 PS, cholesterol		8149.22	
3873	Q37scscauuaaguaggaacacuscasL10	3756 with Cy5 dye	13	8795.9953	
30052	Q38scsgauuaacuagcaagacuscasL10	3756 with Cy5 dye		8746.9181	
Antagomir-143					
3868	asgsagcuacagucucucuscasL10	All 2'OMe, 2 +4 PS, cholesterol		8072.075	
Hsa-mir-146a					
30228	asascccauggaauucagucuscasL10	All 2'OMe, 2 +4 PS, cholesterol		8056.0756	
Hsa-mir-146b					
30229	asgsccuauaggaauucagucuscasL10	All 2'OMe, 2 +4 PS, cholesterol		8073.0598	
Antagomir-mCMV-miR-01-1					
3832	csascgcgcacguguuagcauagsgsasL10	All 2'OMe, 2 +4 PS, cholesterol	74	8509.3855	
Antagomir-mCMV-miR-01-2					

3833	ascsgguuccaaccggauucucsuscsL10	All 2'OMe, 2 +4 PS, cholesterol	60	8264.17	
Antagomir-mCMV-miR-23-1					
3834	cscsgcuugaccgaggcccccscsasL10	All 2'OMe, 2 +4 PS, cholesterol	45	7717.8628	
Antagomir-mCMV-miR-23-2					
3835	ascsgguucccgucgguaccsgsasL10	All 2'OMe, 2 +4 PS, cholesterol		8078.0795	
Antagomir-mCMV-miR-44-1					
3836	ascscgcggcucuggaaaaagsasusL10	All 2'OMe, 2 +4 PS, cholesterol	43	8197.2182	
Antagomir-133					
3850	ascsgcugguugaaggacscsasL10	All 2'OMe, 2 +4 PS, cholesterol	100	8253.2417	
Antagomir-133b					
3987	usascgugguugaaggacscsasL10	All 2'OMe, 2 +4 PS, cholesterol		7910.994	
Antagomir-124					
3866	ususggcauuccaccgugccsusL10	All 2'OMe, 2 +4 PS, cholesterol		8041.0098	
Antagomir-126					
3867	csgscauuauacucacgguascsL10	All 2'OMe, 2 +4 PS, cholesterol		8072.075	

Antagomir-126-3p					
3988	gscsauuauuacucacgguascgsasL10	All 2'OMe, 2 +4 PS, cholesterol	60		7752.8672
Antagomir-126-5p					
3989	csgscguaccaaaaguuauasasusgsl10	All 2'OMe, 2 +4 PS, cholesterol			7822.9717
Antagomir-21					
3935	uscsaacaucagucuguaagscsusasL10	All 2'OMe, 2 +4 PS, cholesterol	80		7736.8678
3332	gsuscaacaucagucugauaagscsusasL10	Parent 3935 with two more purine inserts	200		8439.3322
Antagomir-22					
3696	ascsgauucuucaacuggcagcsususL10	Unmodified			8049.0351
miR-122 Sarnow					
3947	AAACGCCAUUAUCACACUAAUA		45		7266.4274
3948	UGGAGUGUGACAAUGGUGUUUGUL29	Biotin			8138.2676
3949	UGGAGUGUGACAA(aa5U)GGUGUUUGUL29	aa5U=5-allylaminouridine-3'-phosphate and biotin			8193.3461
3950	UGGAGUG(aa5U)GACAAUGGUGUUUGUL29	aa5U=5-allylaminouridine-3'-phosphate and biotin			8193.3461
3951	UGGAGUG(aa5U)GACAA(aa5U)GGUGUUUGUL29	Two aa5U=5-allylaminouridine-3'-phosphate and biotin			8248.4246

3952	UsGGAGUGUGACAA(aa5U)GGUGUUUGUsL29	aa5U=5-allylaminouridine-3'-phosphate, two PS and biotin		8225.4773	
3953	UsGGAGUG(aa5U)GACAAUGGUGUUUGUsL29	aa5U=5-allylaminouridine-3'-phosphate, two PS and biotin		8225.4773	
3954	UsGGAGUG(aa5U)GACAA(aa5U)GGUGUUUGUsL29	Two aa5U=5-allylaminouridine-3'-phosphate two PS and biotin		8280.5558	
3955	UGCAGUGUGACAAUGGUGUUUGUL29	Parent 3948 with one mismatch; G to C		8098.2435	
3975	UGGAGUGUGACAAAY15GGUGUUUGUL29	Y15=5-(psoralencarboxamidoallyl)uridine-3'-phosphate and biotin		8463.583	
3976	UGGAGUGY15GACAAUGGUGUUUGUL29	Y15=5-(psoralencarboxamidoallyl)uridine-3'-phosphate and biotin		8463.583	
3977	UGGAGUGY15GACAAAY15GGUGUUUGUL29	2 X Y15=5-(psoralencarboxamidoallyl)uridine-3'-phosphate		8788.8984	
3978	UsGGAGUGUGACAAAY15GGUGUUUGUsL29	Y15=5-(psoralencarboxamidoallyl)uridine-3'-phosphate, 2 PS and biotin		8495.7142	
3979	UsGGAGUGY15GACAAUGGUGUUUGUsL29	Y15=5-(psoralencarboxamidoallyl)uridine-3'-phosphate, 2 PS and biotin		8495.7142	
3980	UsGGAGUGY15GACAAAY15GGUGUUUGUsL29	2 X Y15=5-(psoralencarboxamidoallyl)uridine-3'-phosphate		8821.0296	
3985	UGCUGUGUGACAAUGGUGUUUGUL29	Biotin, two mismatches; G to C, A to U,		8075.2036	
3986	UGCUGUGUGACAAUGGUGUUUGU	3948 without biotin and two mismatches; G to C, A to U		7359.3225	
mm-antagomir-196a					
3983	cscgccacaccaugcaacuccsgsusL10	All 2'OMe, 2 +4 PS, cholesterol	7	7989.0724	
Antagomir-33					

30004	csasaugcaacuacaaugscsascsL10	All 2'OMe, 2 +4 PS, cholesterol	20	7079.4984	
Target unknown					
30141	ascuaacacgaugacucacuscscasL10	All 2'OMe, 2 +4 PS, cholesterol		8342.2336	
30161	asascuaacaaccuacuagsguscsaL10	All 2'OMe, 2 +4 PS, cholesterol		8063.1161	
30162	asascuaacaagguacuacscsuscscsaL10	Parent 30161, with four mismatches		8063.1161	
Target Let7					
30142	asascuaacaaccuacuacscsuscscsaL10	All 2'OMe, 2 +4 PS, cholesterol		7983.0679	
30143	csgsuacgcggaauacuucgsasasasul10	All 2'OMe, 2 +4 PS, cholesterol		8135.139	

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All capital letters represent ribonucleosides
All small letters represent 2'-methoxyribonucleosides
L10 and Q11 represent 3'-cholesterol
T = 2'-methoxyethylthymidine
A = 2'-methoxyethyadenosine
G = 2'-methoxyethylguanosine
m⁵C = 2'-methoxyethyl 5-methylcytidine
I = Inosine
Y13 = 5-(aminoethyl-3-acrylimido) thymidine
L29=N-(biotinyl-aminododecylcarboxamidocaproyl)-4-hydroxyprolinol
Y14=5-(psoralencarboxamidoethyl-3-acrylimido) thymidine-3'-phosphate

A = C2', C4'-methylene locked adenosine
T = C2', C4'-methylene locked thymidine
^{m5}C = C2', C4'-methylene locked 5-methylcytidine
A = C2', C4'-ethylene locked adenosine
T = C2', C4'-ethylene locked thymidine
^{m5}C = C2', C4'-ethylene locked 5-methylcytidine

In one aspect, the invention features a method of reducing the activity level of an miRNA or pre-miRNA in a cell of a subject, *e.g.*, a human subject. The method includes the step of administering an antagomir to the subject, where the antagomir is substantially single-stranded and includes a sequence that is substantially complementary to 12 to 23 contiguous nucleotides, and preferably 15 to 23 contiguous nucleotides, of a target sequence of an miRNA or pre-miRNA nucleotide sequence. Preferably, the target sequence differs by no more than 1, 2, or 3 nucleotides from a microRNA or pre-microRNA sequence, such as a microRNA sequence shown in Table 1.

In one embodiment, the methods featured in the invention are useful for reducing the level of activity of an endogenous miRNA (*e.g.*, miR-122, miR-16, miR-192 or miR-194) or pre-miRNA in a cell, *e.g.*, in a cell of a subject, such as a human subject. Such methods include contacting the cell with an antagomir, such as a single-stranded oligonucleotide agent, described herein for a time sufficient to allow uptake of the antagomir into the cell.

In another aspect, the invention features a method of making an antagomir, such as a single-stranded oligonucleotide agent, described herein. In one embodiment, the method includes synthesizing an oligonucleotide agent, including incorporating a nucleotide modification that stabilizes the antagomir against nucleolytic degradation.

In another aspect, the invention features a pharmaceutical composition including an antagomir, such as a single-stranded oligonucleotide agent, described herein, and a pharmaceutically acceptable carrier. In a preferred embodiment, the antagomir, such as a single-stranded oligonucleotide agent, included in the pharmaceutical composition hybridizes to miR-122, miR-16, miR-192, or miR-194.

In another aspect the invention features a method of inhibiting miRNA activity levels (*e.g.*, miR-122, miR-16, miR-192, or miR-194 activity) or pre-miRNA activity levels in a cell, *e.g.*, a cell of a subject. The method includes contacting the cell with an effective amount of an antagomir, such as a single-stranded oligonucleotide agent, described herein, which is substantially complementary to the nucleotide sequence of the target miRNA or the target pre-miRNA. Such methods can be performed on a mammalian subject by administering to a subject one of the oligonucleotide agents/pharmaceutical compositions described herein.

In another aspect the invention features a method of increasing levels of an RNA or protein that are encoded by a gene whose expression is down-regulated by an miRNA, *e.g.*,

an endogenous miRNA, such as miR-122, miR-16, miR-192 or mir-194. The method includes contacting the cell with an effective amount of an antagomir, such as a single-stranded oligonucleotide agent, described herein, which is substantially complementary to the nucleotide sequence of the miRNA that binds to and effectively inhibits translation of the RNA transcribed from the gene. For example, the invention features a method of increasing aldolase A protein levels in a cell. Similarly, the invention features a method of increasing Ndr3, Iqgap1, Hmgcr, and/or citrate synthase protein levels in a cell. The methods include contacting the cell with an effective amount of an antagomir described herein (e.g., an antagomire in Table 2 and Table 3), which is substantially complementary to the nucleotide sequence of miR-122 (see Table 1).

Preferably, an antagomir, such as a single-stranded oligonucleotide agent, (a term which is defined below) will include a ligand that is selected to improve stability, distribution or cellular uptake of the agent. Compositions featured in the invention can include conjugated single-stranded oligonucleotide agents as well as conjugated monomers that are the components of or can be used to make the conjugated oligonucleotide agents. The conjugated oligonucleotide agents can modify gene expression by targeting and binding to a nucleic acid, such as an miRNA (e.g., miR-122, miR-16, miR-192, or miR-194) or pre-miRNA.

In a preferred embodiment, the ligand is a lipophilic moiety, *e.g.*, cholesterol, which enhances entry of the antagomir, such as a single-stranded oligonucleotide agent, into a cell, such as a hepatocyte, synoviocyte, myocyte, keratinocyte, leukocyte, endothelial cell (*e.g.*, a kidney cell), B-cell, T-cell, epithelial cell, mesodermal cell, myeloid cell, neural cell, neoplastic cell, mast cell, or fibroblast cell. In some embodiments, a myocyte is a smooth muscle cell or a cardiac myocyte. A fibroblast cell can be a dermal fibroblast, and a leukocyte can be a monocyte. In another embodiment, the cell is from an adherent tumor cell line derived from a tissue, such as bladder, lung, breast, cervix, colon, pancreas, prostate, kidney, liver, skin, or nervous system (*e.g.*, central nervous system). In some preferred embodiments, the ligand is a folate ligand.

In another aspect, the invention provides methods of increasing expression of a target gene by providing an antagomir to which a lipophilic moiety is conjugated, *e.g.*, a lipophilic conjugated antagomir described herein, to a cell. The antagomir preferably hybridizes to an miRNA (e.g., miR-122, miR-16, miR-192, or miR-194) or a pre-miRNA. In a preferred embodiment the conjugated antagomir can be used to increase expression of a target gene in an organism, *e.g.*, a mammal, *e.g.*, a human, or to increase expression of a target gene in a

cell line or in cells which are outside an organism. While not wishing to be bound by theory it is believed that an mRNA transcribed from the target gene hybridizes to an endogenous miRNA, which consequently results in downregulation of mRNA expression. While not wishing to be bound by theory it is believed that an antagomir, such as a single-stranded

5 oligonucleotide agent, featured in the invention hybridizes to the endogenous miRNA and consequently causes an increase in mRNA expression. In the case of a whole organism, the method can be used to increase expression of a gene and treat a condition associated with a low level of expression of the gene. For example, an antagomir, such as a single-stranded oligonucleotide agent, that targets miR-122 (e.g., antagomir-122) can be used to increase
10 expression of an aldolase A gene to treat a subject having, or at risk for developing, hemolytic anemia, arthrogryposis complex congenita, pituitary ectopia, rhabdomyolysis, hyperkalemia, or any other disorder associated with aldolase A deficiency. Administration of an antagomir, such as a single-stranded oligonucleotide agent, that targets miR-122 (e.g., antagomir-122) can be also be used to increase expression of an Ndr3, Iqgap1, Hmgcr, or
15 citrate synthase gene to treat a subject having, or at risk for developing, a disorder associated with a decreased expression of any one of these genes.

In one embodiment, the antagomir, such as a single-stranded oligonucleotide agent, to which a lipophilic moiety is conjugated is used to increase expression of a gene in a cell that is not part of a whole organism, such as when the cell is part of a primary cell line, secondary
20 cell line, tumor cell line, or transformed or immortalized cell line. Cells that are not part of a whole organism can be used in an initial screen to determine if an antagomir, such as a single-stranded oligonucleotide agent, is effective in increasing target gene expression levels, or decreasing levels of a target miRNA or pre-miRNA. A test in cells that are not part of a whole organism can be followed by test of the antagomir in a whole animal. In some
25 embodiments, the antagomir that is conjugated to a lipophilic moiety is administered to an organism, or contacted with a cell that is not part of an organism, in the absence of (or in a reduced amount of) other reagents that facilitate or enhance delivery, e.g., a compound which enhances transit through the cell membrane. (A reduced amount can be an amount of such reagent which is reduced in comparison to what would be needed to get an equal amount of
30 nonconjugated antagomir into the target cell). For example, the antagomir that is conjugated to a lipophilic moiety is administered to an organism, or contacted with a cell that is not part of an organism, in the absence (or reduced amount) of (i) an additional lipophilic moiety; (ii) a transfection agent (e.g., an ion or other substance which substantially alters cell permeability to an oligonucleotide agent); or (iii) a commercial transfecting agent such as

LipofectamineTM (Invitrogen, Carlsbad, CA), Lipofectamine 2000TM, TransIT-TKOTM (Mirus, Madison, WI), FuGENE 6 (Roche, Indianapolis, IN), polyethylenimine, XtremeGENE Q2 (Roche, Indianapolis, IN), DOTAP, DOSPER, MetafecteneTM (Biontex, Munich, Germany), and the like.

5 Exemplary delivery vehicles for an oligonucleotide agent featured herein, include lipid (e.g., cationic lipid) containing vehicles (e.g., liposomes), viral containing vehicles (e.g., vectors), polymer containing vehicles (e.g., biodegradable polymers or dendrimers), and peptide containing vehicles (e.g., a penetration peptide), exosomes, and bacterially-derived, intact minicells. In a preferred example the delivery vehicle includes more than one
10 component. For example, it can include one or more lipid moieties, one or more peptides, one or more polymers, one or more viral vectors, or a combination thereof

In a preferred embodiment, the delivery vehicle is an association complex such as a liposome. A liposome generally includes a plurality of components such as one or more of a cationic lipid (e.g., an amino lipid), a targeting moiety, a fusogenic lipid, a PEGylated lipid.
15 In some embodiments, the PEG-lipid is a targeted PEG-lipid. For example, a liposome can include a nucleic acid and an amine-lipid and a PEGylated lipid. In some embodiments, the PEG-lipid is a targeted PEG-lipid. In some embodiments, the preparation also includes a structural moiety such as cholesterol.

Exemplary delivery vehicles

20 An oligonucleotide agent can be delivered using a variety of delivery vehicles including those containing one or more of the following: cationic lipids, cationic liposomes, neutral and zwitterionic lipids and liposomes, peptides (neutral, anionic and cationic; hydrophobic), dendrimers (neutral, anionic and cationic), polymers, emulsions, intralipids, omega-3 and related natural formulations, microemulsions and nanoemulsions, nanoparticles,
25 nanosystems with targeting groups, nanosystems with endosomal releasing groups, polymeric micelles, polymeric vesicles, PEIs and polyamines, lipophilic polyamines, and hydrogels.

Exemplary delivery vehicles include peptide containing vehicles, collagen containing vehicles, viral vector containing vehicles, polymer containing vehicles, lipid containing (e.g., cationic lipids, PEG containing lipids, etc.). In some embodiments, the delivery vehicle
30 includes a combination of one or more of the delivery components described above.

Exemplary delivery vehicles, which can be evaluated using a screening model described herein include, but are not limited to the following: Exosomes such as those described in US 20070298118; bacterially-derived, intact minicells, for example, as described in US 20070298056; complexes including RNA and peptides such as those described in US

20070293657; cationic lipids, non-cationic lipids, and lipophilic delivery-enhancing compounds such as those described in US 20070293449); Carbohydrate-Derivatized Liposomes (e.g., as described in US 20070292494); siRNA-hydrophilic polymer conjugates (e.g., as described in US 20070287681); lipid and polypeptide based systems such as those
5 described in US 20070281900; organic cation containing systems such as those described in US 20070276134 and US 20070213257; cationic peptide containing systems such as those described in US 20070275923; polypeptide containing systems such as those disclosed in US 20060040882; virus-phage particle containing systems such as those described in US 20070274908; elastin-like polymer containing systems such as those described in US
10 20070265197; non-immunogenic, hydrophilic/cationic block copolymers such as those described in US 20070259828; carrier linked conjugate containing systems such as those described in US 20070258993; biodegradable cationic polymer containing systems such as those described in US 20070243157; chemically modified polycation polymer containing systems such as those described in US 20070231392; collagen containing systems such as
15 those described in US 20070218038; glycopolymer-based particle containing systems such as those described in US 20070202076; biologically active block copolymer containing systems such as those described in US 20070155907; nanoparticle containing systems such as those described in US 20070155658; amphoteric liposome containing systems such as those described in US 20070104775; lipid carrier containing systems such as those described in US
20 20070087045; electroporation systems such as those described in US 20070059832; macromer-melt formulations such as those described in US 20070053954; liposome containing systems such as those described in US 20070042031 and US 20050002999 and US 20050002998; lipid based formulations such as those described in US 20060008910; US 20050014962; US 20060240093, US 20050064595, and US 20060083780; polymer
25 conjugate containing systems such as those described in US 20070041932 and US 20050008617; hydrophobic nanotube and nanoparticle containing systems such as those described in US 20060275371; functional synthetic molecule and macromolecule containing systems such as those described in US 20060241071; polymeric micelle containing systems such as those disclosed in US 20060240092; sugar-modified liposome containing systems
30 such as those described in US 20060193906; cyclic amidinium-containing systems such as those described in US 20060039860 and US 20030220289; peptide containing compositions such as those described in US 20060035815 and US 20050239687; viral vector containing systems such as those described in US 20060009408, US 20030157691, and tRNA vector systems such as those described in US 20050203047; nanocell drug delivery systems such as

those described in US 20050266067; polymerized formamide containing systems such as those described in US 20050265957; intranasal delivery systems such as those described in US 20050265927; nanoparticle systems such as those described in US 20050260276; biodegradable polymer-peptide containing systems such as those described in

5 US 20050191746 and biodegradable polyacetal containing systems such as those described in US 20050080033; biodegradable cationic polymer containing systems such as those described in US 20060258751; biodegradable poly(beta-amino ester) containing systems such as those described in US 20040071654; polyethyleneglycol-modified lipid containing systems such as those described in US 20050175682; virally-encoded RNA systems such as
10 those described in US 20050171041, US 20040023390, US 20030138407; adenoviral vector systems such as those described in US 20040161848 and US 20040096843; carrier complex containing systems such as those described in US 20050158373; compositions comprising amphipathic compounds and polycations such as those described in US 20050143332, US 20040137064, and US 20030125281; delivery peptide and dendrimer containing
15 compositions such as those described in US 20040204377; polyampholyte containing compositions such as those described in US 20040162235; and microcapsule containing systems such as those described in US 20040115254 and formulations described in PCT/US2007/080331. Each of the references above is incorporated by reference herein in its entirety.

20 In some preferred embodiments one or more of the delivery vehicles can be formed into a particle such as a liposome or other association complex. The nucleic acid-based agent can be encapsulated or partially encapsulated in the particle delivery vehicle. In some embodiments, the nucleic acid-based agent is admixed with one or more delivery vehicles described herein.

25 In some embodiments, the nucleic acid-based agent is bound to a delivery vehicle described herein. For example, the nucleic acid-based agent can be bound to a delivery vehicle through hydrostatic interactions, ionic interactions, hydrogen bonding interactions or through a covalent bond.

30 In some embodiments, the nucleic acid-based agent is entrapped or entrained within a delivery vehicle.

Association complexes

Association complexes can be used to administer a nucleic acid based therapy such as an oligonucleotide agent described herein. The association complexes disclosed herein can be useful for packaging an oligonucleotide.

Association complexes can include a plurality of components. In some embodiments, an association complex such as a liposome can include an oligonucleotide agent described herein, a cationic lipid such as an amino lipid. In some embodiments, the association complex can include a plurality of therapeutic agents, for example two or three single or
5 double stranded nucleic acid moieties targeting more than one gene or different regions of the same gene. Other components can also be included in an association complex, including a PEG-lipid or a structural component, such as cholesterol. In some embodiments the association complex also includes a fusogenic lipid or component and/or a targeting molecule. In some preferred embodiments, the association complex is a liposome including
10 an oligonucleotide agent such as dsRNA, a lipid, a PEG-lipid, and a structural component such as cholesterol.

In a preferred embodiment, the antagomir is suitable for delivery to a cell *in vivo*, *e.g.*, to a cell in an organism. In another embodiment, the antagomir is suitable for delivery to a cell *in vitro*, *e.g.*, to a cell in a cell line.

15 An antagomir to which a lipophilic moiety is attached can target any miRNA (*e.g.*, miR-122, miR-16, miR-192, or miR-194) or pre-miRNA described herein and can be delivered to any cell type described herein, *e.g.*, a cell type in an organism, tissue, or cell line. Delivery of the antagomir can be *in vivo*, *e.g.*, to a cell in an organism, or *in vitro*, *e.g.*, to a cell in a cell line.

20 In another aspect, the invention provides compositions including single-stranded oligonucleotide agents described herein, and in particular, compositions including an antagomir to which a lipophilic moiety is conjugated, *e.g.*, a lipophilic conjugated antagomir that hybridizes to miR-122, miR-16, miR-192, or miR-194. In a preferred embodiment the composition is a pharmaceutically acceptable composition.

25 In one embodiment the composition is suitable for delivery to a cell *in vivo*, *e.g.*, to a cell in an organism. In another aspect, the antagomir is suitable for delivery to a cell *in vitro*, *e.g.*, to a cell in a cell line.

30 An “antagomir” or “oligonucleotide agent” of the present invention refers to a single stranded, double stranded or partially double stranded oligomer or polymer of ribonucleic acid (RNA) or deoxyribonucleic acid (DNA) or both or modifications thereof, which is antisense with respect to its target. This term includes oligonucleotides composed of naturally-occurring nucleobases, sugars and covalent internucleoside (backbone) linkages and which contain at least one non-naturally-occurring portions which function similarly. Such modified or substituted oligonucleotides are preferred over native forms because of desirable

properties such as, for example, enhanced cellular uptake, enhanced affinity for nucleic acid target and increased stability in the presence of nucleases. In a preferred embodiment, the antagomir does not include a sense strand, and in another preferred embodiment, the antagomir does not self-hybridize to a significant extent. An antagomir featured in the invention can have secondary structure, but it is substantially single-stranded under physiological conditions. An antagomir that is substantially single-stranded is single-stranded to the extent that less than about 50% (*e.g.*, less than about 40%, 30%, 20%, 10%, or 5%) of the antagomir is duplexed with itself. Figures 5-11 provides representative structures of antagomirs.

The antagomir can include a hairpin segment, *e.g.*, sequence, preferably at the 3' end can self hybridize and form a duplex region, *e.g.*, a duplex region of at least 1, 2, 3, or 4 and preferably less than 8, 7, or 6, nucleotides, *e.g.*, 5 nucleotides. The duplexed region can be connected by a linker, *e.g.*, a nucleotide linker, *e.g.*, 3, 4, 5, or 6 dTs, *e.g.*, modified dTs. In another embodiment the antagomir is duplexed with a shorter oligo, *e.g.*, of 5, 6, 7, 8, 9, or 10 nucleotides in length, *e.g.*, at one or both of the 3' and 5' end or at one end and in the non-terminal or middle of the antagomir.

