

DOUBLE-STRANDED OLIGONUCLEOTIDE AGENTS AND USES THEREOF

CROSS-REFERENCE TO RELATED APPLICATION

[0001] This application claims the benefit of priority to Chinese Patent Application No. 202310900474.0, filed on July 21, 2023, Chinese Patent Application No. 202310904516.8, filed on July 21, 2023, Chinese Patent Application No. 202311532296.7, filed on November 16, 2023, and Chinese Patent Application No. 202410396475.0, filed on April 2, 2024, the contents of which are hereby incorporated by reference in their entirety.

TECHNICAL FIELDS

[0002] Disclosed herein is a double-stranded oligonucleotide agent and uses thereof, capable of inhibiting expression of the target gene via RNA interference (RNAi).

BACKGROUND OF THE INVENTION

[0003] RNA interference (RNAi) technology refers to the highly conserved, efficient and specific degradation of homologous mRNA induced by double-stranded RNA (dsRNA) in the process of evolution. The dsRNA inhibits the expression of a target gene by destroying a target mRNA. The RNAi technology is capable to specifically eliminate or turn off the expression of the target gene, and thus has quickly become one of the most useful research tools in exploring gene functions and therapeutic agents for the treatment of many diseases including metabolic diseases, infectious diseases and malignant tumors, among others.

SUMMARY OF THE INVENTION

[0004] Provided herein is a double-stranded oligonucleotide agent comprising a sense strand and an anti-sense strand, wherein:

the sense strand and the anti-sense strand form a double-stranded portion of 15 to 27 base pairs in length and a 5' extension in the anti-sense strand;

the 5' extension has a length of at least three nucleotides and is cleavable from the most 3' nucleotide of the 5' extension, and the cleaved double-stranded oligonucleotide agent is capable of silencing a target RNA or inhibiting expression of a target gene via RNA interference.

[0005] Provided herein is a double-stranded oligonucleotide agent comprising a structure represented by Formula (C):

sense strand (5'→3'):	second fragment	
anti-sense strand (3'→5'):	first fragment	5' extension

Formula (C),

wherein the first fragment in the anti-sense strand and the second fragment in the sense strand form a double-stranded portion by base pairing, wherein the first fragment and the second fragment are of equal length;

wherein the 5' extension comprises at least three nucleotides in length;

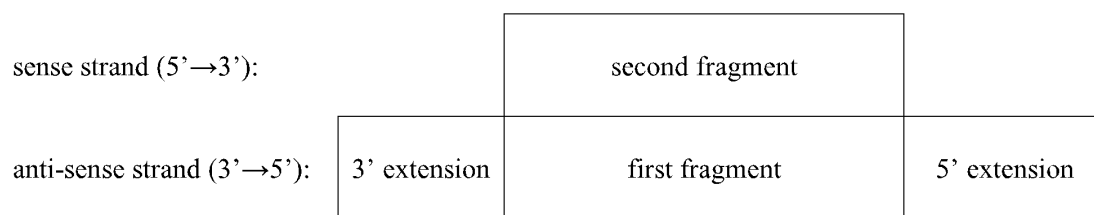
wherein the anti-sense strand comprises a cleavage region comprising the most 5' nucleotide (X2) of the first fragment and the two most 3' nucleotides (Y-Z) of the 5' extension, and the cleavage region comprises a nucleotide sequence set forth in Formula A: (3'-5') X2-Y-Z, which is cleavable between X2 and Y, and the Formula A is as defined below;

wherein the sense strand has a length of 15 to 35, 15 to 23, 15 to 22, or 15 to 21 nucleotides; and

wherein the anti-sense strand has a length of 25 to 35, 26 to 35, 26 to 30, 25 to 27, or 26 to 27 nucleotides.

[0006] A double-stranded oligonucleotide agent of Formula (C) does not require the complementary region and the target region to form a blunt end. It is possible that the targeting region comprising a 3' overhand, or the complementary region comprise a 5' extension, or the complementary region and the target region to form a blunt end.

[0007] Provided herein is a double-stranded oligonucleotide agent comprising a structure represented by Formula (D):



Formula (D),

wherein

the first fragment in the anti-sense strand and the second fragment in the sense strand form a double-stranded portion by base pairing, wherein the first fragment and the second fragment are of equal length;

the 5' extension comprises at least three nucleotides in length;

the anti-sense strand comprises a cleavage region comprising the most 5' nucleotide (X2) of the first fragment and the two most 3' nucleotides (Y-Z) of the 5' extension, and the cleavage region comprises a nucleotide sequence set forth in Formula A: (3'-5') X2-Y-Z, which is cleavable between X2 and Y, and upon cleavage, the 5' extension is removed from its most 3' nucleotide (Y), and the Formula A is as defined below,

wherein the sense strand has a length of 15 to 35, 15 to 23, 15 to 22, 15 to 21, 16 to 25, 17 to 23, 18 to 23, 19 to 23, 19 to 21, 20 to 23, 20 to 21, 21 to 23, such as 17, 18, 19, 20, 21, 22, or 23 nucleotides; and

wherein the anti-sense strand has a length of 25 to 35, 25 to 30, 26 to 35, 26 to 30, 26 to 27, such as 25, 26, 27, 28, 29, or 30 nucleotides.

[0008] Provided herein is a double-stranded oligonucleotide agent comprising a double-stranded oligonucleotide operably linked to a blocking group, wherein the blocking group comprises M03 or M06.

[0009] Provided herein is a double-stranded oligonucleotide agent comprising a double-stranded oligonucleotide operably linked to a ligand, where the ligand comprises a chemical structure selected from the group consisting of: VSDL-01, VSDL-01A, VSDL-02, VSDL-02A, VSDL-03, VSDL-03A, VSDL-04, VSDL-04A, VSDL-05, VSDL-05A, VSDL-06, VSDL-06A, VSDL-07, VSDL-07A, VSDL-08, VSDL-08A, VSDL-09, VSDL-10, VSDL-11, VSDL-12, VSDL-13, and VSDL-14.

[0010] Provided herein is a pharmaceutical composition comprising a double-stranded oligonucleotide agent disclosed herein or a pharmaceutically acceptable salt thereof, and a pharmaceutically acceptable carrier.

[0011] Provided herein is a method of inhibiting the expression of a target gene in a subject in need thereof, comprising administering to the subject a pharmaceutically effective amount of a double-stranded oligonucleotide agent disclosed herein or a pharmaceutically acceptable salt thereof, or a pharmaceutical composition disclosed herein.

[0012] Provided herein is a method of treating a disease or a disorder in a subject in need thereof, comprising administering to the subject a pharmaceutically effective amount of a double-stranded oligonucleotide agent disclosed herein or a pharmaceutically acceptable salt thereof, or a pharmaceutical composition disclosed herein.

[0013] It is to be understood that both the foregoing general description and the following detailed description are exemplary and explanatory only, and are not restrictive of the invention. Further, the accompanying drawings, which are incorporated in and constitute a part of this specification, illustrate embodiments of the invention and together with the description, serve to explain principles of the invention.

[0014] As will be apparent to those of skill in the art upon reading this disclosure, each of the individual embodiments described and illustrated herein has discrete components and features

which may be readily separated from or combined with the features of any of the other several embodiments without departing from the scope or spirit of the present disclosure.

BRIEF DESCRIPTION OF DRAWINGS

[0015] The drawings referenced herein form a part of the specification. Features shown in the drawing illustrate only some embodiments of the application, and not of all embodiments of the application, unless the detailed description explicitly indicates otherwise, and readers of the specification should not make implications to the contrary.

[0016] Fig. 1 shows relative AGT mRNA expression in liver for Test Compounds (ds101 and ds100).

[0017] Fig. 2 shows relative SOD-1 expression in different tissues (frontal cortex, hippocampus, striatum and heart) for Test Compounds (ds110, ds111, ds112 and ds113).

[0018] Fig. 3 shows relative SOD-1 expression in different tissues (prefrontal cortex and lumbar spinal cord) for Test Compounds (ds114 and ds115).

[0019] Fig. 4 shows relative mTTR expression for Test Compounds (ds118 and ds119).

[0020] Fig. 5 shows the results of liver homogenate processing reaction for Test Compound.

[0021] Fig. 6 shows plasma relative AGT levels for Test Compounds (ds101 and ds86) in NHP model.

DETAILED DESCRIPTION OF THE INVENTION

[0022] The following description of the disclosure is merely intended to illustrate various embodiments of the disclosure. As such, the specific modifications discussed are not to be construed as limitations on the scope of the disclosure. It will be apparent to one skilled in the art that various equivalents, changes, and modifications may be made without departing from the scope of the disclosure, and it is understood that such equivalent embodiments are to be included herein. All references cited herein, including publications, patents and patent applications are incorporated herein by reference in their entirety.

[0023] In this application, the use of the singular includes the plural unless specifically stated otherwise. In this application, the use of “or” means “and/or” unless stated otherwise. Furthermore,

the use of the term “including” as well as other forms such as “includes” and “included” is not limiting. In addition, terms such as “element” or “component” encompass both elements and components including one unit, and elements and components that include more than one subunit, unless specifically stated otherwise. Additionally, the section headings used herein are for organizational purposes only, and are not to be construed as limiting the subject matter described.

Definitions

[0024] As used herein, the term “a,” “an,” “the” and similar terms used in the context of the present invention (especially in the context of the claims) are to be construed to cover both the singular and plural unless otherwise indicated herein or clearly contradicted by the context.

[0025] Reference to “about” a value or parameter herein includes (and describes) embodiments that are directed to that value or parameter per se. For example, description referring to “about X” includes description of “X”. Numeric ranges are inclusive of the numbers defining the range. Generally speaking, the term “about” refers to the indicated value of the variable and to all values of the variable that are within the experimental error of the indicated value (*e.g.* within the 95% confidence interval for the mean) or within 10 percent of the indicated value, whichever is greater.

[0026] In this application, the terms “optional” or “optionally” means the described event or condition may or may not occur and includes both the situation where the event or condition occurs and where it does not occur. For example, “optionally modified” includes both unmodified and modified, and further, “nucleotide optionally modified” includes nucleotide both unmodified and modified.

[0027] It is noted that in this disclosure, terms such as “comprises”, “comprised”, “comprising”, “contains”, “containing”, “has”, “having” and the like are intended to be inclusive or open-ended, and do not exclude additional, un-recited elements or method steps.

[0028] As used herein, the term “disorder” refers to any disease, disorder or condition that impairs the normal functioning of a subject (*e.g.*, human).

[0029] The term “effective amount” means the amount of a pharmaceutical agent that produces some desired local or systemic therapeutic effect at a reasonable benefit/risk ratio applicable to any treatment alone or together with further doses. In the case of the treatment of a particular disorder, the desired local or systemic therapeutic effect preferably relates to inhibition of the course of the disorder. This comprises slowing down the progression of the disorder and, in

particular, interrupting or reversing the progress of the disorder. When administered for preventing a disorder, the amount is sufficient to avoid or delay onset of the disorder. An effective amount need not be curative or prevent a disorder from ever occurring. An effective amount of the pharmaceutical agent described herein will depend on the condition to be treated, the severeness of the disorder, the individual parameters of the patient, including age, physiological condition, size and weight, the duration of treatment, the type of an accompanying therapy (if present), the specific route of administration and similar factors. Accordingly, the doses administered of the pharmaceutical agent described herein may depend on various of such parameters. In the case that a reaction in a patient is insufficient with an initial dose, higher doses (or effectively higher doses achieved by a different, more localized route of administration) may be used. In certain embodiments, an effective amount of a pharmaceutical agent will depend on its therapeutic index, solubility, and the like.

[0030] The term “inhibiting,” as used herein, is used interchangeably with “reducing,” “silencing,” “downregulating,” “suppressing” and other similar terms, and includes any level of inhibition, in particular, a statistically significant or clinically significant inhibition.

[0031] As used herein, the term “inhibiting expression” of a target gene refers to any significant level of decrease in the level of target gene expression in cells, cell populations or tissues treated with an agent of interest, compared to those not treated. Gene expression level can be measured by, for example, the level of mRNA transcript of the target gene, or level of protein expressed from the target gene. In certain embodiments, the inhibition of expression of a target gene results in a clinically relevant inhibition of the level of target gene expression, *e.g.*, sufficiently inhibited to permit an effective therapeutic response.

[0032] As used herein, the term “pharmaceutically acceptable” indicates that the substance or composition is compatible chemically and/or toxicologically, with the other ingredients comprising a formulation, and/or the subjects being treated therewith.

