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(54) **Title**: MODIFICATIONS FOR ANTISENSE COMPOUNDS

(57) **Abstract**: The invention pertains to modifications for antisense oligonucleotides, wherein the modifications are used to improve stability and provide protection from nuclease degradation. The modifications could also be incorporated into double-stranded nucleic acids, such as synthetic siRNAs and miRNAs.

MODIFICATIONS FOR ANTISENSE COMPOUNDS

CROSS REFERENCE TO RELATED APPLICATION

[0001]   This application claims the benefit of priority from U.S. Provisional Application No. 61/380,586, filed September 7, 2010, the disclosure of which is incorporated by reference herein in its entirety.

STATEMENT REGARDING FEDERALLY-SPONSORED
RESEARCH OR DEVELOPMENT

FIELD OF THE INVENTION

[0003]   This invention pertains to modifications for antisense oligonucleotides, wherein the modifications are used to improve stability and provide protection from nuclease degradation.

BACKGROUND OF THE INVENTION

[0004]   Antisense oligonucleotides (ASOs) are synthetic nucleic acids that bind to a complementary target and suppress function of that target. Typically ASOs are used to reduce or alter expression of RNA targets, particularly messenger RNA (mRNA) or microRNA (miRNA) species. As a general principle, ASOs can suppress gene expression via two different mechanisms of action, including: 1) by steric blocking, wherein the ASO tightly binds the target nucleic acid and inactivates that species, preventing its participation in cellular biology, or 2) by triggering degradation, wherein the ASO binds the target and leads to activation of a cellular nuclease that degrades the targeted nucleic acid species. One class of "target degrading" ASOs are "RNase H active"; formation of heteroduplex nucleic acids by hybridization of the target RNA with a DNA-containing "RNase H active" ASO forms a substrate for the enzyme RNase H. RNase H degrades the RNA portion of the heteroduplex molecule, thereby reducing expression

of that species. Degradation of the target RNA releases the ASO, which is not degraded, which is then free to recycle and can bind another RNA target of the same sequence. For an overview of antisense strategies, oligonucleotide design, and chemical modifications, *see* Kurreck, 2003, *Eur. J. Biochem.*, 270(8): 1628-44.

[0005]   Unmodified DNA oligonucleotides have a half-life of minutes when incubated in human serum. Therefore, unmodified DNA oligonucleotides have limited utility as ASOs. The primary nuclease present in serum has a 3'-exonuclease activity (Eder *et al.*, 1991, *Antisense Res. Dev.* 1(2): 141-51). Once an ASO gains access to the intracellular compartment, it is susceptible to endonuclease degradation. Historically, the first functional ASOs to gain widespread use comprised DNA modified with phosphorothioate groups (PS). PS modification of the internucleotide linkages confers nuclease resistance, making the ASOs more stable both in serum and in cells. As an added benefit, the PS modification also increases binding of the ASO to serum proteins, such as albumin, which decreases the rate of renal excretion following intravenous injection, thereby improving pharmacokinetics and improving functional performance (Geary *et al.*, 2001, *Curr. Opin. Investig. Drugs*, 2(4): 562–73). However, PS-modified ASOs are limited to a 1-3 day half-life in tissue, and the PS modifications reduce the binding affinity of the ASO for the target RNA, which can decrease potency (Stein *et al.*, 1988, *Nucleic Acids Res.* 16(8): 3209–21).

[0006]   The PS modification is unique in that it confers nuclease stability yet still permits formation of a heteroduplex with RNA that is a substrate for RNase H action. Most other modifications that confer nuclease resistance, such as methyl phosphonates or phosphoramidates, are modifications that do not form heteroduplexes that are RNase H substrates when hybridized to a target mRNA. Improved potency could be obtained using compounds that were both nuclease resistant and showed higher affinity to the target RNA yet retain the ability to activate RNase H mediated degradation pathways.

[0007]   Further design improvements were implemented to increase affinity for the target RNA while still maintaining nuclease resistance (*see* Walder *et al.*, U.S. Patent No. 6,197,944 for designs containing 3'-modifications with a region containing unmodified residues with phosphodiester linkages; *see also* European Patent No. 0618925 for "Gapmer" compounds having 2'-methoxyethylriboses (MOE's) providing 2'-modified 'wings' at the 3' and 5' ends

flanking a central 2'-deoxy gap region). The new strategy allows for chimeric molecules that have distinct functional domains. For example, a single ASO can contain a domain that confers both increased nuclease stability and increased binding affinity but itself does not form an RNase H active substrate; a second domain in the same ASO can be RNase H activating. Having both functional domains in a single molecule improves performance and functional potency in antisense applications. One successful strategy is to build the ASO from different chemical groups with a domain on each end intended to confer increased binding affinity and increased nuclease resistance that flank a central domain comprising different modifications which provides for RNase H activation. This so-called "end blocked" or "gapmer" design is the basis for the improved function "second generation" ASOs. Compounds of this design are typically significantly more potent as gene knockdown agents than the "first generation" PS-DNA ASOs.

[0008] Typically ASOs that function using steric blocking mechanisms of action show higher potency when made to maximize binding affinity. This can be accomplished using chemical modifications that increase binding affinity, such as many of the 2'-ribose modifications discussed herein, minor groove binders, or the internal non-base modifiers of the present invention. Alternatively, increased binding affinity can be achieved by using longer sequences. However, some targets are short, such as miRNAs, which are typically only 20-24 bases long. In this case, making ASOs longer to increase binding affinity is not possible. Further, short synthetic oligonucleotides gain access into cells more efficiently than long oligonucleotides, making it desirable to employ short sequences with modifications that increase binding affinity (see, e.g., Straarup et al., 2010, Nucleic Acids Res. 38(20): 7100-11). The chemical modification and methods of the present invention enable synthesis of relatively short ASOs having increased binding affinity that show improved functional performance.

[0009] ASO modifications that improve both binding affinity and nuclease resistance typically are modified nucleosides that are costly to manufacture. Examples of modified nucleosides include locked nucleic acids (LNA), wherein a methyl bridge connects the 2'-oxygen and the 4'-carbon, locking the ribose in an A-form conformation; variations of LNA are also available, such as ethylene-bridged nucleic acids (ENA) that contain an additional methyl group, amino-LNA and thio-LNA. Additionally, other 2'-modifications, such as 2'-O-methoxyethyl (MOE) or 2'-fluoro (2'-F), can also be incorporated into ASOs. Some modifications decrease

stability, and some can have negative effects such as toxicity (*see* Swayze *et al.*, 2007, *Nucleic Acids Res.* 35(2): 687-700).

[0010]   The present invention provides for non-nucleotide modifying groups that can be inserted between bases in an ASO to improve nuclease resistance and binding affinity, thereby increasing potency. The novel modifications of the present invention can be employed with previously described chemical modifications (such as PS internucleotide linkages, LNA bases, MOE bases, etc.) and with naturally occurring nucleic acid building blocks, such as DNA or 2'-*O*-Methyl RNA (2'OMe), which are inexpensive and non-toxic. These and other advantages of the invention, as well as additional inventive features, will be apparent from the description of the invention provided herein.
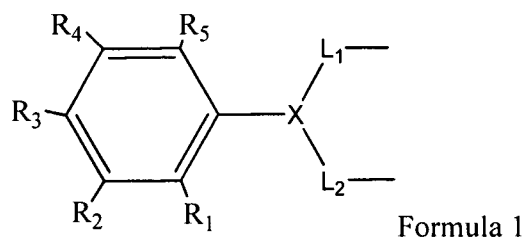

BRIEF SUMMARY OF THE INVENTION

[0011]   The invention provides non-nucleotide modifications for antisense oligonucleotides, wherein the modifications are used to increase binding affinity and provide protection from nuclease degradation.

[0012]   The invention also provides an antisense oligonucleotide comprising at least one modification that is incorporated between two bases of the antisense oligonucleotide, wherein the modification increases binding affinity and nuclease resistance of the antisense oligonucleotide. In one embodiment, the antisense oligonucleotide comprises at least one modification that is located within three bases of a terminal nucleotide. In another embodiment, the antisense oligonucleotide comprises at least one modification that is located between a terminal base and a penultimate base of either the 3'- or the 5'-end of the oligonucleotide. In a further embodiment, the antisense oligonucleotide comprises a modification between the terminal base and the penultimate base of both the 3'- and the 5'-ends of the antisense oligonucleotide.

[0013]   The invention further provides an antisense oligonucleotide comprising at least one modification that is incorporated between two bases of the antisense oligonucleotide, wherein the modification increases binding affinity and nuclease resistance of the antisense oligonucleotide, and wherein the modification is a napthylene-azo compound.
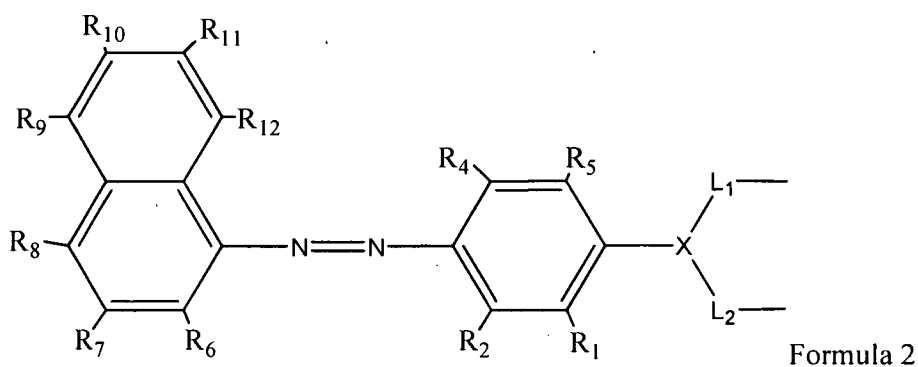
[0014]   The invention further provides an antisense oligonucleotide comprising at least one modification that is incorporated between two bases of the antisense oligonucleotide, wherein the

modification increases binding affinity and nuclease resistance of the antisense oligonucleotide, and wherein the modification has the structure:

Formula 1

wherein the linking groups L_1 and L_2 positioning the modification at an internal position of the oligonucleotide are independently an alkyl, alkynyl, alkenyl, heteroalkyl, substituted alkyl, aryl, heteroaryl, substituted aryl, cycloalkyl, alkylaryl, or alkoxy groups; R_1-R_5 are independently a hydrogen, alkyl, alkynyl, alkenyl, heteroalkyl, substituted alkyl, aryl, heteroaryl, substituted aryl, cycloalkyl, alkylaryl, alkoxy, an electron withdrawing group, an electron donating group, or an attachment point for a ligand; and X is a nitrogen or carbon atom, wherein if X is a carbon atom, the fourth substituent attached to the carbon atom can be hydrogen or a C1-C8 alkyl group.

[0015]   The invention further provides an antisense oligonucleotide comprising at least one modification that is incorporated between two bases of the antisense oligonucleotide, wherein the modification increases binding affinity and nuclease resistance of the antisense oligonucleotide, and wherein the modification has the structure:

Formula 2

wherein the linking groups L_1 and L_2 positioning the modification at an internal position of the oligonucleotide are independently an alkyl, alkynyl, alkenyl, heteroalkyl, substituted alkyl, aryl, heteroaryl, substituted aryl, cycloalkyl, alkylaryl, or alkoxy groups; R_1, R_2, R_4, R_5 are

independently a hydrogen, alkyl, alkynyl, alkenyl, heteroalkyl, substituted alkyl, aryl, heteroaryl, substituted aryl, cycloalkyl, alkylaryl, alkoxy, an electron withdrawing group, or an electron donating group; R_6, R_7, R_9-R_{12} are independently a hydrogen, alkyl, alkynyl, alkenyl, heteroalkyl, substituted alkyl, aryl, heteroaryl, substituted aryl, cycloalkyl, alkylaryl, alkoxy, an electron withdrawing group, or an electron donating group; R_8 is a hydrogen, alkyl, alkynyl, alkenyl, heteroalkyl, substituted alkyl, aryl, heteroaryl, substituted aryl, cycloalkyl, alkylaryl, alkoxy, or an electron withdrawing group; and X is a nitrogen or carbon atom, wherein if X is a carbon atom, the fourth substituent attached to the carbon atom can be hydrogen or a C1-C8 alkyl group. In one embodiment, R_8 is NO_2.
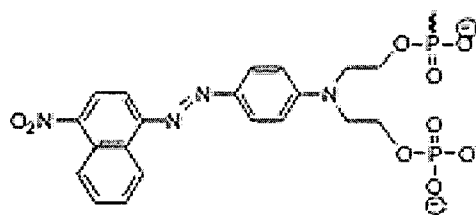
[0016]   The invention further provides an antisense oligonucleotide comprising at least one modification that is incorporated between two bases of the antisense oligonucleotide, wherein the modification increases binding affinity and nuclease resistance of the antisense oligonucleotide, and wherein the modification has the structure:

Formula 3.

[0017]   The antisense oligonucleotides of the invention can include natural, non-natural, or modified bases known in the art. The antisense oligonucleotides of the invention can also include, typically but not necessarily on the 3' or 5' ends of the oligonucleotide, additional modifications such as minor groove binders, spacers, labels, or other non-base entities. In one embodiment, the antisense oligonucleotide further comprises 2'-O-methyl RNA, and optionally comprises at least one napthylene-azo compound. In another embodiment, the antisense oligonucleotide further comprises phosphorothioate linkages. In a further embodiment, the antisense oligonucleotide comprises a region of bases linked through phosphodiester bonds, wherein the region is flanked at one or both ends by regions containing phosphorothioate linkages.

[0018]   The invention further provides an antisense oligonucleotide having the structure:
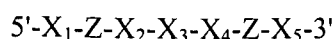
5'-X_1-Z-X_2-X_3-X_4-Z-X_5-3'   Formula 4

wherein X_1 and X_5 are independently 1-3 nucleotides wherein the internucleotide linkages are optionally phosphorothioate; Z is a napthylene-azo compound; X_2 and X_4 are independently 1-5 nucleotides wherein the internucleotide linkages are optionally phosphorothioate; and X_3 is 10-25 nucleotides.

[0019]   In one embodiment, a third modification can be inserted around the middle of the antisense oligonucleotide. For longer nucleotides (greater than 25 bases), additional modifications could be used at intervals to confer greater stability.

[0020]   In the modifications of the invention, a modifying group is inserted between adjacent bases, thereby generating an ASO with reduced toxicity and improved affinity and stability. The bases can be DNA, 2'OMe RNA, or other modified bases. However, modified bases do not need to be employed. Because the modifications are inserted between the bases, they can be added as a phosphoramidite compound using standard phosphoramidite synthesis chemistry.

BRIEF DESCRIPTION OF THE DRAWINGS

[0021]   Figure 1 is a gel photograph that illustrates the levels of degradation of synthetic DNA oligomers in fetal bovine serum. A series of 10-mer single-stranded DNA oligonucleotides were trace labeled with ^{32}P at their 5'-ends and were incubated in serum at 37°C for the indicated times (0-240 minutes). Reaction products were separated by polyacrylamide gel electrophoresis (PAGE) and visualized by phosphorimaging. Samples are identified in Table 4.

[0022]   Figure 2 illustrates relative miR-21 suppression by various anti-miRNA oligonucleotides (AMOs) using a luciferase reporter assay. A reporter plasmid that expresses both *Renilla* luciferase and firefly luciferase was transfected into HeLa cells. Cell extracts were studied for relative activity of both enzymes and *Renilla* luciferase activity was normalized to firefly luciferase activity. The *Renilla* luciferase gene contains a miR-21 binding site and miR-21 is highly expressed in HeLa cells. Different anti-miR-21 oligonucleotides (X-axis) were transfected into the cells and the relative ability of different designs to suppress miR-21 activity directly relate to the increase in *Renilla* luciferase activity (Y-axis).

[0023]   Figure 3 illustrates relative miR-21 suppression by various AMOs using a luciferase reporter assay comparing perfect match and compounds having 1, 2, or 3 mismatches (mismatch pattern 1). A reporter plasmid that expresses both *Renilla* luciferase and firefly luciferase was

transfected into HeLa cells. Cell extracts were studied for relative activity of both enzymes and *Renilla* luciferase activity was normalized to firefly luciferase activity. The *Renilla* luciferase gene contains a miR-21 binding site and miR-21 is highly expressed in HeLa cells. Different anti-miR-21 oligonucleotides (X-axis) were transfected into the cells, and the relative ability of different designs to suppress miR-21 activity directly relate to the increase in *Renilla* luciferase activity (Y-axis).

[0024]   Figure 4 illustrates relative miR-21 suppression by various AMOs using a luciferase reporter assay comparing perfect match and compounds having 1, 2, or 3 mismatches (mismatch pattern 2). A reporter plasmid that expresses both *Renilla* luciferase and firefly luciferase was transfected into HeLa cells. Cell extracts were studied for relative activity of both enzymes, and *Renilla* luciferase activity was normalized to firefly luciferase activity. The *Renilla* luciferase gene contains a miR-21 binding site and miR-21 is highly expressed in HeLa cells. Different anti-miR-21 oligonucleotides (X-axis) were transfected into the cells, and the relative ability of different designs to suppress miR-21 activity directly relate to the increase in *Renilla* luciferase activity (Y-axis).

[0025]   Figure 5 illustrates knockdown of HPRT expression by DNA ASOs, with or without PS bonds or iFQ modification. ASOs were transfected into HeLa cells and RNA was prepared 24 hours post transfection. Relative HPRT levels were assessed by RT-qPCR and are reported on the Y-axis.