"Substantially complementary" means that two sequences are substantially complementary that a duplex can be formed between them. The duplex may have one or more mismatches but the region of duplex formation is sufficient to down-regulate expression of the target nucleic acid. The region of substantial complementarity can be perfectly paired. In other embodiments, there will be nucleotide mismatches in the region of substantial complementarity. In a preferred embodiment, the region of substantial complementarity will have no more than 1, 2, 3, 4, or 5 mismatches.

The antagomirs featured in the invention include oligomers or polymers of ribonucleic acid (RNA) or deoxyribonucleic acid (DNA) or both or modifications thereof. This term includes oligonucleotides composed of naturally occurring nucleobases, sugars, and covalent internucleoside (backbone) linkages as well as oligonucleotides having non-naturally-occurring portions that function similarly. Such modified or substituted oligonucleotides are often preferred over native forms because of desirable properties such as, for example, enhanced cellular uptake, enhanced affinity for nucleic acid target, and/or increased stability in the presence of nucleases. The oligonucleotide agents can be about 12 to about 30 nucleotides long, *e.g.*, about 15 to about 25, or about 18 to about 25 nucleotides long (*e.g.*, about 19, 20, 21, 22, 23, 24 nucleotides long).

The antagomirs featured in the invention can target RNA, *e.g.*, an endogenous pre-miRNA or miRNA of the subject or an endogenous pre-miRNA or miRNA of a pathogen of the subject. For example, the oligonucleotide agents can target an miRNA of the subject, such as miR-122, miR-16, miR-192, or miR-194. Such single-stranded oligonucleotide can be useful for the treatment of diseases involving biological processes that are regulated by miRNAs, including developmental timing, differentiation, apoptosis, cell proliferation, organ development, and metabolism.

MicroRNA-type Oligonucleotide Agents

The antagomirs featured in the invention include microRNA-type (miRNA-type) oligonucleotide agents, *e.g.*, the miRNA-type oligonucleotide agents listed in Table 2 and Table 3. MicroRNAs are small noncoding RNA molecules that are capable of causing post-transcriptional silencing of specific genes in cells such as by the inhibition of translation or through degradation of the targeted mRNA. An miRNA can be completely complementary or can have a region of noncomplementarity with a target nucleic acid, consequently resulting in a “bulge” at the region of non-complementarity. The region of noncomplementarity (the bulge) can be flanked by regions of sufficient complementarity, preferably complete complementarity to allow duplex formation. Preferably, the regions of complementarity are at least 8, 9, or 10 nucleotides long. An miRNA can inhibit gene expression by repressing translation, such as when the microRNA is not completely complementary to the target nucleic acid, or by causing target RNA degradation, which is believed to occur only when the miRNA binds its target with perfect complementarity. The invention also can include double-stranded precursors of miRNAs that may or may not form a bulge when bound to their targets.

An miRNA or pre-miRNA can be 18-100 nucleotides in length, and more preferably from 18-80 nucleotides in length. Mature miRNAs can have a length of 19-30 nucleotides, preferably 21-25 nucleotides, particularly 21, 22, 23, 24, or 25 nucleotides. MicroRNA precursors typically have a length of about 70-100 nucleotides and have a hairpin conformation. MicroRNAs are generated *in vivo* from pre-miRNAs by the enzymes Dicer and Drosha, which specifically process long pre-miRNA into functional miRNA. The miRNA-type oligonucleotide agents, or pre-miRNA-type oligonucleotide agents featured in the invention can be synthesized *in vivo* by a cell-based system or *in vitro* by chemical

synthesis. MicroRNA-type oligonucleotide agents can be synthesized to include a modification that imparts a desired characteristic. For example, the modification can improve stability, hybridization thermodynamics with a target nucleic acid, targeting to a particular tissue or cell-type, or cell permeability, *e.g.*, by an endocytosis-dependent or -independent mechanism. Modifications can also increase sequence specificity, and consequently decrease off-site targeting. Methods of synthesis and chemical modifications are described in greater detail below.

Given a sense strand sequence (*e.g.*, the sequence of a sense strand of a cDNA molecule), an miRNA-type antagomir can be designed according to the rules of Watson and Crick base pairing. The miRNA-type antagomir can be complementary to a portion of an RNA, *e.g.*, an miRNA, pre-miRNA, or mRNA. For example, the miRNA-type antagomir can be complementary to an miRNA endogenous to a cell, such as miR-122, miR-16, miR-192, or miR-194. An miRNA-type antagomir can be, for example, from about 12 to 30 nucleotides in length, preferably about 15 to 28 nucleotides in length (*e.g.*, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, or 27 nucleotides in length).

In particular, an miRNA-type antagomir featured in the invention can have a chemical modification on a nucleotide in an internal (*i.e.*, non-terminal) region having noncomplementarity with the target nucleic acid. For example, a modified nucleotide can be incorporated into the region of an miRNA that forms a bulge. The modification can include a ligand attached to the miRNA, *e.g.*, by a linker. The modification can, for example, improve pharmacokinetics or stability of a therapeutic miRNA-type oligonucleotide agent, or improve hybridization properties (*e.g.*, hybridization thermodynamics) of the miRNA-type antagomir to a target nucleic acid. In some embodiments, it is preferred that the orientation of a modification or ligand incorporated into or tethered to the bulge region of an miRNA-type antagomir is oriented to occupy the space in the bulge region. For example, the modification can include a modified base or sugar on the nucleic acid strand or a ligand that functions as an intercalator. These are preferably located in the bulge. The intercalator can be an aromatic, *e.g.*, a polycyclic aromatic or heterocyclic aromatic compound. A polycyclic intercalator can have stacking capabilities, and can include systems with 2, 3, or 4 fused rings. The universal bases described below can be incorporated into the miRNA-type oligonucleotide agents. In some embodiments, it is preferred that the orientation of a modification or ligand incorporated into or tethered to the bulge region of an miRNA-type

antagomir is oriented to occupy the space in the bulge region. This orientation facilitates the improved hybridization properties or an otherwise desired characteristic of the miRNA-type oligonucleotide agent.

In one embodiment, an miRNA-type antagomir or a pre-miRNA can include an aminoglycoside ligand, which can cause the miRNA-type antagomir to have improved hybridization properties or improved sequence specificity. Exemplary aminoglycosides include glycosylated polylysine; galactosylated polylysine; neomycin B; tobramycin; kanamycin A; and acridine conjugates of aminoglycosides, such as Neo-N-acridine, Neo-S-acridine, Neo-C-acridine, Tobra-N-acridine, and KanaA-N-acridine. Use of an acridine analog can increase sequence specificity. For example, neomycin B has a high affinity for RNA as compared to DNA, but low sequence-specificity. In some embodiments the guanidine analog (the guanidinoglycoside) of an aminoglycoside ligand is tethered to an oligonucleotide agent. In a guanidinoglycoside, the amine group on the amino acid is exchanged for a guanidine group. Attachment of a guanidine analog can enhance cell permeability of an oligonucleotide agent.

In one embodiment, the ligand can include a cleaving group that contributes to target gene inhibition by cleavage of the target nucleic acid. Preferably, the cleaving group is tethered to the miRNA-type antagomir in a manner such that it is positioned in the bulge region, where it can access and cleave the target RNA. The cleaving group can be, for example, a bleomycin (*e.g.*, bleomycin-A₅, bleomycin-A₂, or bleomycin-B₂), pyrene, phenanthroline (*e.g.*, *O*-phenanthroline), a polyamine, a tripeptide (*e.g.*, lys-tyr-lys tripeptide), or metal ion chelating group. The metal ion chelating group can include, *e.g.*, an Lu(III) or EU(III) macrocyclic complex, a Zn(II) 2,9-dimethylphenanthroline derivative, a Cu(II) terpyridine, or acridine, which can promote the selective cleavage of target RNA at the site of the bulge by free metal ions, such as Lu(III). In some embodiments, a peptide ligand can be tethered to an miRNA or a pre-miRNA to promote cleavage of the target RNA, *e.g.*, at the bulge region. For example, 1,8-dimethyl-1,3,6,8,10,13-hexaazacyclotetradecane (cyclam) can be conjugated to a peptide (*e.g.*, by an amino acid derivative) to promote target RNA cleavage. The methods and compositions featured in the invention include miRNA-type oligonucleotide agents that inhibit target gene expression by a cleavage or non-cleavage dependent mechanism.

An miRNA-type antagomir or pre-miRNA-type antagomir can be designed and synthesized to include a region of noncomplementarity (*e.g.*, a region that is 3, 4, 5, or 6 nucleotides long) flanked by regions of sufficient complementarity to form a duplex (*e.g.*, regions that are 7, 8, 9, 10, or 11 nucleotides long) with a target RNA, *e.g.*, an miRNA, such as miR-122, miR-16, miR-192, or miR-194.

For increased nuclease resistance and/or binding affinity to the target, the single-stranded oligonucleotide agents featured in the invention can include 2'-O-methyl, 2'-fluorine, 2'-O-methoxyethyl, 2'-O-aminopropyl, 2'-amino, and/or phosphorothioate linkages. Inclusion of locked nucleic acids (LNA), ethylene nucleic acids (ENA), *e.g.*, 2'-4'-ethylene-bridged nucleic acids, and certain nucleobase modifications such as 2-amino-A, 2-thio (*e.g.*, 2-thio-U), G-clamp modifications, can also increase binding affinity to the target. The inclusion of pyranose sugars in the oligonucleotide backbone can also decrease endonucleolytic cleavage. An antagomir can be further modified by including a 3' cationic group, or by inverting the nucleoside at the 3'-terminus with a 3'-3' linkage. In another alternative, the 3'-terminus can be blocked with an aminoalkyl group, *e.g.*, a 3' C5-aminoalkyl dT. Other 3' conjugates can inhibit 3'-5' exonucleolytic cleavage. While not being bound by theory, a 3' conjugate, such as naproxen or ibuprofen, may inhibit exonucleolytic cleavage by sterically blocking the exonuclease from binding to the 3' end of the oligonucleotide. Even small alkyl chains, aryl groups, or heterocyclic conjugates or modified sugars (D-ribose, deoxyribose, glucose *etc.*) can block 3'-5'-exonucleases.

The 5' -terminus can be blocked with an aminoalkyl group, *e.g.*, a 5'-O-alkylamino substituent. Other 5' conjugates can inhibit 5'-3' exonucleolytic cleavage. While not being bound by theory, a 5' conjugate, such as naproxen or ibuprofen, may inhibit exonucleolytic cleavage by sterically blocking the exonuclease from binding to the 5' end of the oligonucleotide. Even small alkyl chains, aryl groups, or heterocyclic conjugates or modified sugars (D-ribose, deoxyribose, glucose *etc.*) can block 3'-5'-exonucleases.

In one embodiment, an antagomir, such as a single-stranded oligonucleotide agent, includes a modification that improves targeting, *e.g.* a targeting modification described herein. Examples of modifications that target single-stranded oligonucleotide agents to particular cell types include carbohydrate sugars such as galactose, N-acetylgalactosamine, mannose; vitamins such as folates; other ligands such as RGDs and RGD mimics; and small molecules including naproxen, ibuprofen or other known protein-binding molecules.

An antagomir, such as a single-stranded oligonucleotide agent, featured in the invention can be constructed using chemical synthesis and/or enzymatic ligation reactions using procedures known in the art. For example, an antagomir can be chemically synthesized using naturally occurring nucleotides or variously modified nucleotides designed to increase the biological stability of the molecules or to increase the physical stability of the duplex formed between the antagomir and target nucleic acids, *e.g.*, phosphorothioate derivatives and acridine substituted nucleotides can be used. Other appropriate nucleic acid modifications are described herein. Alternatively, the antagomir can be produced biologically using an expression vector into which a nucleic acid has been subcloned in an antisense orientation (*i.e.*, RNA transcribed from the inserted nucleic acid will be of an antisense orientation to a target nucleic acid of interest (*e.g.*, an miRNA or pre-miRNA)).

Chemical 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, C₁-C₁₂ 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 “aralkyl” 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 “aralkyl” include benzyl, 9-fluorenyl, benzhydryl, and trityl 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, allyl, propenyl, 2-butenyl, 3-hexenyl and 3-octenyl 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 sp² and sp³ 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 –NH(alkyl)₂ radicals respectively. The term “aralkylamino” refers to a –NH(aralkyl) radical. The term

“alkoxy” refers to an -O-alkyl radical, and the terms “cycloalkoxy” and “aralkoxy” refer to an -O-cycloalkyl and O-aralkyl radicals respectively. The term “siloxo” refers to a R_3SiO - radical. The term “mercapto” refers to an SH radical. The term “thioalkoxy” refers to an -S-alkyl radical.

5 The term “alkylene” refers to a divalent alkyl (*i.e.*, -R-), *e.g.*, -CH₂-, -CH₂CH₂-, and -CH₂CH₂CH₂-. The term “alkylenedioxy” 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,
10 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.*,
15 spiro-fused rings). Examples of cycloalkyl moieties include, but are not limited to, cyclohexyl, adamantyl, and norbornyl, and decalin.

 The term “heterocyclyl” 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
20 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 heterocyclyl 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 heterocyclyl include, but are not limited to tetrahydrofuranyl, tetrahydropyranyl,
25 piperidiny, morpholino, 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
30 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
5 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 tetrahydropyridyl and dihydropyran.

The term "heteroaryl" refers to an aromatic 5-8 membered monocyclic, 8-12 membered bicyclic, or 11-14 membered tricyclic ring system having 1-3 heteroatoms if
10 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.

15 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.

The term "acyl" refers to an alkylcarbonyl, cycloalkylcarbonyl, arylcarbonyl, heterocyclylcarbonyl, or heteroarylcarbonyl substituent, any of which may be further
20 substituted by substituents.

The term "substituents" refers to a group "substituted" on an alkyl, cycloalkyl, alkenyl, alkynyl, heterocyclyl, heterocycloalkenyl, cycloalkenyl, aryl, or heteroaryl group at any atom of that group. Suitable substituents include, without limitation, alkyl, alkenyl, alkynyl, alkoxy, halo, hydroxy, cyano, nitro, amino, SO₃H, sulfate, phosphate, perfluoroalkyl,
25 perfluoroalkoxy, methylenedioxy, ethylenedioxy, carboxyl, oxo, thioxo, imino (alkyl, aryl, aralkyl), S(O)_nalkyl (where n is 0-2), S(O)_n aryl (where n is 0-2), S(O)_n heteroaryl (where n is 0-2), S(O)_n heterocyclyl (where n is 0-2), amine (mono-, di-, alkyl, cycloalkyl, aralkyl, heteroaralkyl, and combinations thereof), ester (alkyl, aralkyl, heteroaralkyl), amide (mono-, di-, alkyl, aralkyl, heteroaralkyl, and combinations thereof), sulfonamide (mono-, di-, alkyl,
30 aralkyl, heteroaralkyl, and combinations thereof), unsubstituted aryl, unsubstituted heteroaryl, unsubstituted heterocyclyl, and unsubstituted cycloalkyl. In one aspect, the substituents on a group are independently any one single, or any subset of the aforementioned substituents.

The terms "adeninyl, cytosinyl, guaninyl, thyminyl, and uracilyl" and the like refer to radicals of adenine, cytosine, guanine, thymine, and uracil.

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
5 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.

Antagomir Structure

An antagomir, such as a single-stranded oligonucleotide agent, featured in the invention includes a region sufficient complementarity to the target nucleic acid (*e.g.*, target
10 miRNA, pre-miRNA or mRNA), and is of sufficient length in terms of nucleotides, such that the antagomir forms a duplex with the target nucleic acid. The antagomir can modulate the function of the targeted molecule. For example, when the targeted molecule is an miRNA, such as miR-122, miR-16, miR-192, or miR-194, the antagomir can inhibit the gene silencing activity of the target miRNA, which action will up-regulate expression of the mRNA targeted
15 by the target miRNA. When the target is an mRNA, the antagomir can replace or supplement the gene silencing activity of an endogenous miRNA.

For ease of exposition the term nucleotide or ribonucleotide is sometimes used herein in reference to one or more monomeric subunits of an oligonucleotide agent. It will be understood herein that the usage of the term “ribonucleotide” or “nucleotide” herein can, in
20 the case of a modified RNA or nucleotide surrogate, also refer to a modified nucleotide, or surrogate replacement moiety at one or more positions.

An antagomir featured in the invention is, or includes, a region that 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 antagomir and the target, but the
25 correspondence must be sufficient to enable the oligonucleotide agent, or a cleavage product thereof, to modulate (*e.g.*, inhibit) target gene expression.

An antagomir will preferably have one or more of the following properties:

(1) it will be of the Formula 1, 2, 3, or 4 described below;

(2) it will have a 5' modification that includes one or more phosphate groups or one or more analogs of a phosphate group;

(3) it will, despite modifications, even to a very large number of bases
5 specifically base pair and form a duplex structure with a homologous target RNA of sufficient thermodynamic stability to allow modulation of the activity of the targeted RNA;

(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
10 partly, of ribonucleotide-based content. For example, all of the nucleotide sugars can contain *e.g.*, 2'OMe, 2' fluoro 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, an electronegative fluorine prefers an axial orientation when attached to the C2' position of ribose. This spatial preference of fluorine can, in turn, force the sugars to adopt a
15 C_{3'}-*endo* pucker. This is the same puckering mode as observed in RNA molecules and gives rise to the RNA-characteristic A-family-type helix. Further, since fluorine is a good hydrogen bond acceptor, it can 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 hydrogen-bonding which is
20 more characteristic of the 2'-OH moiety of a ribonucleotide than the 2'-H moiety of a deoxyribonucleotide. A preferred antagomir will: exhibit a C_{3'}-*endo* pucker in all, or at least 50, 75, 80, 85, 90, or 95 % of its sugars; exhibit a C_{3'}-*endo* pucker 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 C_{3'}-*endo* pucker structure.

25 Preferred 2'-modifications with C_{3'}-*endo* sugar pucker include:

2'-OH, 2'-O-Me, 2'-O-methoxyethyl, 2'-O-aminopropyl, 2'-F, 2'-O-CH₂-CO-NHMe, 2'-O-CH₂-CH₂-O-CH₂-CH₂-N(Me)₂, and LNA

Preferred 2'-modifications with a C2'-endo sugar pucker include:

2'-H, 2'-Me, 2'-S-Me, 2'-Ethynyl, 2'-ara-F.

Sugar modifications can also include L-sugars and 2'-5'-linked sugars.

As used herein, "specifically hybridizable" and "complementary" are terms that are
5 used to indicate a sufficient degree of complementarity such that stable and specific binding
occurs between an antagomir of the invention and a target RNA molecule, e.g., an miRNA or
a pre-miRNA. Specific binding requires a sufficient lack of complementarity to non-target
sequences under conditions in which specific binding is desired, *i.e.*, under physiological
conditions in the case of *in vivo* assays or therapeutic treatment, or in the case of *in vitro*
10 assays, under conditions in which the assays are performed. It has been shown that a single
mismatch between targeted and non-targeted sequences are sufficient to provide
discrimination for siRNA targeting of an mRNA (Brummelkamp *et al.*, *Cancer Cell*, 2002,
2:243).

In one embodiment, an antagomir is "sufficiently complementary" to a target RNA,
15 such that the antagomir inhibits production of protein encoded by the target mRNA. The
target RNA can be, *e.g.*, a pre-mRNA, mRNA, or miRNA endogenous to the subject. In
another embodiment, the antagomir is "exactly complementary" (excluding the SRMS
containing subunit(s)) to a target RNA, *e.g.*, the target RNA and the antagomir can anneal to
form a hybrid made exclusively of Watson-Crick base pairs in the region of exact
20 complementarity. A "sufficiently complementary" target RNA can include a region (*e.g.*, of
at least 7 nucleotides) that is exactly complementary to a target RNA. Moreover, in some
embodiments, the antagomir specifically discriminates a single-nucleotide difference. In this
case, the antagomir only down-regulates gene expression if exact complementarity is found
in the region of the single-nucleotide difference.

25 Oligonucleotide agents discussed herein include otherwise unmodified RNA and
DNA as well as RNA and DNA that have been modified, *e.g.*, to improve efficacy, and
polymers of nucleoside surrogates. Unmodified RNA refers to a molecule in which the
components of the nucleic acid, namely sugars, bases, and phosphate moieties, are the same

or essentially the same as that which occur in nature, preferably as occur naturally in the human body. The art has referred to rare or unusual, but naturally occurring, RNAs as modified RNAs, see, *e.g.*, Limbach *et al.* (*Nucleic Acids Res.*, 1994, **22**:2183-2196). Such rare or unusual RNAs, often termed modified RNAs, are typically the result of a post-transcriptional modification and are within the term unmodified RNA as used herein.

Modified RNA, as used herein, refers to a molecule in which one or more of the components of the nucleic acid, namely sugars, bases, and phosphate moieties, are different from that which occur in nature, preferably different from that which occurs in the human body. While they are referred to as “modified RNAs” they will of course, because of the modification, include molecules that are not, strictly speaking, RNAs. Nucleoside surrogates are molecules in which the ribophosphate backbone is replaced with a non-ribophosphate construct that allows the bases to be presented in the correct spatial relationship such that hybridization is substantially similar to what is seen with a ribophosphate backbone, *e.g.*, non-charged mimics of the ribophosphate backbone. Examples of all of the above are discussed herein.

In some embodiments, the oligonucleotide agent is modified with one of the following modifications: modification of the nucleotide backbone such as modification of the phosphate linker or replacement of the phosphate linker; modification of the sugar moiety such as modification of the 2' hydroxyl on the ribose; replacement of the sugar moiety such as ribose or deoxyribose with a different chemical structure such as a PNA structure; or modification of the nucleobase for example modification to a universal base or G-clamp.

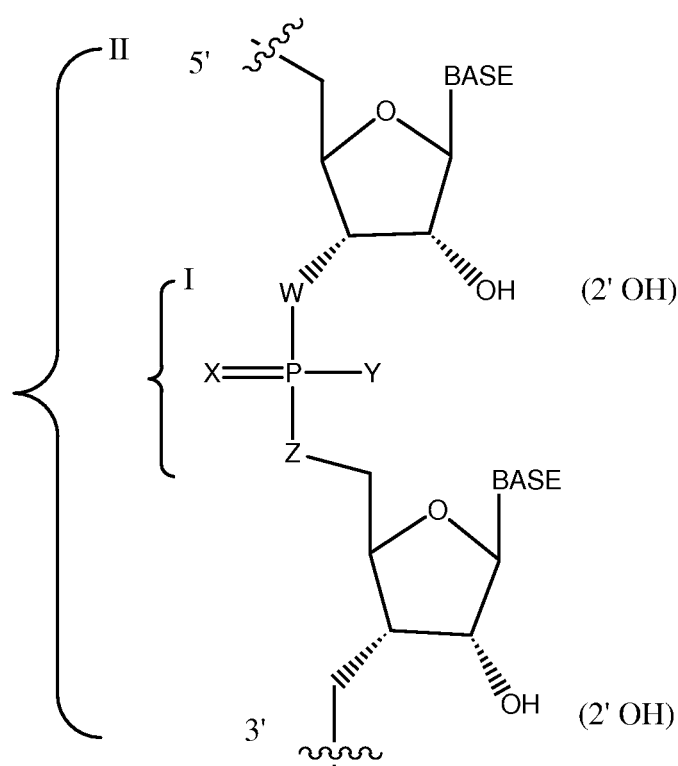
As nucleic acids are polymers of subunits or monomers, many of the modifications described below occur at a position which is repeated within a nucleic acid, *e.g.*, a modification of a base, or a phosphate moiety, or a non-linking O of a phosphate moiety. In some cases the modification will occur at 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, in a terminal region, *e.g.*, at a position on a terminal nucleotide, or in the last 2, 3, 4, 5, or 10 nucleotides of a strand. The ligand can be attached at the 3' end, the 5' end, or at an internal position, or at a combination of these positions. For example, the ligand can be at the 3' end and the 5' end; at the 3' end and at one or more internal positions; at the 5' end and at one or more internal positions; or at the 3' end, the 5' end, and at one or more internal positions. For example, a phosphorothioate modification at a non-linking O position may only occur at one or both termini, or may only occur in a terminal

region, *e.g.*, at a position on a terminal nucleotide or in the last 2, 3, 4, 5, or 10 nucleotides of the oligonucleotide. The 5' end can be phosphorylated.

Exemplary modifications of nucleotides are provided below:

Modifications and nucleotide surrogates are discussed below.

5



FORMULA 1

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.

10

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, for example, 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 oligonucleotide 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 of 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) ligands.

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 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 phosphoroamidate (replacement of a nonlinking oxygen with nitrogen) would be represented by $X = O$ and $Y = N$ in the above figure.

Specific modifications are discussed in more detail below.

(I) The Phosphate Group

The phosphate group is a negatively charged species. The charge is distributed equally over the two non-linking oxygen atoms (*i.e.*, X and Y in Formula 1 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.