[0033] As used herein, the term “pharmaceutically acceptable salt”, unless otherwise indicated, includes salts that retain the biological effectiveness of the free acids and bases of the specified compound and that are not biologically or otherwise undesirable. Contemplated pharmaceutically acceptable salt forms include, but are not limited to, mono, bis, tris, tetrakis, and so on. Pharmaceutically acceptable salts are non-toxic in the amounts and concentrations at which they

are administered. The preparation of such salts can facilitate the pharmacological use by altering the physical characteristics of a compound without preventing it from exerting its physiological effect. Useful alterations in physical properties include lowering the melting point to facilitate transmucosal administration and increasing the solubility to facilitate administering higher concentrations of the drug. Pharmaceutically acceptable salts can include acid addition salts such as those containing sulfate, chloride, fumarate, maleate, phosphate, sulfamate, acetate, citrate, lactate, tartrate, malonate, methanesulfonate, ethanesulfonate, benzenesulfonate, p-toluenesulfonate, cyclohexylsulfamate and quinate. Pharmaceutically acceptable salts can be obtained from acids such as sulfuric acid, hydrochloric acid, fumaric acid, maleic acid, phosphoric acid, sulfamic acid, acetic acid, citric acid, lactic acid, tartaric acid, malonic acid, methanesulfonic acid, ethanesulfonic acid, benzenesulfonic acid, p-toluenesulfonic acid, cyclohexylsulfamic acid, and quinic acid.

[0034] The term “subject” includes human and non-human animals. Non-human animals include all vertebrates, *e.g.*, mammals and non-mammals, such as non-human primates, mouse, rat, cat, rabbit, sheep, dog, cow, chickens, amphibians, and reptiles.

[0035] As used herein, the term “treatment,” “treat” or “treating”, with regard to a disorder, refers to managing, eliminating, reducing or ameliorating a disorder and/or a symptom associated therewith. Although not excluded, treatment of a disorder does not require that the disorder, or symptoms associated therewith be completely eliminated. The term “treatment” as used herein may include “prophylactic treatment” that is applied before development of any symptom or manifestation of a disorder to reduce the possibility of occurrence or recurrence of a disorder, or reducing the possibility of relapse of a previously controlled disorder, in a subject who is not afflicted with a disorder but at risk, or who is susceptible to recurrence of the disorder, or who is at risk or susceptible to relapse of the disorder. Within the meaning of the invention, “treatment” also includes prevention of relapse or prevention stages, as well as treatment of acute or chronic signs, symptoms and/or dysfunction. Treatment can target symptoms, for example, to suppress symptoms. It can function in a short period of time, for a medium period of time, or can be a long-term treatment, such as in the case of maintenance therapy.

[0036] *Nucleotide*

[0037] As used herein, the term “nucleotide” means a base linked to a sugar, with a phosphate group covalently linked to the sugar portion and is intended to encompass both natural (i.e. unmodified) nucleotide and modified nucleotide. In some embodiments, a nucleotide is an unmodified ribonucleotide. In some embodiments, a ribonucleotide is a 3'-ribonucleotide. In some embodiments, a ribonucleotide is a 5'-ribonucleotide. In some embodiments, a modified or unmodified nucleotide may be further modified optionally.

[0038] A natural nucleotide consists of a natural base, a natural sugar moiety, and a phosphoric acid moiety. As used herein, a natural nucleotide refers to an adenine ribonucleotide, adenine deoxyribonucleotide, guanine ribonucleotide, guanine deoxyribonucleotide, cytosine ribonucleotide, cytosine deoxyribonucleotide, uracil ribonucleotide, thymine ribonucleotide or thymine deoxyribonucleotide. “Ribonucleotide” means a nucleotide having a hydroxy at the 2' position of the sugar portion of the nucleotide. “Deoxyribonucleoside” means a nucleotide having a hydrogen at the 2' position of the sugar portion of the nucleotide.

[0039] A natural base of RNA includes A (adenine), G (guanine), C (cytosine), U (uracil), and T (thymine).

[0040] As used herein, unless otherwise specified, nucleotide “G,” “C,” “A,” “T,” and “U” each refers to a natural or a modified nucleotide that contains guanine, cytosine, adenine, thymidine, and uracil as a base, respectively.

[0041] A nucleotide may be replaced by an analogue thereof, including natural and non-natural analogues. Examples of analogues of guanosine includes 6-thioguanosine, 8-azaguanosine, 8-oxoguanosine, 2-aminopurine riboside, and the like. Examples of analogues of adenosine includes cordycepin (3'-deoxyadenosine), N6-benzyladenosine, 2-chloroadenosine, and the like. Examples of analogues of cytidine includes gemcitabine (2',2'-difluoro-2'-deoxycytidine), cytarabine (1-β-D-arabinofuranosylcytosine), decitabine (5-aza-2'-deoxycytidine), and the like. Examples of analogues of uridine includes 5-fluorouridine, pseudo-uridine, 5-bromo-uridine, 4-thiouridine, 5-azido-uridine and the like.

[0042] As used herein, a “ribonucleic acid” (RNA) is a carrier of genetic information that exists in cells and some viruses and viroids. RNA consists of ribonucleotides linked by internucleotide linkages forming a strand, including single-stranded RNA and double-stranded RNA. Natural internucleotide linkage is phosphodiester bond.

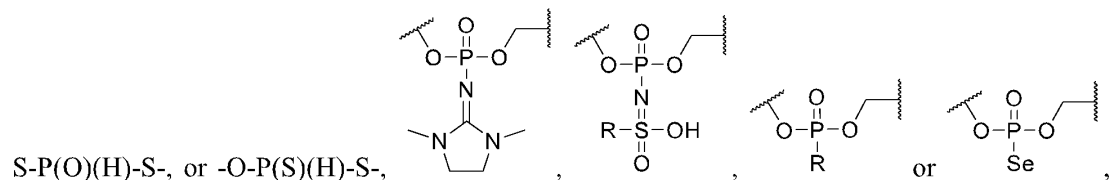
[0043] As used herein, the term “modified nucleotide” refers to a nucleotide having at least a modified base, a modified sugar or a modified phosphate group that can form a modified internucleotide linkage. In some embodiments, a modified nucleotide may comprise one, two, three or more modifications. In some embodiments, a nucleotide may comprise one modification. In some embodiments, a nucleotide may comprise two modifications. In some embodiments, a nucleotide may comprise three modifications.

[0044] Examples of modified bases include, but not limited to, hypoxanthine (I), xanthine, 7-methylguanine, 5, 6-dihydrouracil, 5-methylcytosine (m5C), 5-hydroxymethoylcytosine, N6-methyladenosine (m6A), 3-methyluridine (m3U), 5-methyluridine (m5U), pseudouridine, 2-thiouridine (s2U) and 5-propyluridine (5-pU).

[0045] Examples of modified sugars include, but not limited to, 2'-sugar modified, 3'-sugar modified and 5'-sugar modified, such as 2'-OMe (2'-O-methyl) modification, 2'-F (2'-deoxy-2'-fluoro) modification, 2'-O-MOE (2'-O-methoxyethyl) modification, 2'-deoxy (2'-d) modification, 5'-morpholine (5'-Mo) modification, unlocked nucleic acid (UNA) modification, glycol nucleic acid (GNA) modification, locked nucleic acid (LNA) modification, tricyclo-DNA (tcDNA) modification, (S)-constrained ethyl bridged nucleic acid ((S)-cEt-BNA) modification, 5'-(E)-vinylphosphate (VP) modification, 2'-O-C16 modification, 2'-C16 modification, conjugating with invert abasic nucleotide (invAB) at 5' end or 3' end, replacing with invert abasic nucleotide (invAb), replacing with 2,4-difluorotolyl ribonucleotide (rF), replacing with (S)-glycerol nucleic acid, replacing with inosine (I), conjugating with M03 at 5' end or 3' end, conjugating with M06 at 5' end or 3' end and conjugating with ligand such as a GalNAc ligand, a lipophilic ligand or other ligands targeting a receptor that could facilitate the endocytosis of siRNA conjugate, such as TfR targeting ligands, LDL-R targeting ligands, or integrin targeting ligands.

[0046] Examples of modified internucleotide linkages include, but not limited to, methylphosphonate (MP), methoxypropyl methylphosphonate (MOP), phosphorothioate (PS), phosphorodithioate (PS2), phosphoroselenoate, phosphorodiselenoate, phosphoroanilothioate, phosphoraniladate, phosphoramidate, -O-P(O)(OR)-O-, -O-P(S)(OR)-O-, -O-P(S)(SR)-O-, -S-P(O)(OR)-O-, -O-P(O)(OR)-S-, -S-P(O)(OR)-S-, -O-P(S)(OR)-S-, -S-P(S)(OR)-O-, -O-P(O)(R)-O-, -O-P(S)(R)-O-, -S-P(O)(R)-O-, -S-P(S)(R)-O-, -S-P(O)(R)-S-, -O-P(S)(R)-S-, -O-P(O)(OH)-O-, -O-P(S)(OH)-O-, -O-P(S)(SH)-O-, -S-P(O)(OH)-O-, -O-P(O)(OH)-S-, -S-P(O)(OH)-S-, -O-

P(S)(OH)-S-, -S-P(S)(OH)-O-, -O-P(O)(H)-O-, -O-P(S)(H)-O-, -S-P(O)(H)-O-, -S-P(S)(H)-O-, -



and the like, wherein R is independently selected from H, optional substituted alkyl, optional substituted cycloalkyl, and optional substituted aryl. See, *e.g.*, LaPlanche, et al., *Nucleic Acids Res.* 14:9081 (1986); Stec, et al., *J. Am. Chem. Soc.* 106:6077 (1984), Stein, et al., *Nucleic Acids Res.* 16:3209 (1988), Zon, et al., *Anti Cancer Drug Design* 6:539 (1991); Zon, et al., *Oligonucleotides and Analogues: A Practical Approach*, pp. 87-108 (F. Eckstein, Ed., Oxford University Press, Oxford England (1991)); Stec, et al., U.S. Pat. No. 5,151,510; Uhlmann and Peyman, *Chemical Reviews* 90:543 (1990); *Handbook of RNA Biochemistry: Second, Completely Revised and Enlarged Edition* (Roland K. Hartmann, Albrecht Bindereif, Astrid Schöen, Eric Westhof, Ed., Wiley-VCH Verlag GmbH & Co. KGaA (2014)); *Nucleic Acids in Medicinal Chemistry and Chemical Biology: Drug Development and Clinical Applications* (Lihe Zhang, Xinjing Tang, Zhen Xi, Jyoti Chattopadhyaya, Ed., John Wiley & Sons, Inc. (2023)). In some embodiments, the modified internucleotide linkage does not contain phosphorous but rather contains peptide bonds, for example in a peptide nucleic acid (PNA), or linking groups including carbamate, amides, and linear and cyclic hydrocarbon groups. In some embodiments, the modified internucleotide linkage is a phosphothioate linkage.

[0047] As used herein, the terms “small interfering RNA”, “siRNA”, and “iRNA agent” are used interchangeably to agents that can mediate silencing of a target RNA, *e.g.*, mRNA, *e.g.*, a transcript of a gene that encodes a protein. For convenience, such mRNA is also referred to herein as mRNA to be silenced. Such a gene is also referred to as a target gene. In general, the RNA to be silenced is an endogenous gene or a pathogen gene. In addition, RNAs other than mRNA, *e.g.*, tRNAs, and viral RNAs, can also be targeted.

[0048] Usually, an siRNA comprises a double-stranded region of less than 60, 50, 40 or 30 complementary base pairs; preferably, an siRNA comprises a double-stranded region of 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22 or 23 complementary base pairs.

[0049] In some embodiments of the present application, the sense and/or antisense strands of the siRNA independently have a length of 15-35 nucleotides, forming a complementary double-stranded region with a length of 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, or 25 base pairs. In some embodiments of the present application, the sense strand of the siRNA has a length of 15-35 nucleotides, the antisense strand of the siRNA has a length of 25-35 nucleotides, forming a complementary double-stranded region with a length of 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, or 23 base pairs. In some embodiments of the present application, the sense strand of the siRNA has a length of 17-23 nucleotides, the antisense strand of the siRNA has a length of 25-30 nucleotides. In some embodiments of the present application, the sense strand of the siRNA has a length of 21-23 nucleotides, the antisense strand of the siRNA has a length of 26-30 nucleotides. In some embodiments of the present application, the sense and antisense strands of the siRNA are fully complementary with a length of 15-30 base pairs. In some embodiments of the present application, the sense and antisense strands of the siRNA are fully complementary with a length of 17, 18, 19, 20, 21, 22, or 23 base pairs.

[0050] In some embodiments, the siRNA (*e.g.*, the anti-sense strand) is sufficiently complementary to the target RNA such that the siRNA silences the target RNA, for example inhibits production of protein encoded by the target RNA.