[0026]   Figure 6 illustrates knockdown of HPRT expression by chimeric "gapmer" ASOs, with or without PS bonds and with or without iFQ modification. ASOs were transfected into HeLa cells and RNA was prepared 24 hours post transfection. Relative HPRT levels were assessed by RT-qPCR and are reported on the Y-axis.

[0027]   Figure 7 illustrates knockdown of HPRT using DsiRNAs at doses ranging from 0.01 nM to 1.0 nM. DsiRNAs were modified with iFQ group(s) at positions within the duplexes as indicated in the schematic below the graph. DsiRNAs were transfected into HeLa cells and RNA was prepared 24 hours post transfection. Relative HPRT levels were assessed by RT-qPCR and are reported on the Y-axis.

[0028]   Figure 8 illustrates the toxicity profiles of various AMO chemistries when transfected for 24 hours at 50 nM or 100 nM in HeLa cells. The negative control or "NC1" sequence is not
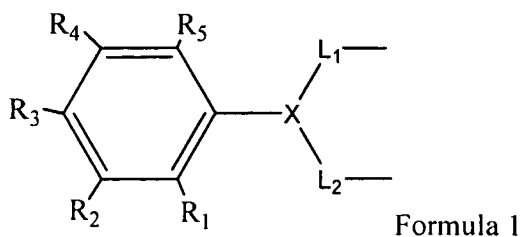
predicted to target any known human miRNAs or mRNAs, and so toxicity effects should be specific to the chemical composition of the oligonucleotide. The MultiTox-Glo Multiplex Cytotoxicity Assay was employed to measure cell viability following treatment with various chemically modified oligonucleotides (X-axis), and cell viability was calculated as a ratio of live/dead cells to normalize the data independent of cell number (Y-axis). A decrease of live/dead cell values correlates with decreased cell viability.

[0029]   Figure 9 illustrates apoptosis induction profiles caused by various AMO chemistries when transfected for 24 hours at 50 nM or 100 nM in HeLa cells. The negative control or "NC1" sequence is not predicted to target any known human miRNAs or mRNAs, and so induction of apoptosis should be specific to the biological effects of chemical composition of the oligonucleotide in the cell. The Caspase-Glo 3/7 Assay was employed to measure the levels of caspase-3 and caspase-7, which are known effectors of apoptosis, using a luciferase assay. Apoptosis induction following treatment with various chemically modified oligonucleotides (X-axis) is proportional to increasing levels of luminescence (Y-axis).

DETAILED DESCRIPTION OF THE INVENTION

[0030]   The antisense oligonucleotides of the invention have modifications placed between nucleotides, wherein the modifications increase affinity to the complementary target and provide nuclease resistance. In one embodiment of the invention, the compounds are the same as those described in U.S. Application No. 13/073,866, the disclosure of which is incorporated by reference herein in its entirety.

[0031]   In another embodiment of the invention, the antisense oligonucleotide comprises at least one modification that has the structure:

$$R_4 \begin{array}{c} R_5 \end{array}$$

L1—
X
L2—

Formula 1

wherein the linking groups L_1 and L_2 positioning the modification at an internal position of the oligonucleotide are independently an alkyl, alkynyl, alkenyl, heteroalkyl, substituted alkyl, aryl, heteroaryl, substituted aryl, cycloalkyl, alkylaryl, or alkoxy groups; R_1-R_5 are independently a hydrogen, alkyl, alkynyl, alkenyl, heteroalkyl, substituted alkyl, aryl, heteroaryl, substituted aryl, cycloalkyl, alkylaryl, alkoxy, an electron withdrawing group, an electron donating group, or an attachment point for a ligand; and X is a nitrogen or carbon atom, wherein if X is a carbon atom, the fourth substituent attached to the carbon atom can be hydrogen or a C1-C8 alkyl group. In a further embodiment of the invention, the antisense oligonucleotide comprises at least one modification that has the structure:

Formula 2

wherein the linking groups L_1 and L_2 positioning the modification at an internal position of the oligonucleotide are independently an alkyl, alkynyl, alkenyl, heteroalkyl, substituted alkyl, aryl, heteroaryl, substituted aryl, cycloalkyl, alkylaryl, or alkoxy groups; R_1, R_2, R_4, R_5 are independently a hydrogen, alkyl, alkynyl, alkenyl, heteroalkyl, substituted alkyl, aryl, heteroaryl, substituted aryl, cycloalkyl, alkylaryl, alkoxy, an electron withdrawing group, or an electron donating group; R_6, R_7, R_9-R_{12} are independently a hydrogen, alkyl, alkynyl, alkenyl, heteroalkyl, substituted alkyl, aryl, heteroaryl, substituted aryl, cycloalkyl, alkylaryl, alkoxy, an electron withdrawing group, or an electron donating group; R_8 is a hydrogen, alkyl, alkynyl, alkenyl, heteroalkyl, substituted alkyl, aryl, heteroaryl, substituted aryl, cycloalkyl, alkylaryl, alkoxy, or an electron withdrawing group; and X is a nitrogen or carbon atom, wherein if X is a carbon atom, the fourth substituent attached to the carbon atom can be hydrogen or a C1-C8 alkyl group.

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[0032] The compositions and methods of the invention involve modification of an oligonucleotide by placing non-base modifying group(s) as insertions between bases while retaining the ability of that sequence to hybridize to a complementary sequence. Typically, insertion of non-base modifying groups between bases results in a significant loss of affinity of the modified sequence to its complement. The unique compositions of the invention increase affinity of the modified sequence to its complement, increasing stability and increasing T_m. Placement of such non-base modifying group(s) between bases prevents nucleases from initiating degradation at the modified linkage(s). When placed between the first and second bases at both ends of the oligonucleotide, the sequence is protected from attack by both 5'-exonucleases and 3'-exonucleases. Placement at central position(s) within the sequence can additionally confer some resistance to endonucleases. In particular, compounds of the class of Formula 2 above impede nuclease attack for several flanking internucleotide phosphate bonds adjacent to the modified linkage, creating a protected "zone" where unmodified linkages are less susceptible to nuclease cleavage. The modifications also prevent nucleases from cleaving terminal bases. Thus the compositions and methods of the invention permit synthesis of ASOs having increased T_m and increased nuclease resistance yet do not employ modified but instead employ a non-base modifying group inserted between residues.

[0033] The ability of the modifying groups of the present invention to increase binding affinity (T_m) of duplexed nucleic acids is demonstrated in Example 1, where melting studies were conducted for a series of unmodified and modified 10-mer duplex DNA oligomers. Using compositions and methods of the present invention, an increase of +11°C was achieved using only two modifying groups (between the two terminal bases on each end of the oligomer). Similar duplexes made with insertions of a propanediol group show significant destabilization, consistent with the expected results for non-base insertions.

[0034] The ability of the modifying groups of the present invention to improve nuclease stability is demonstrated in Example 2, where single-stranded DNA oligomers were incubated in serum (subjected to degradation by serum nucleases) and then examined for integrity by polyacrylamide gel electrophoresis (PAGE). Unmodified DNA oligomers are rapidly degraded in serum whereas a 10-mer DNA oligonucleotide with an insertion of the napthylene-azo modifier between the terminal bases on each end resulted in a compound that was not degraded

after 4 hours incubation. Other modifying groups, such as a propanediol spacer, only slowed the rate of degradation slightly. T_m-enhancing, nuclease blocking modifications (such as the napthylene-azo group) can be inserted into single-stranded oligomers to improve properties. Stabilized, increased binding affinity oligomers of this type can have a variety of uses, as is well appreciated by those with skill in the art. As examples (not meant to be limiting), such oligomers can be used as ASOs to promote reduction of mRNA or miRNA levels in a cell or animal. Such examples are demonstrated in Examples 3 and 4 below.

[0035]   In a further embodiment of the invention, the modifications could also be incorporated into double-stranded nucleic acids, such as synthetic siRNAs and miRNAs. Careful placement of the modifying group should lead to improvements in nuclease stability and could alter local thermal stability, which if employed asymmetrically in an RNA duplex, is well known to influence strand loading into RISC (Peek and Behlke, 2007, *Curr. Opin. Mol. Ther.* 9(2): 110-18), and therefore impact relative biological potency of the compound as a synthetic trigger of RNAi.

[0036]   Oligonucleotides antisense in orientation to miRNAs will bind the miRNA and functionally remove that species from participation in the microRNA-Induced Silencing Complex (miRISC) (Krutzfeldt *et al.*, 2007, *Nucleic Acids Res.* 35(9): 2885-92). Such anti-miRNA oligonucleotides (AMOs) are thought to function by a steric binding mechanism, and compounds with high stability and high affinity generally show improved functional performance compared with low affinity compounds (Lennox and Behlke, 2010, *Pharm. Res.* 27(9): 1788-99). The ASOs of the present invention can function as anti-miRNA oligonucleotides.

[0037]   In the modifications of the present invention, a modifying group is inserted between adjacent bases, thereby generating an ASO with reduced toxicity and improved binding affinity and nuclease stability. The bases can be DNA, 2'OMe RNA, LNA, or other modified bases. However, modified bases do not need to be employed. Because the modifications are inserted between the bases, they can be added as a phosphoramidite compound using standard phosphoramidite synthesis chemistry.

[0038]   In yet another application where ASOs are employed to alter or modify gene expression, the ASOs are designed to be complementary to a pre-mRNA species at sites at or

near an intron/exon splice junction. Binding of the ASO at or near splice sites can alter processing at this intron/exon junction by the nuclear splicing machinery thereby changing splice patterns present in the final mature mRNA (*i.e.*, can be used to alter the exons that are included or excluded in the final processed mRNA). Following mRNA maturation, the altered mRNA will direct synthesis of an altered protein species as a result of this ASO treatment. Methods to design splice-blocking oligonucleotides (SBOs) are well known to those with skill in the art (*see, e.g.*, Aartsma-Rus *et al.*, 2009, *Mol. Ther.* 17(3): 548-53; and Mitrpant *et al.*, 2009, *Mol. Ther.* 17(8): 1418-26). Because SBOs are intended to alter the form of an mRNA but not destroy that mRNA, oligonucleotides of this class are made using chemistries which are compatible with steric blocking antisense mechanism of action and not chemistries or designs that trigger RNA degradation. One example of the use of SBOs induces exon-skipping in the dystrophin gene in individuals having a mutant form of this gene which causes Duchene's Muscular Dystrophy (*see* Muntoni and Wood, 2011, *Nat. Rev. Drug Discov.* 10(8): 621-37; and Goemans *et al.*, 2011, *N. Engl. J. Med.* 364(16): 1513-22). Synthetic oligonucleotides using the design and chemistries of the present invention can be employed as SBOs.

[0039]   In one embodiment, a synthetic oligonucleotide comprises a non-nucleotide modifier of the present invention positioned at or near one or both ends of the sequence. In another embodiment, a synthetic oligonucleotide comprises a non-nucleotide modifier of the present invention positioned between a terminal base and a penultimate base of either the 3'- or the 5'-end of the oligonucleotide. In a further embodiment, the oligonucleotide contains a modification between the terminal base and the penultimate base of both the 3'- and 5'-ends.

[0040]   In one embodiment of the invention, the modification is a napthylene-azo compound. The oligonucleotide is made using modified bases such that the complex of the SBO with the target pre-mRNA does not form a substrate for RNase H, using chemically-modified residues that are well known to those with skill in the art, including, for example, 2'-*O*-methyl RNA, 2'-methyoxyethyl RNA (2'-MOE), 2'-F RNA, LNA, and the like. SBOs made using the non-nucleotide modifiers of the present invention have increased binding affinity compared to the cognate unmodified species. This can permit use of shorter sequences, which can show improved uptake into cells and improved biological activity.

[0041]   In another embodiment of the invention, the modification has the structure:
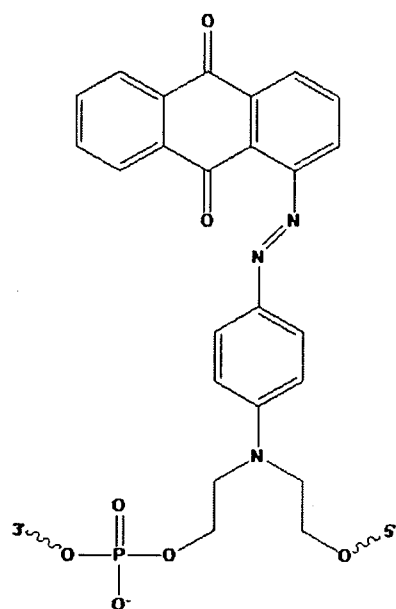
Formula 5

In a further embodiment of the invention, the modification has the structure:
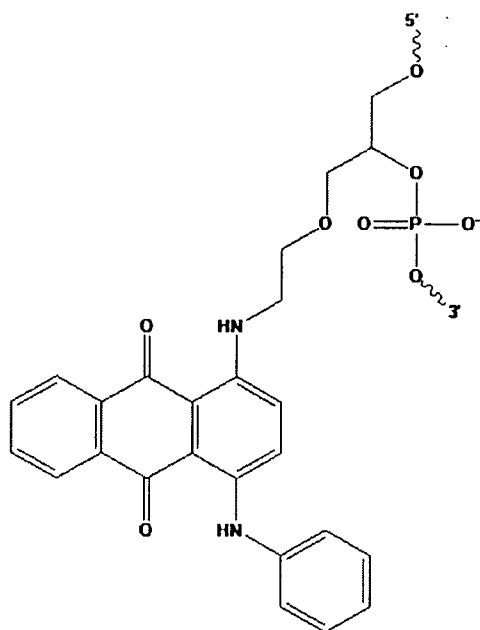
Formula 6

[0042]   The antisense oligonucleotides of the invention may be conjugated to other ligands, which may aid in the delivery of the antisense oligonucleotide to a cell or organism. In one embodiment of the invention, the ligand is 5' cholesterol monoethyleneglycol (/5CholMEG/):
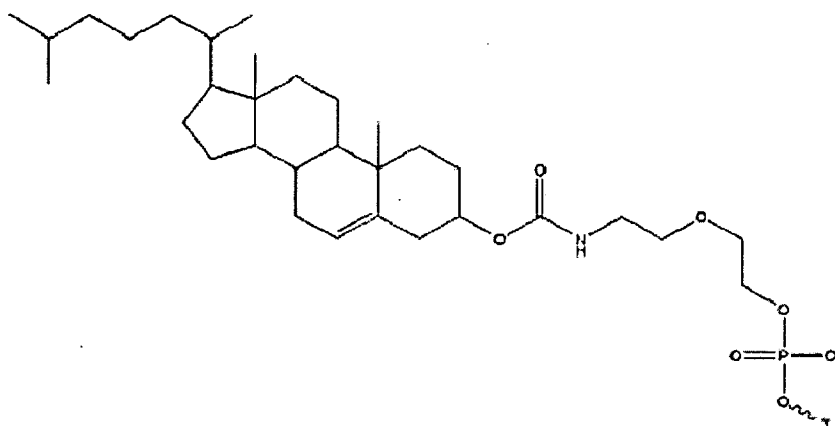
In another embodiment of the invention, the ligand is 5' cholesterol triethyleneglycol (/5Chol-TEG/):
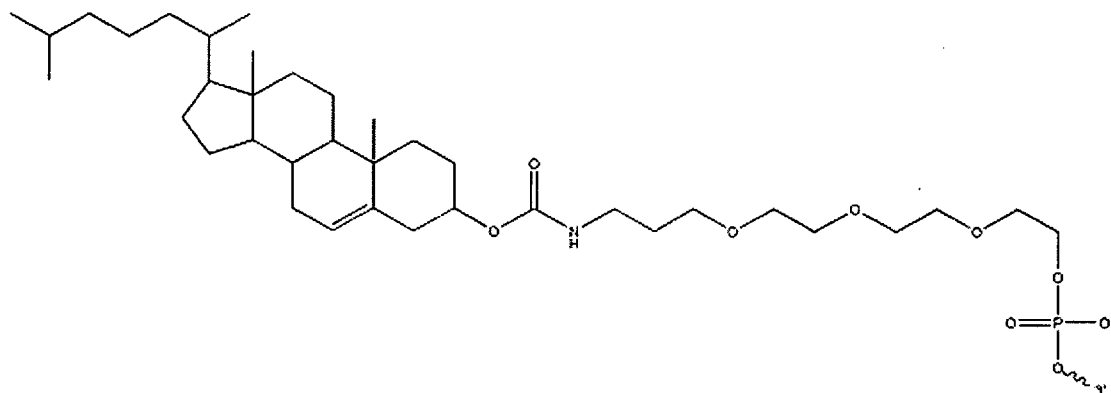
In a further embodiment of the invention, the ligand is 3' cholesterol monoethyleneglycol (/3CholMEG/):
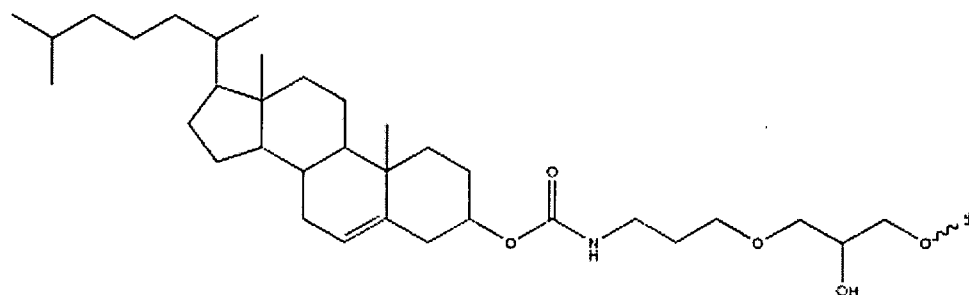
In another embodiment of the invention, the ligand is 3' cholesterol triethyleneglycol (/3CholTEG/):
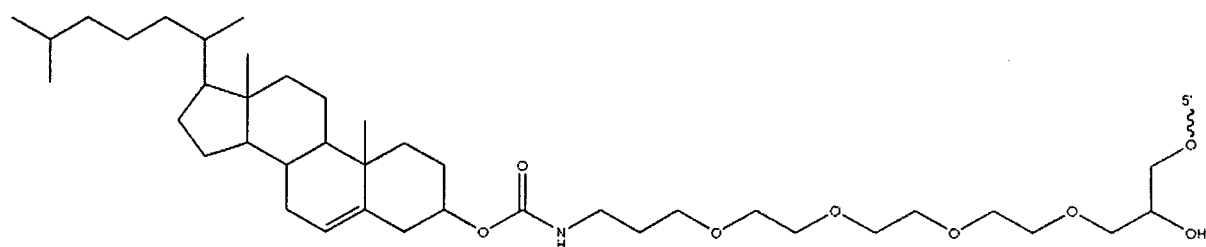
The ligand may be conjugated to the antisense oligonucleotide with or without an additional S18 (hexaethyleneglycol) spacer. In a preferred embodiment, the antisense oligonucleotide is an anti-miRNA oligonucleotide (AMO). In another preferred embodiment, the non-nucleotide modification is a FQ napthylene-azo compound (also referred to as iFQ or ZEN in this disclosure).