Examples of modified phosphate groups include phosphorothioate, phosphoroselenates, borano phosphates, borano phosphate esters, hydrogen phosphonates, phosphoroamidates, alkyl or aryl phosphonates and phosphotriesters. Phosphorodithioates 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 phosphorodithioates 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.*, phosphorodithioate 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. In some preferred embodiments, the phosphate is modified to a phosphorothioate, phosphorodithioate, boranophosphate, N3'-P5' phosphoroamidate,

thiophosphoroamidate, phosphoramidate, cationic phosphoramidate, phosphonoacetate, phosphonothioacetate, 3'-methylene phosphonate, or a methylphosphonate

The phosphate linker can also be modified by replacement of a linking oxygen (i.e., W or Z in Formula 1) with nitrogen (bridged phosphoroamidates), sulfur (bridged phosphorothioates) and carbon (bridged methylenephosphonates). 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.

Exemplary modifications are also found in U.S.S.N. 11/170,798, which is incorporated herein by reference.

(II) The Sugar Group

A modified nucleotide agent can include modification of all or some of the sugar groups of the ribonucleic acid. For example, 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.

Examples of "oxy"-2' hydroxyl group modifications include alkoxy or aryloxy (OR, e.g., R = H, alkyl, cycloalkyl, aryl, aralkyl, heteroaryl or sugar); polyethyleneglycols (PEG), $O(CH_2CH_2O)_nCH_2CH_2OR$; "locked" nucleic acids (LNA) in which the 2' hydroxyl is connected, e.g., by a methylene bridge or ethylene bridge (e.g., 2'-4'-ethylene bridged nucleic acid (ENA)), to the 4' carbon of the same ribose sugar; amino, O-AMINE (AMINE = NH_2 ; alkylamino, dialkylamino, heterocyclyl, arylamino, diaryl amino, heteroaryl amino, or diheteroaryl amino, ethylene diamine, polyamino) and aminoalkoxy, $O(CH_2)_nAMINE$, (e.g., AMINE = NH_2 ; alkylamino, dialkylamino, heterocyclyl, arylamino, diaryl amino, heteroaryl amino, or diheteroaryl amino, ethylene diamine, polyamino). It is noteworthy that oligonucleotides containing only the methoxyethyl group (MOE), $(OCH_2CH_2OCH_3)$, a PEG derivative), exhibit nuclease stabilities comparable to those modified with the robust phosphorothioate modification.

“Deoxy” modifications include hydrogen (*i.e.* deoxyribose sugars); halo (*e.g.*, fluoro); amino (*e.g.* NH₂; alkylamino, dialkylamino, heterocyclyl, arylamino, diaryl amino, heteroaryl amino, diheteroaryl amino, or amino acid); NH(CH₂CH₂NH)_nCH₂CH₂-AMINE (AMINE = NH₂; alkylamino, dialkylamino, heterocyclyl, arylamino, diaryl amino, heteroaryl amino, or diheteroaryl amino), -NHC(O)R (R = alkyl, cycloalkyl, aryl, aralkyl, heteroaryl or sugar), cyano; mercapto; alkyl-thio-alkyl; thioalkoxy; and alkyl, cycloalkyl, aryl, alkenyl and alkynyl, which may be optionally substituted with *e.g.*, an amino functionality. Preferred substituents are 2'-methoxyethyl, 2'-OCH₃, 2'-O-allyl, 2'-C-allyl, and 2'-fluoro.

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 RNA can include nucleotides containing *e.g.*, arabinose, as the sugar.

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.

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.

The modification can also entail the wholesale replacement of a ribose structure with another entity (an SRMS) at one or more sites in the oligonucleotide agent.

Candidate modifications can be evaluated as described below.

In some preferred embodiments, the sugar modification includes one or more of 2'-OMe, 2'-F, 2'-O-MOE, 2'-O-thioMOE, 2'-O-AP, 2'-O-DMAOE, 2'-O-DMAEOE, 2'-O-NMA, 2'-O-DMAEA, 2'-O-GE, 2'-O-AE, 2'-O-DMAE, 2'-O-DMAP, 2'-O-ImBu, 2'-O-allyl, ANA, 2'-F-ANA, 3'-Modifications such as Terminal 3'-modifications, 2'-5' linkages, 4'-Modifications, such as 4'-Thio sugar, 4'-F, 4'-C-aminoethyl, 4'-C-aminoalkyl, 5'-modifications, such as 5'-alkyl, O-alkyl, 5'-terminal modifications, such as 5'-hydroxymethyl, Bicyclic Sugars, LNA, ENA, α -L-LNA, and carbocyclic analogs of LNA.

In some preferred embodiments, the ribose is replaced with one or more of morpholino, a cationic Morpholino, a PNA, a PNA analog, HNA, or CeNA.

(III) Replacement of the Phosphate Group

The phosphate group can be replaced by non-phosphorus containing connectors (*cf.* Bracket I in Formula 1 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 can be desirable, in some embodiment, to introduce alterations in which the charged phosphate group is replaced by a neutral moiety.

5 Examples of moieties which can replace the phosphate group include siloxane, carbonate, carboxymethyl, carbamate, amide, thioether, ethylene oxide linker, sulfonate, sulfonamide, thioformacetal, formacetal, oxime, methyleneimino, methylenemethylimino, methylenehydrazo, methylenedimethylhydrazo and methyleneoxymethylimino. Preferred replacements include the methylenecarbonylamino and methylenemethylimino groups.

10 (IV) The Bases

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
15 natural nucleobases (*e.g.*, inosine, thymine, xanthine, hypoxanthine, nubularine, isoguanisine, or tubercidine) and any one of the above modifications. Alternatively, substituted or modified analogs of any of the above bases, *e.g.*, "unusual 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
20 derivatives of adenine and guanine, 5-halouracil and cytosine, 5-propynyl uracil and cytosine, 6-azo uracil, cytosine and thymine, 5-uracil (pseudouracil), 4-thiouracil, 5-halouracil, 5-(2-aminopropyl)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
25 O-6 substituted purines, including 2-aminopropyladenine, 5-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-pyridinone, 5-nitroindole, 3-nitropyrrole, 5-methoxyuracil, uracil-5-oxyacetic acid, 5-methoxycarbonylmethyluracil, 5-
30 methyl-2-thiouracil, 5-methoxycarbonylmethyl-2-thiouracil, 5-methylaminomethyl-2-thiouracil, 3-(3-amino-3carboxypropyl)uracil, 3-methylcytosine, 5-methylcytosine, N⁴-acetyl cytosine, 2-thiocytosine, N6-methyladenine, N6-isopentyladenine, 2-methylthio-N6-isopentenyladenine, N-methylguanines, 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 Englisch *et al.*, Angewandte Chemie, International Edition, 1991, 30, 613.

In some preferred embodiments, the nucleotide agent includes one or more of the following base modifications: C-5 modified pyrimidine, N-2 modified purine, N-6 modified purine, C-8 modified purine, 2,6-Diaminopurine, a universal base, G-clamp, phenoxazines, or thiophenoxazine.

Exemplary base modifications are described in U.S.S.N: 11/186,915; US 11/197,753; and US 11/119,533, each of which is incorporated herein by reference.

(V) Replacement of Ribophosphate Backbone

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 polyanions (*e.g.* nucleases). Again, while not wishing to be bound by theory, it can be desirable in some embodiment, to introduce alterations in which the bases are tethered by a neutral surrogate backbone.

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

(VI) Ligands

An oligonucleotide agent can be modified to include a ligand. For example, the modification can be at 3' or 5' ends of an oligonucleotide, or internally. 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 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 atom of a nucleotide surrogate (*e.g.*, PNAs). These

spacers or linkers can include *e.g.*, $-(\text{CH}_2)_n-$, $-(\text{CH}_2)_n\text{N}-$, $-(\text{CH}_2)_n\text{O}-$, $-(\text{CH}_2)_n\text{S}-$, $\text{O}(\text{CH}_2\text{CH}_2\text{O})_n\text{CH}_2\text{CH}_2\text{OH}$ (*e.g.*, $n = 3$ or 6), abasic sugars, amide, carboxy, amine, oxyamine, oxyimine, thioether, disulfide, thiourea, sulfonamide, or morpholino, or biotin and fluorescein reagents. 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 alterations (*e.g.*, 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, texaphyrin, 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-O(hexadecyl)glycerol, geranyloxyhexyl group, hexadecylglycerol, borneol, menthol, 1,3-propanediol, heptadecyl group, palmitic acid, myristic acid, O3-(oleoyl)lithocholic acid, O3-(oleoyl)cholenic acid, dimethoxytrityl, or phenoxazine) and peptide conjugates (*e.g.*, antennapedia peptide, Tat peptide), alkylating agents, phosphate, amino, mercapto, PEG (*e.g.*, PEG-40K), MPEG, $[\text{MPEG}]_2$, polyamino, alkyl, substituted alkyl, radiolabeled markers, enzymes, haptens (*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, Eu^{3+} complexes of tetraazamacrocycles).

Modifications (*e.g.*, terminal modifications) can be added for a number of reasons, including as discussed elsewhere herein to modulate activity or to modulate resistance to degradation. Preferred modifications include the addition of a methylphosphonate at the 3'-most terminal linkage; a 3' C5-aminoalkyl-dT; 3' cationic group; or another 3' conjugate to inhibit 3'-5' exonucleolytic degradation.

Modifications (*e.g.*, terminal modifications) useful for modulating activity include modification of the 5' end with phosphate or phosphate analogs. *E.g.*, in preferred embodiments oligonucleotide agents are 5' phosphorylated or include a phosphoryl analog at the 5' terminus. 5'-phosphate modifications include those which are compatible with RISC mediated gene silencing. Suitable modifications include: 5'-monophosphate $((\text{HO})_2(\text{O})\text{P}-\text{O}-5')$; 5'-diphosphate $((\text{HO})_2(\text{O})\text{P}-\text{O}-\text{P}(\text{HO})(\text{O})-\text{O}-5')$; 5'-triphosphate $((\text{HO})_2(\text{O})\text{P}-\text{O}-\text{P}(\text{HO})(\text{O})\text{P}-\text{O}-\text{P}(\text{HO})(\text{O})-\text{O}-5')$; 5'-guanosine cap (7-methylated or non-methylated) $(7\text{m-G}-\text{O}-5'-(\text{HO})(\text{O})\text{P}-\text{O}-\text{P}(\text{HO})(\text{O})\text{P}-\text{O}-\text{P}(\text{HO})(\text{O})-\text{O}-5')$; 5'-adenosine cap (Appp), and any modified or unmodified nucleotide cap structure $(\text{N}-\text{O}-5'-(\text{HO})(\text{O})\text{P}-\text{O}-\text{P}(\text{HO})(\text{O})\text{P}-\text{O}-\text{P}(\text{HO})(\text{O})-\text{O}-5')$; 5'-

monothiophosphate (phosphorothioate; $(\text{HO})_2(\text{S})\text{P}-\text{O}-5'$); 5'-monodithiophosphate (phosphorodithioate; $(\text{HO})(\text{HS})(\text{S})\text{P}-\text{O}-5'$), 5'-phosphorothiolate ($(\text{HO})_2(\text{O})\text{P}-\text{S}-5'$); any additional combination of oxygen/sulfur replaced monophosphate, diphosphate and triphosphates (*e.g.* 5'-alpha-thiotriphosphate, 5'-gamma-thiotriphosphate, etc.), 5'-phosphoramidates ($(\text{HO})_2(\text{O})\text{P}-\text{NH}-5'$, $(\text{HO})(\text{NH}_2)(\text{O})\text{P}-\text{O}-5'$), 5'-alkylphosphonates ($\text{R}=\text{alkyl}=\text{methyl, ethyl, isopropyl, propyl, etc.}$, *e.g.* $\text{RP}(\text{OH})(\text{O})-\text{O}-5'$, $(\text{OH})_2(\text{O})\text{P}-5'-\text{CH}_2-$), 5'-alkyletherphosphonates ($\text{R}=\text{alkylether}=\text{methoxymethyl (MeOCH}_2-$), ethoxymethyl, etc., *e.g.* $\text{RP}(\text{OH})(\text{O})-\text{O}-5'$).

Modifications (*e.g.*, 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 antagomir to another moiety; modifications useful for this include mitomycin C.

Exemplary lipophilic modifications include a cholesterol; a bile acid; and a fatty acid (*e.g.*, lithocholic-oleyl, lauroyl, docosanyl, stearoyl, palmitoyl, myristoyl, oleoyl, linoleoyl). Other exemplary terminal modifications include the following, sugars, carbohydrates, folates and analogs thereof, PEGs, pluronics, PEI, endosomal releasing agents, cell surface targeting small molecules, cell surface targeting peptides (*e.g.* RGD and other phage display derived peptides), cell permeation peptides, nuclear targeting signal peptides (NLS), polymers (for example, polymers with targeting groups, polymers with endosomal releasing agents, polymers with biodegradable properties, polymers with nucleic acid packing groups (by charge interactions, by hydrogen bonding interactions).

The above modifications can be made with the oligonucleotide agent with or without a linker (*e.g.*, a cleavable linker), with or without a tether such as a long alkyl tether, with or without a spacer such as a PEG spacer, with or without a scaffold. The modifications can be made anywhere on the oligonucleotide agent, for example, at 5'-, 3'- or at internal positions.

Evaluation of Candidate Oligonucleotide agents

One can evaluate a candidate single-stranded oligonucleotide agent, *e.g.*, a modified candidate single-stranded oligonucleotide agent, 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 degradant can be evaluated as follows. A candidate modified antagomir (and preferably a control single-

stranded oligonucleotide agent, usually the unmodified form) can be exposed to degradative conditions, *e.g.*, exposed to a milieu, which includes a degradative agent, *e.g.*, a nuclease. For example, 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 can 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 oligonucleotide agents 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.

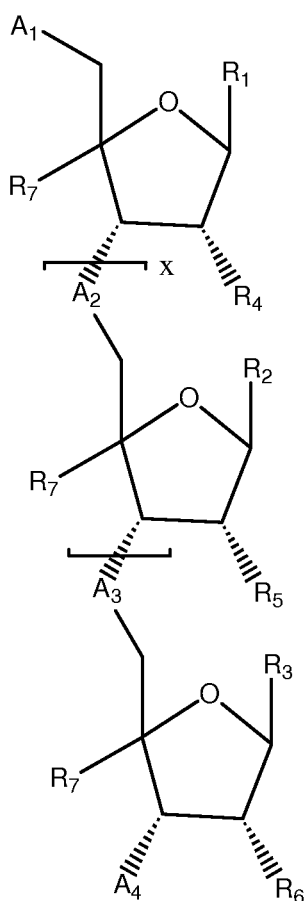
A functional assay can also be used to evaluate the candidate agent. A functional assay can be applied initially or after an earlier non-functional assay, (*e.g.*, assay for resistance to degradation) to determine if the modification alters the ability of the molecule to inhibit gene expression. For 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 antagomir homologous to the transcript encoding the fluorescent protein (see, *e.g.*, WO 00/44914). For example, a modified antagomir 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, in which the transfection did not include the candidate oligonucleotide agent, *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 oligonucleotide agent.

In an alternative functional assay, a candidate antagomir homologous to an endogenous mouse gene, preferably a maternally 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 an antagomir 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 RNA, 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 with no agent is added and/or cells in which a non-modified RNA is added.

Preferred oligonucleotide agents

Preferred single-stranded oligonucleotide agents have the following structure (see Formula 2 below):

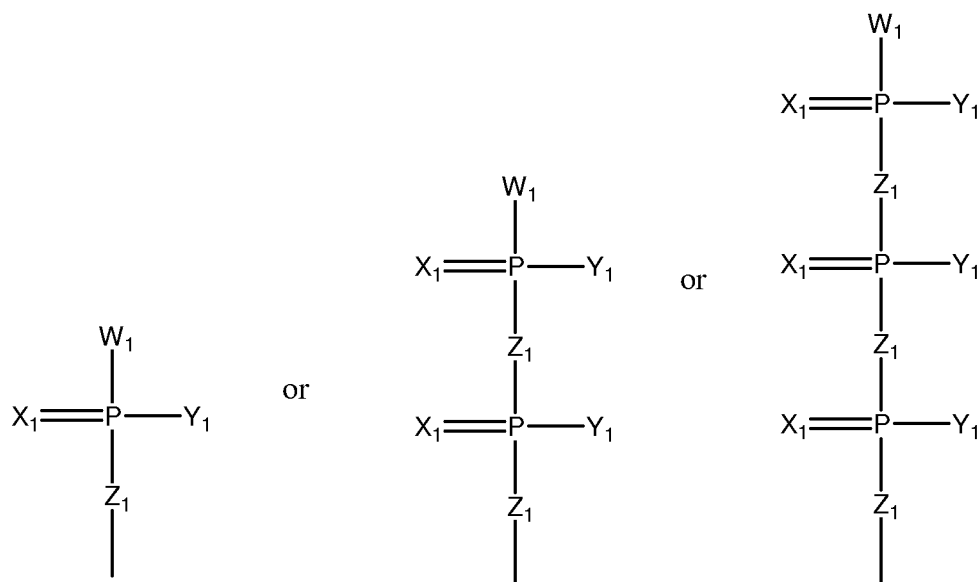


FORMULA 2

Referring to Formula 2 above, R^1 , R^2 , and R^3 are each, independently, H, (*i.e.* abasic nucleotides), adenine, guanine, cytosine and uracil, inosine, thymine, xanthine, hypoxanthine, nubularine, tubercidine, isoguanisine, 2-aminoadenine, 6-methyl and other alkyl derivatives of adenine and guanine, 2-propyl and other alkyl derivatives of adenine and guanine, 5-halouracil and cytosine, 5-propynyl uracil and cytosine, 6-azo uracil, cytosine and thymine, 5-uracil (pseudouracil), 4-thiouracil, 5-halouracil, 5-(2-aminopropyl)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-aminopropyladenine, 5-propynyluracil and 5-propynylcytosine, dihydrouracil, 3-deaza-5-azacytosine, 2-aminopurine, 5-alkyluracil, 7-alkylguanine, 5-alkyl cytosine, 7-deazaadenine, 7-deazaguanine, N6, N6-dimethyladenine, 2,6-diaminopurine, 5-amino-allyl-uracil, N3-methyluracil, substituted 1,2,4-triazoles, 2-pyridinone, 5-nitroindole, 3-nitropyrrole, 5-methoxyuracil, uracil-5-oxyacetic acid, 5-methoxycarbonylmethyluracil, 5-methyl-2-thiouracil, 5-methoxycarbonylmethyl-2-thiouracil, 5-methylaminomethyl-2-thiouracil, 3-(3-amino-3carboxypropyl)uracil, 3-methylcytosine, 5-methylcytosine, N^4 -acetyl cytosine, 2-thiocytosine, N6-methyladenine, N6-isopentyladenine, 2-methylthio-N6-isopentenyladenine, N-methylguanines, or O-alkylated bases.

R^4 , R^5 , and R^6 are each, independently, OR^8 , $O(CH_2CH_2O)_mCH_2CH_2OR^8$; $O(CH_2)_nR^9$; $O(CH_2)_nOR^9$; H; halo; NH_2 ; NHR^8 ; $N(R^8)_2$; $NH(CH_2CH_2NH)_mCH_2CH_2NHR^9$; $NHC(O)R^8$; ; cyano; mercapto, SR^8 ; alkyl-thio-alkyl; alkyl, aralkyl, cycloalkyl, aryl, heteroaryl, alkenyl, alkynyl, each of which may be optionally substituted with halo, hydroxy, oxo, nitro, haloalkyl, alkyl, alkaryl, aryl, aralkyl, alkoxy, aryloxy, amino, alkylamino, dialkylamino, heterocyclyl, arylamino, diaryl amino, heteroaryl amino, diheteroaryl amino, acylamino, alkylcarbamoyle, arylcarbamoyle, aminoalkyl, alkoxy carbonyl, carboxy, hydroxyalkyl, alkanesulfonyl, alkanesulfonamido, arenesulfonamido, aralkylsulfonamido, alkylcarbonyl, acyloxy, cyano, or ureido; or R^4 , R^5 , or R^6 together combine with R^7 to form an [-O-CH₂-] covalently bound bridge between the sugar 2' and 4' carbons.

A¹ is:



5 ; H; OH; OCH₃; W¹; an abasic nucleotide; or absent;

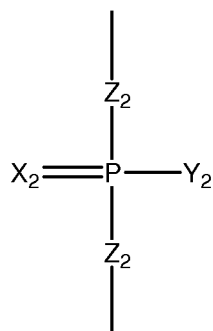
(a preferred A¹, especially with regard to anti-sense strands, is chosen from 5'-monophosphate ((HO)₂(O)P-O-5'), 5'-diphosphate ((HO)₂(O)P-O-P(HO)(O)-O-5'), 5'-triphosphate ((HO)₂(O)P-O-(HO)(O)P-O-P(HO)(O)-O-5'), 5'-guanosine cap (7-methylated or non-methylated) (7m-G-O-5'-(HO)(O)P-O-(HO)(O)P-O-P(HO)(O)-O-5'), 5'-adenosine cap (A_{ppp}), and any modified or unmodified nucleotide cap structure (N-O-5'-(HO)(O)P-O-(HO)(O)P-O-P(HO)(O)-O-5'), 5'-monothiophosphate (phosphorothioate; (HO)₂(S)P-O-5'), 5'-monodithiophosphate (phosphorodithioate; (HO)(HS)(S)P-O-5'), 5'-phosphorothiolate ((HO)₂(O)P-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-5', (HO)(NH₂)(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'-CH₂-),

10

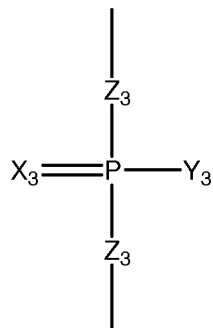
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5'-alkyletherphosphonates (R=alkylether=methoxymethyl (MeOCH₂-), ethoxymethyl, etc., *e.g.* RP(OH)(O)-O-5'-)).

A² is:

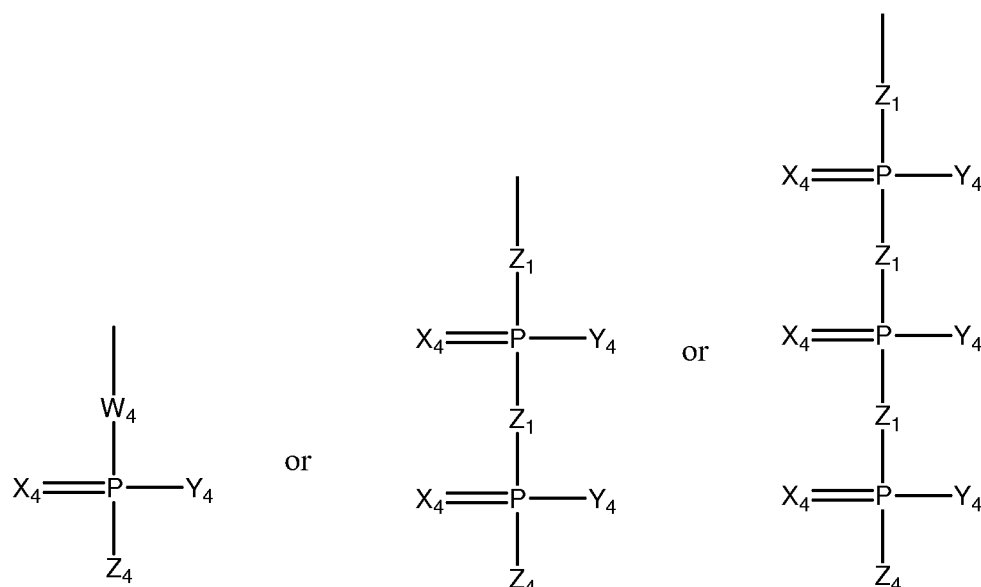


5 A³ is:



; and

A⁴ is:



5 ; H; Z⁴; an inverted nucleotide; an abasic nucleotide; or absent.

W¹ is OH, (CH₂)_nR¹⁰, (CH₂)_nNHR¹⁰, (CH₂)_nOR¹⁰, (CH₂)_nSR¹⁰; O(CH₂)_nR¹⁰; O(CH₂)_nOR¹⁰, O(CH₂)_nNR¹⁰, O(CH₂)_nSR¹⁰; O(CH₂)_nSS(CH₂)_nOR¹⁰, O(CH₂)_nC(O)OR¹⁰, NH(CH₂)_nR¹⁰; NH(CH₂)_nNR¹⁰; NH(CH₂)_nOR¹⁰, NH(CH₂)_nSR¹⁰; S(CH₂)_nR¹⁰, S(CH₂)_nNR¹⁰, S(CH₂)_nOR¹⁰, S(CH₂)_nSR¹⁰ O(CH₂CH₂O)_mCH₂CH₂OR¹⁰; O(CH₂CH₂O)_mCH₂CH₂NHR¹⁰, NH(CH₂CH₂NH)_mCH₂CH₂NHR¹⁰; Q-R¹⁰, O-Q-R¹⁰ N-Q-R¹⁰, S-Q-R¹⁰ or -O-. W⁴ is O, CH₂, NH, or S.

10

X¹, X², X³, and X⁴ are each, independently, O or S.

Y¹, Y², Y³, and Y⁴ are each, independently, OH, O⁻, OR⁸, S, Se, BH₃⁻, H, NHR⁹, N(R⁹)₂ alkyl, cycloalkyl, aralkyl, aryl, or heteroaryl, each of which may be optionally substituted.