[0051] The term “sufficiently complementary” is used to indicate a sufficient degree of complementarity such that stable and specific binding occurs between an siRNA, in particular its anti-sense strand, and a target RNA molecule. Such degree of complementarity is sufficient to avoid non-specific binding of the siRNA to non-target sequences under conditions in which specific binding is desired, *i.e.*, under physiological conditions in the case of assays or therapeutic treatment, or in the case of *in vitro* assays, under conditions in which the assays are performed. The non-target sequences typically differ by at least 4 nucleotides or more. In some embodiments, the non-target sequences differ by at least 8 nucleotides or more. Alternatively, or additionally, such degree of complementarity is sufficient to allow the siRNA to silence target RNA, for example to reduce production of protein encoded by the target mRNA.

[0052] The term “complementary” with respect to two nucleotide sequences means they pair in an antiparallel arrangement, allowing two single-stranded nucleotide sequences to specifically hybridize. The extent to which one oligonucleotide complements another, known as

complementarity, is measured by the percentage of bases in each strand that can form hydrogen bonds with one another, as dictated by established base-pairing rules. The oligonucleotide sequences do not need to be entirely complementary (i.e., perfectly complementary) to their corresponding nucleic acid sequences. For example, a first nucleotide sequence may be deemed complementary to a second nucleotide sequence if it exhibits a certain level of sequence complementarity, such as at least 70%, 75%, 80%, 85%, 90%, 95%, 96%, 97%, 98%, or 99%. In an illustrative embodiment, 18 out of 20 nucleobases of a first nucleotide sequence align with a corresponding region of a second nucleotide sequence, achieving 90 percent complementarity. The non-complementary nucleobases are also referred to as mismatches and might be grouped or interspersed among the complementary bases and do not need to be adjacent to each other or to the complementary nucleobases.

[0053] As used herein, “mismatch” includes, but is not limited to:

- 1) pairing between two opposite (independently natural or non-natural) nucleotides other than A-T, A-U or G-C;
- 2) no hydrogen bond forming between two opposite (independently natural or non-natural) nucleotides;
- 3) lack of bases between two opposite (independently natural or non-natural) nucleotides.

[0054] In some embodiments, mismatches include wobble base pair and Hoogsteen base pair.

[0055] The term “exactly complementary” is intended to mean that a first nucleotide sequence and a second nucleotide sequence form a hybrid made exclusively of Watson-Crick base pairs in the region of exact complementarity.

[0056] A “sufficiently complementary” oligonucleotide can include an internal region (*e.g.*, of at least 7, 8, 9, or 10 nucleotides) that is exactly complementary to a target RNA.

[0057] As used herein, the term “oligonucleotide” refers to a nucleic acid molecule (RNA or DNA) for example of length less than 100, 200, 300, or 400 nucleotides.

[0058] In this application, “monomer” refers to a class of compounds that can be assembled into a ribonucleic acid chain and can perform certain functions. In this application, “monomer” includes, but is not limited to, natural nucleotides, unnatural nucleotides (such as modified nucleotides, nucleotide analogs, abasic deoxyribonucleotides, GNA, LNA, etc.), blocking groups, M03 as disclosed herein and M06 as disclosed herein.

[0059] In this application, “linkage” refers to the connection between the residues of two monomers (such as nucleotides), such as nucleotide residues, via a single bond or a linking group (such as through a phosphodiester linkage, phosphorothioate linkage, or phosphordithioate linkage). In some embodiments, the linkage refers to the connection between the residues of two monomers (such as nucleotides) via an internucleotide linkage, such as phosphodiester linkage, phosphorothioate linkage, or phosphordithioate linkage.

[0060] As used herein, the term “internucleotide linkage” refers the linkage (*e.g.*, a bond or a linking group) between two moieties of the oligonucleotide disclosed herein such as between two monomers, including linkage between to nucleotides, between one nucleotide and one ligand, between one nucleotide and one blocking group and between one nucleotide and an abasic nucleotide of the oligonucleotide disclosed herein.

[0061] The term “alternating motif” or “alternative pattern” as used herein refers to a motif having one or more modifications, each modification occurring on alternating nucleotides of one strand. The alternating nucleotide may refer to one per every other nucleotide or one per every three nucleotides, or a similar pattern. For example, if A, B and C each represent one type of modification to the nucleotide, the alternating motif can be “ABABABABABAB...”, “AABBAABBAABB...”, “AABAABAABAAB...”, “AAABAAABAAAB...”, “AAABBBAABBB...”, or “ABCABCABCABC...”. *etc.*

[0062] The type of modifications contained in the alternating motif may be the same or different. For example, if A, B, C, D each represent one type of modification on the nucleotide, the alternating pattern, *i.e.*, modifications on every other nucleotide, may be the same, but each of the sense strand or antisense strand can be selected from several possibilities of modifications within the alternating motif such as “ABABA...”, “ACACAC...”, “BDBDBD...” or “CDCDCD...”, *etc.*

[0063] As used herein, the term “conjugate” or “conjugating” as a verb refers to residues of two molecules (*e.g.*, two nucleotides) such as residues of two nucleotides are linked via a bond (*e.g.*, a single bond) or a linking group (*e.g.*, phosphodiester, phosphorothioate or phosphordithioate). Correspondingly, the term “conjugate” as a noun refers to a compound or complex formed by the covalent connection between various chemical moieties. For example, “double-stranded RNA conjugate” represents a compound or complex formed by the covalent connection of one or more chemical moieties (such as conjugate groups, ligand groups, or delivery systems) to double-

stranded RNA. As used herein, a double-stranded RNA conjugate disclosed herein may also referred to as “conjugate” for brevity, which should be understood as the full term “double-stranded RNA conjugate” based on the context. In some embodiments, the delivery system, ligand group, or conjugate group can be attached to any available position of any nucleotide of the oligonucleotide agent, including the phosphate group, the sugar ring (including covalent connection of the delivery system, ligand group, or conjugate group to the atom at the 3' or 5' position of the sugar ring of a nucleotide through a phosphodiester linkage), the 2'-hydroxyl group, the 5'-hydroxyl group, and the base. In some embodiments, the ligand group or delivery system can be attached to the 3'-position of a nucleotide, and unless otherwise specified, the nucleotides are connected by 3'-5' phosphodiester linkages. In some embodiments, the delivery system, ligand group, or conjugate group can also be attached to the 2'-position of a nucleotide, and unless otherwise specified, the nucleotides are connected by 2'-5' phosphodiester bonds.

[0064] *Ligand*

[0065] The double-stranded oligonucleotide agent disclosed herein may optionally be conjugated to one or more ligands. The ligand can be attached to the sense strand, antisense strand or both strands, at the 3'-end, 5'-end or both ends. In some embodiments, the ligand may be conjugated to the sense strand, in particular, the 3'-end of the sense strand. In some embodiments, the ligand is conjugated to the anti-sense strand, in particular, the 5'-end of the anti-sense strand.

[0066] A wide variety of entities can be coupled to the oligonucleotides disclosed herein. Preferred moieties are ligands, which are coupled, preferably covalently, either directly or indirectly via an intervening tether.

[0067] In preferred embodiments, a ligand alters the distribution, targeting or lifetime of the molecule into which it is incorporated. In preferred embodiments a ligand provides an enhanced affinity for a selected target, *e.g.*, molecule, cell or cell type, compartment, receptor *e.g.*, a cellular or organ compartment, tissue, organ or region of the body, as, *e.g.*, compared to a species absent such a ligand. Ligands providing enhanced affinity for a selected target are also termed targeting ligands.

[0068] Some ligands can have endosomolytic properties. The endosomolytic ligands promote the lysis of the endosome and/or transport of the composition disclosed herein, or its components, from the endosome to the cytoplasm of the cell.

[0069] Ligands can improve transport, hybridization, and specificity properties and may also improve nuclease resistance of the resultant natural or modified oligoribonucleotide, or a polymeric molecule comprising any combination of monomers described herein and/or natural or modified ribonucleotides.

[0070] Ligands in general can include therapeutic modifiers, *e.g.*, for enhancing uptake; diagnostic compounds or reporter groups *e.g.*, for monitoring distribution; cross-linking agents; and nuclease-resistance conferring moieties. General examples include lipids, steroids, vitamins, sugars, proteins, peptides, polyamines, and peptide mimics. Ligands can also include targeting groups, *e.g.*, a cell or tissue targeting agent, *e.g.*, a lectin, glycoprotein, lipid or protein, *e.g.*, an antibody, that binds to a specified cell type such as a kidney cell.

[0071] Other examples of ligands include dyes, intercalating agents (*e.g.*, acridines), cross-linkers (*e.g.*, psoralene, mitomycin C), porphyrins (*e.g.*, TPPC4, texaphyrin, Sapphyrin), polycyclic aromatic hydrocarbons (*e.g.*, phenazine, dihydrophenazine), artificial endonucleases or a chelator (*e.g.*, EDTA), lipophilic molecules, *e.g.*, cholesterol, cholic acid, adamantane acetic acid, 1-pyrene butyric acid, dihydrotestosterone, 1,3-bis-O-(hexadecyl)glycerol, geranyloxyhexyl group, hexadecylglycerol, borneol, menthol, 1,3-propanediol, heptadecyl group, palmitic acid, myristic acid, O3-(oleoyl)lithocholic acid, O3-(oleoyl)cholenic acid, dimethoxytrityl, or phenoxazine) and peptide conjugates (*e.g.*, antennapedia peptide, Tat peptide), alkylating agents, phosphate, amino, mercapto, PEG (*e.g.*, PEG-40K), MPEG, [MPEG]₂, polyamino, alkyl, substituted alkyl, radiolabeled markers, enzymes, haptens (*e.g.*, biotin), transport/absorption facilitators (*e.g.*, aspirin, vitamin E, folic acid), synthetic ribonucleases (*e.g.*, imidazole, bisimidazole, histamine, imidazole clusters, acridine-imidazole conjugates, Eu³⁺ complexes of tetraazamacrocycles), dinitrophenyl, HRP, or AP.

[0072] Ligands can be proteins, *e.g.*, glycoproteins, or peptides, *e.g.*, molecules having a specific affinity for a co-ligand, or antibodies *e.g.*, an antibody, that binds to a specified cell type such as a cancer cell, endothelial cell, or bone cell. The ligand can be a substance, *e.g.*, a drug, which can increase the uptake of the iRNA agent into the cell, for example, by disrupting the cell's cytoskeleton, *e.g.*, by disrupting the cell's microtubules, microfilaments, and/or intermediate filaments. The ligand can increase the uptake of the oligonucleotide into the cell by activating an inflammatory response, for example. Exemplary ligands that would have such an effect include

tumor necrosis factor alpha (TNFalpha), interleukin-1 beta, or gamma interferon. In one aspect, the ligand is a lipid or lipid-based molecule. A lipid-based ligand can be used to modulate, *e.g.*, control the binding of the conjugate to a target tissue. In another aspect, the ligand is a moiety, *e.g.*, a vitamin, which is taken up by a target cell, *e.g.*, a proliferating cell. In another aspect, the ligand is a cell-permeation agent, preferably a helical cell-permeation agent. The ligand can be a peptide or peptidomimetic. In one embodiment, a targeting peptide can be an amphipathic α -helical peptide.

[0073] The targeting ligand can be any ligand that is capable of targeting a specific receptor. Examples are: folate, GalNAc, galactose, mannose, mannose-6P, clusters of sugars such as GalNAc cluster, mannose cluster, galactose cluster, or an aptamer. A cluster is a combination of two or more sugar units. The targeting ligands also include integrin receptor ligands, Chemokine receptor ligands, transferrin, biotin, serotonin receptor ligands, PSMA, endothelin, GCPH, somatostatin, LDL and HDL ligands. The ligands can also be based on nucleic acid, *e.g.*, an aptamer. The aptamer can be unmodified or have any combination of modifications disclosed herein.

[0074] Other ligand conjugates amenable to the invention are described in U.S. patent applications U.S. Patent Application No. 10/916,185; U.S. Patent Application No. 10/946,873; U.S. Patent Application No. 10/833,934; U.S. Patent Application No. 11/115,989 and U.S. Patent Application No. 11/944,227, which are incorporated by reference in their entireties for all purposes.

[0075] When two or more ligands are present, the ligands can all have same properties, all have different properties or some ligands have the same properties while others have different properties. For example, a ligand can have targeting properties, have endosomolytic activity or have PK modulating properties. In some embodiments, all the ligands have different properties.