[0043]   The following examples further illustrate the invention but, of course, should not be construed as in any way limiting its scope.

EXAMPLE 1

[0044]   This example demonstrates the improved thermal stability of internal napthylene-azo-containing oligomers compared to other compounds.

[0045]   **Oligonucleotide synthesis and preparation.** DNA oligonucleotides were synthesized using solid phase phosphoramidite chemistry, deprotected and desalted on NAP-5 columns (Amersham Pharmacia Biotech, Piscataway, NJ) according to routine techniques (Caruthers *et al.*, 1992, *Methods Enzymol.* 211: 3-20). The oligomers were purified using reversed-phase high performance liquid chromatography (RP-HPLC). The purity of each

oligomer was determined by capillary electrophoresis (CE) carried out on a Beckman P/ACE MDQ system (Beckman Coulter, Inc., Fullerton, CA). All single-strand oligomers were at least 90% pure. Electrospray-ionization liquid chromatography mass spectrometry (ESI-LCMS) of the oligonucleotides was conducted using an Oligo HTCS system (Novatia, Princeton, NJ), which consisted of ThermoFinnigan TSQ7000, Xcalibur data system, ProMass data processing software, and Paradigm MS4™ HPLC (Michrom BioResources, Auburn, CA). Protocols recommended by the manufacturers were followed. Experimental molar masses for all single-strand oligomers were within 1.5 g/mol of expected molar mass. These results confirm identity of the oligomers.

[0046]   **Preparation of DNA samples**. Melting experiments were carried out in buffer containing 3.87 mM NaH_2PO_4, 6.13 mM Na_2HPO_4, 1 mM Na_2EDTA, and 1000 mM NaCl. 1 M NaOH was used to titrate each solution to pH 7.0. Total sodium concentrations were estimated to be 1020 mM. The DNA samples were thoroughly dialyzed against melting buffer in a 28-well Microdialysis System (Life Technologies, Carlsbad, CA) following the manufacturer's recommended protocol. Concentrations of DNA oligomers were estimated from the samples' UV absorbance at 260 nm in a spectrophotometer (Beckman Coulter, Inc., Fullerton, CA), using extinction coefficients for each oligonucleotide that were estimated using the nearest neighbor model for calculating extinction coefficients (*see* Warshaw *et al.*, 1966, *J. Mol. Biol.* 20(1): 29-38).

[0047]   **Internal modifications studied**. The FQ napthylene-azo compound (Formula 3, Integrated DNA Technologies, Inc., sometimes referred to as "iFQ" or "ZEN" in this disclosure), was introduced into oligonucleotides using phosphoramidite reagents at the time of synthesis.

Formula 3
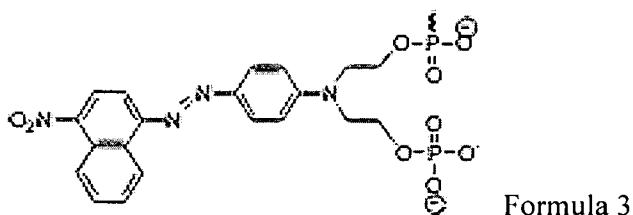
In the first series of duplexes, the iFQ group was placed as an insertion between bases in the duplex so that a 10-base top strand annealed to a 10-base bottom strand and the iFQ group was not aligned to a base. Additionally, 10-mer oligonucleotides with C3 spacer insertions were also

synthesized and studied. The C3 spacer represents the control wherein a linear insertion of a phosphate group plus propanediol is placed between bases, which is similar to the iFQ insertions without having the napthylene-azo ring structures present. Extinction coefficients at 260 nm of iFQ were estimated to be 13340; the C3 spacer does not contribute to UV absorbance.

[0048]   In a second series of duplexes, the iFQ group was placed as a substitution for a base in the duplex so that a 9-base top strand annealed to a 10-base bottom strand and the iFQ group was aligned to a base. Additionally, 10-mer oligonucleotides with C3 spacer substitutions were also synthesized and studied.

[0049]   **Measurement of melting curves.** Oligomer concentrations were measured at least twice for each sample. If the estimated concentrations for any sample differed more than 4%, the results were discarded and new absorbance measurements were performed. To prepare oligonucleotide duplexes, complementary DNA oligomers were mixed in 1:1 molar ratio, heated to 367 K (*i.e.*, 94°C) and slowly cooled to an ambient temperature. Each solution of duplex DNA was diluted with melting buffer to a total DNA concentration (C_T) of 2 μM.

[0050]   Melting experiments were conducted on a single beam Beckman DU 650 spectrophotometer (Beckman-Coulter) with a Micro T_m Analysis accessory, a Beckman High Performance Peltier Controller (to regulate the temperature), and 1 cm path-length cuvettes. Melt data were recorded using a PC interfaced to the spectrophotometer. UV-absorbance values at 268 nm wavelength were measured at 0.1 degree increments in the temperature range from 383 to 368 K (*i.e.*, 10 - 95°C). Both heating (*i.e.*, "denaturation") and cooling (*i.e.*, "renaturation") transition curves were recorded in each sample at a controlled rate of temperature change (24.9 ± 0.3°C per hour). Sample temperatures were collected from the internal probe located inside the Peltier holder, and recorded with each sample's UV-absorbance data. Melting profiles were also recorded for samples of buffer alone (no oligonucleotide), and these "blank" profiles were digitally subtracted from melting curves of the DNA samples. To minimize systematic errors, at least two melting curves were collected for each sample in different cuvettes and in different positions within the Peltier holder.

[0051]   **Determination of melting temperatures.** To determine each sample's melting temperature, the melting profiles were analyzed using methods that have been previously

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described (*see* Doktycz *et al.*, 1992, *Biopolymers* 32(7): 849-64; Owczarzy *et al.*, 1997, *Biopolymers* 44(3): 217-39; and Owczarzy, 2005, *Biophys. Chem.* 117(3): 207-15.). Briefly, the experimental data for each sample was smoothed, using a digital filter, to obtain a plot of the sample's UV-absorbance as a function of its temperature. The fraction of single-stranded oligonucleotide molecules, θ, was then calculated from that plot. The "melting temperature" or "T_m" of a sample was defined as the temperature where $\theta = 0.5$. Table 1 lists the melting temperatures of the oligonucleotides tested.

Table 1: Melting temperatures for nucleic acids containing
a single internal modifying group as an insertion

SEQ ID NO:	Sequence		T_m	ΔT_m	Avg ΔT_m
1	5' ATCGTTGCTA		43.9	-	
2	3' TAGCAACGAT				
3	5' ATC/GTTGCTA	iFQ "/"	48.0	4.1	
2	3' TAG CAACGAT				
4	5' ATCG/TTGCTA	iFQ "/"	48.6	4.7	+3.7
2	3' TAGC AACGAT				
5	5' ATCGT/TGCTA	iFQ "/"	46.3	2.4	
2	3' TAGCA ACGAT				
6	5' A/TCGTTGCTA	iFQ "/"	51.75	7.9	
2	3' T AGCAACGAT				+7.2
7	5' ATCGTTGCT/A	iFQ "/"	50.25	6.4	
2	3' TAGCAACGA T				
8	5' ATC/GTTGCTA	iSpC3 "/"	36.3	-7.6	
2	3' TAG CAACGAT				
9	5' ATCG/TTGCTA	iSpC3 "/"	36.6	-7.3	-8.7
2	3' TAGC AACGAT				
10	5' ATCGT/TGCTA	iSpC3 "/"	32.6	-11.3	
2	3' TAGCA ACGAT				

"/" signifies the site of insertion of a modifying group between bases as indicated.
iFQ = internal FQ azo quencher (ZEN)
iSpC3 = internal C3 spacer

[0052]   When the iFQ (ZEN) modifier was inserted centrally within a 10-mer oligonucleotide (between bases 3/4, 4/5, or 5/6), T_m was increased by an average of 3.7°C. When placed between terminal residues (between bases 1/2 or 9/10), T_m was increased by an average of 7.2°C. In contrast, insertion of a small propanediol group (C3 spacer) had a significant negative impact on the T_m of the duplex (average ΔT_m of -8.7°C).

[0053]   A subset of these sequences were studied using the internal modifications as base substitutions, such that now a 9-base top strand annealed to a 10-base bottom strand with the modification replacing a base and being aligned with a base on the opposing strand. Results are shown in Table 2. In this case, it is evident that the base substitution was significantly destabilizing whereas the insertions (Table 1) were stabilizing (ZEN) or were at least less destabilizing (C3).

Table 2: Melting temperatures for nucleic acids containing
a single internal modifying group comparing substitution vs. insertion

SEQ ID NO:	Duplex Sequence		Ins vs. Subs	T_m	ΔT_m
1 2	5'-ATCGTTGCTA-3' 3'-TAGCAACGAT-5'		-	43.9	0.0
3 2	5'-ATC/GTTGCTA-3' 3'-TAG CAACGAT-5'	iFQ "/"	I	48.0	4.1
8 2	5'-ATC/GTTGCTA-3' 3'-TAG CAACGAT-5'	iSpC3 "/"	I	36.3	-7.6
11 2	5'-ATC/TTGCTA-3' 3'-TAGCAACGAT-5'	iFQ "/"	S	34.7	-9.2
12 2	5'-ATC/TTGCTA-3' 3'-TAGCAACGAT-5'	iSpC3 "/"	S	< 20	
13 2	5'-ATCG/TTGCTA-3' 3'-TAGC AACGAT-5'	iFQ "/"	I	48.6	4.7
14 2	5'-ATCG/TTGCTA-3' 3'-TAGC AACGAT-5'	iSpC3 "/"	I	36.6	-7.3
15 2	5'-ATCG/TGCTA-3' 3'-TAGCAACGAT-5'	iFQ "/"	S	38.2	-5.7

16 2	5'-ATCG/TGCTA-3' iSpC3 "/" 3'-TAGCAACGAT-5'	S	<24	
17 2	5'-ATCGT/TGCTA-3' iFQ "/" 3'-TAGCA ACGAT-5'	I	46.3	2.4
18 2	5'-ATCGT/TGCTA-3' iSpC3 "/" 3'-TAGCA ACGAT-5'	I	32.6	-11.3
19 2	5'-ATCGT/GCTA-3' iFQ "/" 3'-TAGCAACGAT-5'	S	40.8	-3.1
20 2	5'-ATCGT/GCTA-3' iSpC3 "/" 3'-TAGCAACGAT-5'	S	< 26	

[0054] For this series of internal modifications, the average ΔT_m for iFQ (ZEN) insertion was +3.7°C while the average ΔT_m for iFQ (ZEN) substitution was -6°C. The average ΔT_m for iC3 spacer insertion was -8.7°C while the average ΔT_m for iC3 spacer substitution was more than -20°C (accurate measurements were not possible as the T_m was below room temperature). Therefore insertion placement is preferred to substitution placement.

[0055] The napthylene-azo modifier was introduced into the same 10-mer oligomer sequence at 2 or 3 sites, either adjacent to or separated by several bases. Duplexes were formed and T_m values were measured as before. Results are shown in Table 3. Some of the singly modified duplexes from Table 1 are also included in Table 3 to improve clarity of comparisons between modification patterns.

Table 3: Melting temperatures for nucleic acids containing
multiple internal modifying groups as insertions

SEQ ID NO:	Sequence	Tm	ΔTm
1 2	5' ATCGTTGCTA 3' TAGCAACGAT	43.87	-
3 2	5' ATC/GTTGCTA 1x iFQ "/" 3' TAG CAACGAT	48.02	4.15
21 2	5' ATC//GTTGCTA 2x iFQ "//" 3' TAG CAACGAT	39.62	-4.25

22 2	5' ATC/GTT/GCTA 2x iFQ "/.../" 3' TAG CAA CGAT	46.72	2.85
23 2	5' ATC/GT/TG/CTA 3x iFQ "/../../" 3' TAG CA AC GAT	43.36	-0.51
13 2	5' ATCG/TTGCTA 1x iFQ "/" 3' TAGC AACGAT	48.57	4.70
24 2	5' ATCG//TTGCTA 2x iFQ "//" 3' TAGC AACGAT	39.82	-4.05
17 2	5' ATCGT/TGCTA 1x iFQ "/" 3' TAGCA ACGAT	46.32	2.45
25 2	5' ATCGT//TGCTA 2x iFQ "/" 3' TAGCA ACGAT	36.76	-8.90
26 2	5' A/TCGTTGCTA 1x iFQ "/" 3' T AGCAACGAT	51.75	7.88
27 2	5' ATCGTTGCT/A 1x iFQ "/" 3' TAGCAACGA T	50.25	6.38
28 2	5' A/TCGTTGCT/A 2x iFQ "/" 3' T AGCAACGA T	54.91	11.04

"/" signifies the site of insertion of a modifying group between bases as indicated.

[0056]   Insertion of two adjacent napthylene-azo modifiers was destabilizing and T_m was found to change by -4 to -8.9°C depending on sequence context. Placing two napthylene-azo modifying groups in the same sequence separated by 3 bases was slightly stabilizing (T_m +2.9°C); however, this was less stabilizing than use of a single modifier alone (T_m +4.7°C). Use of 3 modifier groups separated by 2 bases between groups was destabilizing. However, when two napthylene-azo modifier groups were placed at the ends (between both bases 1/2 and 9/10), T_m was increased by 11°C. Thus, an additive effect can be obtained by placing multiple insertions of the modifying group into a sequence so long as a sufficient number of bases separate the groups. End effects are particularly potent.

[0057]   Therefore, internal incorporation of the napthylene-azo group within a DNA duplex stabilizes the duplex when placed as an insertion between bases. Certain anthraquinone groups can stabilize a duplex when placed on the ends (Patra *et al.*, 2009, *J. Am. Chem. Soc.* 131(35): 12671-81); however, this effect has not been described for internal placement. Therefore, the

use of napthylene-azo-class compounds would be preferred as an internal modifying group to increase duplex stability.

EXAMPLE 2

[0058]　　This example demonstrates the improved nuclease stability of internal napthylene-azo-containing oligomers compared to other compounds.

[0059]　　**Oligonucleotide synthesis and purification.** DNA oligonucleotides were synthesized using solid phase phosphoramidite chemistry, deprotected and desalted on NAP-5 columns (Amersham Pharmacia Biotech, Piscataway, NJ) according to routine techniques (Caruthers *et al.*, 1992). The oligomers were purified using reversed-phase high performance liquid chromatography (RP-HPLC). The purity of each oligomer was determined by capillary electrophoresis (CE) carried out on a Beckman P/ACE MDQ system (Beckman Coulter, Inc., Fullerton, CA). All single-strand oligomers were at least 90% pure. Electrospray-ionization liquid chromatography mass spectrometry (ESI-LCMS) of the oligonucleotides was conducted using an Oligo HTCS system (Novatia, Princeton, NJ), which consisted of ThermoFinnigan TSQ7000, Xcalibur data system, ProMass data processing software, and Paradigm MS4™ HPLC (Michrom BioResources, Auburn, CA). Protocols recommended by the manufacturers were followed. Experimental molar masses for all single-strand oligomers were within 1.5 g/mol of expected molar mass. These results confirm identity of the oligomers. The synthesized oligonucleotides are listed in Table 4.

Table 4: Synthetic oligomers employed in Example 2

SEQ ID NO:	Name	Sequence
1	DNA	5' ATCGTTGCTA 3'
26	5' iFQ	5' A(iFQ)TCGTTGCTA 3'
27	3' iFQ	5' ATCGTTGCT(iFQ)A 3'
28	5'+3' iFQ	5' A(iFQ)TCGTTGCT(iFQ)A 3'
29	5' iC3	5' A(iSpC3)TCGTTGCTA 3'
30	3' iC3	5' ATCGTTGCT(iSpC3)A 3'

31	5'+3' iC3	5' A(iSpC3)TCGTTGCT(iSpC3)A 3'

[0060]   **Radiolabeling of oligomers.** Single-stranded oligomers were radiolabeled at the 5'-end using polynucleotide kinase. Briefly, 5 pmoles of each oligonucleotide were incubated with 10 units of OptiKinase (USB, Cleveland, OH) and 10 pmoles of alpha ^{32}P γ-ATP (3000 Ci/mmol) (Perkin Elmer, Waltham, MA) for 30 minutes at 37°C, followed by 65°C for 10 minutes. Excess radionucleotide was removed by gel filtration using two sequential passes through MicroSpin G-25 columns (GE Healthcare, Buckinghamshire, UK). Isotope incorporation was measured in a Perkin Elmer TriCarb 2800 TR scintillation counter (Perkin Elmer, Waltham, MA).