15

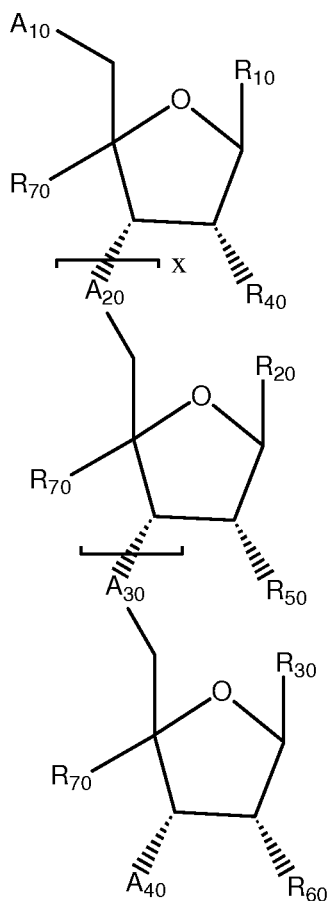
Z^1 , Z^2 , and Z^3 are each independently O, CH_2 , NH, or S. Z^4 is OH, $(\text{CH}_2)_n\text{R}^{10}$, $(\text{CH}_2)_n\text{NHR}^{10}$, $(\text{CH}_2)_n\text{OR}^{10}$, $(\text{CH}_2)_n\text{SR}^{10}$; $\text{O}(\text{CH}_2)_n\text{R}^{10}$; $\text{O}(\text{CH}_2)_n\text{OR}^{10}$, $\text{O}(\text{CH}_2)_n\text{NR}^{10}$, $\text{O}(\text{CH}_2)_n\text{SR}^{10}$, $\text{O}(\text{CH}_2)_n\text{SS}(\text{CH}_2)_n\text{OR}^{10}$, $\text{O}(\text{CH}_2)_n\text{C}(\text{O})\text{OR}^{10}$; $\text{NH}(\text{CH}_2)_n\text{R}^{10}$; $\text{NH}(\text{CH}_2)_n\text{NR}^{10}$; $\text{NH}(\text{CH}_2)_n\text{OR}^{10}$, $\text{NH}(\text{CH}_2)_n\text{SR}^{10}$; $\text{S}(\text{CH}_2)_n\text{R}^{10}$, $\text{S}(\text{CH}_2)_n\text{NR}^{10}$, $\text{S}(\text{CH}_2)_n\text{OR}^{10}$, $\text{S}(\text{CH}_2)_n\text{SR}^{10}$
 5 $\text{O}(\text{CH}_2\text{CH}_2\text{O})_m\text{CH}_2\text{CH}_2\text{OR}^{10}$, $\text{O}(\text{CH}_2\text{CH}_2\text{O})_m\text{CH}_2\text{CH}_2\text{NHR}^{10}$, $\text{NH}(\text{CH}_2\text{CH}_2\text{NH})_m\text{CH}_2\text{CH}_2\text{NHR}^{10}$; Q-R^{10} , O-Q-R^{10} , N-Q-R^{10} , S-Q-R^{10} .

X is 5-100, chosen to comply with a length for an antagomir described herein.

R^7 is H; or is together combined with R^4 , R^5 , or R^6 to form an $[-\text{O}-\text{CH}_2-]$ covalently bound bridge between the sugar 2' and 4' carbons.

10 R^8 is alkyl, cycloalkyl, aryl, aralkyl, heterocyclyl, heteroaryl, amino acid, or sugar; R^9 is NH_2 , alkylamino, dialkylamino, heterocyclyl, arylamino, diaryl amino, heteroaryl amino, diheteroaryl amino, or amino acid; and R^{10} is H; fluorophore (pyrene, TAMRA, fluorescein, Cy3 or Cy5 dyes); sulfur, silicon, boron or ester protecting group; intercalating agents (*e.g.* acridines), cross-linkers (*e.g.* psoralene, mitomycin C), porphyrins (TPPC4, texaphyrin,
 15 Sapphyrin), polycyclic aromatic hydrocarbons (*e.g.*, phenazine, dihydrophenazine), artificial endonucleases (*e.g.* EDTA), lipophilic carriers (cholesterol, cholic acid, adamantane acetic acid, 1-pyrene butyric acid, dihydrotestosterone, 1,3-Bis-O(hexadecyl)glycerol, geranyloxyhexyl group, hexadecylglycerol, borneol, menthol, 1,3-propanediol, heptadecyl group, palmitic acid, myristic acid, O3-(oleoyl)lithocholic acid, O3-(oleoyl)cholenic acid,
 20 dimethoxytrityl, or phenoxazine) and peptide conjugates (*e.g.*, antennapedia peptide, Tat peptide), alkylating agents, phosphate, amino, mercapto, PEG (*e.g.*, PEG-40K), MPEG, $[\text{MPEG}]_2$, polyamino; alkyl, cycloalkyl, aryl, aralkyl, heteroaryl; radiolabelled markers, enzymes, haptens (*e.g.* biotin), transport/absorption facilitators (*e.g.*, aspirin, vitamin E, folic acid), synthetic ribonucleases (*e.g.*, imidazole, bisimidazole, histamine, imidazole clusters,
 25 acridine-imidazole conjugates, Eu^{3+} complexes of tetraazamacrocycles); or an oligonucleotide agent. M is 0-1,000,000, and n is 0-20. Q is a spacer selected from the group consisting of abasic sugar, amide, carboxy, oxyamine, oxyimine, thioether, disulfide, thiourea, sulfonamide, or morpholino, biotin or fluorescein reagents.

Preferred oligonucleotide agents in which the entire phosphate group has been replaced have the following structure (see Formula 3 below):



FORMULA 3

Referring to Formula 3, A¹⁰-A⁴⁰ is L-G-L; A¹⁰ and/or A⁴⁰ 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 CH₂(CH₂)_g; N(CH₂)_g; O(CH₂)_g; S(CH₂)_g. G is a functional group selected from the group consisting of siloxane, carbonate, carboxymethyl, carbamate, amide, thioether,

ethylene oxide linker, sulfonate, sulfonamide, thioformacetal, formacetal, oxime, methyleneimino, methylenemethylimino, methylenehydrazo, methylenedimethylhydrazo and methyleneoxymethylimino.

R^{10} , R^{20} , and R^{30} are each, independently, H, (*i.e.* abasic nucleotides), adenine, guanine, cytosine and uracil, inosine, thymine, xanthine, hypoxanthine, nubularine, tubercidine, isoguanisine, 2-aminoadenine, 6-methyl and other alkyl derivatives of adenine and guanine, 2-propyl and other alkyl derivatives of adenine and guanine, 5-halouracil and cytosine, 5-propynyl uracil and cytosine, 6-azo uracil, cytosine and thymine, 5-uracil (pseudouracil), 4-thiouracil, 5-halouracil, 5-(2-aminopropyl)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-aminopropyladenine, 5-propynyluracil and 5-propynylcytosine, dihydrouracil, 3-deaza-5-azacytosine, 2-aminopurine, 5-alkyluracil, 7-alkylguanine, 5-alkyl cytosine, 7-deazaadenine, 7-deazaguanine, N6, N6-dimethyladenine, 2,6-diaminopurine, 5-amino-allyl-uracil, N3-methyluracil substituted 1,2,4-triazoles, 2-pyridinone, 5-nitroindole, 3-nitropyrrole, 5-methoxyuracil, uracil-5-oxyacetic acid, 5-methoxycarbonylmethyluracil, 5-methyl-2-thiouracil, 5-methoxycarbonylmethyl-2-thiouracil, 5-methylaminomethyl-2-thiouracil, 3-(3-amino-3-carboxypropyl)uracil, 3-methylcytosine, 5-methylcytosine, N^4 -acetyl cytosine, 2-thiocytosine, N6-methyladenine, N6-isopentyladenine, 2-methylthio-N6-isopentenyladenine, N-methylguanines, or O-alkylated bases.

R^{40} , R^{50} , and R^{60} are each, independently, OR^8 , $O(CH_2CH_2O)_mCH_2CH_2OR^8$; $O(CH_2)_nR^9$; $O(CH_2)_nOR^9$, H; halo; NH_2 ; NHR^8 ; $N(R^8)_2$; $NH(CH_2CH_2NH)_mCH_2CH_2R^9$; $NHC(O)R^8$; cyano; mercapto, SR^7 ; alkyl-thio-alkyl; alkyl, aralkyl, cycloalkyl, aryl, heteroaryl, alkenyl, alkynyl, each of which may be optionally substituted with halo, hydroxy, oxo, nitro, haloalkyl, alkyl, alkaryl, aryl, aralkyl, alkoxy, aryloxy, amino, alkylamino, dialkylamino, heterocyclyl, arylamino, diaryl amino, heteroaryl amino, diheteroaryl amino, acylamino, alkylcarbamoyle, arylcarbamoyle, aminoalkyl, alkoxycarbonyl, carboxy, hydroxyalkyl, alkanesulfonyl, alkanesulfonamido, arenesulfonamido, aralkylsulfonamido, alkylcarbonyl, acyloxy, cyano, and ureido groups; or R^{40} , R^{50} , or R^{60} together combine with R^{70} to form an $[-O-CH_2-]$ covalently bound bridge between the sugar 2' and 4' carbons.

X is 5-100 or chosen to comply with a length for an antagomir described herein.

R^{70} is H; or is together combined with R^{40} , R^{50} , or R^{60} to form an [-O-CH₂-] covalently bound bridge between the sugar 2' and 4' carbons.

R^8 is alkyl, cycloalkyl, aryl, aralkyl, heterocyclyl, heteroaryl, amino acid, or sugar; and R^9 is NH₂, alkylamino, dialkylamino, heterocyclyl, arylamino, diaryl amino, heteroaryl amino, diheteroaryl amino, or amino acid. M is 0-1,000,000, n is 0-20, and g is 0-2.

Preferred nucleoside surrogates have the following structure (see Formula 4 below):



FORMULA 4

S is a nucleoside surrogate selected from the group consisting of morpholino, cyclobutyl, pyrrolidine and peptide nucleic acid. L is a linker and is selected from the group consisting of CH₂(CH₂)_g; N(CH₂)_g; O(CH₂)_g; S(CH₂)_g; -C(O)(CH₂)_n- or may be absent. M is an amide bond; sulfonamide; sulfinate; phosphate group; modified phosphate group as described herein; or may be absent.

R^{100} , R^{200} , and R^{300} are each, independently, H (*i.e.*, abasic nucleotides), adenine, guanine, cytosine and uracil, inosine, thymine, xanthine, hypoxanthine, nucleoside, tubercidine, isoguanisine, 2-aminoadenine, 6-methyl and other alkyl derivatives of adenine and guanine, 2-propyl and other alkyl derivatives of adenine and guanine, 5-halouracil and cytosine, 5-propynyl uracil and cytosine, 6-azo uracil, cytosine and thymine, 5-uracil (pseudouracil), 4-thiouracil, 5-halouracil, 5-(2-aminopropyl)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-aminopropyladenine, 5-propynyluracil and 5-propynylcytosine, dihydrouracil, 3-deaza-5-azacytosine, 2-aminopurine, 5-alkyluracil, 7-alkylguanine, 5-alkyl cytosine, 7-deazaadenine, 5-7-deazaguanine, N6, N6-dimethyladenine, 2,6-diaminopurine, 5-amino-allyl-uracil, N3-methyluracil substituted 1, 2, 4,-triazoles, 2-pyridinones, 5-nitroindole, 3-nitropyrrole, 5-methoxyuracil, uracil-5-oxyacetic acid, 5-methoxycarbonylmethyluracil, 5-methyl-2-thiouracil, 5-methoxycarbonylmethyl-2-thiouracil, 5-methylaminomethyl-2-thiouracil, 3-(3-amino-3carboxypropyl)uracil, 3-methylcytosine, 5-methylcytosine, N⁴-acetyl cytosine, 2-thiocytosine, N6-methyladenine, N6-isopentyladenine, 2-methylthio-N6-isopentenyladenine, N-methylguanines, or O-alkylated bases.

X is 5-100, or chosen to comply with a length for an antagomir described herein; and g is 0-2.

An antagomir can include an internucleotide linkage (*e.g.*, the chiral phosphorothioate linkage) useful for increasing nuclease resistance. In addition, or in the alternative, an antagomir can include a ribose mimic for increased nuclease resistance. Exemplary internucleotide linkages and ribose mimics for increased nuclease resistance are described in co-owned PCT Application No. PCT/US2004/07070 filed on March 8, 2004.

An antagomir can include ligand-conjugated monomer subunits and monomers for oligonucleotide synthesis. Exemplary monomers are described in co-owned U.S. Application No. 10/916,185, filed on August 10, 2004.

An antagomir can have a ZXY structure, such as is described in co-owned PCT Application No. PCT/US2004/07070 filed on March 8, 2004.

An antagomir can be complexed with an amphipathic moiety. Exemplary amphipathic moieties for use with oligonucleotide agents are described in co-owned PCT Application No. PCT/US2004/07070 filed on March 8, 2004.

In another embodiment, the antagomir can be complexed to a delivery agent that features a modular complex. The complex can include a carrier agent linked to one or more of (preferably two or more, more preferably all three of): (a) a condensing agent (*e.g.*, an agent capable of attracting, *e.g.*, binding, a nucleic acid, *e.g.*, through ionic or electrostatic interactions); (b) a fusogenic agent (*e.g.*, an agent capable of fusing and/or being transported through a cell membrane); and (c) a targeting group, *e.g.*, a cell or tissue targeting agent, *e.g.*,

a lectin, glycoprotein, lipid or protein, *e.g.*, an antibody, that binds to a specified cell type. oligonucleotide agents complexed to a delivery agent are described in co-owned PCT Application No. PCT/US2004/07070 filed on March 8, 2004.

Enhanced nuclease resistance

5 An antagomir, such as a single-stranded oligonucleotide agent, featured in the invention can have enhanced resistance to nucleases.

For increased nuclease resistance and/or binding affinity to the target, an oligonucleotide agent, *e.g.*, the oligonucleotide agent, can include, for example, 2'-modified ribose units and/or phosphorothioate linkages. *E.g.*, the 2' hydroxyl group (OH) can be
 10 modified or replaced with a number of different "oxy" or "deoxy" substituents.

Examples of "oxy"-2' hydroxyl group modifications include alkoxy or aryloxy (OR, *e.g.*, R = H, alkyl, cycloalkyl, aryl, aralkyl, heteroaryl or sugar); polyethyleneglycols (PEG), $O(CH_2CH_2O)_nCH_2CH_2OR$; "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; amine, O-
 15 AMINE and aminoalkoxy, $O(CH_2)_nAMINE$, (*e.g.*, AMINE = NH_2 ; alkylamino, dialkylamino, heterocyclyl amino, arylamino, diaryl amino, heteroaryl amino, or diheteroaryl amino, ethylene diamine, polyamino). It is noteworthy that oligonucleotides containing only the methoxyethyl group (MOE), $(OCH_2CH_2OCH_3)_n$, a PEG derivative, exhibit nuclease stabilities comparable to those modified with the robust phosphorothioate modification.

20 "Deoxy" modifications include hydrogen (*i.e.* deoxyribose sugars); halo (*e.g.*, fluoro); amino (*e.g.* NH_2 ; alkylamino, dialkylamino, heterocyclyl, arylamino, diaryl amino, heteroaryl amino, diheteroaryl amino, or amino acid); $NH(CH_2CH_2NH)_nCH_2CH_2-AMINE$ (AMINE = NH_2 ; alkylamino, dialkylamino, heterocyclyl amino, arylamino, diaryl amino, heteroaryl amino, or diheteroaryl amino), $-NHC(O)R$ (R = alkyl, cycloalkyl, aryl, aralkyl, heteroaryl or
 25 sugar), cyano; mercapto; alkyl-thio-alkyl; thioalkoxy; and alkyl, cycloalkyl, aryl, alkenyl and alkynyl, which may be optionally substituted with *e.g.*, an amino functionality.

Preferred substituents are 2'-methoxyethyl, 2'-OCH₃, 2'-O-allyl, 2'-C- allyl, and 2'-fluoro.

One way to increase resistance is to identify cleavage sites and modify such sites to
 30 inhibit cleavage, as described in co-owned U.S. Application No. 60/559,917, filed on May 4, 2004. For example, the dinucleotides 5'-UA-3', 5'-UG-3', 5'-CA-3', 5'-UU-3', or 5'-CC-3'

can serve as cleavage sites. Enhanced nuclease resistance can therefore be achieved by modifying the 5' nucleotide, resulting, for example, in at least one 5'-uridine-adenine-3' (5'-UA-3') dinucleotide wherein the uridine is a 2'-modified nucleotide; at least one 5'-uridine-guanine-3' (5'-UG-3') dinucleotide, wherein the 5'-uridine is a 2'-modified nucleotide; at least one 5'-cytidine-adenine-3' (5'-CA-3') dinucleotide, wherein the 5'-cytidine is a 2'-modified nucleotide; at least one 5'-uridine-uridine-3' (5'-UU-3') dinucleotide, wherein the 5'-uridine is a 2'-modified nucleotide; or at least one 5'-cytidine-cytidine-3' (5'-CC-3') dinucleotide, wherein the 5'-cytidine is a 2'-modified nucleotide. The antagomir can include at least 2, at least 3, at least 4 or at least 5 of such dinucleotides. In certain embodiments, all the pyrimidines of an antagomir carry a 2'-modification, and the antagomir therefore has enhanced resistance to endonucleases.

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.

The inclusion of furanose sugars in the oligonucleotide backbone can also decrease endonucleolytic cleavage. An antagomir can be further modified by including a 3' cationic group, or by inverting the nucleoside at the 3'-terminus with a 3'-3' linkage. In another alternative, the 3'-terminus can be blocked with an aminoalkyl group, *e.g.*, a 3' C5-aminoalkyl dT. Other 3' conjugates can inhibit 3'-5' exonucleolytic cleavage. While not being bound by theory, a 3' conjugate, such as naproxen or ibuprofen, may inhibit exonucleolytic cleavage by sterically blocking the exonuclease from binding to the 3'-end of oligonucleotide. Even small alkyl chains, aryl groups, or heterocyclic conjugates or modified sugars (D-ribose, deoxyribose, glucose *etc.*) can block 3'-5'-exonucleases.

Similarly, 5' conjugates can inhibit 5'-3' exonucleolytic cleavage. While not being bound by theory, a 5' conjugate, such as naproxen or ibuprofen, may inhibit exonucleolytic cleavage by sterically blocking the exonuclease from binding to the 5'-end of oligonucleotide. Even small alkyl chains, aryl groups, or heterocyclic conjugates or modified sugars (D-ribose, deoxyribose, glucose *etc.*) can block 3'-5'-exonucleases.

Thus, an antagomir can include modifications so as to inhibit degradation, *e.g.*, by nucleases, *e.g.*, endonucleases or exonucleases, found in the body of a subject. These monomers are referred to herein as NRMs, or Nuclease Resistance promoting Monomers, the corresponding modifications as NRM modifications. In many cases these modifications will

modulate other properties of the antagomir as well, e.g., the ability to interact with a protein, e.g., a transport protein, e.g., serum albumin, or a member of the RISC, or the ability of the antagomir to form a duplex with another sequence, e.g., a target molecule, such as an miRNA or pre-miRNA.

5 One or more different NRM modifications can be introduced into an antagomir or into a sequence of an oligonucleotide agent. An NRM modification can be used more than once in a sequence or in an oligonucleotide agent.

 NRM modifications include some which can be placed only at the terminus and others which can go at any position. Some NRM modifications that can inhibit hybridization are
10 preferably used only in terminal regions, and more preferably not at the cleavage site or in the cleavage region of the oligonucleotide agent.

 Modifications which interfere with 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 Elbashir *et al.*, *Genes and Dev.* 15: 188, 2001, hereby
15 incorporated by reference). Cleavage of the target occurs about in the middle of a 20 or 21 nt oligonucleotide agent, or about 10 or 11 nucleotides upstream of the first nucleotide on the target mRNA which is complementary to the oligonucleotide agent. As used herein, cleavage site refers to the nucleotides on either side of the site of cleavage, on the target mRNA or on the antagomir which hybridizes to it. Cleavage region means the nucleotides within 1, 2, or 3
20 nucleotides of the cleavage site, in either direction.

 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.

Delivery of single-stranded oligonucleotide agents to tissues and cells

25 Formulation

 The single-stranded oligonucleotide agents described herein can be formulated for administration to a subject.

 For ease of exposition, the formulations, compositions, and methods in this section are discussed largely with regard to unmodified oligonucleotide agents. It should be

understood, however, that these formulations, compositions, and methods can be practiced with other oligonucleotide agents, *e.g.*, modified oligonucleotide agents, and such practice is within the invention.

5 A formulated antagomir composition can assume a variety of states. In some examples, the composition is at least partially crystalline, uniformly crystalline, and/or anhydrous (*e.g.*, less than 80, 50, 30, 20, or 10% water). In another example, the antagomir is in an aqueous phase, *e.g.*, in a solution that includes water, this form being the preferred form for administration via inhalation.

10 The aqueous phase or the crystalline compositions can be incorporated into a delivery vehicle, *e.g.*, a liposome (particularly for the aqueous phase), or a particle (*e.g.*, a microparticle as can be appropriate for a crystalline composition). Generally, the antagomir composition is formulated in a manner that is compatible with the intended method of administration.

15 An antagomir preparation can be formulated in combination with another agent, *e.g.*, another therapeutic agent or an agent that stabilizes an oligonucleotide agent, *e.g.*, a protein that complexes with the oligonucleotide agent. Still other agents include chelators, *e.g.*, EDTA (*e.g.*, to remove divalent cations such as Mg^{2+}), salts, RNase inhibitors (*e.g.*, a broad specificity RNase inhibitor such as RNasin) and so forth.

20 In one embodiment, the antagomir preparation includes another antagomir, *e.g.*, a second antagomir that can down-regulate expression of a second gene. Still other preparations can include at least three, five, ten, twenty, fifty, or a hundred or more different oligonucleotide species. In some embodiments, the agents are directed to the same target nucleic acid but different target sequences. In another embodiment, each antagomir is directed to a different target. In one embodiment the antagomir preparation includes a double
25 stranded RNA that targets an RNA (*e.g.*, an mRNA) for downregulation by an RNAi silencing mechanism.

Treatment Methods and Routes of Delivery

A composition that includes an antagomir featured in the invention, e.g., an antagomir that targets an miRNA or pre-miRNA (e.g., miR-122, miR-16, miR-192, or miR-194) can be delivered to a subject by a variety of routes. Exemplary routes include inhalation, intrathecal, parenchymal, intravenous, nasal, oral, and ocular delivery.

An antagomir can be incorporated into pharmaceutical compositions suitable for administration. For example, compositions can include one or more oligonucleotide agents and a pharmaceutically acceptable carrier. As used herein the language “pharmaceutically acceptable carrier” is intended to include any and all solvents, dispersion media, coatings, antibacterial and antifungal agents, isotonic and absorption delaying agents, and the like, compatible with pharmaceutical administration. The use of such media and agents for pharmaceutically active substances is well known in the art. Except insofar as any conventional media or agent is incompatible with the active compound, use thereof in the compositions is contemplated. Supplementary active compounds can also be incorporated into the compositions.

The pharmaceutical compositions featured in the invention may be administered in a number of ways depending upon whether local or systemic treatment is desired and upon the area to be treated. Administration may be topical (including ophthalmic, intranasal, transdermal, intrapulmonary), oral or parenteral. Parenteral administration includes intravenous drip, subcutaneous, intraperitoneal or intramuscular injection, or intrathecal or intraventricular administration.

In general, delivery of an antagomir featured in the invention directs the agent to the site of infection in a subject. The preferred means of delivery is through local administration directly to the site of infection, or by systemic administration, e.g. parental administration.

Formulations for direct injection and parenteral administration are well known in the art. Such formulations may include sterile aqueous solutions which may also contain buffers, diluents and other suitable additives. For intravenous use, the total concentration of solutes should be controlled to render the preparation isotonic.

Administration of Oligonucleotide Agents A patient who has been diagnosed with a disorder characterized by unwanted miRNA activity (e.g., unwanted activity of miR-122, miR-16, miR-192, or miR-194) can be treated by administration of an antagomir described herein to block the negative effects of the miRNA, thereby alleviating the symptoms associated with the unwanted miRNA activity. Similarly, a human who has or is at risk for

deleveloping a disorder characterized by underexpression of a gene that is regulated by an miRNA can be treated by the administration of an antagomir that targets the miRNA. For example, a human diagnosed with hemolytic anemia, and who carries a mutation in the aldolase A gene, expresses a compromised form of the enzyme. The patient can be

5 administered an antagomir that targets endogenous miR-122, which binds aldolase A RNA *in vivo*, presumably to downregulate translation of the aldolase A mRNA and consequently downregulate aldolase A protein levels. Administration of an antagomir that targets the endogenous miR-122 in a patient having hemolytic anemia will decrease miR-122 activity, which will result in the upregulation of aldolase A expression and an increase in aldolase A
10 protein levels. Although the enzyme activity of the mutant aldolase A is suboptimal, an increase in protein levels may be sufficient to relieve the disease symptoms. A human who has or who is at risk for developing arthrogryposis multiplex congenital, pituitary ectopia, rhabdomyolysis, or hyperkalemia, or who suffers from a myopathic symptom, is also a suitable candidate for treatment with an antagomir that targets miR-122. A human who
15 carries a mutation in the aldolase A gene can be a candidate for treatment with an antagomir that targets miR-122. A human who carries a mutation in the aldolase A gene can have a symptom characterizing aldolase A deficiency including growth and developmental retardation, midfacial hypoplasia, and hepatomegaly.