[0076] Ligands can be coupled to the oligonucleotides at various places, for example, 3'-end, 5'-end, and/or at an internal position. In preferred embodiments, the ligand is attached to the oligonucleotides via an intervening tether. The ligand or tethered ligand may be present on a monomer when said monomer is incorporated into the growing strand. In some embodiments, the ligand may be incorporated via coupling to a "precursor" monomer after said "precursor" monomer has been incorporated into the growing strand. For example, a monomer having, *e.g.*, an amino-terminated tether (*i.e.*, having no associated ligand), *e.g.*, TAP-(CH₂)_nNH₂ may be

incorporated into a growing oligonucleotide strand. In a subsequent operation, *i.e.*, after incorporation of the precursor monomer into the strand, a ligand having an electrophilic group, *e.g.*, a pentafluorophenyl ester or aldehyde group, can subsequently be attached to the precursor monomer by coupling the electrophilic group of the ligand with the terminal nucleophilic group of the precursor monomer's tether.

[0077] In another example, a monomer having a chemical group suitable for taking part in Click Chemistry reaction may be incorporated *e.g.*, an azide or alkyne terminated tether/linker. In a subsequent operation, *i.e.*, after incorporation of the precursor monomer into the strand, a ligand having complementary chemical group, *e.g.*, an alkyne or azide can be attached to the precursor monomer by coupling the alkyne and the azide together.

[0078] For double-stranded oligonucleotides, ligands can be attached to one or both strands. In some embodiments, a double-stranded iRNA agent contains a ligand conjugated to the sense strand. In other embodiments, a double-stranded iRNA agent contains a ligand conjugated to the anti-sense strand.

[0079] In some embodiments, ligand can be conjugated to bases, sugars, or internucleotide linkages of nucleic acid molecules. Conjugation to purine bases or derivatives thereof can occur at any position including, endocyclic and exocyclic atoms. In some embodiments, the 2-, 6-, 7-, or 8-positions of a purine base are attached to a conjugate moiety. Conjugation to pyrimidine bases or derivatives thereof can also occur at any position. In some embodiments, the 2-, 5-, and 6-positions of a pyrimidine base can be substituted with a conjugate moiety. Conjugation to sugars of nucleosides can occur at any carbon atom. Example carbon atoms of a sugar moiety that can be attached to a conjugate moiety include the 2', 3', and 5' carbon atoms. The 1' position can also be attached to a conjugate moiety, such as in an abasic residue. Internucleotide linkages can also bear conjugate moieties. For phosphorus-containing linkages (*e.g.*, phosphodiester, phosphorothioate, phosphorodithiotate, phosphoroamidate, and the like), the conjugate moiety can be attached directly to the phosphorus atom or to an O, N, or S atom bound to the phosphorus atom. For amine- or amide-containing internucleotide linkages (*e.g.*, PNA), the conjugate moiety can be attached to the nitrogen atom of the amine or amide or to an adjacent carbon atom.

[0080] Any suitable ligand in the field of RNA interference may be used, although the ligand is typically a carbohydrate *e.g.*, monosaccharide (such as GalNAc), disaccharide, trisaccharide, tetrasaccharide, polysaccharide.

[0081] Linkers that conjugate the ligand to the nucleic acid include those discussed above. For example, the ligand can be one or more GalNAc (N-acetylglucosamine) derivatives attached through a bivalent or trivalent branched linker.

[0082] In some embodiments, the ligand is conjugated to the oligonucleotide (*e.g.*, the 5' end of the anti-sense strand) via an internucleotide linkage, and the internucleotide linkage is optionally modified as described above.

[0083] In some embodiments, the ligand is a GalNAc ligand, a lipophilic ligand or other ligands targeting a receptor that could facilitate the endocytosis of siRNA conjugate, such as TfR targeting ligands, LDL-R targeting ligands, or integrin targeting ligands.

[0084] *Blocking Group*

[0085] The double-stranded oligonucleotide agent disclosed herein may optionally be conjugated to one or more blocking groups. The blocking group can be attached to the sense strand, antisense strand or both strands, at the 3'-end, 5'-end or both ends. In some embodiments, the blocking group is conjugated to the anti-sense strand, in particular, the 5'-end of the anti-sense strand.

[0086] In some embodiments, the blocking group is conjugated to the oligonucleotide (*e.g.*, the 5' end of the anti-sense strand) via an internucleotide linkage, and the internucleotide linkage is optionally modified as described above. In some embodiments, the blocking group is linked to the double-stranded oligonucleotide agent with a phosphorothioate. In some embodiments, the blocking group is linked to the double-stranded oligonucleotide agent with a phosphodiester.

[0087] As used herein, a "blocking group" refers to a group may be conjugated to an oligonucleotide disclosed herein, *e.g.*, at the 5' end of the anti-sense strand, which may reduce or inhibit exonuclease catabolism. In some embodiments, the blocking group may reduce or inhibit the RNA interference effect of the oligonucleotide. In some embodiment, the blocking group is cleaved from the oligonucleotide before providing the RNA interference effect.

[0088] Examples of blocking groups include but not limited to an abasic residue, an inverted abasic residue, M03 and M06.

[0089] **I. Double-Stranded Oligonucleotide Agent**

[0090] The present disclosure provides a double-stranded oligonucleotide agent comprising a sense strand and an anti-sense strand. The sense strand and the anti-sense strand form a double-stranded portion and a 5' extension in the anti-sense strand. In certain embodiments, the 5' extension has a length of at least three nucleotides.

[0091] The inventors surprisingly discovered that 5' extension in the anti-sense strand can be designed such that it can be cleaved at a specific cleavage site. In particular, the double-stranded oligonucleotide agent provided herein is particularly advantageous in that the 5' extension is cleavable from the most 3' nucleotide of the 5' extension. In other words, when the double-stranded oligonucleotide agent reaches the target tissue, it can be converted into the cleaved product. Such a cleaved double-stranded oligonucleotide agent is found to be capable of inhibiting expression of a target gene via RNA interference.

[0092] As used herein, the phrase "RNA interference" refers to the ability to silence, in a sequence specific manner, a target RNA, by a double-stranded short interfering RNA (siRNA). The first step in RNA interference (RNAi) involves activating the RNA-induced silencing complex (RISC), which necessitates the degradation of the sense strand of the double-stranded RNA (dsRNA) duplex. The sense strand serves as the initial substrate for RISC and is cleaved by Argonaute 2 (Ago2) in the middle of the duplex region. Once the cleaved 5'-end and 3'-end fragments of the sense strand are removed by Ago2, the RISC is activated by the antisense strand (Rand et al. (2005) Cell 123, 621).

[0093] In some embodiments, the double-stranded oligonucleotide agents provided herein have enhanced activity in inhibition of the target gene expression than a reference siRNA having otherwise comparable anti-sense sequence except for the 5' extension or the cleavage region provided herein.

[0094] In some embodiments, the double-stranded oligonucleotide agents provided herein comprises, essentially consists of or consists of ribonucleic acids (RNA). In some embodiments, the sense strand of the double-stranded oligonucleotide agents provided herein comprises, essentially consists of or consists of RNA. In some embodiments, the anti-sense strand of the double-stranded oligonucleotide agents provided herein comprises, essentially consists of or consists of RNA.

[0095] In some embodiments, the double-stranded oligonucleotide agents provided herein can be converted into an siRNA, optionally after delivery to a target tissue or a target cell. In some embodiments, the double-stranded oligonucleotide agents provided herein is can be converted into an siRNA by an enzyme, or optionally by RNAase III, or optionally by dicer. In some embodiments, the cleaved double-stranded oligonucleotide agents provided herein is an siRNA.

[0096] a. Double-stranded Portion and 5' Extension

[0097] In certain embodiments, the double-stranded portion is formed by base pairing between a first fragment in the anti-sense strand and a second fragment in the sense strand, wherein the first fragment and the second fragment are of equal length.

[0098] In some embodiments, the base pairing between the first fragment and the second fragment can include base pairs in a Watson-Crick manner and/or in any other manner that allows the formation of a stable double strand. In Watson-Crick base pairing, adenine (A) is paired with thymine (T) in DNA and with uracil (U) in RNA; Guanine (G) is paired with cytosine (C). Base pairs can also be formed by non-Watson-Crick base pairs, which include, but not limited to, G-U wobble base pair and Hoogsteen base pair. In some embodiments, a nucleotide containing hypoxanthine as its base may be paired with a nucleotide base containing adenine, cytosine, or uracil. In some embodiments, a nucleotide containing uracil, guanine, or adenine may be replaced by a nucleotide containing, for example, inosine (as used herein, "I" may indicate a hypoxanthine base, inosine, or an inosine-containing nucleotide depending on its context). This substitution is referred to "I modification". In some embodiments, adenine and cytosine anywhere in the oligonucleotide can be replaced by guanine and uracil, respectively, to form a G-U wobble base pair with the target mRNA.

[0099] In some embodiments, the base pairs formed in the double-stranded portion can comprise or consist essentially of Watson-Crick base pairing, and/or non-Watson-Crick base pairs such as G-U wobble base pair and Hoogsteen base pair, or a combination thereof.

[0100] In some embodiments, the first fragment in the sense strand and the second fragment in the anti-sense strand are of equal length, and are complementary to form the double-stranded portion. In some embodiments, the first fragment and the second fragment are at least 80%, 85%, 90%, 95%, or 100% complementary.

[0101] In certain embodiments, the double-stranded portion is of 15 to 27 nucleotide pairs in length. In certain embodiments, the double-stranded portion has a length of 16 to 27 nucleotide pairs, 16 to 26 nucleotide pairs, 16 to 25 nucleotide pairs, 16 to 24 nucleotide pairs, 16 to 23 nucleotide pairs, 16 to 22 nucleotide pairs, 16 to 21 nucleotide pairs, 16 to 20 nucleotide pairs, 17 to 23 nucleotide pairs, 17 to 22 nucleotide pairs, 17 to 21 nucleotide pairs, 17 to 20 nucleotide pairs, 18 to 23 nucleotide pairs, 18 to 22 nucleotide pairs, 18 to 21 nucleotide pairs, 18 to 20 nucleotide pairs, 19 to 23 nucleotide pairs, 19 to 22 nucleotide pairs, 19 to 21 nucleotide pairs. In certain embodiments, the double-stranded portion has a length of 16 nucleotide pairs, 17 nucleotide pairs, 18 nucleotide pairs, 19 nucleotide pairs, 20 nucleotide pairs, 21 nucleotide pairs, 22 nucleotide pairs, or 23 nucleotide pairs.

[0102] In certain embodiments, the sense strand of the double-stranded oligonucleotide agent has a length of 15 to 35 nucleotides (nt), 16 to 35, 16 to 30, 16 to 27, 16 to 26, 16 to 25, 16 to 24, 16 to 23, 16 to 22, 16 to 21, 17 to 35, 17 to 30, 17 to 25, 17 to 23, 17 to 22, 17 to 21, 18 to 35, 18 to 30, 18 to 25, 18 to 23, 18 to 22, 18 to 21, 19 to 35, 19 to 30, 19 to 25, 19 to 23, 19 to 22, 19 to 21, 20 to 35, 20 to 30, 20 to 25, 20 to 23, 21 to 35, 21 to 30, 21 to 25, 21 to 23, such as 35, 30, 29, 28, 27, 26, 25, 24, 23, 22, 21, 20, 19, 18, 17, 16, or 15 nt.

[0103] In certain embodiments, the sense strand of the double-stranded oligonucleotide agent has a length of 15 to 23, 15 to 22, or 15 to 21 nt.

[0104] In certain embodiments, the 5' extension has a length of at least 3, 4, 5, 6, 7 or more nucleotides.

[0105] In certain embodiments, the double-stranded oligonucleotide agent further comprises a 3' extension in the anti-sense strand. In certain embodiments, the 3' extension has a length of at least 1, 2, or more nucleotides. In certain embodiments, the 3' extension can be an overhang having a length of at least 1, 2, 3, 4, or 5 nucleotides.

[0106] An extension (*e.g.*, 3' extension) can be 1-6 nucleotides in length, for instance 2-6 nucleotides in length, 1-5 nucleotides in length, 2-5 nucleotides in length, 1-4 nucleotides in length, 2-4 nucleotides in length, 1-3 nucleotides in length, 2-3 nucleotides in length, or 1-2 nucleotides in length. The extensions (*e.g.*, 5' extension or 3' extension) can be the result of one strand being longer than the other, or the result of two strands of the same length being staggered.

[0107] In one embodiment, the nucleotides in the extension region can each independently be a modified or unmodified nucleotide including, but not limited to 2'-sugar modification, such as, 2'-F, or 2'-OMe.

[0108] The 5'- or 3'-extensions at the sense strand, antisense strand or both strands may be phosphorylated. In some embodiments, the extension region contains two nucleotides having a phosphorothioate linkage between the two nucleotides, where the two nucleotides can be the same or different. In one embodiment, this 3'-extension is present in the antisense strand.