[0061]   **Serum degradation of oligomers.** As labeling efficiencies varied (lower specific activity was obtained for the oligomers with a modification near the 5'-end), equivalent numbers of dpms of radiolabeled oligomers were mixed with unlabeled oligomers to a final concentration of 8 μM in the presence of 50% fetal bovine serum (not heat inactivated; Invitrogen, Carlsbad, CA). Samples were incubated at 37°C for 0, 30, 60, or 240 minutes; aliquots were removed at the indicated time points, an equal volume of 90% formamide was added, and samples flash frozen on dry ice. Degradation products were separated by PAGE using a 20% polyacrylamide, 7 M Urea denaturing gel and visualized on a Cyclone phosphorimager (Perkin Elmer, Waltham, MA). Results are shown in Figure 1.

[0062]   The unmodified DNA oligomer was rapidly degraded and no intact full-length material was present after 30 minutes incubation. The sample was fully degraded by 4 hours. A similar pattern of degradation was seen for the oligomer having a single internal C3 spacer positioned near the 5'-end. In contrast, only incomplete degradation was observed for the oligomer bearing a single internal FQ modifier near the 5'-end. The degradation pattern observed is most consistent with processive 3'-exonuclease cleavage that stopped before the oligomer was fully degraded. This suggests the possibility that the iFQ modifier protects neighboring DNA residues from exonuclease degradation, providing a small zone of protection around the 5'-end.

[0063]   The oligomer having a single internal C3 spacer near the 3'-end shows prompt removal of what appears to be a single base and then was slowly degraded. Slightly greater

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protection was seen in the oligomer having an internal C3 spacer placed near both ends. In contrast, no evidence was seen for single base cleavage at the 3'-end of the oligomer having a single internal FQ modifier near the 3'-end, and no evidence for degradation was observed after 4 hours incubation in 50% serum for the oligomer having an internal FQ modifier placed near both ends.

[0064]   Therefore, the FQ modifier will block exonuclease attack from the enzymes present in fetal bovine serum, and can confer relative nuclease resistance to neighboring unmodified bases, creating a protected "zone" in its vicinity.

EXAMPLE 3

[0065]   This example demonstrates improved functional activity of internal napthylene-azo-containing ASOs at reducing microRNA activity compared to other compounds.

[0066]   **Oligonucleotide synthesis and purification**. DNA, 2'OMe RNA, and LNA containing oligonucleotides were synthesized using solid phase phosphoramidite chemistry, deprotected and desalted on NAP-5 columns (Amersham Pharmacia Biotech, Piscataway, NJ) according to routine techniques (Caruthers *et al.*, 1992). The oligomers were purified using reversed-phase high performance liquid chromatography (RP-HPLC). The purity of each oligomer was determined by capillary electrophoresis (CE) carried out on a Beckman P/ACE MDQ system (Beckman Coulter, Inc., Fullerton, CA). All single-strand oligomers were at least 85% pure. Electrospray-ionization liquid chromatography mass spectrometry (ESI-LCMS) of the oligonucleotides was conducted using an Oligo HTCS system (Novatia, Princeton, NJ), which consisted of ThermoFinnigan TSQ7000, Xcalibur data system, ProMass data processing software, and Paradigm MS4™ HPLC (Michrom BioResources, Auburn, CA). Protocols recommended by the manufacturers were followed. Experimental molar masses for all single-strand oligomers were within 1.5 g/mol of expected molar mass. These results confirm identity of the oligomers. Table 5 lists the synthetic oligomers used in this Example.

Table 5: Synthetic oligomers employed in Example 3 (miR21 AMOs)

SEQ ID NO:	Name	Sequence

32	2'OMe	U C A A C A U C A G U C U G A U A A G C U A
33	2'OMe PSends	U*C*A*A C A U C A G U C U G A U A A G*C*U*A
34	2'OMe PS	U*C*A*A*C*A*U*C*A*G*U*C*U*G*A*U*A*A*G*C*U*A
35	2'OMe 5'+3'iFQ	U$_z$C A A C A U C A G U C U G A U A A G C U$_z$A
36	2'OMePS 5'+3'iFQ	U$_z$C*A*A*C*A*U*C*A*G*U*C*U*G*A*U*A*A*G*C*U$_z$A
37	2'OMe 3'iFQ	U C A A C A U C A G U C U G A U A A G C U$_z$A
38	2'OMe 5'iFQ	U$_z$C A A C A U C A G U C U G A U A A G C U A
39	2'OMe 5'+I+3'iFQ	U$_z$C A A C A U C A G U$_z$C U G A U A A G C U$_z$A
40	DNA/LNA PS	t*<u>C</u>*a*a*<u>C</u>*a*t*<u>C</u>*a*g*<u>T</u>*c*t*<u>G</u>*a*t*<u>A</u>*a*g*<u>C</u>*t*a
41	2'OMe/LNA PS	U*<u>C</u>*A*A*<u>C</u>*A*U*<u>C</u>*A*G*<u>T</u>*C*U*<u>G</u>*A*U*<u>A</u>*A*G*<u>C</u>*U*A

Uppercase = 2'OMe RNA
Lowercase = DNA
Uppercase with underscore = LNA
"*" = phosphorothioate linkage
"z" = napthylene-azo modifier (iFQ)

[0067]   **Plasmid preparation.** The psiCHECK™-2 vector (Promega, Madison, WI) was
restriction enzyme digested sequentially with Xho1 and Not1 (New England Biolabs, Ipswitch,
MA) and purified with a Qiaquick PCR purification column (Qiagen, Valencia, CA). A perfect
complement hsa-miR-21 binding site was created by annealing two synthetic duplexed
oligonucleotides (Integrated DNA Technologies, Coralville, IA) and was cloned into the
Xho1/Not1 sites in the 3'UTR of *Renilla* luciferase. This miR-21 reporter construct was
sequence verified on a 3130 Genetic Analyzer (AB, Foster City, CA). Plasmids were purified
using a Plasmid Midiprep Kit (Bio-Rad, Hercules, CA) and treated twice for endotoxin removal

with the MiraCLEAN Endotoxin Removal Kit (Mirus Corporation, Madison, WI). Plasmids were filtered through a 0.2 µ filter and quantified by measurement of the absorbance at 260 nm using UV spectrophotometry. This reporter plasmid having a perfect match miRNA binding site is denoted as psiCHECK™-2-miR21.

[0068]   **Cell culture, transfections, and luciferase assays.** HeLa cells were plated in a 100 mm dish in DMEM containing 10% FBS to achieve 90% confluency the next day. The following morning, 5 µg of the psiCHECK™-2-miR21 plasmid was transfected with Lipofectamine™ 2000 (Invitrogen, Carlsbad, CA). After 6 hours, cells were washed with 1X PBS, trypsinized, counted, and replated in DMEM with 10% FBS in 48-well plates to achieve ~70% confluency the next day. The following morning, the miR-21 AMOs were transfected at various concentrations in triplicate with 1 µl TriFECTin® (Integrated DNA Technologies) per well in DMEM without serum. After 6 hours, the transfection media was removed and replenished with DMEM containing 10% FBS. The following morning, (48 hours after plasmid transfection, 24 hours after miRNA AMO transfection) the cells were analyzed for luciferase luminescence using the Dual-Luciferase® Reporter Assay System (Promega, Madison, WI) per the manufacturer's instructions. *Renilla* luciferase was measured as a fold increase in expression compared to the TriFECTin® reagent-only negative controls. Values for *Renilla* luciferase luminescence were normalized to levels concurrently measured for firefly luciferase, which is present as a separate expression unit on the same plasmids as an internal control (RLuc/FLuc ratio).

[0069]   **Results.** The RLuc/FLuc ratios obtained from transfections done with the various AMOs are shown in Figure 2. In the untreated state, HeLa cells contain large amounts of miRNA 21 that suppress expression of the RLuc reporter. Any treatment that decreases miR-21 levels leads to an increase in RLuc expression and thus increases the relative RLuc/FLuc ratio (with FLuc serving as an internal normalization control for transfection efficiency).

[0070]   The unmodified 2'OMe RNA AMO showed essentially no inhibition of miR-21 activity, probably due to rapid nuclease degradation of this unprotected oligomer during transfection or in the intracellular environment. The addition of 3 PS linkages on each end of the AMO blocks exonuclease attack and the "2'OMe-PSends" AMO showed good potency for functional knockdown of miR-21. When this AMO is changed to be fully PS modified ("2'OMe-

PS"), potency drops, which is probably due to having lower binding affinity (lower T_m) that accompanies extensive PS modification. Each substitution of a PS bond for a standard phosphodiester bond reduces T_m, and there are 21 PS bonds in this oligomer compared with only 6 PS bonds in the "2'OMe PSends" version.

[0071] A desirable modification chemistry or modification pattern is one that both increases nuclease stability and increases T_m. The internal napthylene-azo modifier meets these criteria. The 2'OMe oligomer having an internal napthylene-azo modifier placed between the terminal and adjacent bases on each end (2'OMe 5'+3'iFQ) showed markedly improved anti-miR21 activity and was more potent than any of the PS modified 2'OMe AMOs tested. Adding PS modification to this design (2'OMePS 5'+3'iFQ) reduced potency, likely due to the lower binding affinity caused by the addition of 19 PS linkages. This compound was nevertheless still significantly more potent than the 2'OMe-PS version without the 2 iFQ modifications.

[0072] Protecting only one end of the anti-miR-21 AMO with an internal napthylene-azo modifier showed improved potency when compared with the unmodified 2'OMe AMO; however, the performance was much reduced compared with the dual-modified version. Interestingly, modification at the 5'-terminal linkage had more effect than modification at the 3'-terminal linkage, the exact opposite of the results anticipated from the relative serum stability profiles demonstrated in Example 2. This result is explained by measured effects of T_m (*see* Table 6).

[0073] Addition of a third iFQ modification into the end-blocked version (2'OMe 5'+I+3'iFQ) showed reduced potency compared with the original end-blocked version (2'OMe 5'+3'iFQ), which is likely due to a reduction of T_m seen with placing this many iFQ modifying groups in a single, short 22-mer sequence.

[0074] The "DNA/LNA-PS" AMO is a design employed by Exiqon as its preferred anti-miRNA agent and is widely accepted as the "gold standard" for miRNA knockdown studies performed today. The DNA/LNA compound showed the same potency as the dual-modified "2'OMe 5'+3'iFQ" AMO. The "2'OMe/LNA-PS" AMO showed highest potency within the set studied. The LNA modification confers nuclease resistance and gives very large increases in T_m, resulting in AMOs with higher potency but also having lower specificity than AMOs without LNA bases with lower binding affinity. The relative specificity of the different AMOs is

presented in Example 4 below. Of note, the LNA-PS modified AMOs show some toxicity and cell cultures transfected with the highest doses (50 nM) had dysmorphic, unhealthy appearing cells at the time of harvest. The "2'OMe 5'+3'iFQ" AMO did not show any visual evidence for toxicity at any of the doses tested. In subsequent experimentation, toxicity effects were evaluated at high doses by measuring cell viability, cytotoxicity, and induction of apoptosis (*see* Example 7). The "2'OMe 5'+3'iFQ" chemistry showed no cellular toxicity, compared to the substantial cellular toxicity that occurred upon transfection of single-stranded oligonucleotides containing LNA bases, extensive PS modification (all 21 linkages), or both LNA and PS modifications (the "gold standard" AMO). Thus, the "2'OMe 5'+3'iFQ" may be a new class of AMO that achieves high potency yet maintains low toxicity.

[0075]   The melting temperatures, T_m, of the AMOs described above were measured using the same methods described in Example 1. Synthetic AMO oligonucleotides were annealed to a synthetic RNA complement (mature miR21 sequence). Measurements were done at 2 μM duplex concentration in 150 mM NaCl to approximate intracellular ion concentration.

Table 6: T_m of synthetic miR21 AMOs in 150 mM NaCl

SEQ ID NO:	Name	Sequence	T_m	ΔT_m
32	2'OMe	U C A A C A U C A G U C U G A U A A G C U A	72.1	--
33	2'OMe PSends	U*C*A*A C A U C A G U C U G A U A A G*C*U*A	70.9	-1.2
34	2'OMe PS	U*C*A*A*C*A*U*C*A*G*U*C*U*G*A*U*A*A*G*C*U*A	67.1	-5.0
35	2'OMe 5'+3'iFQ	U$_z$C A A C A U C A G U C U G A U A A G C U$_z$A	75.4	+3.3
36	2'OMePS 5'+3'iFQ	U$_z$C*A*A*C*A*U*C*A*G*U*C*U*G*A*U*A*A*G*C*U$_z$A	70.6	-1.5
37	2'OMe 3'iFQ	U C A A C A U C A G U C U G A U A A G C U$_z$A	72.4	+0.3

38	2'OMe 5'iFQ	U_zC A A C A U C A G U C Ů G A U A A G C U A	74.3	+2.2
39	2'OMe 5'+I+3'iFQ	U_zC A A C A U C A G U_zC U G A U A A G C U_zA	71.3	-0.8
40	DNA/ LNA PS	t*C̲*a*a*C̲*a*t*C̲*a*g*T̲*c*t*G̲*a*t*A̲*a*g*C̲*t*a	74.0	+1.9
41	2'OMe/ LNA PS	U*C̲*A*A*C̲*A*U*C̲*A*G*T̲*C*U*G̲*A*U*A̲*A*G*C̲*U*A	85.9	+13.8

Uppercase = 2'OMe RNA
Lowercase = DNA
Uppercase with underscore = LNA
"*" = phosphorothioate linkage
"z" = insertion of napthylene-azo modifier (iFQ)

[0076]   The 22-mer 2'OMe miR21 AMO showed a T_m of 72.1°C when hybridized to an RNA perfect complement in 150 mM NaCl. Substitution of 6 PS bonds for native PO linkages lowered T_m by 1.2°C ("2'OMe PSends") and complete PS modified lowered T_m by 5.0°C ("2'OMe PS"), a change of -0.20 to -0.25°C per modified internucleotide linkage. In contrast, insertion of an iFQ group at the 3'-terminal linkage ("2'OMe 3'iFQ") resulted in a T_m increase of +0.3°C and at the 5'-terminal linkage ("2'OMe 5'iFQ") resulted in a T_m increase of +2.2°C. Combining these two designs, addition of two iFQ modifications (one at each terminal linkage, "2'OMe 5'+3'iFQ") increased T_m to 75.4°C, which is a change of +3.3°C compared with the unmodified sequence or +4.5°C relative to the PS-end blocked sequence (which is the most relevant comparison). This dual-end-modification pattern results in good nuclease resistance (Figure 1) and when employed in a 2'OMe AMO shows increased T_m (Table 6) and is a very potent anti-miR21 agent (Figure 2). Interestingly, addition of a third iFQ group centrally placed ("2'OMe 5'+I+3' iFQ") resulted in a T_m decrease of 0.8°C relative to the unmodified compound, or a decrease of 4.1°C relative to the two-end insertion version ("2'OMe 5'+3'iFQ"). Thus while inserting the iFQ modifier between terminal bases increases T_m, adding a third modification in the center of the sequence leads to a decrease in T_m, even though these modifications are fully 10 bases distant from each other. This loss of T_m results in a loss of functional potency (Figure 2). Therefore the dual-modified end-insertion pattern is preferred.

[0077]   As a general rule, the relative potency of the various miR21 AMOs correlated with increased binding affinity (T_m). All variations in potency observed between compounds could be explained by relative contributions of improvements in binding affinity and nuclease stability between the different modification patterns studied. The AMO having 2'OMe bases with an iFQ modification placed near each end ("2'OMe 5'+3'iFQ") provided an excellent balance of nuclease stability with increased T_m and the only AMO showing higher potency was the "2'OMe/LNA-PS" compound. The "2'OMe/LNA-PS" compound, however, showed reduced specificity due to its extreme elevation in binding affinity (*see* Example 4) and increased cellular toxicity (*see* Example 7). Therefore, the novel "2'OMe 5'+3'iFQ" design of the present invention is superior.

EXAMPLE 4

[0078]   This example demonstrates improved specificity of internal napthylene-azo-containing oligomers when reducing microRNA activity compared to other compounds containing modifications that increase binding affinity.

[0079]   Three of the more potent AMO designs from the functional study performed in Example 3 were examined in greater detail to assess their relative ability to discriminate mismatches between the synthetic anti-miRNA oligonucleotide and their target. In general, high affinity oligonucleotides show high potency but usually show reduced specificity as the high affinity permits hybridization even in the presence of one or more mismatches in complementarity. The designs "2'OMe 5'+3'iFQ", "DNA/LNA-PS", and "2'OMe/LNA-PS" were synthesized as variants having 1, 2, or 3 mismatches to the miR-21 target sequence. Sequences are shown in Table 7. Studies were performed as described in Example 3.