The single-stranded oligonucleotide agents featured in the invention can be
20 administered systemically, *e.g.*, orally or by intramuscular injection or by intravenous injection, in admixture with a pharmaceutically acceptable carrier adapted for the route of administration. An antagomir can include a delivery vehicle, such as liposomes, for administration to a subject, carriers and diluents and their salts, and/or can be present in pharmaceutically acceptable formulations. Methods for the delivery of nucleic acid
25 molecules are described in Akhtar *et al.*, *Trends in Cell Bio.* 2:139, 1992; Delivery Strategies for Antisense Oligonucleotide Therapeutics, ed. Akhtar, 1995; Maurer *et al.*, *Mol. Membr. Biol.*, 16:129, 1999; Hofland and Huang, *Handb. Exp. Pharmacol.* 137:165, 1999; and Lee *et al.*, *ACS Symp. Ser.* 752:184, 2000, all of which are incorporated herein by reference. Beigelman *et al.*, U.S. Pat. No. 6,395,713 and Sullivan *et al.*, PCT WO 94/02595 further
30 describe the general methods for delivery of nucleic acid molecules. Nucleic acid molecules can be administered to cells by a variety of methods known to those of skill in the art, including, but not restricted to, encapsulation in liposomes, by ionophoresis, or by incorporation into other vehicles, such as hydrogels, cyclodextrins (see for example Gonzalez *et al.*, *Bioconjugate Chem.* 10:1068, 1999), biodegradable nanocapsules, and bioadhesive

microspheres, or by proteinaceous vectors (O'Hare and Normand, International PCT Publication No. WO 00/53722).

In the present methods, the antagomir can be administered to the subject either as a naked oligonucleotide agent, in conjunction with a delivery reagent, or as a recombinant plasmid or viral vector which expresses the oligonucleotide agent. Preferably, the antagomir is administered as a naked oligonucleotide agent.

An antagomir featured in the invention can be administered to the subject by any means suitable for delivering the agent to the cells of the tissue at or near the area of unwanted target nucleic acid activity (e.g., target miRNA or pre-miRNA activity). For example, an antagomir that targets miR-122 can be delivered directly to the liver, or can be conjugated to a molecule that targets the liver. Exemplary delivery methods include administration by gene gun, electroporation, or other suitable parenteral administration route.

Suitable enteral administration routes include oral delivery.

Suitable parenteral administration routes include intravascular administration (e.g., intravenous bolus injection, intravenous infusion, intra-arterial bolus injection, intra-arterial infusion and catheter instillation into the vasculature); peri- and intra-tissue injection (e.g., intraocular injection, intra-retinal injection, or sub-retinal injection); subcutaneous injection or deposition including subcutaneous infusion (such as by osmotic pumps); direct application to the area at or near the site of neovascularization, for example by a catheter or other placement device (e.g., a retinal pellet or an implant comprising a porous, non-porous, or gelatinous material).

An antagomir featured in the invention can be delivered using an intraocular implant. Such implants can be biodegradable and/or biocompatible implants, or may be non-biodegradable implants. The implants may be permeable or impermeable to the active agent, and may be inserted into a chamber of the eye, such as the anterior or posterior chambers, or may be implanted in the sclera, transchoroidal space, or an avascularized region exterior to the vitreous. In a preferred embodiment, the implant may be positioned over an avascular region, such as on the sclera, so as to allow for transscleral diffusion of the drug to the desired site of treatment, e.g., the intraocular space and macula of the eye. Furthermore, the site of transscleral diffusion is preferably in proximity to the macula.

An antagomir featured in the invention can also be administered topically, for example, by patch or by direct application to the eye, or by iontophoresis. Ointments, sprays, or droppable liquids can be delivered by ocular delivery systems known in the art such as applicators or eyedroppers. The compositions can be administered directly to the surface of

the eye or to the interior of the eyelid. Such compositions can include mucomimetics such as hyaluronic acid, chondroitin sulfate, hydroxypropyl methylcellulose or poly(vinyl alcohol), preservatives such as sorbic acid, EDTA or benzylchromium chloride, and the usual quantities of diluents and/or carriers.

5 An antagomir featured in the invention may be provided in sustained release compositions, such as those described in, for example, U.S. Patent Nos. 5,672,659 and 5,595,760. The use of immediate or sustained release compositions depends on the nature of the condition being treated. If the condition consists of an acute or over-acute disorder, treatment with an immediate release form will be preferred over a prolonged release
10 composition. Alternatively, for certain preventative or long-term treatments, a sustained release composition may be appropriate.

An antagomir can be injected into the interior of the eye, such as with a needle or other delivery device.

An antagomir featured in the invention can be administered in a single dose or in
15 multiple doses. Where the administration of the antagomir is by infusion, the infusion can be a single sustained dose or can be delivered by multiple infusions. Injection of the agent can be directly into the tissue at or near the site of aberrant or unwanted target gene expression (e.g., aberrant or unwanted miRNA or pre-miRNA activity). Multiple injections of the agent can be made into the tissue at or near the site.

20 Dosage levels on the order of about 1 µg/kg to 100 mg/kg of body weight per administration are useful in the treatment of a disease. One skilled in the art can also readily determine an appropriate dosage regimen for administering the antagomir of the invention to a given subject. For example, the antagomir can be administered to the subject once, *e.g.*, as a single injection or deposition at or near the site on unwanted target nucleic acid expression.
25 Alternatively, the antagomir can be administered once or twice daily to a subject for a period of from about three to about twenty-eight days, more preferably from about seven to about ten days. In a preferred dosage regimen, the antagomir is injected at or near a site of unwanted target nucleic acid expression once a day for seven days. Where a dosage regimen comprises multiple administrations, it is understood that the effective amount of antagomir
30 administered to the subject can include the total amount of antagomir administered over the entire dosage regimen. One skilled in the art will appreciate that the exact individual dosages may be adjusted somewhat depending on a variety of factors, including the specific antagomir being administered, the time of administration, the route of administration, the nature of the formulation, the rate of excretion, the particular disorder being treated, the

severity of the disorder, the pharmacodynamics of the oligonucleotide agent, and the age, sex, weight, and general health of the patient. Wide variations in the necessary dosage level are to be expected in view of the differing efficiencies of the various routes of administration. For instance, oral administration generally would be expected to require higher dosage levels than administration by intravenous or intravitreal injection. Variations in these dosage levels can be adjusted using standard empirical routines of optimization, which are well-known in the art. The precise therapeutically effective dosage levels and patterns are preferably determined by the attending physician in consideration of the above-identified factors.

In addition to treating pre-existing diseases or disorders, oligonucleotide agents featured in the invention (*e.g.*, single-stranded oligonucleotide agents targeting miR-122, miR-16, miR-192, or miR-194) can be administered prophylactically in order to prevent or slow the onset of a particular disease or disorder. In prophylactic applications, an antagomir is administered to a patient susceptible to or otherwise at risk of a particular disorder, such as disorder associated with aberrant or unwanted activity of an miRNA or pre-miRNA.

The oligonucleotide agents featured by the invention are preferably formulated as pharmaceutical compositions prior to administering to a subject, according to techniques known in the art. Pharmaceutical compositions featured in the present invention are characterized as being at least sterile and pyrogen-free. As used herein, "pharmaceutical formulations" include formulations for human and veterinary use. Methods for preparing pharmaceutical compositions are within the skill in the art, for example as described in Remington's Pharmaceutical Science, 18th ed., Mack Publishing Company, Easton, Pa. (1990), and The Science and Practice of Pharmacy, 2003, Gennaro *et al.*, the entire disclosures of which are herein incorporated by reference.

The present pharmaceutical formulations include an antagomir featured in the invention (*e.g.*, 0.1 to 90% by weight), or a physiologically acceptable salt thereof, mixed with a physiologically acceptable carrier medium. Preferred physiologically acceptable carrier media are water, buffered water, normal saline, 0.4% saline, 0.3% glycine, hyaluronic acid and the like.

Pharmaceutical compositions featured in the invention can also include conventional pharmaceutical excipients and/or additives. Suitable pharmaceutical excipients include stabilizers, antioxidants, osmolality adjusting agents, buffers, and pH adjusting agents. Suitable additives include physiologically biocompatible buffers (*e.g.*, tromethamine hydrochloride), additions of chelants (such as, for example, DTPA or DTPA-bisamide) or calcium chelate complexes (as for example calcium DTPA, CaNaDTPA-bisamide), or,

optionally, additions of calcium or sodium salts (for example, calcium chloride, calcium ascorbate, calcium gluconate or calcium lactate). Pharmaceutical compositions can be packaged for use in liquid form, or can be lyophilized.

For solid compositions, conventional non-toxic solid carriers can be used; for example, pharmaceutical grades of mannitol, lactose, starch, magnesium stearate, sodium saccharin, talcum, cellulose, glucose, sucrose, magnesium carbonate, and the like.

For example, a solid pharmaceutical composition for oral administration can include any of the carriers and excipients listed above and 10-95%, preferably 25%-75%, of one or more single-stranded oligonucleotide agents featured in the invention.

By "pharmaceutically acceptable formulation" is meant a composition or formulation that allows for the effective distribution of the nucleic acid molecules of the instant invention in the physical location most suitable for their desired activity. Non-limiting examples of agents suitable for formulation with the nucleic acid molecules of the instant invention include: P-glycoprotein inhibitors (such as PluronicP85), which can enhance entry of drugs into the CNS (Jolliet-Riant and Tillement, *Fundam. Clin. Pharmacol.* 13:16, 1999); biodegradable polymers, such as poly (DL-lactide-coglycolide) microspheres for sustained release delivery. Other non-limiting examples of delivery strategies for the nucleic acid molecules featured in the instant invention include material described in Boado *et al.*, *J. Pharm. Sci.* 87:1308, 1998; Tyler *et al.*, *FEBS Lett.* 421:280, 1999; Pardridge *et al.*, *PNAS USA*, 92:5592, 1995; Boado, *Adv. Drug Delivery Rev.* 15:73, 1995; Aldrian-Herrada *et al.*, *Nucleic Acids Res.* 26:4910, 1998; and Tyler *et al.*, *PNAS USA* 96:7053, 1999.

The invention also features the use of a composition that includes surface-modified liposomes containing poly (ethylene glycol) lipids (PEG-modified, or long-circulating liposomes or stealth liposomes). These formulations offer a method for increasing the accumulation of drugs in target tissues. This class of drug carriers resists opsonization and elimination by the mononuclear phagocytic system (MPS or RES), thereby enabling longer blood circulation times and enhanced tissue exposure for the encapsulated drug (Lasic *et al.*, *Chem. Rev.* 95:2601, 1995; Ishiwata *et al.*, *Chem.Phare. Bull.* 43:1005, 1995).

Such liposomes have been shown to accumulate selectively in tumors, presumably by extravasation and capture in the neovascularized target tissues (Lasic *et al.*, *Science* 267:1275, 1995; Oku *et al.*, *Biochim. Biophys. Acta* 1238:86, 1995). The long-circulating liposomes enhance the pharmacokinetics and pharmacodynamics of DNA and RNA, particularly compared to conventional cationic liposomes which are known to accumulate in tissues of the MPS (Liu *et al.*, *J. Biol. Chem.* 42:24864, 1995; Choi *et al.*, International PCT

Publication No. WO 96/10391; Ansell *et al.*, International PCT Publication No. WO 96/10390 ; Holland *et al.*, International PCT Publication No. WO 96/10392). Long-circulating liposomes are also likely to protect drugs from nuclease degradation to a greater extent compared to cationic liposomes, based on their ability to avoid accumulation in metabolically aggressive MPS tissues such as the liver and spleen.

The present invention also features compositions prepared for storage or administration that include a pharmaceutically effective amount of the desired oligonucleotides in a pharmaceutically acceptable carrier or diluent. Acceptable carriers or diluents for therapeutic use are well known in the pharmaceutical art, and are described, for example, in Remington's Pharmaceutical Sciences, Mack Publishing Co. (A. R. Gennaro edit. 1985), hereby incorporated by reference herein. For example, preservatives, stabilizers, dyes and flavoring agents can be provided. These include sodium benzoate, sorbic acid and esters of p-hydroxybenzoic acid. In addition, antioxidants and suspending agents can be used.

The nucleic acid molecules of the present invention can also be administered to a subject in combination with other therapeutic compounds to increase the overall therapeutic effect. The use of multiple compounds to treat an indication can increase the beneficial effects while reducing the presence of side effects.

Alternatively, certain single-stranded oligonucleotide agents featured in the instant invention can be expressed within cells from eukaryotic promoters (*e.g.*, Izant and Weintraub, *Science* 229:345, 1985; McGarry and Lindquist, *Proc. Natl. Acad. Sci. USA* 83:399, 1986; Scanlon *et al.*, *Proc. Natl. Acad. Sci. USA* 88:10591, 1991; Kashani-Sabet *et al.*, *Antisense Res. Dev.* 2:3, 1992; Dropulic *et al.*, *J. Virol.* 66:1432, 1992; Weerasinghe *et al.*, *J. Virol.* 65:5531, 1991; Ojwang *et al.*, *Proc. Natl. Acad. Sci. USA* 89:10802, 1992; Chen *et al.*, *Nucleic Acids Res.* 20:4581, 1992; Sarver *et al.*, *Science* 247:1222, 1990; Thompson *et al.*, *Nucleic Acids Res.* 23:2259, 1995; Good *et al.*, *Gene Therapy* 4:45, 1997). Those skilled in the art realize that any nucleic acid can be expressed in eukaryotic cells from the appropriate DNA/RNA vector. The activity of such nucleic acids can be augmented by their release from the primary transcript by a enzymatic nucleic acid (Draper *et al.*, PCT WO 93/23569, and Sullivan *et al.*, PCT WO 94/02595; Ohkawa *et al.*, *Nucleic Acids Symp. Ser.* 27:156, 1992; Taira *et al.*, *Nucleic Acids Res.* 19:5125, 1991; Ventura *et al.*, *Nucleic Acids Res.* 21:3249, 1993; Chowrira *et al.*, *J. Biol. Chem.* 269:25856, 1994).

In another aspect of the invention, RNA molecules of the present invention can be expressed from transcription units (see for example Couture *et al.*, *Trends in Genetics* 12:510, 1996) inserted into DNA or RNA vectors. The recombinant vectors can be DNA plasmids or

viral vectors. Oligonucleotide agent-expressing viral vectors can be constructed based on, but not limited to, adeno-associated virus, retrovirus, adenovirus, or alphavirus. In another embodiment, pol III based constructs are used to express nucleic acid molecules of the invention (see for example Thompson, U. S. Pats. Nos. 5,902,880 and 6,146,886). The recombinant vectors capable of expressing the oligonucleotide agents can be delivered as described above, and can persist in target cells. Alternatively, viral vectors can be used that provide for transient expression of nucleic acid molecules. Such vectors can be repeatedly administered as necessary. Once expressed, the antagomir interacts with the target RNA (e.g., miRNA or pre-miRNA) and inhibits miRNA activity. In a preferred embodiment, the antagomir forms a duplex with the target miRNA, which prevents the miRNA from binding to its target mRNA, which results in increased translation of the target mRNA. Delivery of oligonucleotide agent-expressing vectors can be systemic, such as by intravenous or intramuscular administration, by administration to target cells ex-planted from a subject followed by reintroduction into the subject, or by any other means that would allow for introduction into the desired target cell (for a review see Couture *et al.*, *Trends in Genetics* 12:510, 1996).

The term “therapeutically effective amount” is the amount present in the composition that is needed to provide the desired level of drug in the subject to be treated to give the anticipated physiological response.

The term “physiologically effective amount” is that amount delivered to a subject to give the desired palliative or curative effect.

The term “pharmaceutically acceptable carrier” means that the carrier can be taken into the subject with no significant adverse toxicological effects on the subject.

The term “co-administration” refers to administering to a subject two or more single-stranded oligonucleotide agents. The agents can be contained in a single pharmaceutical composition and be administered at the same time, or the agents can be contained in separate formulation and administered serially to a subject. So long as the two agents can be detected in the subject at the same time, the two agents are said to be co-administered.

The types of pharmaceutical excipients that are useful as carrier include stabilizers such as human serum albumin (HSA), bulking agents such as carbohydrates, amino acids and polypeptides; pH adjusters or buffers; salts such as sodium chloride; and the like. These carriers may be in a crystalline or amorphous form or may be a mixture of the two.

Bulking agents that are particularly valuable include compatible carbohydrates, polypeptides, amino acids or combinations thereof. Suitable carbohydrates include monosaccharides such as galactose, D-mannose, sorbose, and the like; disaccharides, such as

lactose, trehalose, and the like; cyclodextrins, such as 2-hydroxypropyl-.beta.-cyclodextrin; and polysaccharides, such as raffinose, maltodextrins, dextrans, and the like; alditols, such as mannitol, xylitol, and the like. A preferred group of carbohydrates includes lactose, threhalose, raffinose maltodextrins, and mannitol. Suitable polypeptides include aspartame.

5 Amino acids include alanine and glycine, with glycine being preferred.

Suitable pH adjusters or buffers include organic salts prepared from organic acids and bases, such as sodium citrate, sodium ascorbate, and the like; sodium citrate is preferred.

Dosage. An antagomir can be administered at a unit dose less than about 75 mg per kg of bodyweight, or less than about 70, 60, 50, 40, 30, 20, 10, 5, 2, 1, 0.5, 0.1, 0.05, 0.01, 10 0.005, 0.001, or 0.0005 mg per kg of bodyweight, and less than 200 nmol of antagomir (e.g., about 4.4×10^{16} copies) per kg of bodyweight, or less than 1500, 750, 300, 150, 75, 15, 7.5, 1.5, 0.75, 0.15, 0.075, 0.015, 0.0075, 0.0015, 0.00075, 0.00015 nmol of antagomir per kg of bodyweight. The unit dose, for example, can be administered by injection (e.g., intravenous or intramuscular, intrathecally, or directly into an organ), inhalation, or a topical application.

15 Delivery of an antagomir directly to an organ (e.g., directly to the liver) can be at a dosage on the order of about 0.00001 mg to about 3 mg per organ, or preferably about 0.0001-0.001 mg per organ, about 0.03- 3.0 mg per organ, about 0.1-3.0 mg per organ or about 0.3-3.0 mg per organ.

The dosage can be an amount effective to treat or prevent a disease or disorder.

20 In one embodiment, the unit dose is administered less frequently than once a day, e.g., less than every 2, 4, 8 or 30 days. In another embodiment, the unit dose is not administered with a frequency (e.g., not a regular frequency). For example, the unit dose may be administered a single time. Because oligonucleotide agent-mediated silencing can persist for several days after administering the antagomir composition, in many instances, it is possible 25 to administer the composition with a frequency of less than once per day, or, for some instances, only once for the entire therapeutic regimen.

In one embodiment, a subject is administered an initial dose, and one or more maintenance doses of an antagomir. The maintenance dose or doses are generally lower than the initial dose, e.g., one-half less of the initial dose. A maintenance regimen can include 30 treating the subject with a dose or doses ranging from 0.01 µg to 75 mg/kg of body weight per day, e.g., 70, 60, 50, 40, 30, 20, 10, 5, 2, 1, 0.5, 0.1, 0.05, 0.01, 0.005, 0.001, or 0.0005

mg per kg of bodyweight per day. The maintenance doses are preferably administered no more than once every 5, 10, or 30 days. Further, the treatment regimen may last for a period of time which will vary depending upon the nature of the particular disease, its severity and the overall condition of the patient. In preferred embodiments the dosage may be delivered
5 no more than once per day, *e.g.*, no more than once per 24, 36, 48, or more hours, *e.g.*, no more than once every 5 or 8 days. Following treatment, the patient can be monitored for changes in his condition and for alleviation of the symptoms of the disease state. The dosage of the compound may either be increased in the event the patient does not respond significantly to current dosage levels, or the dose may be decreased if an alleviation of the
10 symptoms of the disease state is observed, if the disease state has been ablated, or if undesired side-effects are observed.

The effective dose can be administered in a single dose or in two or more doses, as desired or considered appropriate under the specific circumstances. If desired to facilitate repeated or frequent infusions, implantation of a delivery device, *e.g.*, a pump, semi-
15 permanent stent (*e.g.*, intravenous, intraperitoneal, intracisternal or intracapsular), or reservoir may be advisable.

Following successful treatment, it may be desirable to have the patient undergo maintenance therapy to prevent the recurrence of the disease state, wherein the compound of the invention is administered in maintenance doses, ranging from 0.01 μg to 100 g per kg of
20 body weight (see US 6,107,094).

The concentration of the antagomir composition is an amount sufficient to be effective in treating or preventing a disorder or to regulate a physiological condition in humans. The concentration or amount of antagomir administered will depend on the parameters determined for the agent and the method of administration, *e.g.* direct administration to the
25 eye. For example, eye formulations tend to require much lower concentrations of some ingredients in order to avoid irritation or burning of the ocular tissues. It is sometimes desirable to dilute an oral formulation up to 10-100 times in order to provide a suitable ocular formulation.

Certain factors may influence the dosage required to effectively treat a subject,
30 including but not limited to the severity of the disease or disorder, previous treatments, the general health and/or age of the subject, and other diseases present. It will also be

appreciated that the effective dosage of the antagomir used for treatment may increase or decrease over the course of a particular treatment. Changes in dosage may result and become apparent from the results of diagnostic assays. For example, the subject can be monitored after administering an antagomir composition. Based on information from the monitoring, an
5 additional amount of the antagomir composition can be administered.

Dosing is dependent on severity and responsiveness of the disease condition to be treated, with the course of treatment lasting from several days to several months, or until a cure is effected or a diminution of disease state is achieved. Optimal dosing schedules can be calculated from measurements of drug accumulation in the body of the patient. Persons of
10 ordinary skill can easily determine optimum dosages, dosing methodologies and repetition rates. Optimum dosages may vary depending on the relative potency of individual compounds, and can generally be estimated based on EC₅₀s found to be effective in *in vitro* and *in vivo* animal models.

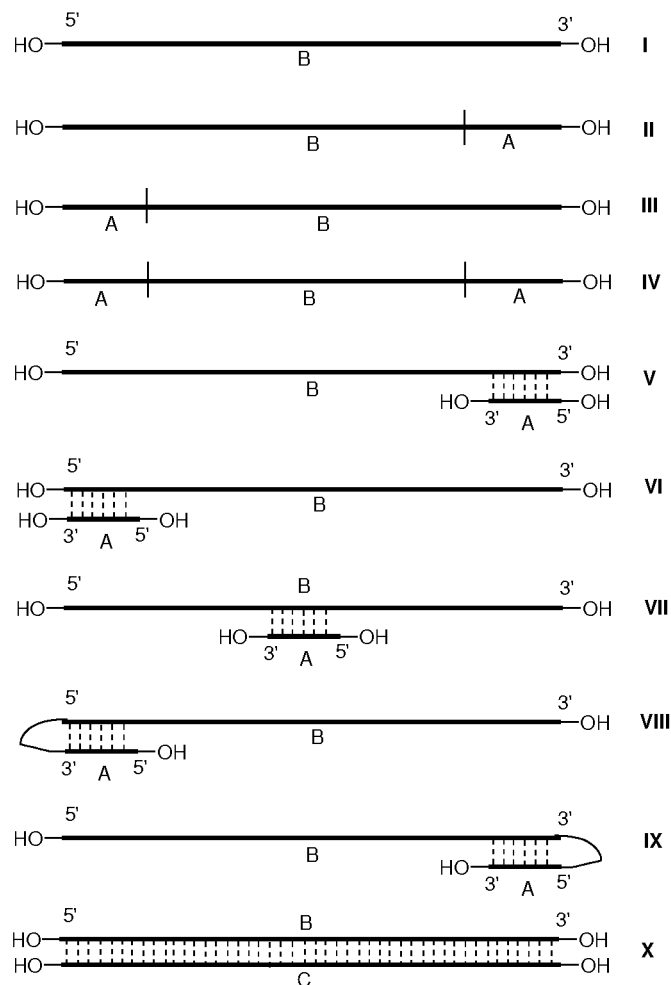
The invention is further illustrated by the following examples, which should not be
15 construed as further limiting.