[0109] In certain embodiments, the anti-sense strand of the double-stranded oligonucleotide agent has a length of at least 25 to 35, 25 to 34, 25 to 33, 25 to 32, 25 to 31, 25 to 30, 25 to 29, 25 to 28, 25 to 27, 25 to 26, 26 to 35, 26 to 34, 26 to 33, 26 to 32, 26 to 31, 26 to 30, 26 to 29, 26 to 28, 26 to 27 nt. In certain embodiments, the anti-sense strand of the double-stranded oligonucleotide agent has a length of 25 nt, 26 nt, 27 nt, 28 nt, 29 nt, 30 nt.

[0110] In certain embodiments, the first fragment comprises a targeting region which is sufficiently complementary to part of a target RNA, optionally a mRNA encoding the target gene.

[0111] In one embodiment, the targeting region disclosed herein is at least 90%, at least 95%, or 100% complementary to the part of the target RNA. In some embodiments, the targeting region binds to the target RNA and forms a duplex made exclusively of Watson-Crick base pairs in the region of complementarity. In one embodiment, the target region can include an internal region (*e.g.*, of at least 10 nucleotides) that is 100% complementary to a target RNA. In one embodiment, the internal region 100% complementary to a target RNA can be the seed region which is from position 2 to position 8 from the 5' end of the targeting region in the anti-sense strand. In another embodiment, the target region is 100% complementary to a target RNA, *e.g.*, the target RNA and the dsRNA duplex agent anneal, for example to form a hybrid made exclusively of Watson-Crick base pairs in the region of exact complementarity. Moreover, in some embodiments, the dsRNA agent of the invention specifically discriminates a single-nucleotide difference. In this case, the dsRNA agent only mediates RNAi if exact complementarity is found in the region (*e.g.*, within 7 nucleotides of) the single-nucleotide difference.

[0112] **b. Cleavage Region**

[0113] In certain embodiments, the anti-sense strand of the double-stranded oligonucleotide agent comprises a cleavage region that allows specific cleavage from the most 3' nucleotide of the 5' extension.

[0114] In some embodiments, a double-stranded oligonucleotide agent disclosed herein (*e.g.*, the 5' extension) undergoes cleavage mediated by an enzyme (*e.g.*, an endonuclease) before mediates RNAi. In some embodiments, the cleavage is mediated by a ribonuclease (RNase). In some embodiments, the cleavage is mediated by an endonuclease. In some embodiments, the cleavage is mediated by a RNAase III. RNase III represents a class of endoribonucleases producing small RNAs for RNA silencing pathways. In some embodiments, the RNAase III is dicer. In some embodiments, the cleavage is a specific cleavage.

[0115] As used herein, the term "specific" or "specifically" with respect to cleavage of an oligonucleotide agent (*e.g.*, an siRNA agent), refers to controlled or selective cleavage at a specific position, *i.e.*, between two desired nucleotides of the oligonucleotide strand undergoing specific cleavage. The products of a specific cleavage may comprise a plurality of cleavage products, wherein the desired cleavage product (*i.e.*, the product obtained by cleavage at the specific or desired position) is the most abundant cleavage product. In some embodiments, the desired cleavage product counts for at least 30%, 40%, 50%, 60%, 70%, 80% or 90% of all cleavage products (by molar or weight). In some embodiments, the amount of desired cleavage product (by molar or weight) is at least 2-fold, 3-fold, 4-fold, 5-fold, 6-fold, 7-fold, 8-fold, 9-fold, 10-fold of any other cleavage products. While not wishing to be bound by theory, a specific cleavage is based on the recognition of a certain sequence, features, motifs or a combination thereof of the oligonucleotide agent undergoing cleavage by the enzyme mediating the cleavage.

[0116] In certain embodiments, the cleavage region comprises a nucleotide sequence set forth in Formula A: (3'-5') X2-Y-Z, which is cleavable between X2 and Y, wherein X2 is the most 5' nucleotide of the first fragment and Y-Z are the two most 3' nucleotides of the 5' extension.

[0117] In certain embodiments, the cleavage region further comprises a fourth nucleotide (N1) which is the third nucleotide from the 3' end of the 5' extension, and the cleavage region comprises a nucleotide sequence set forth in Formula B: (3'-5') X2-Y-Z-N1, which is cleavable between X2 and Y.

[0118] In certain embodiments, the cleavage region further comprises a third fragment (N), the third fragment comprises at least one nucleotide, wherein N1 is the most 3' nucleotide of the third fragment, and the cleavage region comprises a nucleotide sequence set forth in Formula B': (3'-5') X2-Y-Z-N, which is cleavable between X2 and Y, the length of N is 1-10 nucleotides, preferably the length of N is 1-5 nucleotides, further preferably the length of N is 1 nucleotides.

[0119] Without wishing to be bound by any theory, it is believed that the enzymatic cleavage can be determined using target tissue (*e.g.*, liver, eye, lung, kidney, brain, spin cord, muscle, fat, *etc.*) homogenate processing reaction assay, see for example the methods described in Example 4.6, or using Dicer Endonuclease Treatment assay.

[0120] Without wishing to be bound by any theory, it is believed that certain nucleotides at certain positions at the cleavage region provide superior effects.

[0121] In certain embodiments, Z is selected from G or A or a natural or non-natural analogue thereof. In certain embodiments, Z is selected from G or a natural or non-natural analogue thereof.

[0122] In certain embodiments, X2 is selected from A or U, or a natural or non-natural analogue thereof.

[0123] Without wishing to be bound by any theory, a double-stranded oligonucleotide agent disclosed herein is capable of being specifically cleaved between X2 and Y, to remove the 5' extension, thereby generating a cleaved double-stranded oligonucleotide agent. The cleaved double-stranded oligonucleotide is believed to be capable of mediating RNAi.

[0124] The cleaved double-stranded oligonucleotide agent has a blunt end at the 5' end of the anti-sense strand, where the first base pair at the 5' end the anti-sense strand composed of X2 and its complementary nucleotide in the sense strand. In some embodiments, such first base pair at the 5' end of the anti-sense strand is an AU base pair.

[0125] In certain embodiments, the Formula A has the nucleotide sequence from 3'-5' selected from the group consisting of: UUG, UAG, AUG, AAG, UUA, UAA, AUA, AAA, UCG, UGG, ACG, AGG, UCA, UGA, ACA, and AGA, or a natural or non-natural analogue thereof.

[0126] In certain embodiments, the nucleotide Y is selected from A or U, or a natural or non-natural analogue thereof.

[0127] In certain embodiments, the Formula A has the nucleotide sequence from 3'-5' selected from the group consisting of: UUG, UAG, AUG, AAG, UUA, UAA, AUA, and AAA.

[0128] In certain embodiments, at least one nucleotide in the cleavage region is a modified nucleotide, preferably all nucleotides in the cleavage region are modified nucleotides.

[0129] In certain embodiments, the modified nucleotide has a modified base, a modified sugar or a modified internucleotide linkage. In certain embodiments, the modified nucleotide has a modified base, and optionally the modified base comprises hypoxanthine (I), xanthine, 7-methylguanine, 5, 6-dihydrouracil, 5-methylcytosine (m5C), 5-hydroxymethoylcytosine, N6-methyladenosine (m6A), 3-methyluridine (m3U), 5-methyluridine (m5U), pseudouridine, 2-thiouridine (s2U) or 5-propyluridine (5-pU).

[0130] In certain embodiments, the modified nucleotide has modified sugar, and optionally the modified sugar comprises 2'-sugar modification, 3'-sugar modification and 5'-sugar modification, such as 2'-OMe (2'-O-methyl) modification, 2'-F (2'-deoxy-2'-fluoro) modification, 2'-O-MOE (2'-O-methoxyethyl) modification, 2'-deoxy (2'-d) modification, 5'-morpholine (5'-Mo) modification, unlocked nucleic acid (UNA) modification, glycol nucleic acid (GNA) modification, locked nucleic acid (LNA) modification, tricyclo-DNA (tcDNA) modification, (S)-constrained ethyl bridged nucleic acid ((S)-cEt-BNA) modification, 5'-(E)-vinylphosphate (VP) modification, 2'-O-C16 modification, conjugating with invert abasic nucleotide (invAB) at 5' end or 3' end, replacing with invert abasic nucleotide (invAb), replacing with 2,4-difluorotolyl ribonucleotide (rF), replacing with (S)-glycerol nucleic acid, replacing with inosine (I), conjugating with M03 at 5' end or 3' end, conjugating with M06 at 5' end or 3' end and conjugating with ligand such as a GalNAc ligand, a lipophilic ligand or other ligands targeting a receptor that could facilitate the endocytosis of siRNA conjugate, such as TfR targeting ligands, LDL-R targeting ligands, or integrin targeting ligands.

[0131] In certain embodiments, the modified nucleotide has modified internucleotide linkage, and optionally the modified internucleotide linkage comprises methylphosphonate (MP), methoxypropyl methylphosphonate (MOP), phosphorothioate (PS), phosphorodithioate (PS2), phosphoroselenoate, phosphorodiselenoate, phosphoroanilothioate, phosphoraniladate, phosphoramidate and PNA.

[0132] In certain embodiments, at least one nucleotide in the cleavage region is 2'-OMe modified or 2'-F modified. In certain embodiments, all nucleotides in the cleavage region are modified nucleotides selected from 2'-OMe modified and 2'-F modified. In certain embodiments, at least

one nucleotide in the cleavage region is 2'-F modified. In certain embodiments, no more than two nucleotides in the cleavage region are 2'-F modified.

[0133] In certain embodiments, Z in the Formula A or Formula B is 2'-F modified, and optionally X2 in the Formula A or Formula B is 2'-F modified. In certain embodiments, N1 in the Formula A or Formula B is 2'-F modified. In certain embodiments, both X2 and Y in the Formula A or Formula B are 2'-OMe modified, and both Z and N1 in the Formula A or Formula B are 2'-F modified. In certain embodiments, all of X2, Y and Z in the Formula A or Formula B are 2'-OMe modified, and N1 in the Formula A or Formula B is 2'-F modified.

[0134] Without wishing to be bound by any theory, it is believed that certain internucleotide linkage at certain positions at the cleavage region provide superior effects.

[0135] In certain embodiments, at least one of the internucleotide linkages between the nucleotides in the cleavage region is not phosphorothioate linkage. In certain embodiments, the internucleotide linkage between X2 and Y is not phosphorothioate linkage. In certain embodiments, the internucleotide linkage between Y and Z is not phosphorothioate linkage. In certain embodiments, none of the internucleotide linkages between the nucleotides in the cleavage region is phosphorothioate linkage. In certain embodiments, at least one of the internucleotide linkages between the nucleotides in the cleavage region is phosphodiester linkage. In certain embodiments, the internucleotide linkage between X2 and Y is phosphodiester linkage. In certain embodiments, the internucleotide linkage between Y and Z is phosphodiester linkage. In certain embodiments, each of the internucleotide linkages between the nucleotides in the cleavage region is phosphodiester linkage.

[0136] c. Modification of the Double-Stranded Oligonucleotide Agent

[0137] In certain embodiments, the double-stranded oligonucleotide agent further comprises at least one phosphorothioate or methylphosphonate internucleotide linkage.

[0138] In certain embodiments, the double-stranded oligonucleotide agent provided herein comprises at least one phosphorothioate or methylphosphonate internucleotide linkage at position 1 and/or 2 of the first fragment (counting from its 5'-end). In certain embodiments, the double-stranded oligonucleotide agent provided herein comprises at least one phosphorothioate or methylphosphonate internucleotide linkage at position 1 and/or 2 of the first fragment (counting from its 3'-end). In certain embodiments, the double-stranded oligonucleotide agent provided

herein comprises phosphorothioate or methylphosphonate internucleotide linkage between the first fragment (*e.g.* the nucleotide at position 1 counting from its 5'-end) and the X2 of Formula A or Formula B.

[0139] In certain embodiments, the double-stranded oligonucleotide agent provided herein comprises at least one phosphorothioate or methylphosphonate internucleotide linkage at positions 1 to 8, or at positions 1 to 6, or at position 1 and/or 2 of the second fragment (counting from its 5'-end). In certain embodiments, the double-stranded oligonucleotide agent provided herein comprises at least one phosphorothioate or methylphosphonate internucleotide linkage at positions 1 to 8, or at positions 1 to 6, or at position 1 and/or 2 of the second fragment (counting from its 5'-end).

[0140] In certain embodiments, each nucleotide of the first fragment of the anti-sense strand is modified. In certain embodiments, each nucleotide of the anti-sense strand is modified. In certain embodiments, each nucleotide of the second fragment of the sense strand is modified. In certain embodiments, each nucleotide of the sense strand is modified.