Table 7

SEQ ID NO:	Name	Sequence
35	2'OMe 5'+3'iFQ	U_zC A A C A U C A G U C U G A U A A G C U_zA
42	2'OMe 5'+3'iFQ 1MUT	U_zC A A C A U C A G U C U *C* A U A A G C U_zA

43	2'OMe 5'+3'iFQ 2MUT	U_zC A A *G* A U C A G U C U *C* A U A A G C U_zA
44	2'OMe 5'+3'iFQ 3MUT	U_zC A A *G* A U C A G U C U *C* A U A A G *G* U_zA
40	DNA/ LNA PS	t*<u>C</u>*a*a*<u>C</u>*a*t*<u>C</u>*a*g*<u>T</u>*c*t*<u>G</u>*a*t*<u>A</u>*a*g*<u>C</u>*t*a
45	DNA/ LNA PS 1MUT	t*<u>C</u>*a*a*<u>C</u>*a*t*<u>C</u>*a*g*<u>T</u>*c*t*<u>***C***</u>*a*t*<u>A</u>*a*g*<u>C</u>*t*a
46	DNA/ LNA PS 2MUT	t*<u>C</u>*a*a*<u>***G***</u>*a*t*<u>C</u>*a*g*<u>T</u>*c*t*<u>***C***</u>*a*t*<u>A</u>*a*g*<u>C</u>*t*a
47	DNA/ LNA PS 3MUT	t*<u>C</u>*a*a*<u>***G***</u>*a*t*<u>C</u>*a*g*<u>T</u>*c*t*<u>***C***</u>*a*t*<u>A</u>*a*g*<u>***G***</u>*t*a
41	2'OMe/ LNA PS	U*<u>C</u>*A*A*<u>C</u>*A*U*<u>C</u>*A*G*<u>T</u>*C*U*<u>G</u>*A*U*<u>A</u>*A*G*<u>C</u>*U*A
48	2'OMe/ LNA PS 1MUT	U*<u>C</u>*A*A*<u>C</u>*A*U*<u>C</u>*A*G*<u>T</u>*C*U*<u>***C***</u>*A*U*<u>A</u>*A*G*<u>C</u>*U*A
49	2'OMe/ LNA PS 2MUT	U*<u>C</u>*A*A*<u>***G***</u>*A*U*<u>C</u>*A*G*<u>T</u>*C*U*<u>***C***</u>*A*U*<u>A</u>*A*G*<u>C</u>*U*A
50	2'OMe/ LNA PS 3MUT	U*<u>C</u>*A*A*<u>***G***</u>*A*U*<u>C</u>*A*G*<u>T</u>*C*U*<u>***C***</u>*A*U*<u>A</u>*A*G*<u>***G***</u>*U*A

Uppercase = 2'OMe RNA
Lowercase = DNA
Uppercase with underscore = LNA
"*" = phosphorothioate linkage
"z" = napthylene-azo modifier (iFQ)
Mutations are identified with bold italic font

[0080]   **Results.** The RLuc/FLuc ratios obtained from transfections done with the various
AMOs are shown in Figure 3. In the untreated state, HeLa cells contain large amounts of
miRNA 21 that suppress expression of the RLuc reporter. Any treatment that decreases miR-21

levels leads to an increase in RLuc expression and thus increases the relative RLuc/FLuc ratio (with FLuc serving as an internal normalization control for transfection efficiency). For each of the chemistries studied, the parent wild-type sequence is followed by variants having 1, 2, or 3 mutations.

[0081]   In all cases, the perfect match AMO showed significant suppression of miR-21 activity as evidenced by increases in luciferase levels (increase in the RLuc to FLuc ratio indicating de-repression of the RLuc mRNA). As in Example 3 (Figure 3), the "2'OMe/LNA-PS" compound showed the highest potency as evidenced by suppression of miR-21 at low dose (1 nM and 5 nM data points). The "2'OMe 5'+3'iFQ" and "DNA/LNA-PS" AMOs showed relatively similar performance both in wild-type (perfect match) and mutant (mismatch) versions. In both cases, a single mismatch showed a partial reduction of anti-miR-21 activity, the double mismatch showed almost complete elimination of anti-miR-21 activity, and the triple mismatch did not show any anti-miR-21 activity. In contrast, the higher affinity "2'OMe/LNA-PS" compound showed significant anti-miR-21 activity for both the single and double mismatch compounds and even showed some activity at high dose (50 nM) for the triple mismatch compound. Thus, while the "2'OMe/LNA-PS" compound is most potent, it is also the least specific of the reagents studied.

[0082]   Of note, the above experiments were performed using AMOs that placed the mismatches at positions that are LNA modified (in the LNA containing AMOs). This design may influence the likelihood that a mismatch will affect activity as it disrupts a high affinity LNA:RNA base pair. Thus, these results represent the best case scenario for specificity of the LNA-modified AMOs. The experiment was repeated using a new set of reagents where the mismatches were all positioned at non-LNA bases. This new series of AMO reagents is shown in Table 8.

Table 8: Synthetic oligomers employed in Example 4 (miR21 AMOs)

SEQ ID NO:	Name	Sequence
35	2'OMe 5'+3'iFQ	U_zC A A C A U C A G U C U G A U A A G C U_zA

51	2'OMe 5'+3'iFQ 1MUT v2	U_zC A A C A U C A G U C **A** G A U A A G C U_zA
52	2'OMe 5'+3'iFQ 2MUT v2	U_zC A A C **C** U C A G U C **A** G A U A A G C U_zA
53	2'OMe 5'+3'iFQ 3MUT v2	U_zC A A C **C** U C A G U C **A** G A U A A **C** C U_zA
40	DNA/ LNA PS	t*<u>C</u>*a*a*<u>C</u>*a*t*<u>C</u>*a*g*<u>T</u>*c*t*<u>G</u>*a*t*<u>A</u>*a*g*<u>C</u>*t*a
54	DNA/ LNA PS 1MUT v2	t*<u>C</u>*a*a*<u>C</u>*a*t*<u>C</u>*a*g*<u>T</u>*c***a***<u>G</u>*a*t*<u>A</u>*a*g*<u>C</u>*t*a
55	DNA/ LNA PS 2MUT v2	t*<u>C</u>*a*a*<u>C</u>***c***t*<u>C</u>*a*g*<u>T</u>*c***a***<u>G</u>*a*t*<u>A</u>*a*g*<u>C</u>*t*a
56	DNA/ LNA PS 3MUT v2	t*<u>C</u>*a*a*<u>C</u>***c***t*<u>C</u>*a*g*<u>T</u>*c***a***<u>G</u>*a*t*<u>A</u>*a***c***<u>C</u>*t*a
41	2'OMe/ LNA PS	U*<u>C</u>*A*A*<u>C</u>*A*U*<u>C</u>*A*G*<u>T</u>*C*U*<u>G</u>*A*U*<u>A</u>*A*G*<u>C</u>*U*A
57	2'OMe/ LNA PS 1MUT v2	U*<u>C</u>*A*A*<u>C</u>*A*U*<u>C</u>*A*G*<u>T</u>*C***A***<u>G</u>*A*U*<u>A</u>*A*G*<u>C</u>*U*A
58	2'OMe/ LNA PS 2MUT v2	U*<u>C</u>*A*A*<u>C</u>***C***U*<u>C</u>*A*G*<u>T</u>*C***A***<u>G</u>*A*U*<u>A</u>*A*G*<u>C</u>*U*A
59	2'OMe/ LNA PS 3MUT v2	U*<u>C</u>*A*A*<u>C</u>***C***U*<u>C</u>*A*G*<u>T</u>*C***A***<u>G</u>*A*U*<u>A</u>*A***C***<u>C</u>*U*A

Uppercase = 2'OMe RNA
Lowercase = DNA
Uppercase with underscore = LNA
"*" = phosphorothioate linkage
"z" = napthylene-azo modifier (iFQ)
Mutations are identified with bold italic font

[0083]   **Results.** The RLuc/FLuc ratios obtained from transfections done with the various

AMOs are shown in Figure 4. In the untreated state, HeLa cells contain large amounts of

miRNA 21 that suppress expression of the RLuc reporter. Any treatment that decreases miR-21

levels leads to an increase in RLuc expression and thus increases the relative RLuc/FLuc ratio

(with FLuc serving as an internal normalization control for transfection efficiency). For each of

the chemistries studied, the parent wild-type sequence is followed by variants having 1, 2, or 3

mutations.

[0084]   The results were nearly identical to those obtained with the original mutation

mismatch placement (Figure 3). In all cases, the perfect match AMO showed significant

suppression of miR-21 activity as evidenced by increases in luciferase levels (increase in the

RLuc to FLuc ratio indicating de-repression of the RLuc mRNA). As in Example 3 (Figure 3),

the "2'OMe/LNA-PS" compound showed the highest potency as evidenced by suppression of

miR-21 at low dose (1 nM and 5 nM data points). The "2'OMe 5'+3'iFQ" and "DNA/LNA-PS"

AMOs showed relatively similar performance both in wild-type (perfect match) and mutant

(mismatch) versions. In both cases, a single mismatch showed a partial reduction of anti-miR-21

activity, the double mismatch showed almost complete elimination of anti-miR-21 activity, and

the triple mismatch did not show any anti-miR-21 activity. In contrast, the higher affinity

"2'OMe/LNA-PS" compound showed significant anti-miR-21 activity for both the single and

double mismatch compounds and even showed some activity at high dose (50 nM) for the triple

mismatch compound. Thus, while the "2'OMe/LNA-PS" compound is most potent, it is also the

least specific of the reagents studied.

EXAMPLE 5

[0085]   This example demonstrates improved functional activity of internal napthylene-azo-

containing oligomers at reducing cellular mRNA levels when incorporated into RNase H active

ASOs as compared to other related compounds.

[0086]   Oligonucleotides antisense in orientation to cellular messenger RNAs (mRNAs) will

hybridize to the mRNA and form an RNA/DNA heteroduplex, which is a substrate for cellular

RNase H. Degradation by RNase H leads to a cut site in the mRNA and subsequently to total

degradation of that RNA species, thereby functionally lowering effective expression of the

targeted transcript and the protein it encodes. ASOs of this type require a domain containing at least 4 bases of DNA to be a substrate for RNase H, and maximal activity is not seen until 8-10 DNA bases are present. ASOs must be chemically modified to resist degradation by serum and cellular nucleases. Phosphorothioate (PS) modification of the internucleotide linkages is compatible with RNase H activation, however most other nuclease resistant modifications prevent RNase H activity, including all 2'-modifications, such as 2'OMe RNA, LNA, MOE, etc. The PS modification lowers binding affinity (T_m). In general, modifications that lower T_m decrease potency while modifications that increase T_m improve potency. One strategy to improve potency of ASOs is to employ a chimeric design where a low T_m, RNase H activating domain made of PS-modified DNA is flanked by end domains that contain 2'-modified sugars which confer high binding affinity but are not RNase H activating ("gapmer" design). One commonly employed strategy is to place five 2'-modified bases at the 5'-end, ten PS-modified DNA bases in the middle, and five 2'-modified bases at the 3'-end of the ASO (so called "5-10-5" design). A modification that confers nuclease resistance, increases binding affinity, and does not impair the reagent's ability to activate RNase H would be ideal. The present example demonstrates the utility of the internal napthylene-azo modifier to improve the nuclease stability and increase binding affinity of ASOs, enhancing their function as gene knockdown reagents.

[0087]   **Oligonucleotide synthesis and purification.** DNA, 2'OMe RNA, and LNA containing oligonucleotides were synthesized using solid phase phosphoramidite chemistry, deprotected and desalted on NAP-5 columns (Amersham Pharmacia Biotech, Piscataway, NJ) according to routine techniques (Caruthers *et al.*, 1992). The oligomers were purified using reversed-phase high performance liquid chromatography (RP-HPLC). The purity of each oligomer was determined by capillary electrophoresis (CE) carried out on a Beckman P/ACE MDQ system (Beckman Coulter, Inc., Fullerton, CA). All single-strand oligomers were at least 85% pure. Electrospray-ionization liquid chromatography mass spectrometry (ESI-LCMS) of the oligonucleotides was conducted using an Oligo HTCS system (Novatia, Princeton, NJ), which consisted of ThermoFinnigan TSQ7000, Xcalibur data system, ProMass data processing software, and Paradigm MS4™ HPLC (Michrom BioResources, Auburn, CA). Protocols recommended by the manufacturers were followed. Experimental molar masses for all single-

strand oligomers were within 1.5 g/mol of expected molar mass. These results confirm identity of the oligomers.

Table 9: Synthetic oligomers employed in Example 5 (anti-HPRT ASOs)

SEQ ID NO:	Name	Sequence
60	HPRT#1 DNA	a t a g g a c t c c a g a t g t t t c c
61	HPRT#1 DNA 5'iFQ	a_zt a g g a c t c c a g a t g t t t c c
62	HPRT#1 DNA 3'iFQ	a t a g g a c t c c a g a t g t t t c_zc
63	HPRT#1 DNA 5'+3'iFQ	a_zt a g g a c t c c a g a t g t t t c_zc
64	HPRT#1 DNA 5'+i+3'iFQ	a_zt a g g a c t c c_za g a t g t t t c_zc
65	HPRT#1 DNA PS	a*t*a*g*g*a*c*t*c*c*a*g*a*t*g*t*t*t*c*c
66	HPRT#1 DNA PS 5'iFQ	a_zt*a*g*g*a*c*t*c*c*a*g*a*t*g*t*t*t*c*c
67	HPRT#1 DNA PS 3'iFQ	a*t*a*g*g*a*c*t*c*c*a*g*a*t*g*t*t*t*c_zc
68	HPRT#1 DNA PS 5'+3'iFQ	a_zt*a*g*g*a*c*t*c*c*a*g*a*t*g*t*t*t*c_zc
69	HPRT#1 DNA PS 5'+I+3'iFQ	a_zt*a*g*g*a*c*t*c*c*c_za*g*a*t*g*t*t*t*c_zc
70	HPRT#1 5-10-5	A U A G G a c t c c a g a t g U U U C C
71	HPRT#1 5-10-5 2x iFQ	A_zU A G G a c t c c a g a t g U U U C_zC

72	HPRT#1 5-10-5 3x iFQ	A$_z$U A G G a c t c c$_z$a g a t g U U U C$_z$C
73	HPRT#1 5-10-5 PS	A*U*A*G*G*a*c*t*c*c*a*g*a*t*g*U*U*U*C*C
74	HPRT#1 5-10-5 PS 2x iFQ	A$_z$U*A*G*G*a*c*t*c*c*a*g*a*t*g*U*U*U*C$_z$C
75	HPRT#1 5-10-5 gapPS	A U A G G a*c*t*c*c*a*g*a*t*g*U U U C C
76	HPRT#1 5-10-5 gapPS 2x iFQ	A$_z$U A G G a*c*t*c*c*a*g*a*t*g*U U U C$_z$C
77	HPRT#1 5-10-5 gapPS 3x iFQ	A$_z$U A G G a*c*t*c*c$_z$a*g*a*t*g*U U U C$_z$C
78	HPRT#1 5-10-5 LNA PS	A*U*A*G*G*a*c*t*c*c*a*g*a*t*g*U*U*U*C*C

Uppercase = 2'OMe RNA
Lowercase = DNA
Uppercase with underscore = LNA
"*" = phosphorothioate linkage
"z" = insertion of napthylene-azo modifier (iFQ)

[0088]   **HeLa cell culture, transfections, and RT-qPCR methods.** HeLa cells were split into 48-well plates and were transfected the next day at ~60% confluency in serum-free Dulbecco's Modified Eagle Medium (Invitrogen, Carlsbad, CA) using TriFECTin® (Integrated DNA Technologies, Coralville, IA) at a concentration of 2% (1 µL per 50 µL OptiMEM® I) (Invitrogen, Carlsbad, CA) with ASOs at the indicated concentrations. All transfections were performed in triplicate. After 6 hours, media was exchanged with Dulbecco's Modified Eagle Medium containing 10% fetal bovine serum. RNA was prepared 24 hours after transfection using the SV96 Total RNA Isolation Kit (Promega, Madison, WI). cDNA was synthesized using 150 ng total RNA with SuperScript™-II Reverse Transcriptase (Invitrogen, Carlsbad, CA) per the manufacturer's instructions using both random hexamer and oligo-dT priming. Transfection experiments were all performed a minimum of three times.

[0089]   Quantitative real-time PCR was performed using 10 ng cDNA per 10 μL reaction

with Immolase™ DNA Polymerase (Bioline, Randolph, MA), 200 nM primers, and 200 nM

probe. Hypoxanthine phosphoribosyltransferase 1 (HPRT1) (GenBank Acc. No. NM_000194)

specific primers were:

HPRT-For 5' GACTTTGCTTTCCTTGGTCAGGCA (SEQ ID NO: 79),

HPRT-Rev 5' GGCTTATATCCAACACTTCGTGGG (SEQ ID NO: 80), and probe

HPRT-P 5' MAX- ATGGTCAAGGTCGCAAGCTTGCTGGT-IowaBlackFQ (IBFQ) (SEQ ID

NO: 81)

and were normalized to levels of an internal control gene, human acidic ribosomal

phosphoprotein P0 (RPLP0) (GenBank Acc. No. NM_001002), which was measured in a

multiplexed reaction using primers:

RPLP0-For 5' GGCGACCTGGAAGTCCAACT (SEQ ID NO: 82),

RPLP0-Rev 5' CCATCAGCACCACAGCCTTC (SEQ ID NO: 83), and probe

RPLP0-P 5' FAM-ATCTGCTGCATCTGCTTGGAGCCCA-IBFQ (SEQ ID NO: 84)

(Bieche *et al.*, 2000, *Clin. Cancer Res.* 6(2): 452-59). Cycling conditions employed were: 95°C

for 10 minutes followed by 40 cycles of 2-step PCR with 95°C for 15 seconds and 60°C for 1

minute. PCR and fluorescence measurements were done using an ABI Prism™ 7900 Sequence

Detector (Applied Biosystems Inc., Foster City, CA). All reactions were performed in triplicate.