EXAMPLES

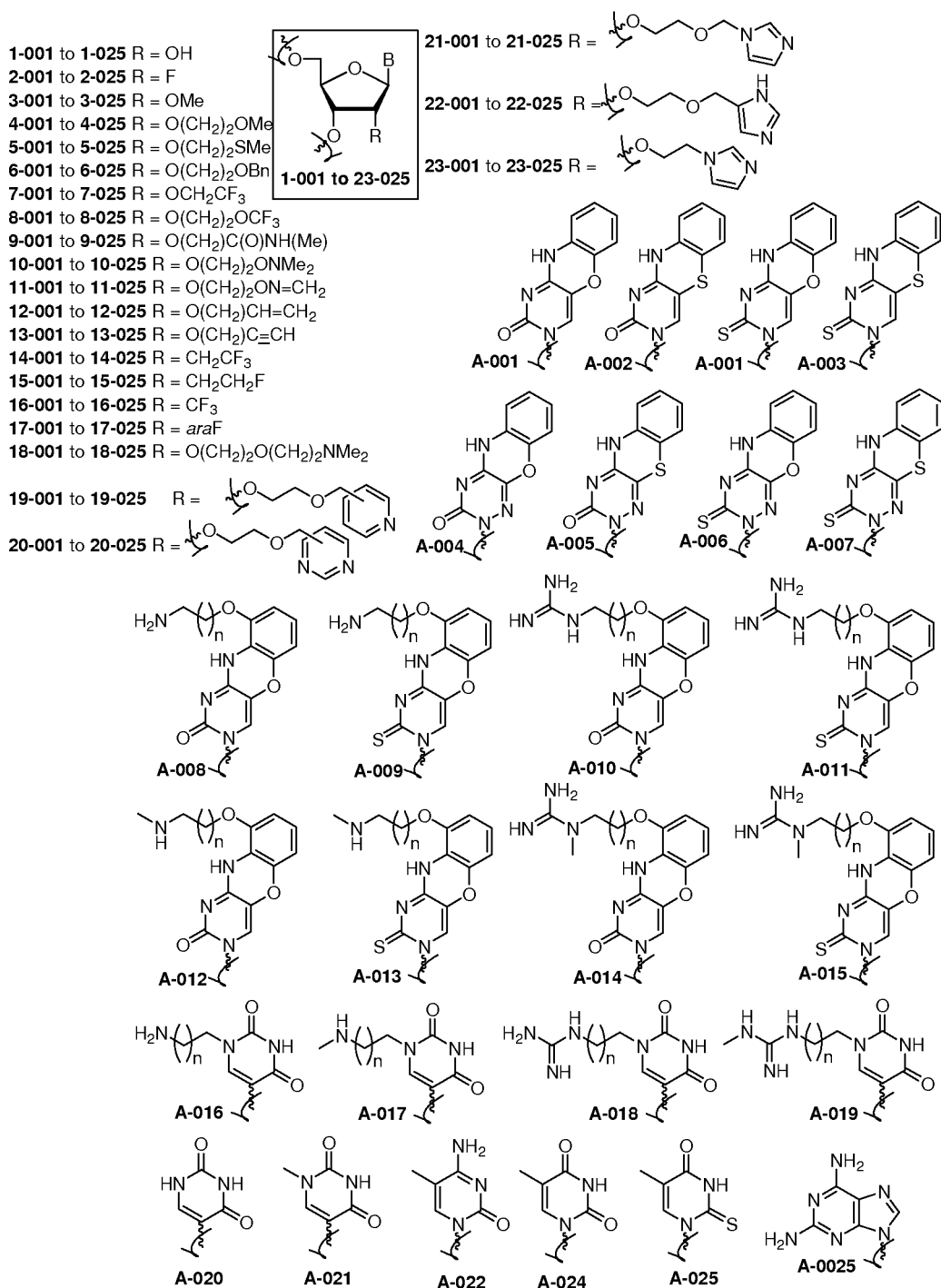
Example 1. Oligonucleotide agents containing high affinity nucleoside modifications

High affinity sugar-base modifications

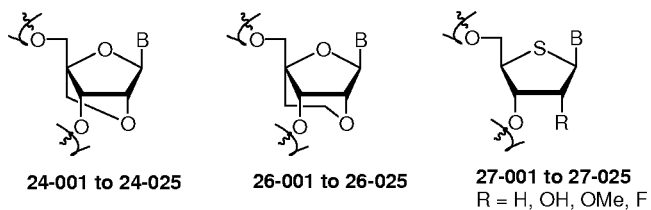
At least one of the listed nucleotide in Exemplification 2 is present in the
oligonucleotide agent shown in Exemplification 1.



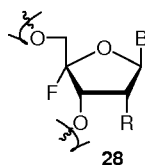
Exemplification 1. Oligonucleotide agent designs. **I:** Oligonucleotide agent; **II:** Oligonucleotide agent with 3'-ribose and phosphate (2-8 nucleotide in length); **III:** Oligonucleotide agent with 5'-ribose and phosphate (2-8 nucleotide in length); **IV:** Oligonucleotide agent with 3' and 5'-ribose and phosphate (2-8 nucleotide in length); **V:** Oligonucleotide agent with 3'- end partial duplex with oligoribonucleotide (4-8 nucleotide in length); **VI:** Oligonucleotide agent with 5'- end partial duplex with oligoribonucleotide (4-8 nucleotide in length); **VII:** Oligonucleotide agent with internal partial duplex with oligoribonucleotide (4-8 nucleotide in length); **VIII:** Oligonucleotide agent with 5'- end partial hairpin with oligoribonucleotide (4-8 nucleotide in length); **IX:** Oligonucleotide agent with 3'- end partial hairpin with oligoribonucleotide (4-8 nucleotide in length); **X:** Oligonucleotide agent with inactivated complementary antisense strand. Segment A indicates oligoribonucleotide with phosphate backbone; segment B indicates Oligonucleotide agent and segment C indicates inactivated antisense strand complementary to a Oligonucleotide agent.



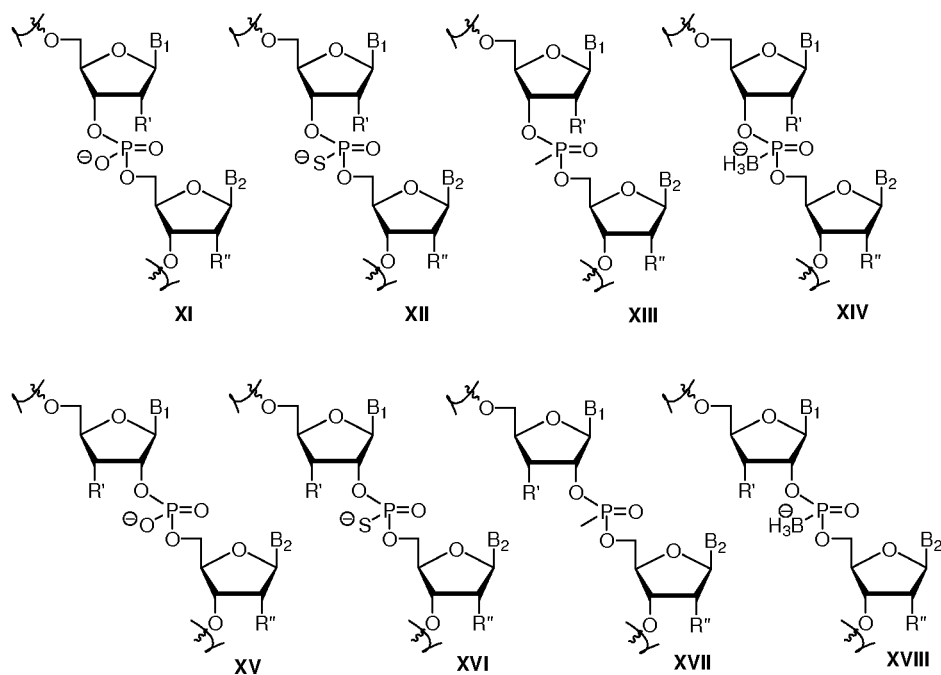
Exemplification 1. Compound **1-001** to **23-025** represents oligonucleotide agents with corresponding nucleoside modification. Lower case 'n' = 0-11 and uppercase A = 1 to 24.



Exemplification 2. Sugar modifications. **24**: LNA; **25**: ENA and **26**: 4'-Thio. B is from A-001 to A-025 of Figure 2.

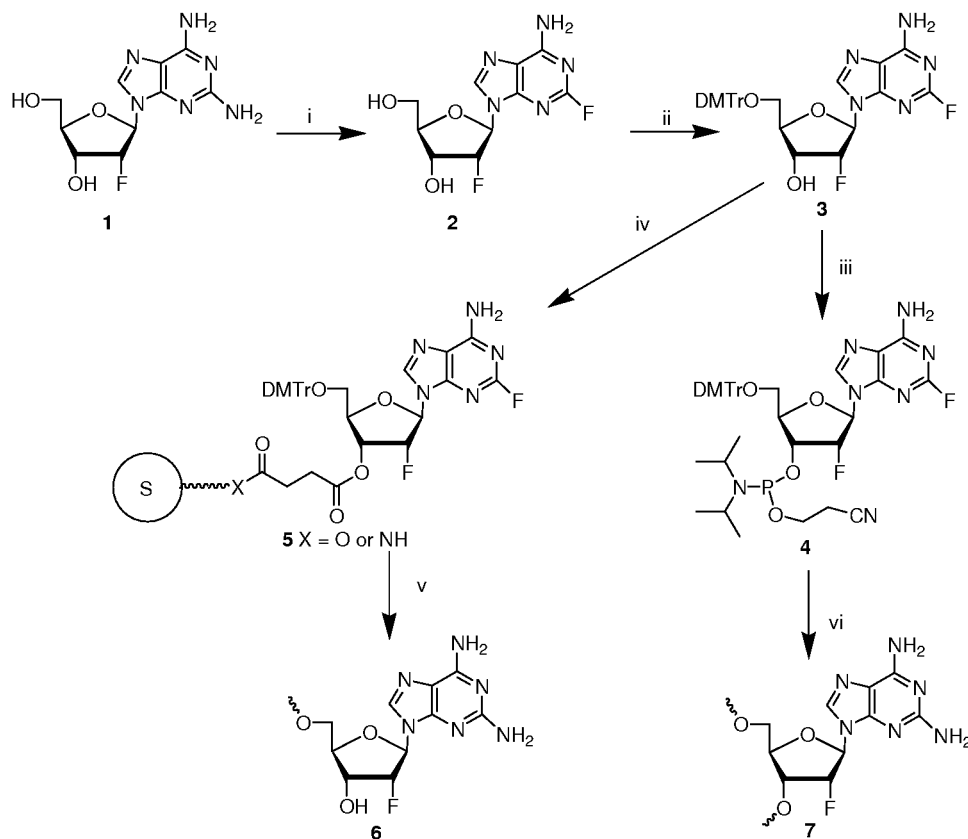


Exemplification 3. 4'-Fluoro sugar modification



Exemplification 4. Backbone linkages. XI: 3'-5' Phosphate; XII: 3',5' phosphorothioate; XIII 3',5'methylphosphonate; XIV: 3',5'boranophosphate; XV: 2'-5' Phosphate; XVI: 2',5' phosphorothioate; XVII 2',5'methylphosphonate; XVIII: 2',5'boranophosphate

Example 2. High affinity oligonucleotides containing 2-amino-2'-deoxy-2'-fluoro-adenosine



Scheme 1. Synthesis of oligonucleotide containing 2-amino-2'-deoxy-2'-fluoro-adenosine. (i)

Step 1 Compound 2: A 2 L polyethylene bottle was equipped with a magnetic stirrer, thermometer, dry ice/acetone bath and a stream of argon gas. Anhydrous pyridine (500 mL) was added and the solution was cooled to -20°C . To this was added 70% hydrogen fluoride in pyridine (400 mL). 2'-fluoro-2,6-diaminopurine riboside (**1**, 90 g, 0.317 mol) was dissolved in the solution. Tert-butylnitrite (75 mL, 0.63 mol) was added in one portion and the reaction was stirred at $6-12^{\circ}\text{C}$ until the reaction was complete as judged by TLC (3 h). Sodium bicarbonate (2000 g) was suspended with manual stirring in water (2 L) in a 20 L bucket. The reaction solution was slowly poured (to allow for evolution of carbon dioxide) into the aqueous layer with vigorous stirring. The resulting solution was extracted with ethyl acetate (3x500 mL). The organic layers were combined and concentrated to a solid. The

solid was mostly dissolved in methanol (300 mL) at reflux. The solution was cooled in a ice water bath and the resulting solid was collected, rinsed with methanol (2x50 mL) and dried under vacuum (1 mm Hg, 25 °C, 24 h) to give 55 g of compound **2** as a dark gold solid. The mother liquor was concentrated and purified by column chromatography to give an additional
5 15.1 g of product for a total of 70.1 g (77%).

Step 2 Compound 3: 2, 2'-Difluoro-2'-deoxyadenosine (**2**, 70 g, 0.244 mol) and dimethoxytrityl chloride (91.0 g, 0.268 mol) were dissolved in anhydrous pyridine (700 mL) and stirred at ambient temperature for 3 h. The reaction was quenched by the addition of methanol (50 mL) and then concentrated under reduced pressure to an oil. The residue was
10 partitioned between ethyl acetate (1 L) and sat'd sodium bicarbonate (1 L mL). The aqueous layer was extracted with ethyl acetate (500 mL) once more and the combined extracts were concentrated under reduced pressure. The resulting solid was purified by crystallization from hexane - ethyl acetate (1:1) to give a light brown solid **3** (120.1 g, 83%).

Step 3 Compound 4: 5'-O-(4,4'-Dimethoxytrityl)- 2, 2'-difluoro-2'-deoxyadenosine
15 (**3**, 90 g, 0.153 mol), 2-cyanoethyl tetraisopropylphosphorodiamidite (55.0 g, 0.183 mol), diisopropylamine tetrazolide (17.0 g, 0.1 mol) were dissolved in anhydrous dichloromethane (1 L) and allowed to stir at ambient temperature under an argon atmosphere for 16 h. The reaction was concentrated under reduced pressure to a thin oil and then directly applied to a silica gel column (200 g). The product was eluted with ethyl acetate-triethylamine (99:1).
20 The appropriate fractions were combined, concentrated under reduced pressure, coevaporated with anhydrous acetonitrile and dried (1 mm Hg, 25 °C, 24 h) to 109.1 g (90%) of light yellow foam of compound **4**.

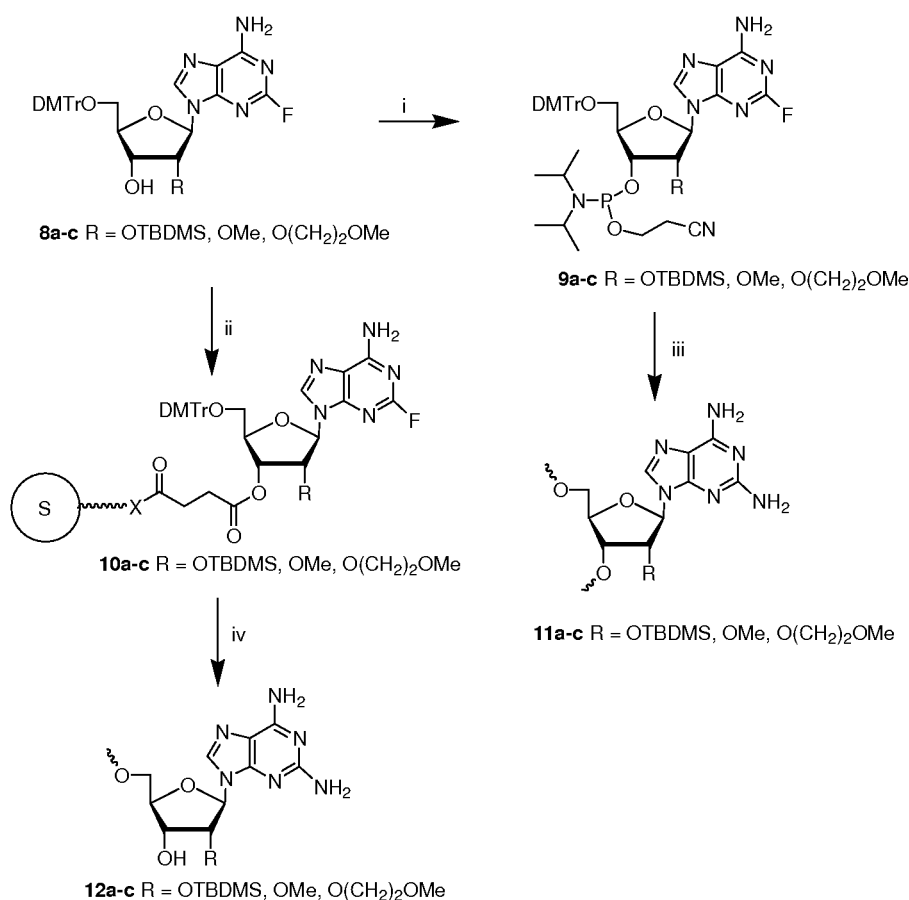
Step 4 Compound 5: 5'-O-(4,4'-Dimethoxytrityl)-2, 2'-difluoro-2'-deoxyadenosine (**4**, 10 g, 16.9 mmol), dimethylaminopyridine (0.32 g, 2.6 mmol) and succinic anhydride (3.4
25 g, 34 mmol) were dissolved in anhydrous pyridine (50 mL) and stirred at ambient temperature under an argon atmosphere for 6 h. The reaction was quenched by the addition of water (20 mL) and then concentrated under reduced pressure to an oil. The oil was purified by column chromatography, eluted with methanol-dichloromethane-triethylamine (7:92:1). The appropriate fractions were collected and evaporated to give the product as a
30 light yellow solid (10.4 g, 78%) of the corresponding succinate. The succinate is subsequently attached to solid support as reported in the literature to obtain the desired solid support **5**.

Step 5 Compound 6: Oligonucleotide containing 2-Amino-2'-fluoro-adenosine at the 3'-end is synthesized starting from the solid support **5** according to the standard solid phase

oligonucleotide synthesis and deprotection protocols. (Ref: WO00012563 and WO01002608). Oligonucleotide with phosphodiester and phosphorothioate backbone are prepared as reported by Ross *et al.* (WO00012563 and WO01002608)

Step 6 Compound 7: Oligonucleotide containing 2-amino-adenosine with 2'-deoxy-
 5 2'-fluoro sugar modification is synthesized and characterized as reported by Manoharan and Cook (Ref: WO01002608).

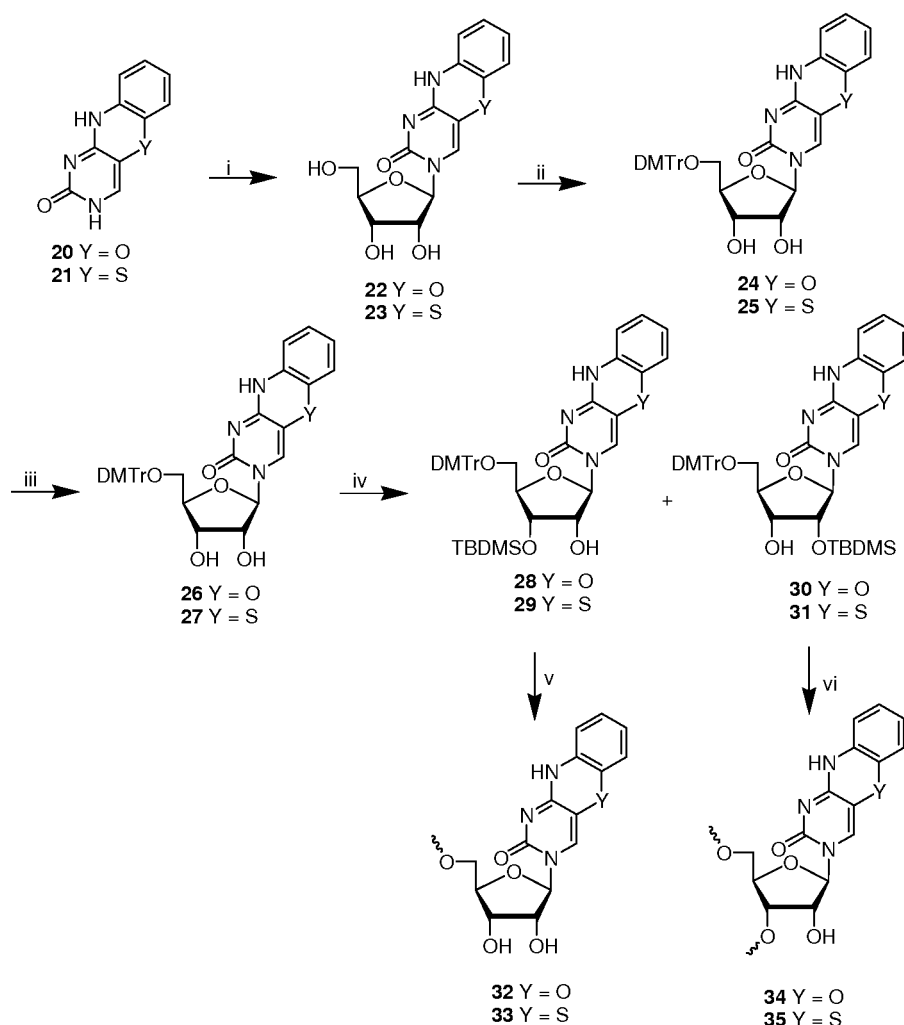
Example 3. High affinity oligonucleotides containing other 2-amino-adenosine modifications



Scheme 2. Synthesis of oligonucleotide agents containing 2-amino-adenosines

Compounds **8a-c** are prepared as reported by Manoharan and Cook (Ref: WO01002608). Oligonucleotides with 2-amino-adenosine base modification (**11a-c** and **12a-c**) are prepared as described in Example 1.

Example 4. High affinity oligonucleotides containing high affinity phenoxazine and thiophenoxazine.



Scheme 3. Synthesis of oligonucleotide containing phenoxazine and thiophenoxazine base modifications.

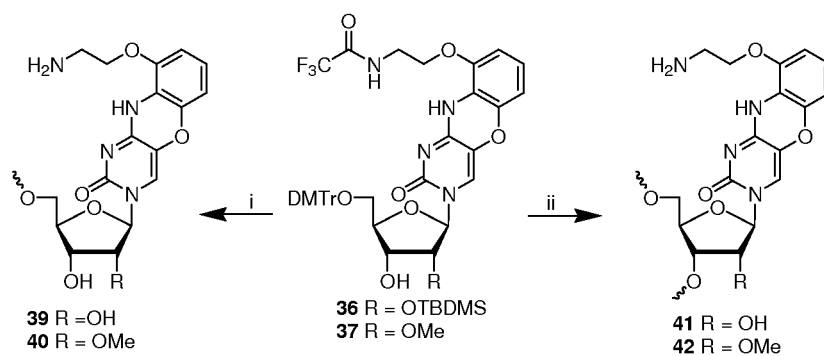
5

Step 1 Compounds 22 and 23: Compounds **20** and **21** are prepared according to the literature procedure (Sandin, Peter; Lincoln, Per; Brown, Tom; Wilhelmsson, L. Marcus. *Nature Protocols*, **2007**, 2(3), 615-623). Nucleosides **22** and **23** are obtained respectively from **20** and **21** according to the procedures reported by Rajeev and Broom (*Organic Lett.*, **2000**, 2, 3595).

10 Compounds **28** to **31** are obtained from respectively from **22** and **23** according to procedured reported by Xia *et al.* (*ACS Chem. Biol.*, **2006**, 1, 176). Oligonucleotides **32** to **35** are synthesized from corresponding precursors **28** to **31** as described in Example 1 and as reported by Sandin *et al.* (*Nature Protocols*, **2007**, 2(3), 615-623).

15

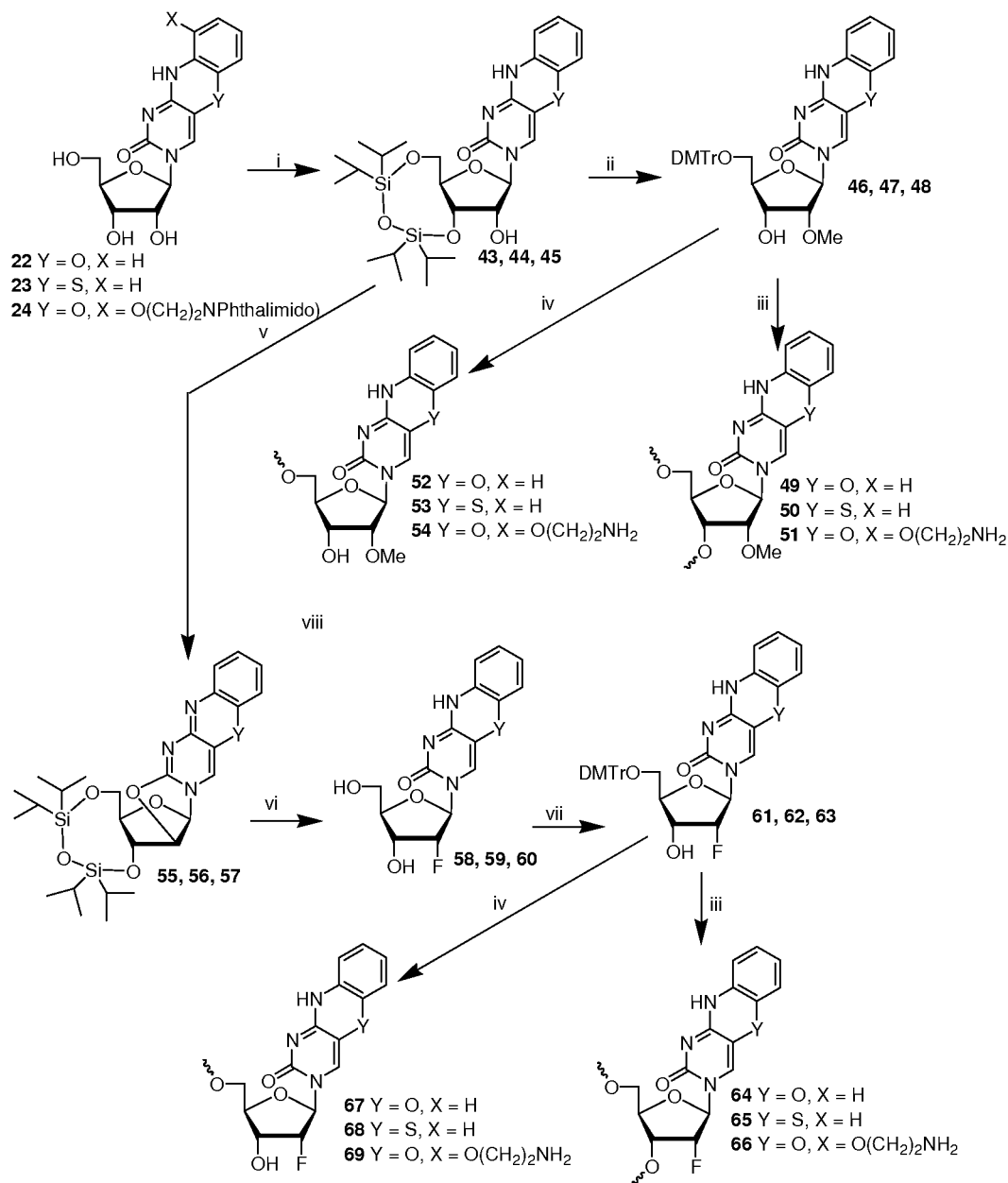
Example 5. High affinity oligonucleotides with G-clamp modification



Scheme 4. Synthesis of oligonucleotides containing G-clamp base modification

Compound **36** and **37** are prepared as reported by Holmes *et al.*, *Nucleic Acids Res.*,
 5 **2003**, *31*, 2759. Oligonucleotides **39** to **42** are obtained from corresponding precursors **36** and
37 as described in Examples 3 and 1.