[0141] In certain embodiments, each nucleotide of the anti-sense strand is either 2'-F modified or 2'-OMe modified; and/or each nucleotide of the sense strand is either 2'-F modified or 2'-OMe modified.

[0142] In certain embodiments, the sense strand further comprises a) a motif of three consecutive 2'-F modified nucleotides, optionally the motif occurs at positions 9, 10 and 11 counting from 5' end nucleotide of the sense strand; and/or b) 2'-F modified nucleotide at position 7 and/or 18 counting from 5' end nucleotide of the sense strand. In certain embodiments, the sense strand further comprises an alternating motif from position 9 to 13 counting from 5' end, optionally 2'-F modification and 2'-OMe modification occur on alternating nucleotides in the motif.

[0143] In certain embodiments, the first fragment of the anti-sense strand and/or the second fragment of the sense strand further comprises an alternating motif where modification occurs on alternating nucleotides in the motif, optionally 2'-F modification occurs on alternating nucleotides in the motif and/or 2'-OMe modification occurs on alternating nucleotides in the motif.

[0144] In certain embodiments, the first fragment of the anti-sense strand and/or the second fragment of the sense strand comprises modification selected from the group consisting of 2'-OMe (2'-O-methyl) modification, 2'-F (2'-deoxy-2'-fluoro) modification, 2'-O-MOE (2'-O-

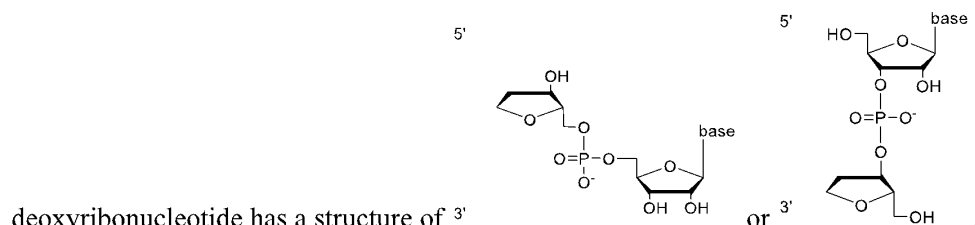
methoxyethyl) modification, 2'-deoxy (2'-d) modification, 5'-morpholine (5'-Mo) modification, unlocked nucleic acid (UNA) modification, glycol nucleic acid (GNA) modification, locked nucleic acid (LNA) modification, tricyclo-DNA (tcDNA) modification, (S)-constrained ethyl bridged nucleic acid ((S)-cEt-BNA) modification, 5'-(E)-vinylphosphate (VP) modification, 2'-O-C16 modification, conjugating with invert abasic nucleotide (invAB) at 5' end or 3' end, replacing with invert abasic nucleotide (invAb), replacing with 2,4-difluorotolyl ribonucleotide (rF), replacing with (S)-glycerol nucleic acid and replacing with inosine (I).

[0145] d. Blocking Group

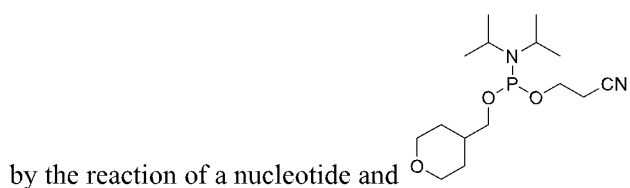
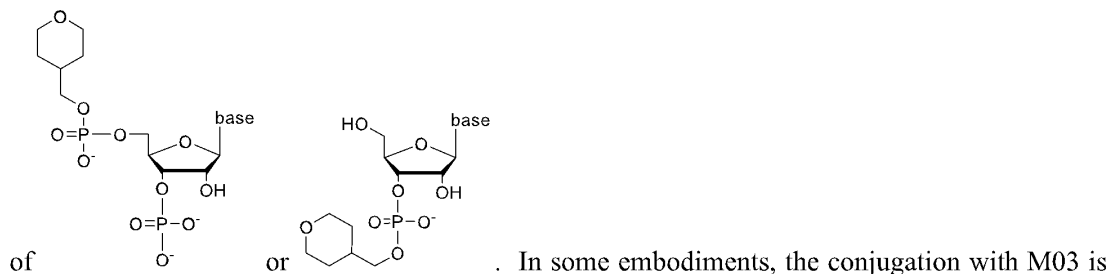
[0146] The double-stranded oligonucleotide agent disclosed herein may optionally be linked to one or more blocking groups. The blocking group can be attached to the sense strand, antisense strand or both strands, at the 3'-end, 5'-end or both ends. In some embodiments, the blocking group is linked to the anti-sense strand, in particular, the 5'-end of the anti-sense strand.

[0147] In some embodiments, the blocking group is linked to the oligonucleotide (*e.g.*, the 5' end of the anti-sense strand) via an internucleotide linkage, and the internucleotide linkage is optionally modified as described above. In some embodiments, the blocking group is linked to the double-stranded oligonucleotide agent with a phosphorothioate. In some embodiments, the blocking group is linked to the double-stranded oligonucleotide agent with a phosphodiester.

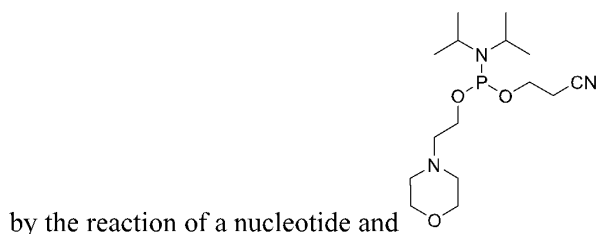
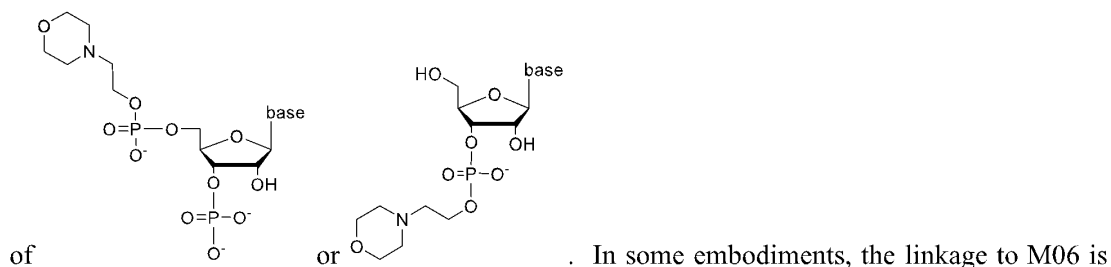
[0148] For example, a nucleotide linked (*e.g.*, linked at 5' end or 3' end) to an inverted abasic



[0149] For example, a nucleotide linked (*e.g.*, linked at 5' end or 3' end) with M03 has a structure



[0150] For example, a nucleotide linked (*e.g.*, linked at 5' end or 3' end) with M06 has a structure



[0151] Also provided herein is a method of reducing or inhibiting the exonulcease catabolism of an oligonucleotide (*e.g.*, a double-stranded oligonucleotide such as a siRNA), by conjugating or linking a blocking group to the oligonucleotide. In some embodiments, the blocking group is an abasic residue, an inverted abasic residue, M03 or M06. In some embodiments, the blocking group is M03 or M06. In some embodiments, the blocking group (*e.g.*, M03 or M06) is linked to an anti-sense strand of the oligonucleotide, in particular, the 5' end or 3' end of the anti-sense strand. In some embodiments, the blocking group (*e.g.*, M03 or M06) is linked to the 5' end of

the anti-sense strand. In some embodiments, the blocking group may reduce or inhibit the RNA interference effect of the oligonucleotide.

[0152] Also provided herein is use of a blocking group in reducing or inhibiting the exonulcease catabolism of an oligonucleotide (e.g., a double-stranded oligonucleotide such as a siRNA), wherein the blocking group is conjugated or linked to the oligonucleotide, in particular, the 5' end or 3' end of the anti-sense strand. In some embodiments, the blocking group is an abasic residue, an inverted abasic residue, M03 or M06. In some embodiments, the blocking group is M03 or M06. In some embodiments, the blocking group is M03 or M06. In some embodiments, the blocking group (e.g., M03 or M06) is linked to the 5' end of the anti-sense strand. In some embodiments, the blocking group may reduce or inhibit the RNA interference effect of the oligonucleotide.

[0153] **e. Specific Embodiments of the Double-Stranded Oligonucleotide Agent**

[0154] In certain embodiments, the double-stranded oligonucleotide agent provided herein comprises a structure represented by Formula (C):

sense strand (5'→3'):	second fragment	
anti-sense strand (3'→5'):	first fragment	5' extension

Formula (C),

wherein the first fragment in the anti-sense strand and the second fragment in the sense strand form a double -stranded portion by base pairing, wherein the first fragment and the second fragment are of equal length;

wherein the 5' extension comprises at least three nucleotides in length;

wherein the anti-sense strand comprises a cleavage region comprising the most 5' nucleotide (X2) of the first fragment and the two most 3' nucleotides (Y-Z) of the 5' extension, and the cleavage region comprises a nucleotide sequence set forth in Formula

A: (3'-5') X2-Y-Z, which is cleavable between X2 and Y, and the Formula A is as defined herein.

wherein the sense strand has a length of 15 to 35, 15 to 23, 15 to 22, or 15 to 21 nucleotides; and

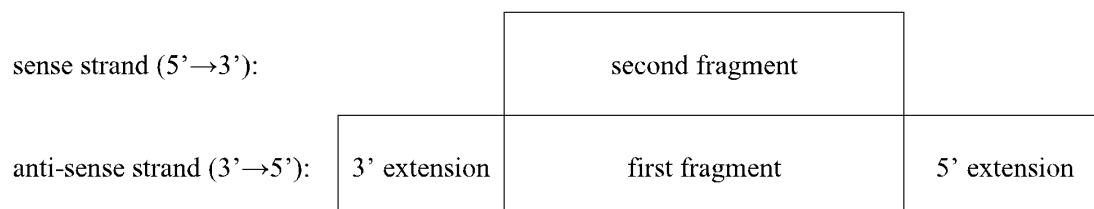
wherein the anti-sense strand has a length of 25 to 35, 26 to 35, 26 to 30, 25 to 27, or 26 to 27 nucleotides.

[0155] In some embodiments, the sense strand of the double-stranded oligonucleotide agent of Formula (C) has a length of 17 to 23, 17 to 22, or 17 to 21 nucleotides; and the anti-sense strand of the double-stranded oligonucleotide agent of Formula (C) has a length of 20-26, 20 to 25, 20 to 24 nucleotides.

[0156] In some embodiments, the sense strand of the double-stranded oligonucleotide agent of Formula (C) and the anti-sense strand of the double-stranded oligonucleotide agent of Formula (C) have a length of a) 17nt and 20nt respectively; b) 18nt and 21nt, respectively; c) 19nt and 22nt, respectively; d) 20nt and 23nt, respectively; or e) 21nt and 24nt, respectively.

[0157] In some embodiments, the double-stranded oligonucleotide agent disclosed herein may optionally be linked to one or more blocking groups. Any suitable blocking group provided in the present disclosure can be used. The blocking group can be attached to the sense strand, antisense strand or both strands, at the 3'-end, 5'-end or both ends. In some embodiments, the blocking group is linked to the anti-sense strand, in particular, the 5'-end of the anti-sense strand. In some embodiments, the blocking group is linked to the 5' end of the 5' extension.

[0158] Also provided herein is a double-stranded oligonucleotide agent comprising a structure represented by Formula (D):



Formula (D),

wherein

the first fragment in the anti-sense strand and the second fragment in the sense strand form a double-stranded portion by base pairing, wherein the first fragment and the second fragment are of equal length;

the 5' extension comprises at least three nucleotides in length;

the anti-sense strand comprises a cleavage region comprising the most 5' nucleotide (X2) of the first fragment and the two most 3' nucleotides (Y-Z) of the 5' extension, and the cleavage region comprises a nucleotide sequence set forth in Formula A: (3'-5') X2-Y-Z, which is cleavable between X2 and Y, and upon cleavage, the 5' extension is removed from its most 3' nucleotide (Y), and the Formula A is as defined herein,

wherein the sense strand has a length of 15 to 35, 15 to 23, 15 to 22, 15 to 21, 16 to 25, 17 to 23, 18 to 23, 19 to 23, 19 to 21, 20 to 23, 20 to 21, 21 to 23, such as 17, 18, 19, 20, 21, 22, or 23 nucleotides; and

wherein the anti-sense strand has a length of 25 to 35, 25 to 30, 26 to 35, 26 to 30, 26 to 27, such as 25, 26, 27, 28, 29, or 30 nucleotides.