Expression data were normalized. Copy number standards were multiplexed using linearized

cloned amplicons for both the HPRT and RPLP0 assays. Unknowns were extrapolated against

standards to establish absolute quantitative measurements.

[0090]   **Results.** ASOs were transfected into HeLa cells at 1 nM, 5 nM, and 20 nM

concentrations. RNA was prepared 24 hours post transfection, converted to cDNA, and HPRT

expression levels were measured using qPCR. Results are shown in Figure 5 for the set of anti-

HPRT ASOs made from DNA bases. Unmodified single-stranded DNA oligos are rapidly

degraded by exonucleases and endonucleases. No knockdown of HPRT was observed using this

design (HPRT DNA), presumably due to rapid degradation of the unprotected compound. ASOs

with a single iFQ modification near the 3'-end (HPRT DNA 3'iFQ), a single iFQ modification

near the 5'-end (HPRT DNA 5'iFQ), two iFQ modifications inserted near both ends (HPRT DNA

5'+3'iFQ), and three iFQ modifications inserted in the center and near both ends (HPRT DNA

5'+I+3'iFQ) were also tested and similarly showed no functional gene knockdown at the doses examined. Therefore, the addition of even up to 3 iFQ modifications does not provide sufficient nuclease stabilization to permit otherwise unmodified DNA oligos to function as antisense gene-knockdown agents.

[0091]   The same series of oligonucleotides was synthesized having phosphorothioate (PS) internucleotide bonds throughout the sequence (except where the phosphate connects to an iFQ modifier). Historically, DNA-PS oligos were among the first effective antisense compounds studied. This modification increases nuclease stability; however, it also lowers binding affinity (T_m) and as a result this so-called "first generation" antisense chemistry usually shows relatively low potency. The "DNA-PS" ASO reduced HPRT levels by 50% at 20 nM concentration; however, no reduction in HPRT levels was observed at lower doses. Addition of the iFQ modification, which increases binding affinity and blocks exonuclease action, improved function of the DNA-PS ASOs. The "DNA-PS 5'+I+3'iFQ" compound showed the best results within this series, with HPRT knockdown of 70% at 20 nM and 40% at 5 nM observed (Figure 5).

[0092]   A second set of ASOs was synthesized using a chimeric "5-10-5 gapmer" design where five base end domains were made of 2'OMe RNA and a central ten base RNase H active domain were made of DNA. Oligonucleotides had zero, one, two, or three iFQ modifiers inserted at the same positions as the DNA ASOs in Figure 5. These oligonucleotides were transfected into HeLa cells as before and HPRT mRNA levels were examined 24 hours post-transfection. Results are shown in Figure 6. The three gapmer ASOs with a phosphodiester DNA central domain showed no activity in reducing HPRT mRNA levels, regardless of whether the sequence was modified with the iFQ group or not ("5-10-5", 5-10-5 2x iFQ" and "5-10-5 3x iFQ"). The same sequence fully PS modified ("5-10-5-PS") showed good potency with 75% knockdown of HPRT at 20 nM concentration. The addition of two iFQ groups near the ends of this design ("5-10-5-PS 2xiFQ") showed the best potency of this set, with >90% knockdown of HPRT at 20 nM and >70% knockdown at 5 nM concentration.

[0093]   Although 2'OMe RNA is somewhat resistant to endonuclease attack, gapmer ASOs of this design are usually made with full PS modification to prevent exonuclease degradation. Consistent with this idea, an ASO with the DNA domain protected by PS internucleotide linkages but having phosphodiester bonds in the 2'OMe flanking domains showed no gene

knockdown activity ("5-10-5 gapPS"). Use of the iFQ modification at the ends, however, permits use of this new design by providing protection from exonuclease attack; this new design should also increase binding affinity and lower toxicity by reducing PS content. This strategy was effective and the ASO ("5-10-5 gapPS 2xiFQ") showed knockdown of HPRT levels by >90% at 20 nM and by >70% at 5 nM. Potency was very similar to the full PS modified ASO. This design is expected to have reduced toxicity; however, toxicity is not easily tested in this system as HeLa cells are tolerant to fairly high doses of PS modified oligonucleotides. Benefit from reduced PS content will be better appreciated *in vivo*.

[0094]   Although it did not increase functional potency, addition of a third centrally placed iFQ group ("5-10-5 gapPS-3x-iFQ") was compatible with gene knockdown in this RNase H active antisense design. It is generally accepted that maximal activity of RNase H active ASOs requires a DNA domain having at least 8 uninterrupted DNA residues. It was unexpected that the 3xiFQ design (where the 10 base DNA domain is interrupted by a central iFQ group) would work without reducing potency compared with the 2xiFQ design (where the 10 base DNA domain is continuous). It is possible that unique properties of the iFQ group allow its insertion to remain compatible with RNase H activity, possibly due to the same postulated base stacking interactions that result in increased T_m in these compounds.

[0095]   The most potent antisense design in current use are LNA-modified gapmers, where very strong T_m enhancing LNA modifications are used in the flanking domains in place of the 2'OMe RNA bases used in the present example. While potent, this design is expensive and can show significant toxicity in certain contexts. The same anti-HPRT sequence was made as an LNA 5-10-5 gapmer (fully PS modified). As expected, this compound showed the highest relative potency of any of the ASOs tested ("5-10-5 LNA PS") but the observed potency was only marginally higher than the best of the iFQ compositions ("5-10-5-PS 2xiFQ"). The very high binding affinity LNA reagents usually result in decreased specificity, so use of the iFQ designs of the present invention may show improved specificity at a small cost in potency.

EXAMPLE 6

[0096]   This example demonstrates use of the iFQ modification in RNA duplexes with application in suppressing gene expression via an RNAi mechanism of action.

[0097]   The use of double-stranded RNA (dsRNA) to trigger gene suppression via RNA interference (RNAi) is a well-described technique. Synthetic dsRNAs that mimic natural cellular products (small interfering RNAs, or siRNAs) are usually 21 bases long with a central 19 base duplex domain with 2-base 3'-overhangs. Alternatively, slightly larger synthetic oligonucleotides can be used that are substrates for the cytoplasmic nuclease Dicer, which processes these species into 21-mer siRNAs. Typically these reagents are asymmetric and have a 25 base top (Sense strand, "S") and a 27 base bottom strand (Antisense strand, "AS") with a single 2-base 3'-overhang on the AS strand. These longer siRNAs are called Dicer-substrate siRNAs, or DsiRNAs. Although dsRNA is far more stable to nuclease attack than single-stranded RNA (ssRNA), degradation of the synthetic siRNAs can significantly limit potency of the compounds, especially when used *in vivo*. Incorporation of chemical modifications, such as 2'OMe RNA, 2'F RNA, or LNA bases, improves nuclease stability and can improve function of the siRNA. Selective placement of nuclease-resistant phosphorothioate bonds (PS) can also help stabilize the siRNA, especially when used near the terminal 3'-internucleotide linkages. Unfortunately, careful placement of modified groups is essential as extensive chemical modification usually lowers functional potency of the compound even though nuclease stabilization has been achieved, probably through disrupting interaction of the RNA duplex with key protein mediators of RNAi, like Dicer or Ago2.

[0098]   The present example demonstrates that the iFQ modifier can be introduced into DsiRNAs. Like other chemical modifiers, iFQ insertion can lead to increased potency, decreased potency, or no change in potency depending upon placement.

[0099]   **Oligonucleotide synthesis and purification.** RNA and modified RNA oligonucleotides were synthesized using solid phase phosphoramidite chemistry, deprotected and desalted according to routine techniques (Caruthers *et al.*, 1992). The oligomers were purified using ion-exchange high performance liquid chromatography (IE-HPLC) and were handled under RNase-free conditions. All RNA oligonucleotides were prepared as a sodium salt. The purity of each oligomer was determined by capillary electrophoresis (CE) carried out on a Beckman P/ACE MDQ system (Beckman Coulter, Inc., Fullerton, CA). All single-strand oligomers were at least 85% pure. Electrospray-ionization liquid chromatography mass spectrometry (ESI-LCMS) of the oligonucleotides was conducted using an Oligo HTCS system

(Novatia, Princeton, NJ), which consisted of ThermoFinnigan TSQ7000, Xcalibur data system, ProMass data processing software, and Paradigm MS4™ HPLC (Michrom BioResources, Auburn, CA). Protocols recommended by the manufacturers were followed. Experimental molar masses for all single-strand oligomers were within 1.5 g/mol of expected molar mass. These results confirm identity of the oligomers.

[00100] Duplexes were formed by mixing equal molar amounts of the top and bottom strands in 30 mM Hepes, pH 7.5, 100 mM potassium acetate, heating at 95°C for 2 minutes, then cooling to room temperature. Table 10 lists the duplexes synthesized for Example 6.

Table 10: Synthetic RNA duplexes employed in Example 6 (anti-HPRT DsiRNAs)

SEQ ID NO:	Name	Sequence		
85	NC1 Negative Control	5' CGUUAAUCGCGUAUAAUACGCGUat 3'		S
86		3' CAGCAAUUAGCGCAUAUUAUGCGCAUA 5'		AS
87	HPRT unmod	5' GCCAGACUUUGUUGGAUUUGAAAtt 3'		S
88		3' UUCGGUCUGAAACAACCUAAACUUUAA 5'		AS
89	HPRT iFQ v1	5' GCCAGACUUUGUUGGAUUUGAAAtt 3'		S
90		3' U^{z}UCGGUCUGAAACAACCUAAACUUUAA 5'		AS
91	HPRT iFQ v2	5' G^{z}CCAGACUUUGUUGGAUUUGAAAtt 3'		S
92		3' UUC GGUCUGAAACAACCUAAACUUUAA 5'		AS
93	HPRT iFQ v3	5' G^{z}CCAGACUUUGUUGGAUUUGAAAt^{z}t 3'		S
94		3' UUC GGUCUGAAACAACCUAAACUUUA A 5'		AS
95	HPRT iFQ v4	5' G CCAGACUUUGUUGGAUUUGAAAtt 3'		S
96		3' UUC^{z}GGUCUGAAACAACCUAAACUUUAA 5'		AS
97	HPRT iFQ v5	5' G CCAGACUUUGUUGGAUUUGAAAt t 3'		S
98		3' UUC^{z}GGUCUGAAACAACCUAAACUUUA^{z}A 5'		AS
99	HPRT iFQ v6	5' G^{z}CCAGACUUUGUUGGAUUUGAAAt^{z}t 3'		S
100		3' UUC^{z}GGUCUGAAACAACCUAAACUUUA A 5'		AS
101	HPRT iFQ v7	5' G^{z}CCAGACUUUGUUGGAUUUGAAAt^{z}t 3'		S
102		3' UUC^{z}GGUCUGAAACAACCUAAACUUUA^{z}A 5'		AS

Uppercase = RNA
Lowercase = DNA
"z" = insertion of napthylene-azo modifier (iFQ)

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Note: gaps have been introduced in sequences for the purpose of alignment only and do not represent any modification to sequence.

[00101]   HeLa cell culture, transfections, and RT-qPCR methods. HeLa cells were transfected in "reverse format" at ~60% confluency (Invitrogen, Carlsbad, CA) using 1 μL Lipofectamine™ RNAiMAX per 50 μL OptiMEM™ I (Invitrogen, Carlsbad, CA) with RNA duplexes at the indicated concentrations. All transfections were performed in triplicate. RNA was prepared 24 hours after transfection using the SV96 Total RNA Isolation Kit (Promega, Madison, WI); cDNA was synthesized using 150 ng total RNA with SuperScript™-II Reverse Transcriptase (Invitrogen, Carlsbad, CA) per the manufacturer's instructions using both random hexamer and oligo-dT priming.

**[00102]   Quantitative real-time PCR reactions were done using 10 ng cDNA per 10 μL reaction, Immolase™ DNA Polymerase (Bioline, Randolph, MA), 500 nM primers, and 250 nM probe. Hypoxanthine phosphoribosyltransferase 1 (HPRT1) (GenBank Acc, No. NM_000194) specific primers were:

HPRT-For 5' GACTTTGCTTTCCTTGGTCAGGCA (SEQ ID NO: 79),

HPRT-Rev 5' GGCTTATATCCAACACTTCGTGGG (SEQ ID NO: 80), and probe

HPRT-P 5' FAM- ATGGTCAAGGTCGCAAGCTTGCTGGT-IowaBlackFQ (IBFQ) (SEQ ID NO: 81).

Cycling conditions employed were: 95°C for 10 minutes followed by 40 cycles of 2-step PCR with 95°C for 15 seconds and 60°C for 1 minute. PCR and fluorescence measurements were done using an ABI Prism™ 7900 Sequence Detector (Applied Biosystems Inc., Foster City, CA). All data points were performed in triplicate. Expression data were normalized to levels of an internal control gene, human splicing factor, arginine/serine-rich 9 (SFRS9) (GenBank Acc. No. NM_003769), which was measured in a multiplexed reaction using primers:

SFRS9-For 5' TGTGCAGAAGGATGGAGT (SEQ ID NO: 103),

SFRS9-Rev 5' CTGGTGCTTCTCTCAGGATA (SEQ ID NO: 104), and probe

SFRS9-P 5' MAX-TGGAATATGCCCTGCGTAAACTGGA-IBFQ (SEQ ID NO: 105), setting the baseline to cells transfected with a scrambled negative control RNA duplex (NC1). Copy number standards were run in parallel using linearized cloned amplicons for both the HPRT and

SFRS9 assays. Unknowns were extrapolated against standards to establish absolute quantitative measurements.

[00103]   **Results.** The anti-HPRT DsiRNA employed in the present study is extremely potent and typically shows detectable knockdown of target mRNA at low picomolar levels. Consistent with this expectation, the unmodified duplex reduced HPRT levels by ~40% at a 10 pM dose at 24 hours post-transfection in HeLa cells. A series of modified duplexes containing the iFQ group positioned at various locations in the S strand, AS strand, or both were similarly transfected into HeLa cells and HPRT mRNA levels were measured 24 hour post-transfection. Results are shown in Figure 7.

[00104]   Placing the iFQ group near the 3'-end of the AS strand was well tolerated; insertion between bases 1 and 2 from the 3'-end (in the single-stranded 3'-overhang domain) (duplex HPRT iFQ v1) or between bases 3 and 4 from the 3'-end (at the start of the duplex domain) (duplex HPRT iFQ v4) showed similar potency to the unmodified duplex. Placing the iFQ group near the 5'-end of the S strand was similarly well tolerated (duplex HPRT iFQ v2) as was placing the iFQ group near both ends of the S strand (duplex HPRT iFQ v3). In contrast, duplexes having an iFQ group near the 5'-end of the AS strand showed reduced potency (duplexes HPRT iFQ v5 and v7), so modification at this position should be avoided.

[00105]   Within the error of the system studied, the iFQ modified and unmodified duplexes showed similar potency (except for those duplexes modified at the 5'-end of the AS strand, as noted above). Benefit from the iFQ group is most likely to be evident in settings where nuclease stabilization is needed, which is not appreciated in the present *in vitro* system, but based on the results of Examples 1 and 2, greater benefit would be expected from use of this modification when used *in vivo* where exposure to serum nucleases is more problematic.

EXAMPLE 7

[00106]   This example demonstrates decreased cellular toxicity from lipid transfected internal napthylene-azo-containing oligomers compared with other compounds.

[00107]   Toxicity from chemical modification of synthetic oligomers can be problematic as it can give unwanted side effects, cause unreliable results, and limit therapeutic utility of the oligomer. Cellular death can result from toxic chemical modifications by inducing necrosis or

apoptosis. Toxicity was ascertained with oligomers containing a non-targeting, negative control

("NC1") sequence using chemical modification patterns employed in the AMOs examined in

Examples 3 and 4 (*see* Table 11). Generalized cytotoxicity (from necrosis and/or apoptosis) was

measured by quantifying the relative number of live and dead cells after treatment with the

chemically modified oligomers, while cytotoxicity resulting from the induction of the apoptotic

pathway was determined by measuring the levels of caspase-3 and -7 after oligomer treatment.

[00108]   **Oligonucleotide synthesis and preparation**. DNA oligonucleotides were

synthesized using solid phase phosphoramidite chemistry, deprotected and desalted on NAP-5

columns (Amersham Pharmacia Biotech, Piscataway, NJ) according to routine techniques

(Caruthers *et al.*, 1992). The oligomers were purified using reversed-phase high performance

liquid chromatography (RP-HPLC). The purity of each oligomer was determined by capillary

electrophoresis (CE) carried out on a Beckman P/ACE MDQ system (Beckman Coulter, Inc.,

Fullerton, CA). All single-strand oligomers were at least 90% pure. Electrospray-ionization

liquid chromatography mass spectrometry (ESI-LCMS) of the oligonucleotides was conducted

using an Oligo HTCS system (Novatia, Princeton, NJ), which consisted of ThermoFinnigan

TSQ7000, Xcalibur data system, ProMass data processing software, and Paradigm MS4™ HPLC

(Michrom BioResources, Auburn, CA). Protocols recommended by the manufacturers were

followed. Experimental molar masses for all single-strand oligomers were within 1.5 g/mol of

expected molar mass. These results confirm identity of the oligomers.