Example 6. High affinity oligonucleotides containing 2'-OMe and 2'-deoxy-2'-F sugar modified phenoxazine nucleosides

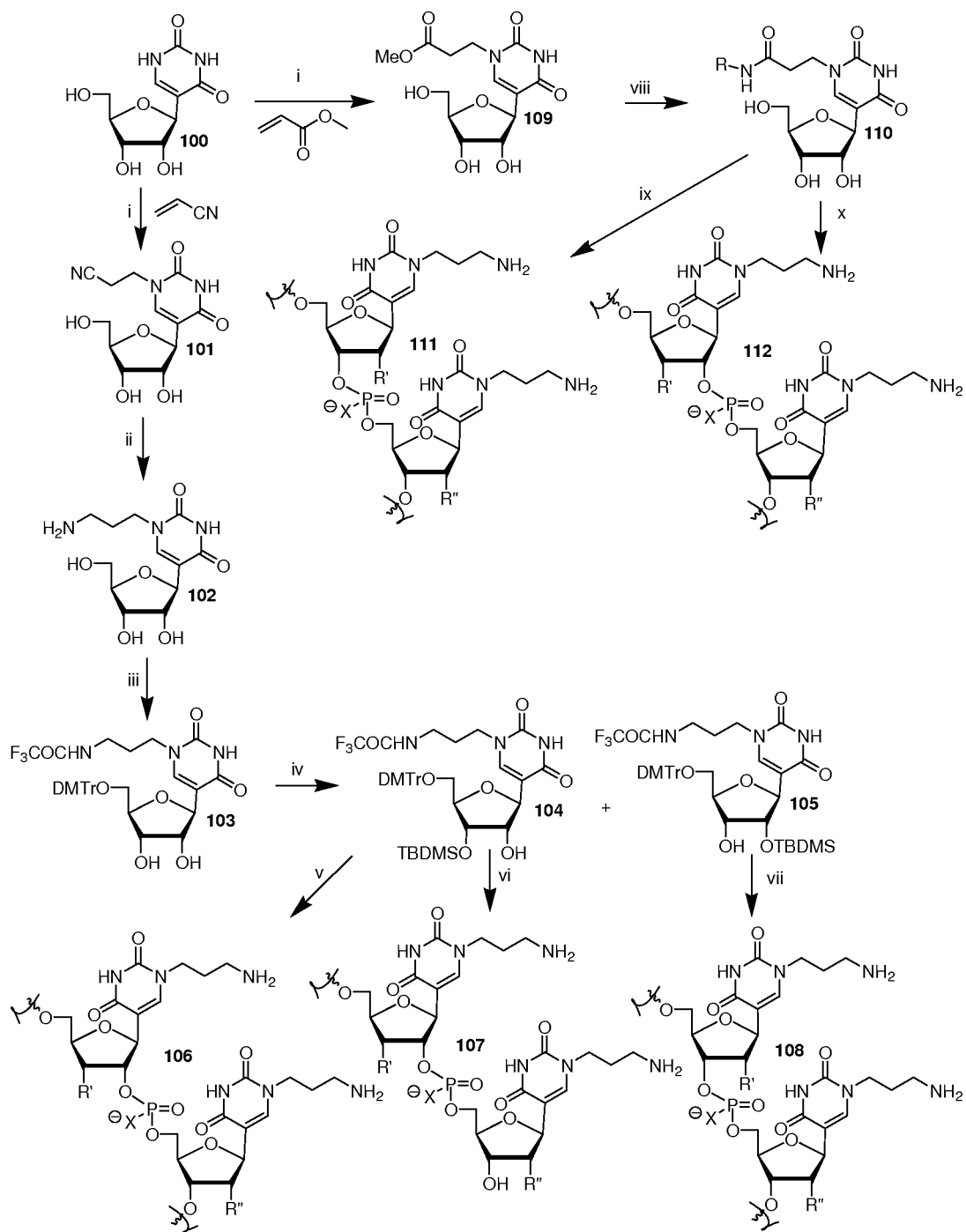


Scheme 5. Synthesis of oligonucleotides containing 2'-deoxy-2'-fluoro and

5 nucleobase modifications

Compounds **46**, **47** and **48** are prepared according to reported procedures (Holmes *et al.*, *Nucleic Acids Res.*, **2003**, *31*, 2759). Oligonucleotides **49** to **54** are obtained from corresponding precursors **47**, **47** and **48** as described in Example 1

10 Compounds **58**, **59** and **60** are prepared according to reported procedures (Shi *et al.*, *Bioorg. Med. Chem.*, **2005**, *13*, 1641). Oligonucleotides **64** to **69** are obtained from corresponding precursors **58**, **59** and **60** as described in Example 1

Example 7. High affinity oligonucleotides containing pseudouridine base modifications

Scheme 6. Synthesis of oligonucleotides containing pseudouridine analogues

Example 8: Synthesis of Antagomirs

Step 1. Oligonucleotide Synthesis

All oligonucleotides were synthesized on an AKTAoligopilot synthesizer or on an ABI 394 DNA/RNA synthesizer. Commercially available controlled pore glass solid supports (rU-CPG, 2'-*O*-methly modified rA-CPG and 2'-*O*-methyl modified rG-CPG from Prime Synthesis) or the in-house synthesized solid support hydroxyprolinol-cholesterol-CPG (described in patent xxxx) were used for the synthesis. RNA phosphoramidites and 2'-*O*-methyl modified RNA phosphoramidites with standard protecting groups (5'-*O*-dimethoxytrityl-N6-benzoyl-2'-*t*-butyldimethylsilyl-adenosine-3'-*O*-N,N'-diisopropyl-2-cyanoethylphosphoramidite, 5'-*O*-dimethoxytrityl-N4-acetyl-2'-*t*-butyldimethylsilyl-cytidine-3'-*O*-N,N'-diisopropyl-2-cyanoethylphosphoramidite, 5'-*O*-dimethoxytrityl-N2-isobutryl-2'-*t*-butyldimethylsilyl-guanosine-3'-*O*-N,N'-diisopropyl-2-cyanoethylphosphoramidite, 5'-*O*-dimethoxytrityl-2'-*t*-butyldimethylsilyl-uridine-3'-*O*-N,N'-diisopropyl-2-cyanoethylphosphoramidite, 5'-*O*-dimethoxytrityl-N6-benzoyl-2'-*O*-methyl-adenosine-3'-*O*-N,N'-diisopropyl-2-cyanoethylphosphoramidite, 5'-*O*-dimethoxytrityl-N4-acetyl-2'-*O*-methyl-cytidine-3'-*O*-N,N'-diisopropyl-2-cyanoethylphosphoramidite, 5'-*O*-dimethoxytrityl-N2-isobutryl-2'-*O*-methyl-guanosine-3'-*O*-N,N'-diisopropyl-2-cyanoethylphosphoramidite, 5'-*O*-dimethoxytrityl-2'-*O*-methyl-uridine-3'-*O*-N,N'-diisopropyl-2-cyanoethylphosphoramidite and 5'-*O*-dimethoxytrityl-2'-deoxy-thymidine-3'-*O*-N,N'-diisopropyl-2-cyanoethylphosphoramidite) were obtained from Pierce Nucleic Acids Technologies and ChemGenes Research. The Quasar 570 phosphoramidite was obtained from Biosearch Technologies. The 5'-*O*-dimethoxytrityl- 2'-*t*-butyldimethylsilyl-inosine-3'-*O*-N,N'-diisopropyl-2-cyanoethylphosphoramidite was obtained from ChemGenes Research. The 5'-*O*-dimethoxytrityl- 2'-*t*-butyldimethylsilyl-(2,4)-difluorotolyl-3'-*O*-N,N'-diisopropyl-2-cyanoethylphosphoramidite (DFT-phosphoramidite) and the 5'-*O*-dimethoxytrityl- 2'-*t*-

butyldimethylsilyl-9-(2-aminoethoxy)-phenoxazine-3'-O-N,N'-diisopropyl-2-cyanoethylphosphoramidite (G-clamp phosphoramidite) were synthesized in house.

For the syntheses on AKTAoligopilot synthesizer, all phosphoramidites were used at a concentration of 0.2 M in CH₃CN except for guanosine and 2'-O-methyl-uridine, which were used at 0.2 M concentration in 10% THF/CH₃CN (v/v). Coupling/recycling time of 16 minutes was used for all phosphoramidite couplings. The activator was 5-ethyl-thio-tetrazole (0.75 M, American International Chemicals). For the PO-oxidation, 50 mM iodine in water/pyridine (10:90 v/v) was used and for the PS-oxidation 2% PADS (GL Synthesis) in 2,6-lutidine/CH₃CN (1:1 v/v) was used. For the syntheses on ABI 394 DNA/RNA synthesizer, all phosphoramidites, including DFT and G-clamp phosphoramidites were used at a concentration of 0.15 M in CH₃CN except for 2'-O-methyl-uridine, which was used at 0.15 M concentration in 10% THF/CH₃CN (v/v). Coupling time of 10 minutes was used for all phosphoramidite couplings. The activator was 5-ethyl-thio-tetrazole (0.25 M, Glen Research). For the PO-oxidation, 20 mM iodine in water/pyridine (Glen Research) was used and for the PS-oxidation 0.1M DDTT (AM Chemicals) in pyridine was used. Coupling of the Quasar 570 phosphoramidite was carried out on the ABI DNA/RNA synthesizer. The Quasar 570 phosphoramidite was used at a concentration of 0.1M in CH₃CN with a coupling time of 10 mins. The activator was 5-ethyl-thio-tetrazole (0.25 M, Glen Research) and 0.1M DDTT (AM Chemicals) in pyridine was used for PS oxidation.

Step 2. Deprotection of oligonucleotides

A. Sequences synthesized on the AKTAoligopilot synthesizer

After completion of synthesis, the support was transferred to a 100 mL glass bottle (VWR). The oligonucleotide was cleaved from the support with simultaneous deprotection of base and phosphate groups with 40 mL of a 40% aq. methyl amine (Aldrich) 90 mins at 45°C. The bottle was cooled briefly on ice and then the methylamine was filtered

into a new 500 mL bottle. The CPG was washed three times with 40 mL portions of DMSO. The mixture was then cooled on dry ice.

In order to remove the *tert*-butyldimethylsilyl (TBDMS) groups at the 2' position, 60 mL triethylamine trihydrofluoride (Et₃N-HF) was added to the above mixture.

5 The mixture was heated at 40°C for 60 minutes. The reaction was then quenched with 220 mL of 50 mM sodium acetate (pH 5.5) and stored in the freezer until purification.

B. Sequences synthesized on the ABI DAN/RNA synthesizer

After completion of synthesis, the support was transferred to a 15 mL tube (VWR). The oligonucleotide was cleaved from the support with simultaneous deprotection of
10 base and phosphate groups with 7 mL of a 40% aq. methyl amine (Aldrich) 15 mins at 65°C. The bottle was cooled briefly on ice and then the methylamine was filtered into a 100 mL bottle (VWR). The CPG was washed three times with 7 mL portions of DMSO. The mixture was then cooled on dry ice.

In order to remove the *tert*-butyldimethylsilyl (TBDMS) groups at the 2'
15 position, 10.5 mL triethylamine trihydrofluoride (Et₃N-HF) was added to the above mixture. The mixture was heated at 60°C for 15 minutes. The reaction was then quenched with 38.5 mL of 50 mM sodium acetate (pH 5.5) and stored in the freezer until purification.

Step 3. Quantitation of Crude Oligonucleotides

For all samples, a 10 µL aliquot was diluted with 990 µL of deionised nuclease free water
20 (1.0 mL) and the absorbance reading at 260 nm was obtained.

Step 4. Purification of Oligonucleotides

(a) Unconjugated oligonucleotides

The unconjugated crude oligonucleotides were first analyzed by HPLC (Dionex PA 100). The buffers were 20 mM phosphate, pH 11 (buffer A); and 20 mM phosphate, 1.8 M NaBr,
25 pH 11 (buffer B). The flow rate 1.0 mL/min and monitored wavelength was 260-280 nm. Injections of 5-15 µL were done for each sample.

The unconjugated samples were purified by HPLC on a TSK-Gel SuperQ-5PW (20) column packed in house (17.3 x 5 cm) or on a commercially available TSK-Gel SuperQ-5PW column (15 x 0.215cm) available from TOSOH Bioscience. The buffers were 20 mM phosphate in 10% CH₃CN, pH 8.5 (buffer A) and 20 mM phosphate, 1.0 M NaBr in 10% CH₃CN, pH 8.5 (buffer B). The flow rate was 50.0 mL/min for the in house packed column and 10.0ml/min for the commercially obtained column. Wavelengths of 260 and 294 nm were monitored. The fractions containing the full-length oligonucleotides were pooled together, evaporated, and reconstituted to ~100 mL with deionised water.

(b) Cholesterol-conjugated oligonucleotides

The cholesterol-conjugated crude oligonucleotides were first analyzed by LC/MS to determine purity. The cholesterol conjugated sequences were HPLC purified on RPC-Source15 reverse-phase columns packed in house (17.3 x 5 cm or 15 x 2 cm). The buffers were 20 mM NaOAc in 10 % CH₃CN (buffer A) and 20 mM NaOAc in 70% CH₃CN (buffer B). The flow rate was 50.0 mL/min for the 17.3x 5cm column and 12.0ml/min for the 15 x 2 cm column. Wavelengths of 260 and 284 nm were monitored. The fractions containing the full-length oligonucleotides were pooled, evaporated, and reconstituted to 100 mL with deionised water.

Step 5. Desalting of Purified Oligonucleotides

The purified oligonucleotides were desalted on either an AKTA Explorer or an AKTA Prime system (Amersham Biosciences) using a Sephadex G-25 column packed in house. First, the column was washed with water at a flow rate of 40 mL/min for 20-30 min. The sample was then applied in 40-60 mL fractions. The eluted salt-free fractions were combined, dried, and reconstituted in ~50 mL of RNase free water.

Step 6. Purity Analysis by Capillary Gel Electrophoresis (CGE), Ion-exchange HPLC (IEX), and Electrospray LC/MS

Approximately 0.3 OD of each of the desalted oligonucleotides were diluted in water to 300 μ L and were analyzed by CGE, ion exchange HPLC, and LC/MS.

5

Table 1: Oligonucleotides synthesized to modulate microRNAs

AL-SQ #	Sequence	Target	Calc Mass	Found Mass	Purity (%)
3227	oCsoGsoCoCoAoAoUoAoUoUoAoCoGoUoGoCoUoGsoCsoUsoA s-Chol	miR-16	8047.82	8048.88	94.0*
3228	oGsoGsoCoUoGoUoCoAoAoUoUoCoAoUoAoGoGoUsoCsoAsoGs- Chol	miR-192	7807.68	7808.49	97.1*
3229	oUsoCsoCoAoCoAoUoGoGoAoGoUoUoGoCoUoGoUoUsoAsoCsoA s-Chol	miR-194	8088.84	8089.69	92.7*
3230	oUsoCsoAoCoGoCoGoAoGoCoCoGoAoAoCoGoAoAoCsoAsoAsoA s-Chol	miR-375	8178.03	8178.77	100*
3332	oGsoUsoCoAoAoCoAoUoCoAoGoUoCoUoGoAoUoAoAoGsoCsoUs oAs-Chol	miR-21	8438.13	8438.56	91.5*
3537	oAsoAsoCoUoCoAoCoCoGoAoCoAoGoCoGoUoUoGoAoAoUsoGso UsoUs-Chol	miR-181	8773.34	8773.70	88*
3538	oAsoAsoCoUoAoAoCoCoGoAoCoCoGoCoUoUoUoGoAoAoGsoGso UsoUs-Chol	miR-181	8773.34	8773.73	85.3*
3548	oGsoGsoCoCoGoUoCoCoAoUoUoAoAoUoAoGoAoUsoCsoAsoGs- Chol	miR-192	7790.69	7791.21	85.8*
3549	oUsoCsoCoCoCoAoUoAoGoAoGoCoUoGoCoUoGoCoUsoAsoCsoAs -Chol	miR-194	8045.84	8046.47	88.8**
3550	eAseCseAeAeAeCeAeCeCeAeUeUeGeUeCeAeCeAeCeUseCseCseAs -Chol	miR-122	9533.79	9536.30	82
3551	eAseCseAseAseAseCseAseCseCseAseUseUseGseUseCseAseCseAseC seUseCseCseAs-Chol	miR-122	9805.79	9805.25	99.3

3595	oUsoAsoCoAoUoAoCoUoUoCoUoUoAoCoAoUsoUsoCsoCsoA	miR-1	6890.05	6890.01	92
3596	oUsoAsoCsoAsoUsoAsoCsoUsoUsoCsoUsoUsoUsoAsoCsoAsoUsoUs oCsoCsoA	miR-1	7114.5	7114.12	97*
3597	oUsoAsoCoAoUoAoCoUoUoCoUoUoAoCoAoUsoCsoCsoAs- Chol	miR-1	7594.5	7594.5	90*
3598	oUsoAsoCsoAsoUsoAsoCsoUsoUsoCsoUsoUsoUsoAsoCsoAsoUsoUs oCsoCsoAs-Chol	miR-1	7834.5	7834.5	94**
3600	oUsoGsoCoGoCoUoCoCoUoGoGoAoCoGoUoAoGoCsoCsoUsoUs- Chol	GFP	7735.59	7736.05	79**
3675	oCsoAsoGoAoCoUoCoCoGoGoUoGoGoAoAoUoGoAoAsoGsoGsoA s-Chol	miR-205	8252.04	8252.68	81
3676	oCsoAsoGsoAsoCsoUsoCsoCsoGsoGsoUsoGsoGsoAsoAsoUsoGsoAs oAsoGsoGsoAs-Chol	miR-205	8508.04	8509.65	88*
3687	oAoCoGoUoAoAoA	miR-16	2309.61	2308.38	96.4
3688	oUoAoGoCoAoGoC	miR-16	2301.58	2301.37	92.8*
3689	oAsoAsoCoUoCoCoGoGoGoGoCoUoGoAoUoCoAoGoGsoUsoUsoA s-Chol	ebv- BHRF1-1	8165.93	8166.04	98.5*
3690	oUsoUsoCoAoAoUoUoCoUoGoCoCoGoCoAoAoAoAoGsoAsoUs oAs-Chol	ebv- BHRF1-2	8399.09	8399.29	84.1*
3691	oGsoCsoUoUoAoCoAoCoCoAoGoUoUoCoCoUoGoUsoAsoAs oUs-Chol	kshv-K12- 1	8327.99	8328.08	87.5*
3692	oUsoCsoGoCoUoGoCoCoGoUoCoCoUoCoAoGoAoAoUoGsoUsoGs oAs-Chol	kshv-K3	8422.07	8422.95	86.8*
3693	oUsoCsoAoGoCoUoAoGoGoCoCoUoCoAoGoUoAoUoUsoCsoUsoA s-Chol	kshv-K4- 3p	8047.82	8047.86	84.8*
3694	oAsoCsoCoGoCoCoUoGoAoAoGoUoCoUoCoUoGoAoUsoUsoAsoA s-Chol	luc-1309	8070.86	8071.08	89.1*
3695	oCsoAsoGoCoUoAoUoGoCoCoAoGoCoAoUoCoUoUoGsoCsoCsoUs -Chol	miR-31	8022.8	8023.15	98**
3696	oAsoCsoAoGoUoUoCoUoUoCoAoAoCoUoGoGoCoAoGsoCsoUsoU s-Chol	miR-22	8047.82	8047.74	91**
3697	oAsoCsoUoCoAoCoCoGoAoCoAoGoCoGoUoUoGoAoAoUsoGsoUs oUs-Chol	miR-181	8430.1	8431.06	95

3698	Q38soAsoCoUoCoAoCoCoGoAoCoAoGoCoGoUoUoGoAoAoUsoGs oUsoUs-Chol	miR-181	9050.92	9050.1	79**
3699	oCsoCsoAoAoCoAoAoCoAoUoGoAoAoAoCoUoAoCsoCsoUsoAs- Chol	miR-196	7724.71	7725.3	81*
3700	oCoGoCoCoAoAoUoAoUoUoUoAoCoGoUoGoCoUoGoCoUoAdTdT dTdTToUoAoGoCs-Chol	miR-16	10526.3 4	10527.25	89.1*
3701	oCoGoCoCoAoAoUoAoUoUoUoAoCoGoUoGoCoUoGoCoUoAsdTs dTsdTsdTsoUoAoGoCs-Chol	miR-16	10606.6 7	10607.32	76.3*
3753	oGsoUsoCoUoGoUoCoAoAoAoUoCoAoUoAoGoGoUsoCsoAsoUs- Chol	mR-215	7752.64	7753.31	94
3754	oCsoCsoCoCoUoAoUoCoAoCoAoAoUoUoAoGoCoAoUsoUsoAsoAs- Chol	miR-155	7998.83	7999.73	95**
3755	oGsoUsoAoGoUoGoCoUoUoUoCoUoAoCoUoUoUsoAsoUsoGs-Chol	miR-142-5p	7364.31	7364.80	95**
3756	oCsoCsoAoUoAoAoAoGoUoAoGoGoAoAoAoCoAoCoUsoAsoCsoA s-Chol	miR-142-3p	8148.01	8148.45	100**
3812	oCsoGsoCoCoAoAoUoAoUoUoUoAoCoGoUoGoCoUsoGsoCsoUsoA	miR-16	7343.82	7343.91	96
3832	oCsoAsoCoGoCoGoCoAoCoGoUoGoUoUoAoGoCoAoUoAsoGsoGs oAs-Chol	mCMV- miR-01-1	8508.18	8507.52	98
3833	oAsoCsoCoGoUoUoCoCoAoAoCoCoCoGoAoUoUoCoUoCsoUsoUso Cs-Chol	mCMV- miR-01-2	8262.94	8262.69	100
3834	oCsoCsoGoCoUoUoGoAoCoCoGoAoGoGoCoCoCoCsoCsoAsoUs- Chol	mCMV- miR-23-1	7716.62	7716.33	100
3835	oAsoCsoGoGoUoUoCoCoCoCoGoUoCoCoGoUoAoCoCsoGsoAsoGs -Chol	mCMV- miR-23-2	8076.85	8076.48	98**
3836	oAsoCsoCoGoCoGoGoCoUoCoUoGoGoAoAoAoAoAoGsoAsoUsoA s-Chol	mCMV- miR-44-1	8196.01	8195.64	100
3849	oUsoGsoGoAoGoUoGoUoGoAoCoAoAoUoGoGoUoGoUsoUsoUsoG soU	miR-122	7841.13	7840.13	96*
3850	oAsoCsoAoGoCoUoGoGoUoUoGoAoAoGoGoGoGoAoCsoCsoAsoA s-Chol	miR-133	8252.04	8251.35	97**
3866	oUsoUsoGoGoCoAoUoUoCoAoCoCoGoCoGoUoGoCoCsoUsoUsoAs -Chol	miR-124	8039.79	8039.01	99**
3867	oCsoGsoCoAoUoUoAoUoUoAoCoUoCoAoCoGoGoUoAsoCsoGsoAs -Chol	miR-126	8070.86	8070.17	95**
3868	oAsoGsoAoGoCoUoAoCoAoGoUoGoCoUoUoCoAoUoCsoUsoCsoAs -Chol	miR-143	8070.86	8070.24	97**
3869	Q37soAsoCoUoCoAoCoCoGoAoCoAoGoCoGoUoUoGoAoAoUsoGs oUsoUs-Chol	miR-181	9076.92	9075.89	91**
3870	Q37soAsoCoUoCoAoCoCoGoAoCoAoGoGoUoUoGoAoAoUsoGsoU soUs-Chol	miR-181	8757.71	8756.76	90**

3871	Q37soCsoCoCoCoUoAoUoCoAoCoAoAoUoUoAoGoCoAoUsoUsoA soAs-Chol	miR-155	8645.65	8644.77	93**
3872	Q37soGsoUoAoGoUoGoCoUoUoUoCoUoAoCoUoUoUsoAsoUsoGs- Chol	miR-142-5p	8011.13	8010.25	95**
3873	Q37soCsoCoAoUoAoAoAoGoUoAoGoGoAoAoAoCoAoCoUsoAsoC soAs-Chol	miR-142-3p	8794.83	8793.63	98**
3874	Q38soAsoCoUoCoAoCoCoGoAoCoAoGoCoGoUoUoUoUoUsoUs oAsoUs-Chol	miR-181	8972.84	8971.53	99**
3875	Q38soAsoCoUoCoAoCoCoGoAoCoAoGoGoUoUoGoAoAoUsoGsoU soUs-Chol	miR-181	8731.71	8370.34	99**
3935	oUsoCsoAoAoCoAoUoCoAoGoUoCoUoGoUoAoAoGsoCsoUsoAs- Chol	miR-21	7735.65	7735.46	99
3983	oCsoCsoCoCoAoCoAoCoCoAoUoGoCoAoAoCoUoCoCsoGsoUsoAs -Chol	miR-196	7987.83	7987.83	92
3987	oUsoAsoGoCoUoGoGoUoUoGoAoAoGoGoGoGoAoCsoCsoAsoAs- Chol	miR-133	7909.79	7909.56	94*
3988	oGsoCsoAoUoUoAoUoUoAoCoUoCoAoCoGoGoUoAsoCsoGsoAs- Chol	miR-126-3p	7751.65	7751.65	99
3989	oCsoGsoCoGoUoAoCoCoAoAoAoAoGoUoAoAoUoAsoAsoUsoGs- Chol	miR-126-5p	7821.76	7821.69	79
30004	oCsoAsoAoUoGoCoAoAoCoUoAoCoAoAoUoGsoCsoAsoCs-Chol	miR-33	7078.26	7078.37	82*
30031	oCsoAsoGoAoUoCoGoAoCoCoCoGoGoAoCoUoAoCoAsoGsoUsoUs -Chol	kshv-mir- K2	8108.91		
30032	oCsoCsoGoGoCoAoAoGoUoUoCoCoAoGoGoCoAoUoCsoCsoUsoAs -Chol	kshv-mir- K5	8084.88		
30033	oCsoUsoCoAoAoCoAoGoCoCoCoGoAoAoAoAoCoCoAsoUsoCsoAs -Chol	kshv-mir- K6-3p	8058.93		
30034	oUsoGsoAoGoCoGoCoCoAoGoCoAoAoCoAoUoGoGoGoAsoUsoCs oAs-Chol	kshv-mir- K7	8531.22		
30035	oUsoCsoGoGoAoCoAoCoAoGoGoCoUoAoAoGoCoAoUsoUsoAsoA s-Chol	kshv-mir- K11	8156.97		
30036	oUsoCsoGoCoAoGoAoCoUoGoGoGoUoAoAoGoCoAoUsoUsoAsoA s-Chol	kshv-mir- mm-K11	8173.96		

The strands are shown written 5' to 3'. Lower case "s" indicates a phosphorothioate linkage. "Chol-" indicates a hydroxyprolinol cholesterol conjugate. Subscript "OMe" indicates a 2'-O-methyl sugar. Purity was determined by CGE except for the where indicated by (* and **), in these cases purity was determined by anion-exchange HPLC (*) or LC/MS (**).