[0159] In some embodiments, the sense strand of the double-stranded oligonucleotide agent of Formula (D) has a length of 17 to 23, 17 to 22, or 17 to 21 nucleotides; and wherein the anti-sense strand of the double-stranded oligonucleotide agent of Formula (D) has a length of 22-28, 22 to 27, or 22 to 26 nucleotides.

[0160] In some embodiments, the sense strand of the double-stranded oligonucleotide agent of Formula (D) and the anti-sense strand of the double-stranded oligonucleotide agent of Formula (D) have a length of a) 17nt and 22nt respectively; b) 18nt and 23nt, respectively; c) 19nt and 24nt, respectively; d) 20nt and 25nt, respectively; or e) 21nt and 26nt, respectively.

[0161] In some embodiments, the double-stranded oligonucleotide agent disclosed herein may optionally be linked to one or more blocking groups. Any suitable blocking group provided in the present disclosure can be used. The blocking group can be attached to the sense strand, antisense strand or both strands, at the 3'-end, 5'-end or both ends. In some embodiments, the blocking

group is linked to the anti-sense strand, in particular, the 5'-end of the anti-sense strand. In some embodiments, the blocking group is linked to the 5' end of the 5' extension.

[0162] In some embodiments, the sense strand of the oligonucleotide agent disclosed herein comprises at least 19, 20, 21 or 22 consecutive nucleotides of any one of SEQ ID NOs: 1-97. In some embodiments, the sense strand of the oligonucleotide agent provided herein is any one of SEQ ID NOs: 1-97.

[0163] In some embodiments, the anti-sense strand of the oligonucleotide agent disclosed herein comprises at least 19, 20, 21 or 22 consecutive nucleotides of any one of SEQ ID NOs: 98-194. In some embodiments, the anti-sense strand of the oligonucleotide agent provided herein is any one of SEQ ID NOs: 98-194.

[0164] In some embodiments, the anti-sense strand of the oligonucleotide agent disclosed herein comprises a pair of sense strand or anti-sense strand as set forth in Table 1.

Table 1: Exemplary oligonucleotides

SEQ ID NO	SS (5'-3')	SEQ ID NO	AS (5'-3')
1	ACGGGACAGUAUUCUCAGUGA	98	ACGAUCACUGAGAAUACUGUCCCCGUUU
2	ACCAGUCGUCCCAGAAUAACU	99	CUGAAGUUAUUCUGGGACGACUGGUCU
3	UGAGCAAGAUUUUGCAACUUGA	100	ACGAUCAAGUUGCAAAUCUUGCUC AUG
4	GGUUAACACCAUUUACUUCAA	101	CCGAUUGAAGUAAAUGGUGUUAACCAG
5	GGCUACGAAAAUCCAACCUAA	102	UCGAUUAGGUUGGAUUUUCGUAGCCGU
6	CACUUUAAUCCUCUAUCCAGA	103	AGGAUCUGGAUAGAGGAUUAAGUGAG
7	CCUUUGGAAUAAAGCUGCCUU	104	CCGUAAGGCAGCUUUAUUCCAAAGGGC
8	GCAGGAAGCACUGAGAUUCGU	105	CCGAACGAAUCUCAGUGCUUCCUGCAC
9	CUAGACCUGUUUUGCUUUUGU	106	UGGAACAAAAGCAAAACAGGUCUAGAA
10	AUGCAGAUUAUGCGGAUCAAA	107	ACGAUUUGAUCCGCAUAAUCUGCAUGG
11	GCUCAACAUAUUUGAUCAGUA	108	CCGAUACUGAUCAAAUAUGUUGAGCUU
12	GAGCCGUUCUCUACAAUUACU	109	GCGAAGUAAUUGUAGAGAACGGCUCGG
13	AAGCAAGAUUUUUUAUAAUA	110	ACGUUAUUUAAAAUAUCUUGCUUUU
14	UGGGAUUUCAUGUAACCAAGA	111	CCGAUCUUGGUUACAUGAAAUCCCAUC
15	GUCAUCCACAAUGAGAGUACA	112	CUUUGUACGCUCAUUGUGGAUGACGA
16	GUCAUCCACAAUGAGAGUACA	113	CAUUGUACGCUCAUUGUGGAUGACGA
17	GUCAUCCACAAUGAGAGUACA	114	CCUUGUACGCUCAUUGUGGAUGACGA

18	GUCAUCCACAAUGAGAGUACA	115	CGUUGUACGCUCAUUGUGGAUGACGA
19	GUCAUCCACAAUGAGAGUACA	116	CUAUGUACGCUCAUUGUGGAUGACGA
20	GUCAUCCACAAUGAGAGUACA	117	CAAUGUACGCUCAUUGUGGAUGACGA
21	GUCAUCCACAAUGAGAGUACA	118	CCAUGUACGCUCAUUGUGGAUGACGA
22	GUCAUCCACAAUGAGAGUACA	119	CGAUGUACGCUCAUUGUGGAUGACGA
23	GUCAUCCACAAUGAGAGUACU	120	CUUAGUACGCUCAUUGUGGAUGACGA
24	GUCAUCCACAAUGAGAGUACU	121	CAUAGUACGCUCAUUGUGGAUGACGA
25	GUCAUCCACAAUGAGAGUACU	122	CCUAGUACGCUCAUUGUGGAUGACGA
26	GUCAUCCACAAUGAGAGUACU	123	CGUAGUACGCUCAUUGUGGAUGACGA
27	GUCAUCCACAAUGAGAGUACU	124	CUAAGUACGCUCAUUGUGGAUGACGA
28	GUCAUCCACAAUGAGAGUACU	125	CAAAGUACGCUCAUUGUGGAUGACGA
29	GUCAUCCACAAUGAGAGUACU	126	CCAAGUACGCUCAUUGUGGAUGACGA
30	GUCAUCCACAAUGAGAGUACU	127	CGAAGUACGCUCAUUGUGGAUGACGA
31	GCUCAACAUAUUUGAUCAGUA	128	CUUUACUGAUCAAAUAUGUUGAGCAA
32	GCUCAACAUAUUUGAUCAGUA	129	CAUUACUGAUCAAAUAUGUUGAGCAA
33	GCUCAACAUAUUUGAUCAGUA	130	CCUUACUGAUCAAAUAUGUUGAGCAA
34	GCUCAACAUAUUUGAUCAGUA	131	CGUUACUGAUCAAAUAUGUUGAGCAA
35	GCUCAACAUAUUUGAUCAGUA	132	CUAUACUGAUCAAAUAUGUUGAGCAA
36	GCUCAACAUAUUUGAUCAGUA	133	CAAUACUGAUCAAAUAUGUUGAGCAA
37	GCUCAACAUAUUUGAUCAGUA	134	CCAUACUGAUCAAAUAUGUUGAGCAA
38	GCUCAACAUAUUUGAUCAGUA	135	CGAUACUGAUCAAAUAUGUUGAGCAA
39	GCUCAACAUAUUUGAUCAGUU	136	CUUAAACUGAUCAAAUAUGUUGAGCAA
40	GCUCAACAUAUUUGAUCAGUU	137	CAUAAACUGAUCAAAUAUGUUGAGCAA
41	GCUCAACAUAUUUGAUCAGUU	138	CCUAAACUGAUCAAAUAUGUUGAGCAA
42	GCUCAACAUAUUUGAUCAGUU	139	CGUAAACUGAUCAAAUAUGUUGAGCAA
43	GCUCAACAUAUUUGAUCAGUU	140	CUAAACUGAUCAAAUAUGUUGAGCAA
44	GCUCAACAUAUUUGAUCAGUU	141	CAAAACUGAUCAAAUAUGUUGAGCAA
45	GCUCAACAUAUUUGAUCAGUU	142	CCAAACUGAUCAAAUAUGUUGAGCAA
46	GCUCAACAUAUUUGAUCAGUU	143	CGAAACUGAUCAAAUAUGUUGAGCAA
47	GUCAUCCACAAUGAGAGUACU	144	CGAAGUACGCUCAUUGUGGAUGACGA
48	GUCAUCCACAAUGAGAGUACU	145	CGUAGUACGCUCAUUGUGGAUGACGA
49	GUCAUCCACAAUGAGAGUACU	146	CGGAGUACGCUCAUUGUGGAUGACGA
50	GUCAUCCACAAUGAGAGUACU	147	CGCAGUACGCUCAUUGUGGAUGACGA
51	GUCAUCCACAAUGAGAGUACU	148	GGAAGUACGCUCAUUGUGGAUGACGA
52	GUCAUCCACAAUGAGAGUACU	149	UGAAGUACGCUCAUUGUGGAUGACGA

53	GUCAUCCACAAUGAGAGUACG	150	CGACGUACGCUCAUUGUGGAUGACGA
54	GUCAUCCACAAUGAGAGUACC	151	CGAGGUACGCUCAUUGUGGAUGACGA
55	GUCAUCCACAAUGAGAGUACU	152	CCGCCGAAGUACGCUCAUUGUGGAUGACGA
56	CUAGACCUGUTUUGCUUUUGU	153	CGAACAAAAGCAAAACAGGUCUAGAA
57	CUAGACCUGUTUUGCUUUUGU	154	CGUACAAAAGCAAAACAGGUCUAGAA
58	CUAGACCUGUTUUGCUUUUGU	155	CGGACAAAAGCAAAACAGGUCUAGAA
59	CUAGACCUGUTUUGCUUUUGU	156	CGCACAAAAGCAAAACAGGUCUAGAA
60	CUAGACCUGUTUUGCUUUUGU	157	AGAACAAAAGCAAAACAGGUCUAGAA
61	CUAGACCUGUTUUGCUUUUGU	158	GGUACAAAAGCAAAACAGGUCUAGAA
62	CUAGACCUGUTUUGCUUUUGG	159	CGACCAAAAAGCAAAACAGGUCUAGAA
63	CUAGACCUGUTUUGCUUUUGC	160	CGAGCAAAAAGCAAAACAGGUCUAGAA
64	CUAGACCUGUTUUGCUUUUGU	161	GCUUCGAACAAAAGCAAAACAGGUCUAGAA
65	GUCAUCCACAAUGAGAGUACU	162	CGAAGUACGCUCAUUGUGGAUGACGA
66	GUCAUCCACAAUGAGAGUACU	163	CGAAGUACGCUCAUUGUGGAUGACGA
67	GUCAUCCACAAUGAGAGUACU	164	CGAAGUACGCUCAUUGUGGAUGACGA
68	GUCAUCCACAAUGAGAGUACU	165	CGAAGUACGCUCAUUGUGGAUGACGA
69	GUCAUCCACAAUGAGAGUACU	166	CGAAGUACGCUCAUUGUGGAUGACGA
70	GUCAUCCACAAUGAGAGUACU	167	CGAAGUACGCUCAUUGUGGAUGACGA
71	GUCAUCCACAAUGAGAGUACU	168	CGAAGUACGCUCAUUGUGGAUGACGA
72	GUCAUCCACAAUGAGAGUACU	169	CGAAGUACGCUCAUUGUGGAUGACGA
73	GUCAUCCACAAUGAGAGUACU	170	CGAAGUACGCUCAUUGUGGAUGACGA
74	GUCAUCCACAAUGAGAGUACU	171	CGAAGUACGCUCAUUGUGGAUGACGA
75	GUCAUCCACAAUGAGAGUACU	172	CGAAGUACGCUCAUUGUGGAUGACGA
76	GUCAUCCACAAUGAGAGUACU	173	CGAAGUACGCUCAUUGUGGAUGACGA
77	GUCAUCCACAAUGAGAGUACU	174	CGAAGUACGCUCAUUGUGGAUGACGA
78	GUCAUCCACAAUGAGAGUACU	175	CGAAGUACGCUCAUUGUGGAUGACGA
79	GUCAUCCACAAUGAGAGUACA	176	UGUACGCUCAUUGUGGAUGACGA
80	ACCAGUCGUCCCAGAAUAAU	177	AGUUAUTCUGGGACGACUGGUCU
81	ACCAGUCGUCCCAGAAUAAU	178	CGAAGUUAUTCUGGGACGACUGGUCU
82	AAGCAAGAUUUUUUAUAAUA	179	UAUUUAUUUUUAUUCUUGCUUUU
83	AAGCAAGAUUUUUUAUAAUA	180	CGAUUUUAUUUUUAUUCUUGCUUUU
84	GGUUAACACCAUUUACUCAA	181	UUGAAGUAAAUGGUGUUAACCAG
85	GGUUAACACCAUUUACUCAA	182	CGAUUGAAGUAAAUGGUGUUAACCAG
86	GAGCCGUUCUCUACAAUUACU	183	AGUAAUUGUAGAGAACGGCUCGG
87	GAGCCGUUCUCUACAAUUACU	184	CGAAGUAAUUGUAGAGAACGGCUCGG