Table 11: Synthetic oligomers employed in Example 7 (NC1 AMOs)

SEQ ID NO:	Name	Sequence
106	2'OMe 5'+3' iFQ	G$_z$C G U A U U A U A G C C G A U U A A C G$_z$A
107	2'OMe PSends	G*C*G*U A U U A U A G C C G A U U A A*C*G*A
108	DNA/PS	g*c*g*t*a*t*t*a*t*a*g*c*c*g*a*t*t*a*a*c*g*a
109	DNA/LNA PO	g C g t A t t A t a G c c G a t T a a C g a

110	DNA/LNA PS	g*C̲*g*t*A̲*t*t*A̲*t*a*G̲*c*c*G̲*a*t*T̲*a*a*C̲*g*a
111	2'OMe/LNA PO	G C̲ G T A̲ T T A̲ T A G̲ C C G̲ A T T̲ A A C̲ G A
112	2'OMe/LNA PS	G*C̲*G*T*A̲*T*T*A̲*T*A*G̲*C*C*G̲*A*T*T̲*A*A*C̲*G*A

Uppercase = 2'OMe RNA
Lowercase = DNA
Uppercase with underscore = LNA
"*" = phosphorothioate linkage
"z" = napthylene-azo modifier (iFQ)

[00109]   **Cell culture, transfections, and luciferase assays.** HeLa cells were plated in 48-well plates in DMEM containing 10% FBS to achieve 90% confluency the next day. The following morning, NC1 AMOs were transfected at 100 nM or 50 nM concentrations in triplicate wells in two sets (one for measuring general cytotoxicity, one for measuring apoptosis induction) with 1 μl TriFECTin® (Integrated DNA Technologies) per well in DMEM containing 10% FBS. An apoptosis-inducing agent, Staurosporine (1 mM in DMSO), was incubated at 1 μM on the cells for 24 hours as a positive control. After 24 hours of NC1 AMO treatment, the first set of cells was analyzed for viability using the MultiTox-Glo Multiplex Cytotoxicity Assay (Promega, Madison, WI) with the peptide-substrate GF-AFC (glycyl-phenylalanylaminofluorocoumarin), which generates a fluorescence signal upon cleavage by a "live-cell" specific protease, measured at $405nm_{Ex}/505nm_{Em}$ in a SpectraFluor Microplate Reader (Tecan Group Ltd, Männedorf, Switzerland). Continuing to use the MultiTox-Glo Multiplex Cytotoxicity Assay, the same cells were subsequently analyzed for cytotoxicity by detecting a "dead-cell" protease activity in a luciferase-based assay measured on a GloMax® 96 Microplate Luminometer (Promega) per the manufacturer's recommendations. To assess cytotoxicity derived from induction of the apoptosis pathway, the Caspase-Glo® 3/7 Assay (Promega) was performed with the second set of cells to measure caspase-3 and -7 levels according to the manufacturer's recommendations on a GloMax® 96 Microplate Luminometer (Promega).

[00110]   **Results.** For the cytoxocity analysis graphed in Figure 8, data is presented as a ratio of live/dead cells as calculated from the abundance of "live-cell" and "dead-cell" proteases described above. The ratio of live/dead cells serves as an internal normalizing control providing

data independent of cell number, and a reduction of live/dead cells correlates with cytotoxicity. The "2'OMe 5'+3' iFQ" and "2'OMe PSends" compounds are the least toxic oligomers and there is minimal toxicity even at the high 100 nM dose. The "DNA/PS" oligomer, which is entirely comprised of PS linkages, shows substantial cell death at both doses suggesting that PS modification is toxic to the cells. When LNA bases are incorporated into the NC1 AMO, such as in the "DNA/LNA PO" oligomer, cell death is seen at the high 100 nM dose suggesting that LNA modification is toxic to the cells. Importantly, additive cell death is seen after combining these two chemistries in the "DNA/LNA PS" oligomer, demonstrating toxicity which also correlates with the dysmorphic, unhealthy cells seen during the visual analysis at the time the assay was performed. Substituting DNA with 2'OMe bases in the "2'OMe/LNA PO" and "2'OMe/LNA PS" NC1 AMOs reduces toxicity compared with their DNA counterparts; however, cell death is still seen at the 100 nM dose.

[00111]   In parallel, HeLa cells treated with the NC1 AMOs were assessed for apoptosis induction by evaluating the levels of caspase-3 and -7 in a luciferase-based assay (Figure 9). Luminescence is proportional to the abundance of the apoptosis effectors and an increase in RLUs correlates with an increase in apoptosis. The data in Figure 9 mirrors the cytotoxicity profiles from the NC1 AMOs assayed in Figure 8. The NC1 AMOs that do not trigger apoptosis are the "2'OMe 5'+3' iFQ" and "2'OMe PSends" compounds. Both extensive PS modification ("DNA/PS") and incorporation of LNA bases ("DNA/LNA PO") induce apoptosis, while an additive effect is seen when these two chemistries are combined ("DNA/LNA PS"). Again, substitution of DNA bases for 2'OMe bases ("2'OMe/LNA PO" and "2'OMe/LNA PS") reduces apoptosis induction. However, the "2'OMe/LNA PS" still demonstrates apoptosis induction at the 100 nM dose.

[00112]   This cytotoxicity profiling analysis clearly exemplifies that certain chemical modification strategies can be detrimental to cell viability. The "2'OMe 5'+3' iFQ" AMO and the "DNA/LNA PS" AMOs, which demonstrated similar high potency in Example 3, have significantly different toxicity profiles. The "2'OMe 5'+3' iFQ" oligomer was non-toxic in this system, and the "DNA/LNA PS" oligomer caused substantial cell death in Figure 8 and was shown to induce apoptosis in Figure 9. These data confirm the superiority of the "2'OMe 5'+3'

iFQ" AMO when compared to other standard AMOs with comparable potency (Example 3),
increased specificity (Example 4), and reduced toxicity.

[00113]   All references, including publications, patent applications, and patents, cited herein
are hereby incorporated by reference to the same extent as if each reference were individually
and specifically indicated to be incorporated by reference and were set forth in its entirety herein.

[00114]   The use of the terms "a" and "an" and "the" and similar referents in the context of
describing the invention (especially in the context of the following claims) are to be construed to
cover both the singular and the plural, unless otherwise indicated herein or clearly contradicted
by context. The terms "comprising," "having," "including," and "containing" are to be construed
as open-ended terms (*i.e.*, meaning "including, but not limited to") unless otherwise noted.
Recitation of ranges of values herein are merely intended to serve as a shorthand method of
referring individually to each separate value falling within the range, unless otherwise indicated
herein, and each separate value is incorporated into the specification as if it were individually
recited herein. All methods described herein can be performed in any suitable order unless
otherwise indicated herein or otherwise clearly contradicted by context. The use of any and all
examples, or exemplary language (*e.g.*, "such as") provided herein, is intended merely to better
illuminate the invention and does not pose a limitation on the scope of the invention unless
otherwise claimed. No language in the specification should be construed as indicating any non-
claimed element as essential to the practice of the invention.

[00115]   Preferred embodiments of this invention are described herein, including the best
mode known to the inventors for carrying out the invention. Variations of those preferred
embodiments may become apparent to those of ordinary skill in the art upon reading the
foregoing description. The inventors expect skilled artisans to employ such variations as
appropriate, and the inventors intend for the invention to be practiced otherwise than as
specifically described herein. Accordingly, this invention includes all modifications and
equivalents of the subject matter recited in the claims appended hereto as permitted by applicable
law. Moreover, any combination of the above-described elements in all possible variations
thereof is encompassed by the invention unless otherwise indicated herein or otherwise clearly
contradicted by context.

WHAT IS CLAIMED IS:

Claim 1:   An antisense oligonucleotide comprising at least one modification that is incorporated between two bases of the antisense oligonucleotide, wherein the modification increases binding affinity and nuclease resistance of the antisense oligonucleotide.
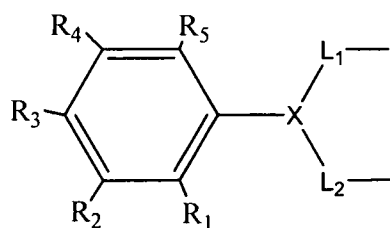
Claim 2:   The antisense oligonucleotide of claim 1, wherein the modification is located within three bases of a terminal nucleotide.

Claim 3:   The antisense oligonucleotide of claim 1, wherein the modification is located between a terminal base and a penultimate base of either the 3' or the 5' end of the antisense oligonucleotide.

Claim 4:   The antisense oligonucleotide of claim 1, wherein a modification is located between the terminal base and the penultimate base at both the 3' and the 5' ends of the antisense oligonucleotide.

Claim 5:   The antisense oligonucleotide of claim 1, wherein the modification is a napthylene-azo compound.

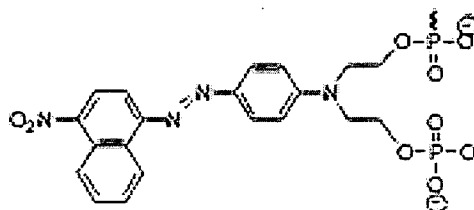Claim 6:   The antisense oligonucleotide of claim 1 wherein the modification has the structure:

wherein the linking groups L_1 and L_2 positioning the modification at an internal position of the oligonucleotide are independently an alkyl, alkynyl, alkenyl, heteroalkyl, substituted alkyl, aryl, heteroaryl, substituted aryl, cycloalkyl, alkylaryl, or alkoxy groups; R_1-R_5 are independently a hydrogen, alkyl, alkynyl, alkenyl, heteroalkyl, substituted alkyl, aryl, heteroaryl, substituted aryl, cycloalkyl, alkylaryl, alkoxy, an electron withdrawing group, an electron donating group, or an attachment point for a ligand; and X is a nitrogen or carbon atom, wherein if X is a carbon atom, the fourth substituent attached to the carbon atom can be hydrogen or a C1-C8 alkyl group.

Claim 7:   The antisense oligonucleotide of claim 1, wherein the modification has the structure:

wherein the linking groups L_1 and L_2 positioning the modification at an internal position of the oligonucleotide are independently an alkyl, alkynyl, alkenyl, heteroalkyl, substituted alkyl, aryl, heteroaryl, substituted aryl, cycloalkyl, alkylaryl, or alkoxy groups; R_1, R_2, R_4, R_5 are independently a hydrogen, alkyl, alkynyl, alkenyl, heteroalkyl, substituted alkyl, aryl, heteroaryl, substituted aryl, cycloalkyl, alkylaryl, alkoxy, an electron withdrawing group, or an electron donating group; R_6, R_7, R_9-R_{12} are independently a hydrogen, alkyl, alkynyl, alkenyl, heteroalkyl, substituted alkyl, aryl, heteroaryl, substituted aryl, cycloalkyl, alkylaryl, alkoxy, an electron withdrawing group, or an electron donating group; R_8 is a hydrogen, alkyl, alkynyl, alkenyl, heteroalkyl, substituted alkyl, aryl, heteroaryl, substituted aryl, cycloalkyl, alkylaryl, alkoxy, or an electron withdrawing group; and X is a nitrogen or carbon atom, wherein if X is a carbon atom, the fourth substituent attached to the carbon atom can be hydrogen or a C1-C8 alkyl group.

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Claim 8:   The antisense oligonucleotide of claim 7, wherein R_8 is NO_2.

Claim 9:   The antisense oligonucleotide of claim 1, wherein the modification has the
structure:

Claim 10:   The antisense oligonucleotide of claim 1, wherein the antisense oligonucleotide
further comprises at least one 2'-O-methyl RNA.

Claim 11:   The antisense oligonucleotide of claim 1, wherein the antisense oligonucleotide
further comprises one or more phosphorothioate linkages.

Claim 12:   The antisense oligonucleotide of claim 1, wherein the antisense oligonucleotide
comprises a region of bases linked through phosphodiester bonds, wherein the region is flanked
at one or both ends by regions containing phosphorothioate linkages.

Claim 14:   An antisense oligonucleotide having the structure:
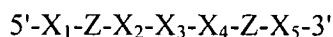
$$5'-X_1-Z-X_2-X_3-X_4-Z-X_5-3'$$

wherein X_1 and X_5 are independently 1-3 nucleotides, wherein the internucleotide linkages are
optionally phosphorothioate; Z is a napthylene-azo compound; X_2 and X_4 are independently 1-5
nucleotides wherein the internucleotide linkages are optionally phosphorothioate; and X_3 is 10-
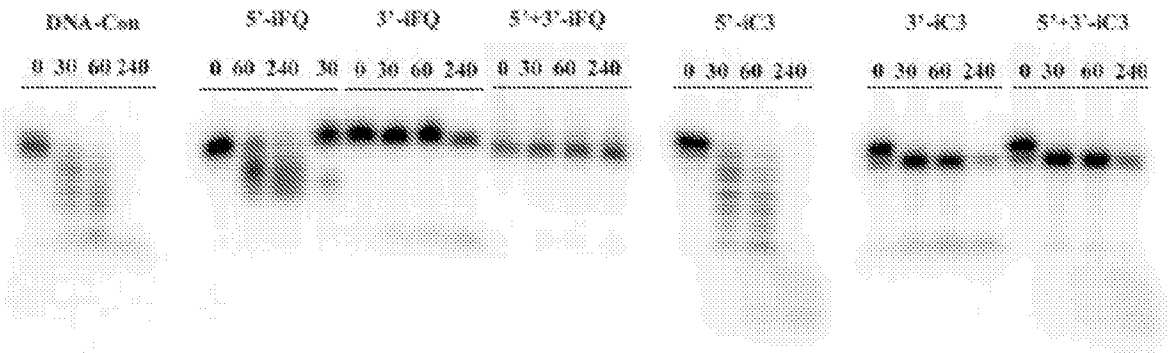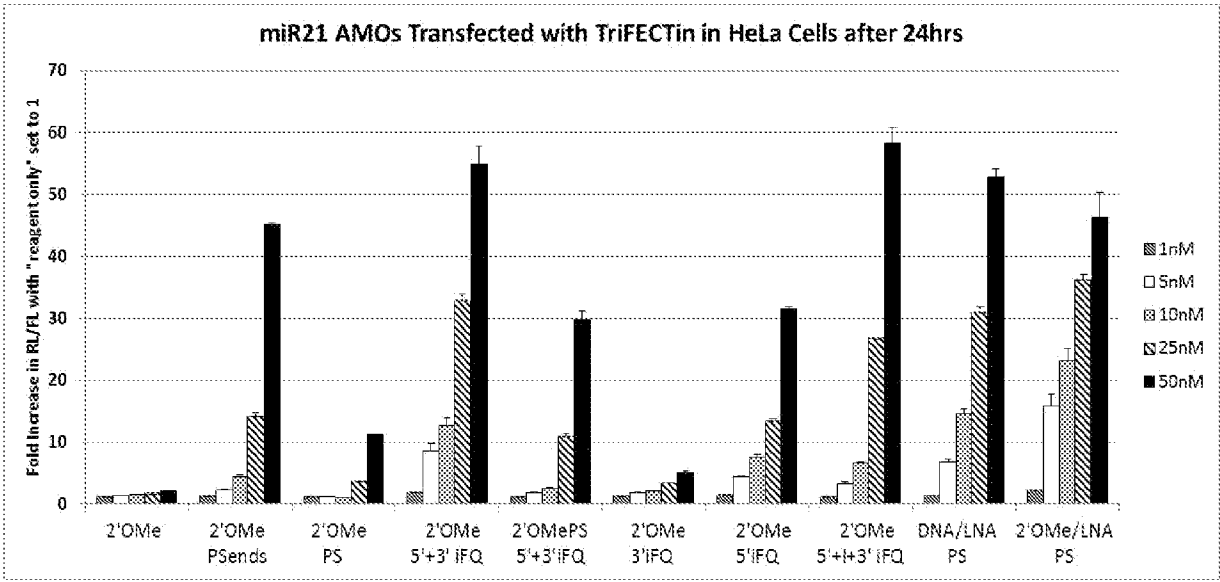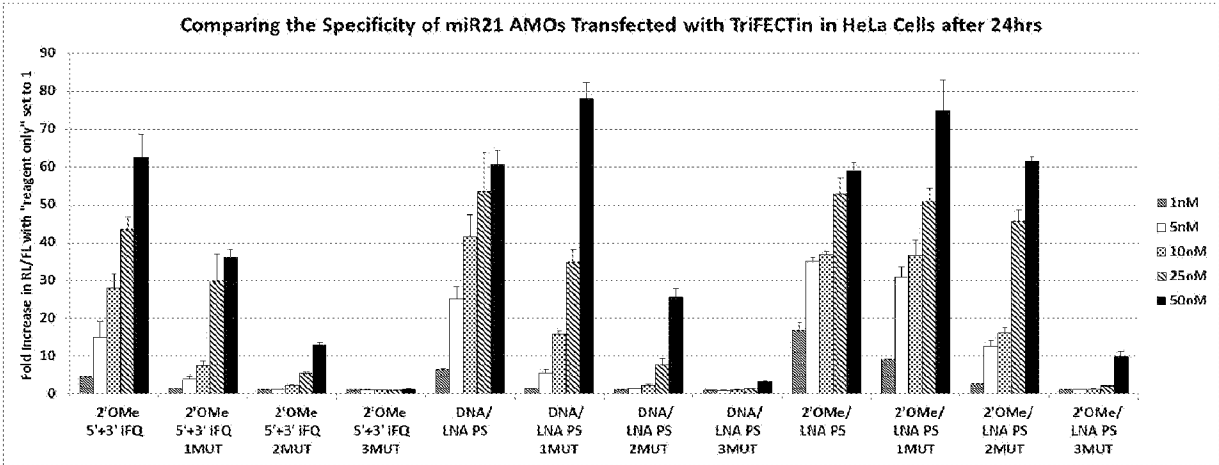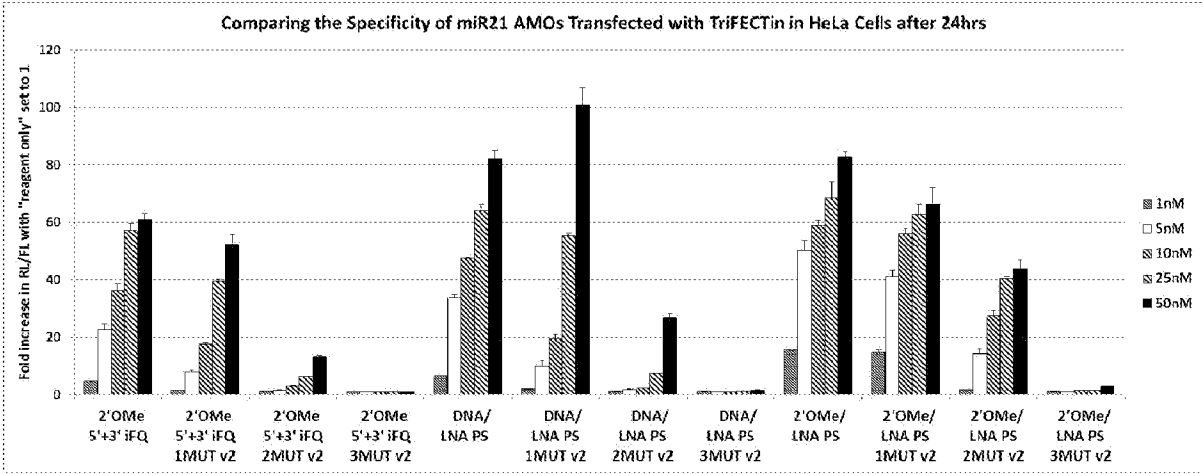25 nucleotides.