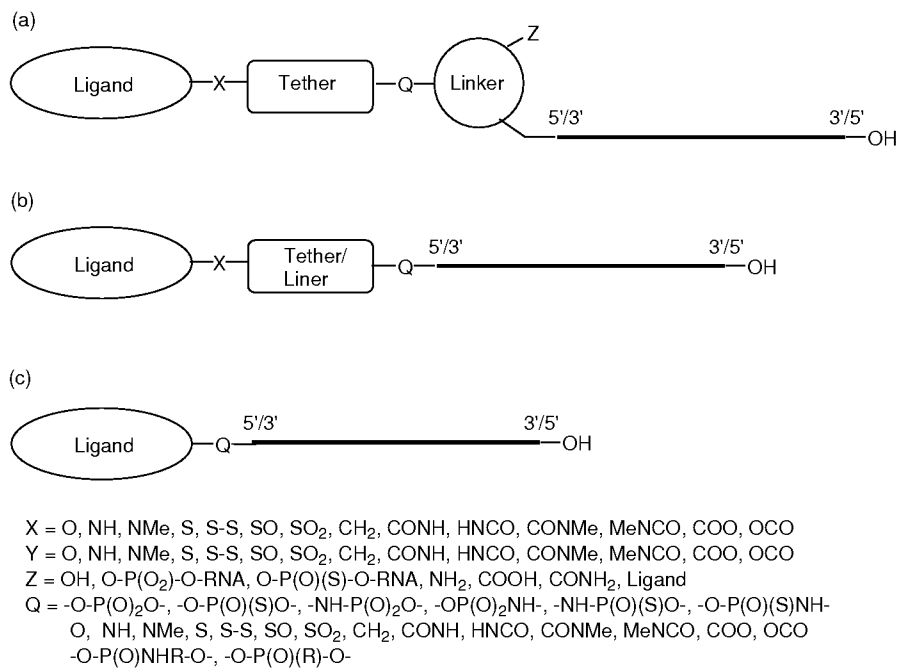
OTHER EMBODIMENTS

5 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. Accordingly, other embodiments are within the scope of the following claims.

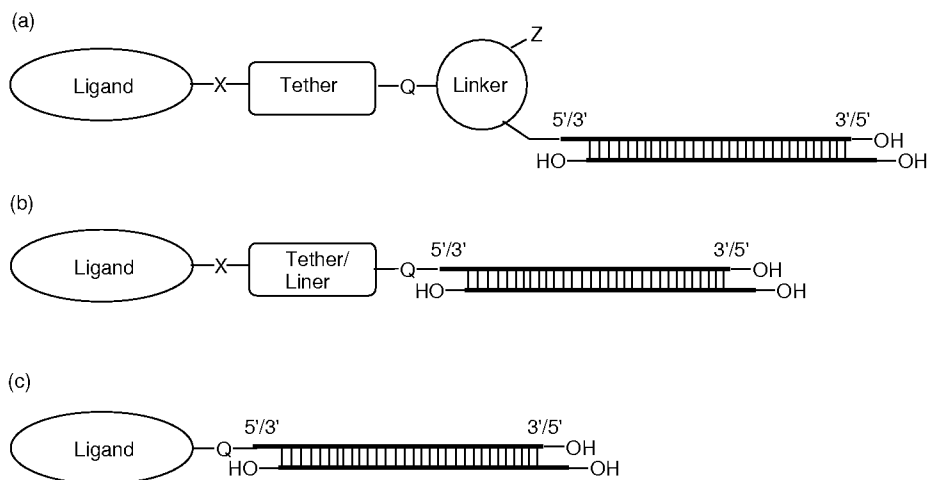
WHAT IS CLAIMED IS:

1. A method of reducing the amount of a microRNA activity in a cell in a subject comprising the step of administering antagomir 3631 to the subject.

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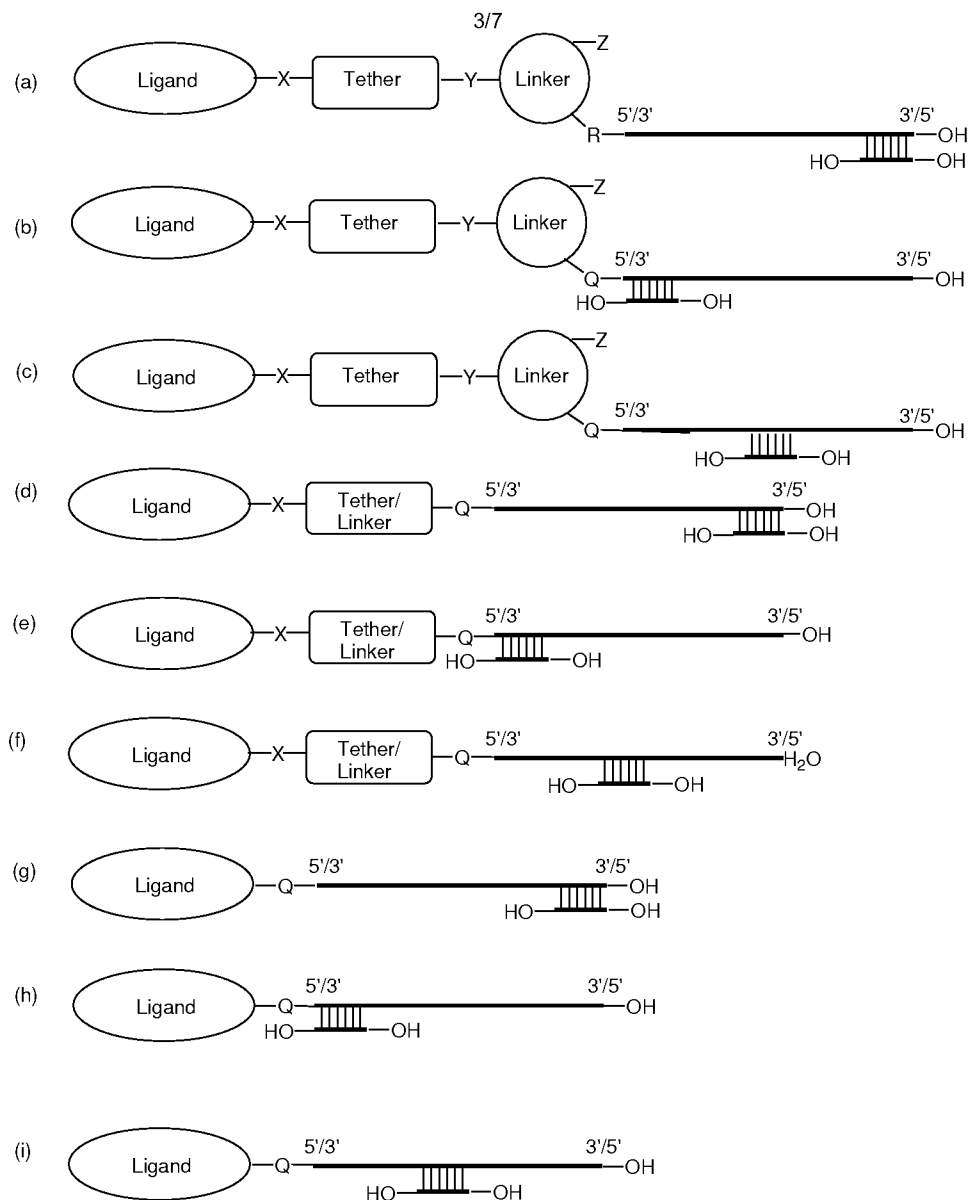
**FIG. 5**

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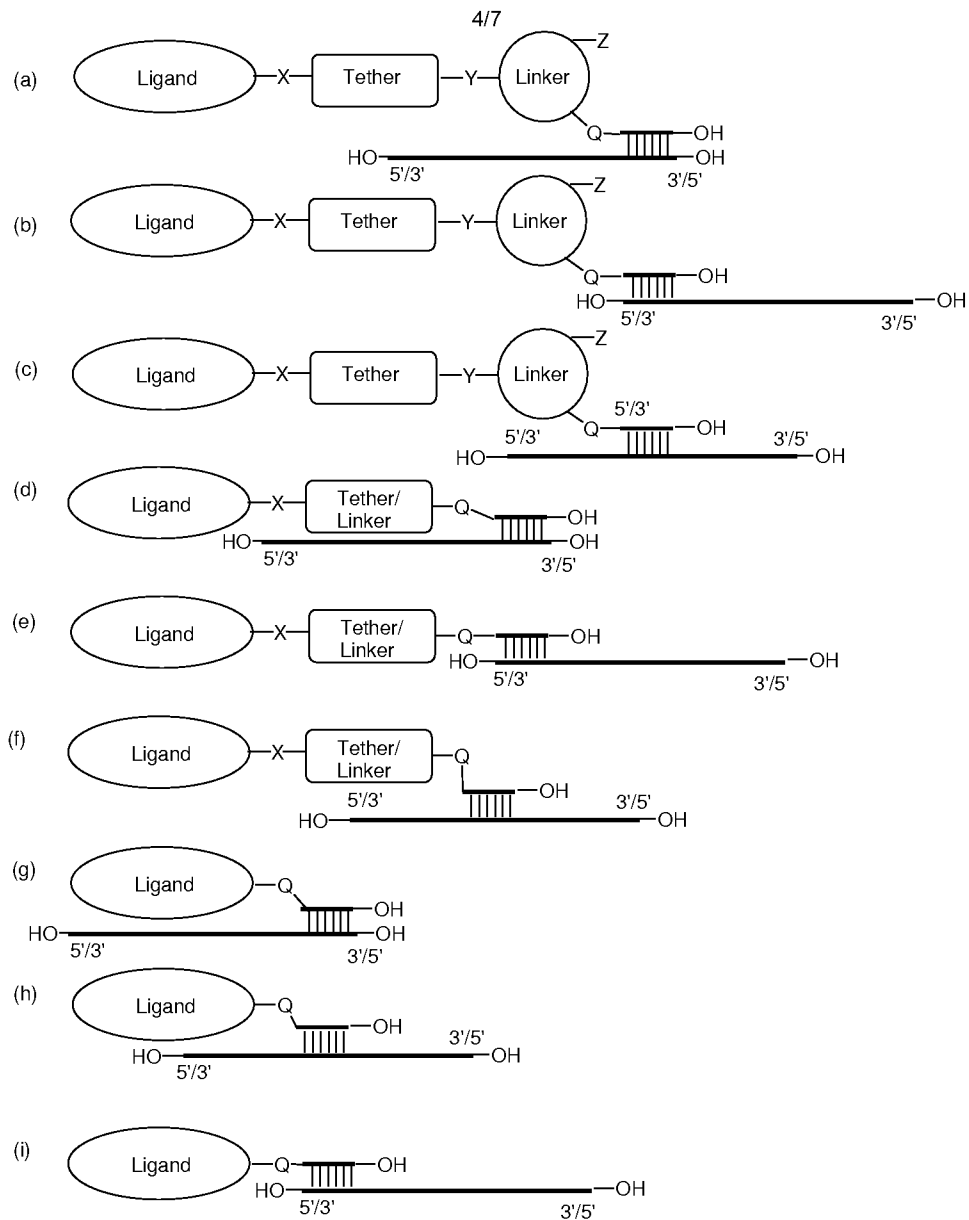
X = O, NH, NMe, S, S-S, SO, SO₂, CH₂, CONH, HNCO, CONMe, MeNCO, COO, OCO
 Y = O, NH, NMe, S, S-S, SO, SO₂, CH₂, CONH, HNCO, CONMe, MeNCO, COO, OCO
 Z = OH, O-P(O₂)-O-RNA, O-P(O)(S)-O-RNA, NH₂, COOH, CONH₂, Ligand
 Q = -O-P(O)₂O-, -O-P(O)(S)O-, -NH-P(O)₂O-, -OP(O)₂NH-, -NH-P(O)(S)O-, -O-P(O)(S)NH-
 O, NH, NMe, S, S-S, SO, SO₂, CH₂, CONH, HNCO, CONMe, MeNCO, COO, OCO
 -O-P(O)NHR-O-, -O-P(O)(R)-O-

FIG. 6



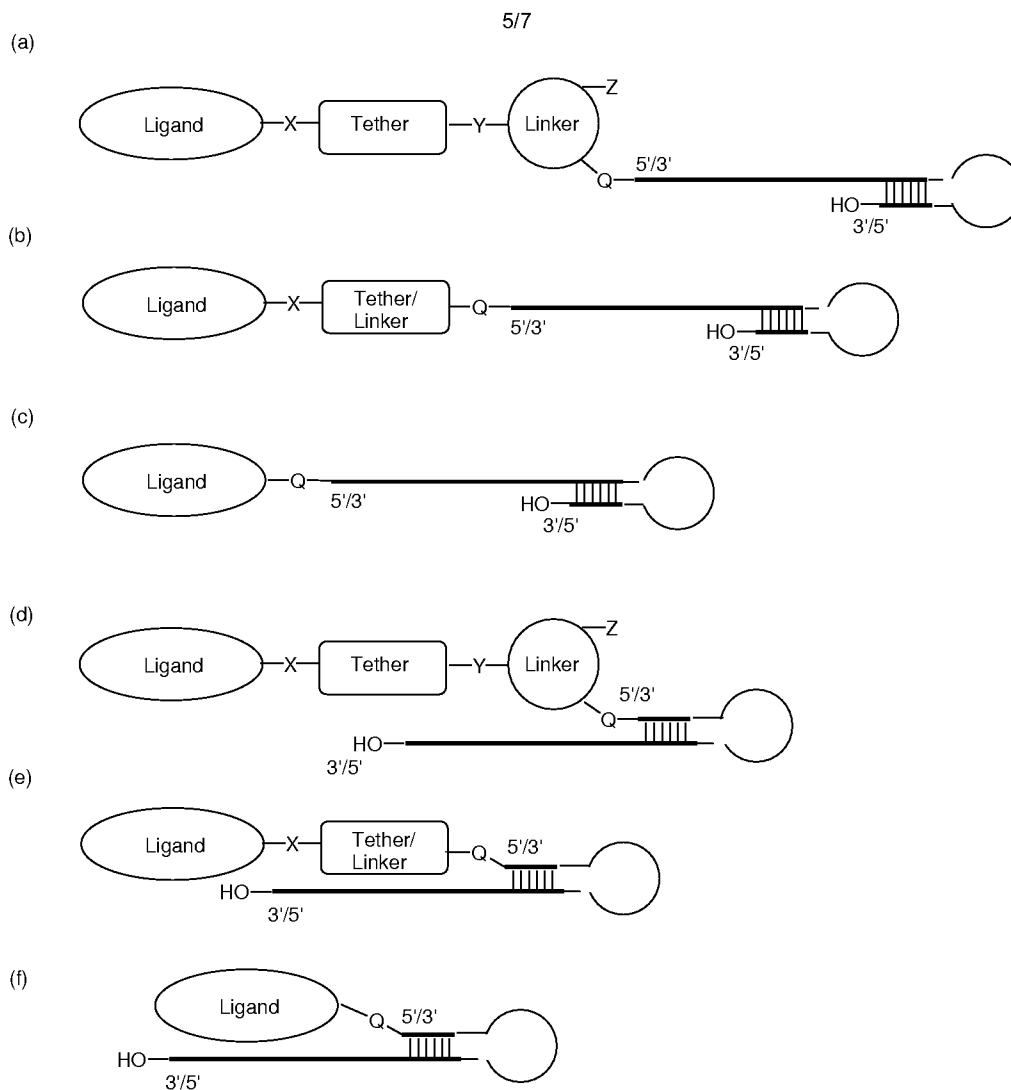
X = O, NH, NMe, S, S-S, SO, SO₂, CH₂, CONH, HNCO, CONMe, MeNCO, COO, OCO
Y = O, NH, NMe, S, S-S, SO, SO₂, CH₂, CONH, HNCO, CONMe, MeNCO, COO, OCO
Z = OH, O-P(O)₂-O-RNA, O-P(O)(S)-O-RNA, NH₂, COOH, CONH₂, Ligand
Q = -O-P(O)₂O-, -O-P(O)(S)O-, -NH-P(O)₂O-, -OP(O)₂NH-, -NH-P(O)(S)O-, -O-P(O)(S)NH-
O, NH, NMe, S, S-S, SO, SO₂, CH₂, CONH, HNCO, CONMe, MeNCO, COO, OCO
-O-P(O)NHR-, -O-P(O)(R)O-

FIG. 7



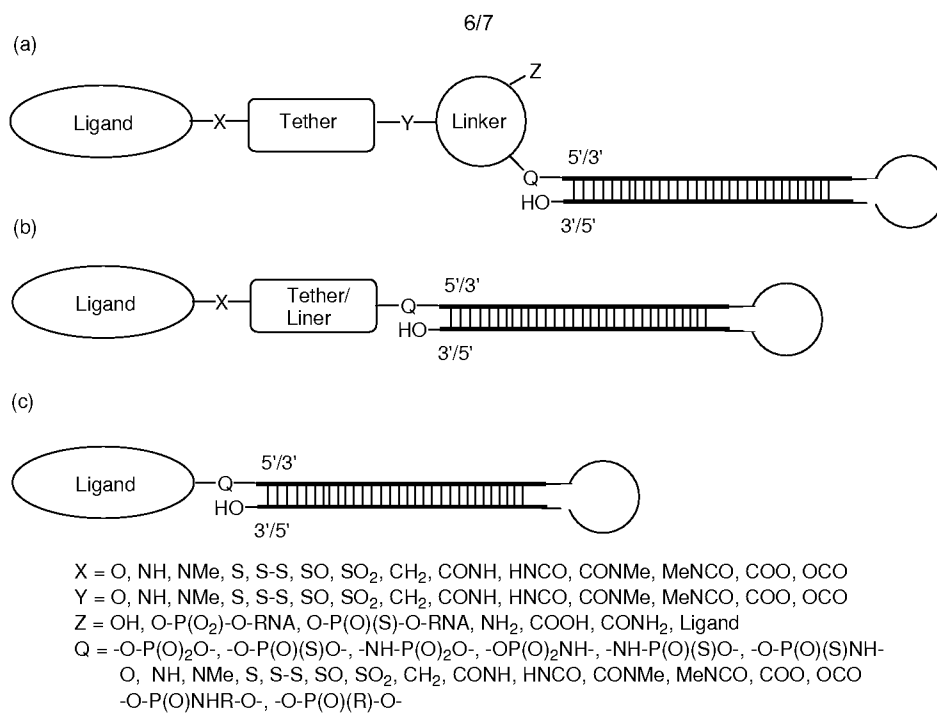
X = O, NH, NMe, S, S-S, SO, SO₂, CH₂, CONH, HNCO, CONMe, MeNCO, COO, OCO
Y = O, NH, NMe, S, S-S, SO, SO₂, CH₂, CONH, HNCO, CONMe, MeNCO, COO, OCO
Z = OH, O-P(O)₂-O-RNA, O-P(O)(S)-O-RNA, NH₂, COOH, CONH₂, Ligand
Q = -O-P(O)₂O-, -O-P(O)(S)O-, -NH-P(O)₂O-, -OP(O)₂NH-, -NH-P(O)(S)O-, -O-P(O)(S)NH-
O, NH, NMe, S, S-S, SO, SO₂, CH₂, CONH, HNCO, CONMe, MeNCO, COO, OCO
-O-P(O)NHR-O-, -O-P(O)(R)-O-

FIG. 8



X = O, NH, NMe, S, S-S, SO, SO₂, CH₂, CONH, HNCO, CONMe, MeNCO, COO, OCO
 Y = O, NH, NMe, S, S-S, SO, SO₂, CH₂, CONH, HNCO, CONMe, MeNCO, COO, OCO
 Z = OH, O-P(O)₂-O-RNA, O-P(O)(S)-O-RNA, NH₂, COOH, CONH₂, Ligand
 Q = -O-P(O)₂O-, -O-P(O)(S)O-, -NH-P(O)₂O-, -OP(O)₂NH-, -NH-P(O)(S)O-, -O-P(O)(S)NH-
 O, NH, NMe, S, S-S, SO, SO₂, CH₂, CONH, HNCO, CONMe, MeNCO, COO, OCO
 -O-P(O)NHR-O-, -O-P(O)(R)-O-

FIG. 9

**FIG. 10**

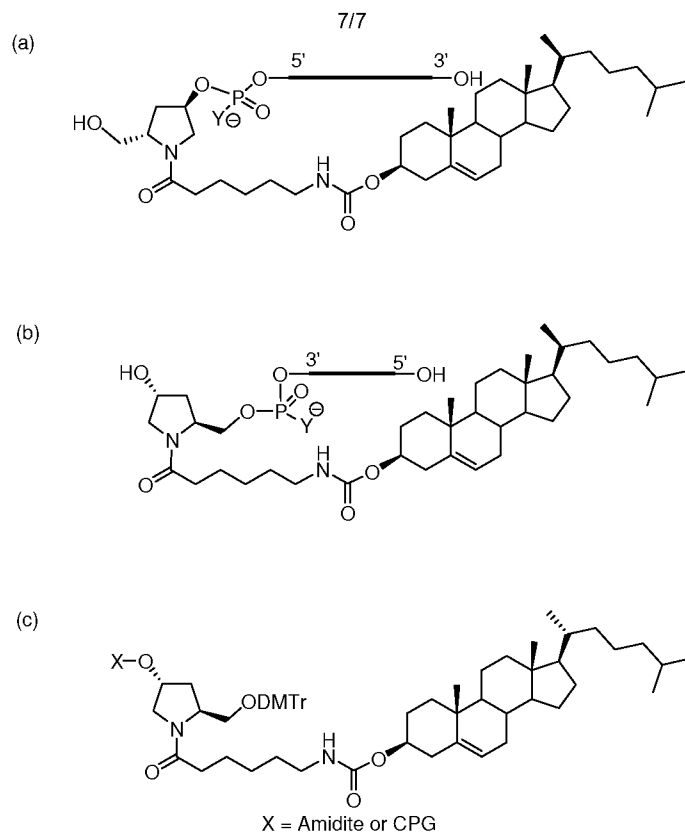


FIG. 11