FIG 1

FIG. 2

FIG. 3

FIG. 4
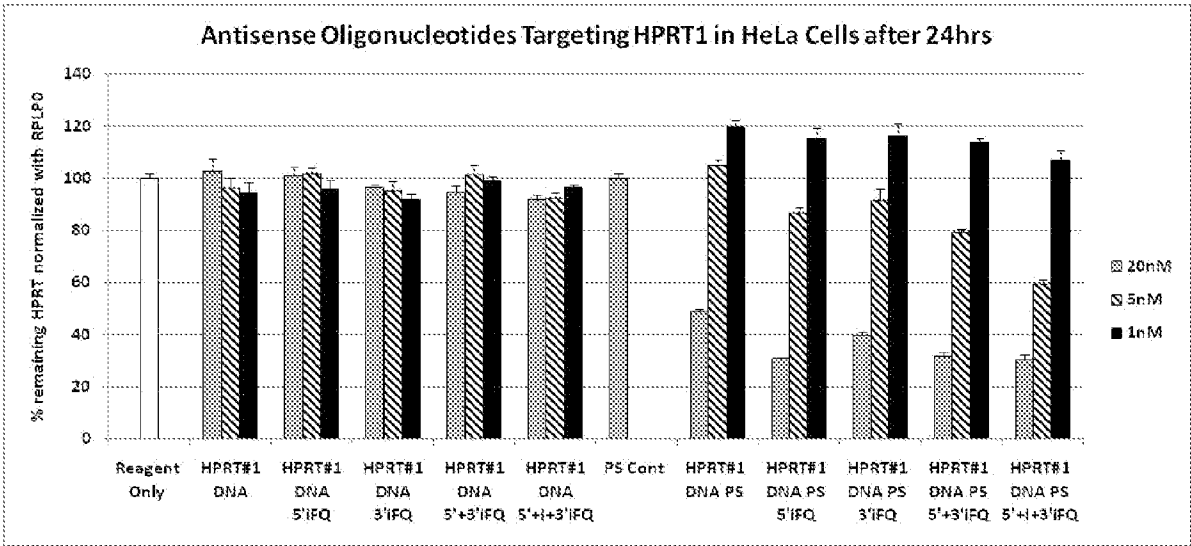
FIG. 5
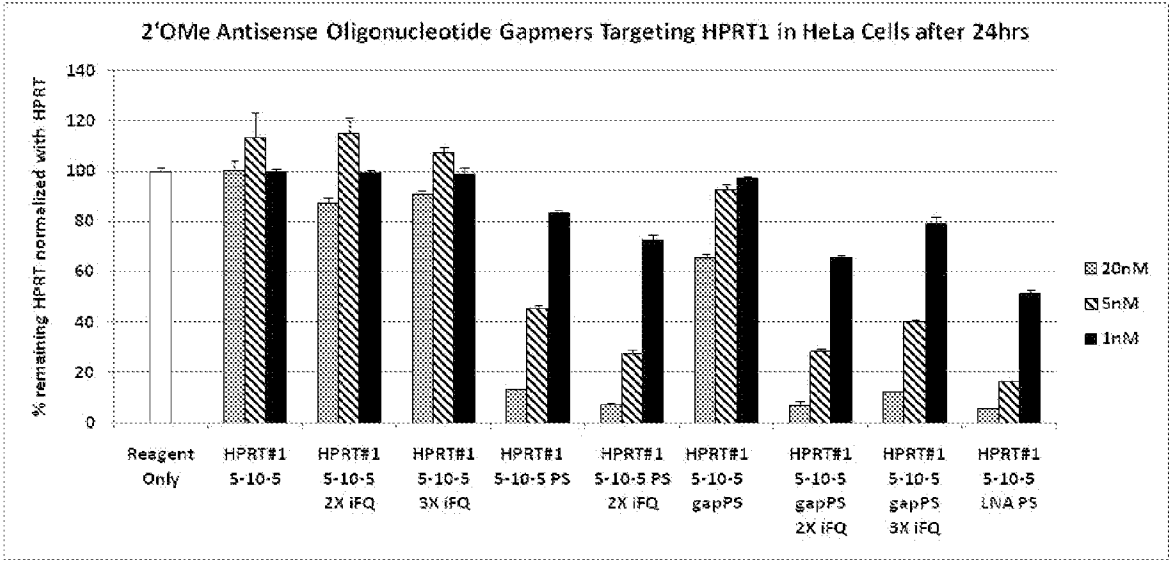
FIG. 6
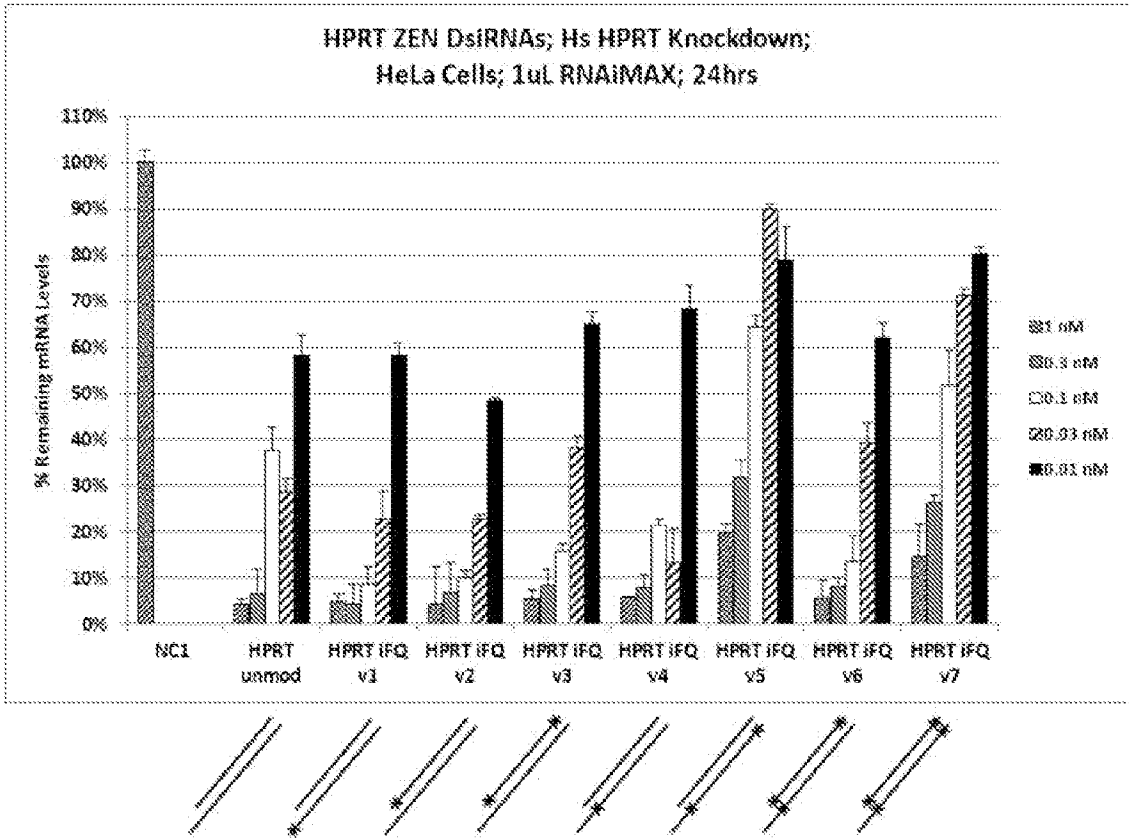
FIG. 7
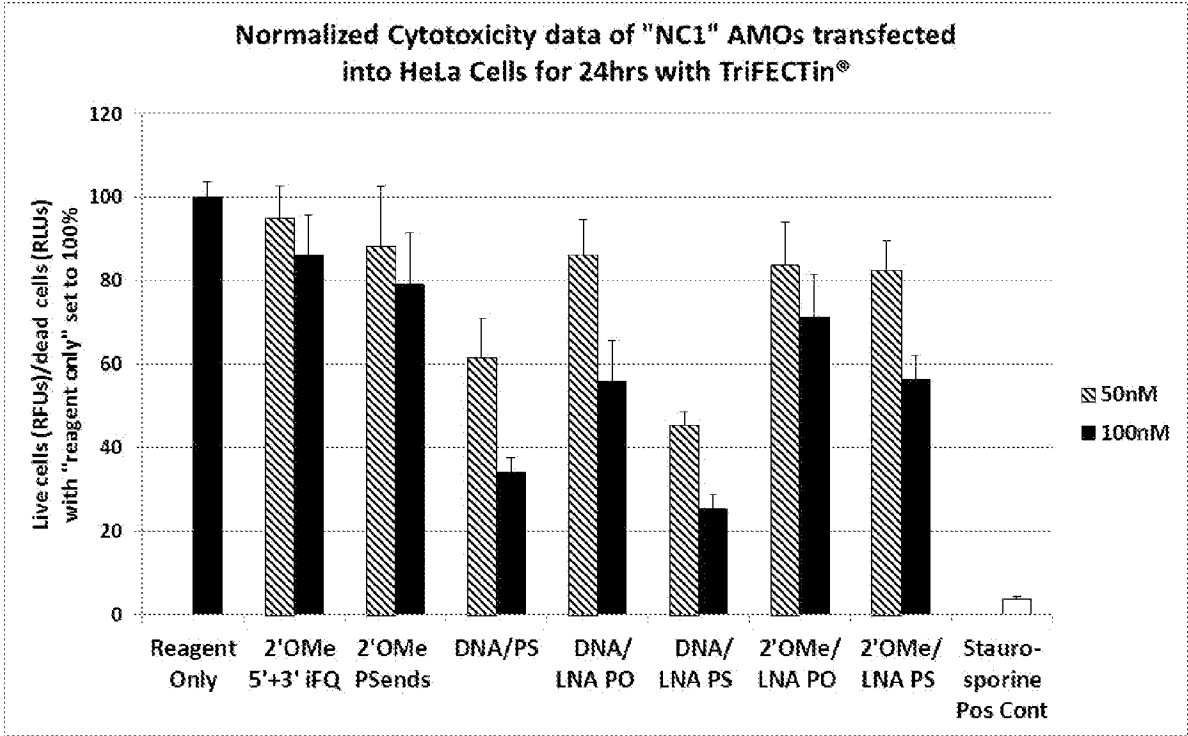
FIG. 8
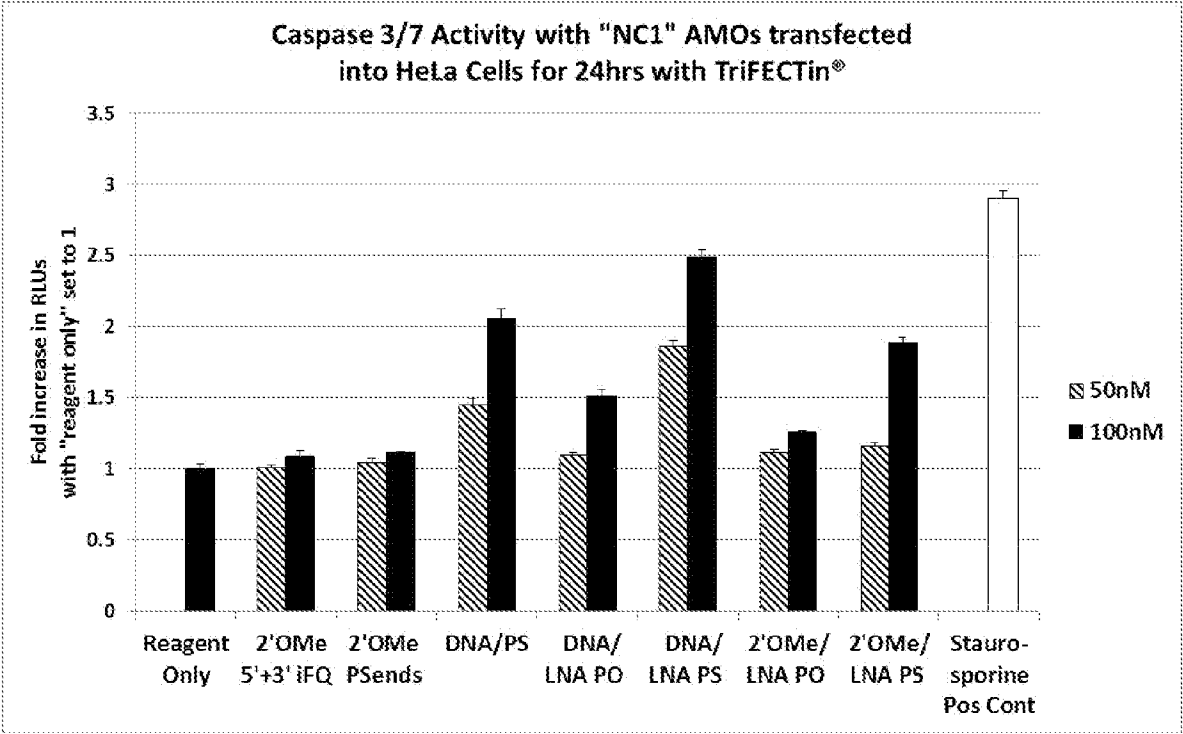
FIG. 9

INTERNATIONAL SEARCH REPORT

A. CLASSIFICATION OF SUBJECT MATTER
INV. C12N15/113
ADD.

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

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

C12N

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

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

EPO-Internal, BIOSIS, MEDLINE, Sequence Search, WPI Data

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	JEPSEN J S ET AL: "LNA-ANTISENSE RIVALS SIRNA FOR GENE SILENCING", CURRENT OPINION IN DRUG DISCOVERY AND DEVELOPMENT, CURRENT DRUGS, LONDON, GB, vol. 7, no. 2, 1 January 2004 (2004-01-01) , pages 188-194, XP009083873, ISSN: 1367-6733 the whole document	1-12,14
A	US 2006/263816 A1 (LAIKHTER ANDREI [US] ET AL) 23 November 2006 (2006-11-23) the whole document	1-12,14
A	WO 2005/115479 A2 (AVI BIOPHARMA INC [US]; IVERSEN PATRICK L [US]; WELLER DWIGHT D [US];) 8 December 2005 (2005-12-08) page 15 - page 17; claim 11; figures 1-3; example 5; table 1	1-12,14

-/--

| X | Further documents are listed in the continuation of Box C. | X | See patent family annex. |

* Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
5 December 2011	12/12/2011

Name and mailing address of the ISA/	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Fax: (+31-70) 340-3016	Spindler, Mark-Peter

Form PCT/ISA/210 (second sheet) (April 2005)

C(Continuation).	DOCUMENTS CONSIDERED TO BE RELEVANT	
Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	US 2003/045488 A1 (BROWN BOB D [US] ET AL) 6 March 2003 (2003-03-06) the whole document -----	1-12,14

Patent document cited in search report			Publication date	Patent family member(s)			Publication date
US 2006263816	A1		23-11-2006	US	2006263816	A1	23-11-2006
				US	2009118482	A1	07-05-2009
				US	2009299041	A1	03-12-2009
				US	2010076181	A1	25-03-2010
				US	2010298554	A1	25-11-2010
WO 2005115479	A2		08-12-2005	AT	529513	T	15-11-2011
				AU	2005247460	A1	08-12-2005
				CA	2565103	A1	08-12-2005
				EP	1765414	A2	28-03-2007
				JP	2008509701	A	03-04-2008
				JP	2011172596	A	08-09-2011
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