88	CAUUUUAAUCCUCACUCUAAA	185	UUUAGAGUGAGGAUUAAAAUGAG
89	CAUUUUAAUCCUCACUCUAAU	186	CGAAUUAGAGUGAGGAUUAAAAUGAG
90	CAUUUUAAUCCUCACUCUAAU	187	CGAAUUAGAGUGAGGAUUAAAAUGAG
91	CAUUUUAAUCCUCACUCUAAU	188	CGAAUUAGAGUGAGGAUUAAAAUGAG
92	CAUUUUAAUCCUCACUCUAAA	189	UUUAGAGUGAGGAUUAAAAUGAG
93	CAUUUUAAUCCUCACUCUAAU	190	CGAAUUAGAGUGAGGAUUAAAAUGAG
94	GGCUACGAAAAUCCAACCUAA	191	UUAGGUGGGAUTUUCGUAGCCGU
95	GGCUACGAAAAUCCAACCUAA	192	CGAUUAGGUGGGAUTUUCGUAGCCGU
96	AACAGUGUUCUUGCUCUAUAA	193	UUAUAGAGCAAGAACACUGUUUU
97	AACAGUGUUCUUGCUCUAUAA	194	CGAUUAUAGAGCAAGAACACUGUUUU

[0165] In some embodiments, the sense strand of the oligonucleotide agent provided herein is any one of SEQ ID NOs: 1-97, the anti-sense strand of the oligonucleotide agent provided herein is any one of SEQ ID NOs: 98-194, and

- a) at least one of the internucleotide linkages between the nucleotides in the cleavage region is not phosphorothioate linkage;
- b) the internucleotide linkage between X2 and Y is not phosphorothioate linkage;
- c) the internucleotide linkage between Y and Z is not phosphorothioate linkage;
- d) none of the internucleotide linkages between the nucleotides in the cleavage region is phosphorothioate linkage;
- e) at least one of the internucleotide linkages between the nucleotides in the cleavage region is phosphodiester linkage;
- f) the internucleotide linkage between X2 and Y is phosphodiester linkage;
- g) the internucleotide linkage between Y and Z is phosphodiester linkage; and/or
- h) each of the internucleotide linkages between the nucleotides in the cleavage region is phosphodiester linkage.

[0166] In some embodiments, the sense strand of the oligonucleotide agent provided herein is any one of SEQ ID NOs: 1-97, the anti-sense strand of the oligonucleotide agent provided herein is any one of SEQ ID NOs: 98-194, and

- a) at least one nucleotide in the cleavage region is 2'-OMe modified or 2'-F modified;
- b) each nucleotide in the cleavage region is either 2'-OMe modified or 2'-F modified;
- c) at least one nucleotide in the cleavage region is 2'-F modified;

- d) no more than two nucleotides in the cleavage region are 2'-F modified;
- e) Z in the Formula A or Formula B is 2'-F modified, and optionally X2 in the Formula A or Formula B is 2'-F modified;
- f) N1 in the Formula A or Formula B is 2'-F modified;
- g) both X2 and Y in the Formula A or Formula B are 2'-OMe modified, and both Z and N1 in the Formula A or Formula B are 2'-F modified; and/or
- h) all of X2, Y and Z in the Formula A or Formula B are 2'-OMe modified, and N1 in the Formula A or Formula B is 2'-F modified.

[0167] In some embodiments, the oligonucleotide agent disclosed herein is any one of ds1-ds121.

[0168] In some embodiments, the double-stranded oligonucleotide agent disclosed herein may optionally be linked to one or more blocking groups. Any suitable blocking group provided in the present disclosure can be used. The blocking group can be attached to the sense strand, antisense strand or both strands, at the 3'-end, 5'-end or both ends. In some embodiments, the blocking group is linked to the anti-sense strand, in particular, the 5'-end of the anti-sense strand.

[0169] f. Cleaved Double-Stranded Oligonucleotide agent

[0170] In some embodiments, the cleaved double-stranded oligonucleotide agent comprises a 3' extension on the anti-sense strand.

[0171] Each nucleotide of the cleaved double-stranded oligonucleotide agent may be independently modified or unmodified as describe above. In some embodiments, all nucleotides of the cleaved double-stranded oligonucleotide agent are independently modified. In some embodiments, all nucleotides of the cleaved double-stranded oligonucleotide agent are independently modified with 2'-F or 2'-OMe.

[0172] Each internucleotide linkage of the cleaved double-stranded oligonucleotide agent may independently modified or unmodified as describe above. In some embodiments, the cleaved double-stranded oligonucleotide agent comprises at least one modified internucleotide linkage. In some embodiments, the modified internucleotide linkage is phosphorothioate (PS) or phosphorodithioate (PS2).

[0173] In some embodiments, the cleaved double-stranded oligonucleotide agent comprises modifications of an alternating pattern as described above.

[0174] In some embodiments, the cleaved double-stranded oligonucleotide agent comprises phosphorothioate or methylphosphonate internucleotide linkage. The phosphorothioate or methylphosphonate internucleotide linkage modification may occur on any nucleotide of the sense strand or antisense strand or both in any position of the strand. For instance, the internucleotide linkage modification may occur on every nucleotide on the sense strand and/or antisense strand; each internucleotide linkage modification may occur in an alternating pattern on the sense strand or antisense strand; or the sense strand or antisense strand comprises both internucleotide linkage modifications in an alternating pattern. The alternating pattern of the internucleotide linkage modification on the sense strand may be the same or different from the antisense strand, and the alternating pattern of the internucleotide linkage modification on the sense strand may have a shift relative to the alternating pattern of the internucleotide linkage modification on the antisense strand.

[0175] In one embodiment, the cleaved double-stranded oligonucleotide agent comprises the phosphorothioate or methylphosphonate internucleotide linkage modification in the extension (*e.g.*, 3' extension) region. For example, the extension (*e.g.*, 3' extension) region comprises two nucleotides having a phosphorothioate or methylphosphonate internucleotide linkage between the two nucleotides. Internucleotide linkage modifications also may be made to link the extension nucleotides with the terminal paired nucleotides within duplex region. For example, at least 2, 3, 4, or all the extension (*e.g.*, 3' extension) nucleotides may be linked through phosphorothioate or methylphosphonate internucleotide linkage, and optionally, there may be additional phosphorothioate or methylphosphonate internucleotide linkages linking the extension nucleotide with a paired nucleotide that is next to the extension nucleotide. For instance, there may be at least two phosphorothioate internucleotide linkages between the terminal three nucleotides, in which two of the three nucleotides are extension nucleotides, and the third is a paired nucleotide next to the extension nucleotide. Preferably, these terminal three nucleotides may be at the 3'-end of the antisense strand.

[0176] In one embodiment the sense strand of the cleaved double-stranded oligonucleotide agent comprises 1-10 blocks of two to ten phosphorothioate or methylphosphonate internucleotide linkages separated by 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15 or 16 phosphate internucleotide linkages, wherein one of the phosphorothioate or methylphosphonate internucleotide linkages is placed at any position in the oligonucleotide sequence and the said sense strand is paired with an

antisense strand comprising any combination of phosphorothioate, methylphosphonate and phosphate internucleotide linkages or an antisense strand comprising either phosphorothioate or methylphosphonate or phosphate linkage.

[0177] In one embodiment the antisense strand of the cleaved double-stranded oligonucleotide agent comprises two blocks of two phosphorothioate or methylphosphonate internucleotide linkages separated by 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, or 18 phosphate internucleotide linkages, wherein one of the phosphorothioate or methylphosphonate internucleotide linkages is placed at any position in the oligonucleotide sequence and the said antisense strand is paired with a sense strand comprising any combination of phosphorothioate, methylphosphonate and phosphate internucleotide linkages or an antisense strand comprising either phosphorothioate or methylphosphonate or phosphate linkage.

[0178] In one embodiment, the cleaved double-stranded oligonucleotide agent further comprises one to five phosphorothioate or methylphosphonate internucleotide linkage modification(s) within position 1-5 and one to five phosphorothioate or methylphosphonate internucleotide linkage modification(s) within position 18-23 of the sense strand (counting from the 5'-end), and one to five phosphorothioate or methylphosphonate internucleotide linkage modification at positions 1 and 2 and one to five within positions 18-23 of the antisense strand (counting from the 5'-end).

[0179] In one embodiment, the cleaved double-stranded oligonucleotide agent comprises the pattern of the alternating motif of 2'-OMe modification and 2'-F modification on the sense strand initially has a shift relative to the pattern of the alternating motif of 2'-OMe modification and 2'-F modification on the antisense strand initially, *i.e.*, the 2'-OMe modified nucleotide on the sense strand base pairs with a 2'-F modified nucleotide on the antisense strand and vice versa. For example, the 1 position of the sense strand may start with the 2'-F modification, and the 1 position of the antisense strand may start with the 2'-OMe modification. The introduction of one or more motifs of three identical modifications on three consecutive nucleotides to the sense strand and/or antisense strand interrupts the initial modification pattern present in the sense strand and/or antisense strand. This interruption of the modification pattern of the sense and/or antisense strand by introducing one or more motifs of three identical modifications on three consecutive nucleotides to the sense and/or antisense strand surprisingly enhances the gene silencing activity to the target gene.

[0180] g. Ligand

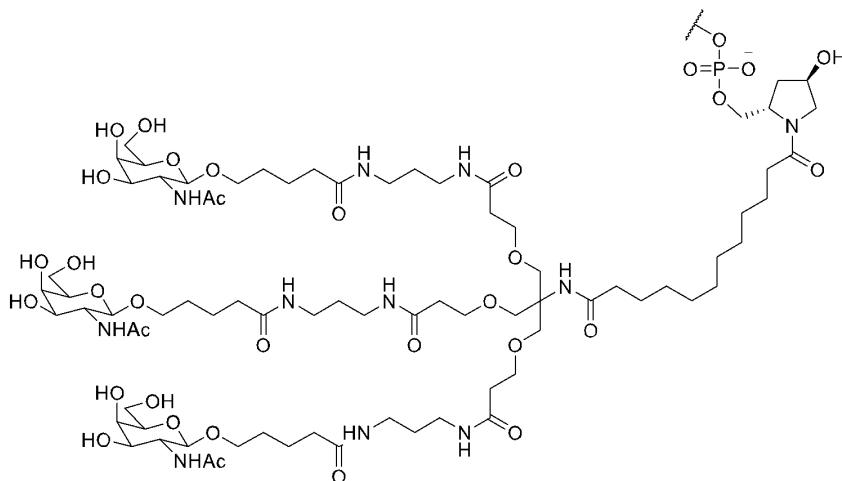
[0181] In certain embodiments, the double-stranded oligonucleotide agent disclosed herein may optionally be conjugated to one or more ligands. The ligand can be attached to the sense strand, antisense strand or both strands, at the 3'-end, 5'-end or both ends or at any available nucleotides (*e.g.*, at the sugar ring). In some embodiments, the ligand may be conjugated to the sense strand, in particular, the 3'-end of the sense strand. In some embodiments, the ligand is conjugated to the anti-sense strand, in particular, the 5'-end of the anti-sense strand.

[0182] In some embodiments, a nucleotide (*e.g.*, of the sense stand or anti-sense strand) is replaced with a nucleotide conjugate to a ligand. In this case, the nucleotide conjugate to a ligand is also encompassed in the range of a “ligand”.

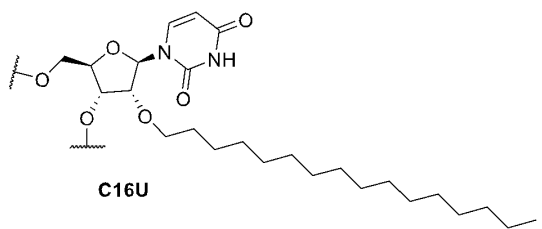
[0183] In some embodiments, the ligand is a GalNAc ligand, a lipophilic ligand or other ligands targeting a receptor that could facilitate the endocytosis of siRNA conjugate, such as TfR targeting ligands, LDL-R targeting ligands, or integrin targeting ligands.

[0184] In some embodiments, the ligand is conjugated to the oligonucleotide (*e.g.*, the 5' end of the anti-sense strand) via an internucleotide linkage, and the internucleotide linkage is optionally modified as described above. In some embodiments, the ligand is linked to the double-stranded oligonucleotide agent with a phosphorothioate.

[0185] In some embodiments, the ligand is L96, having a structure of

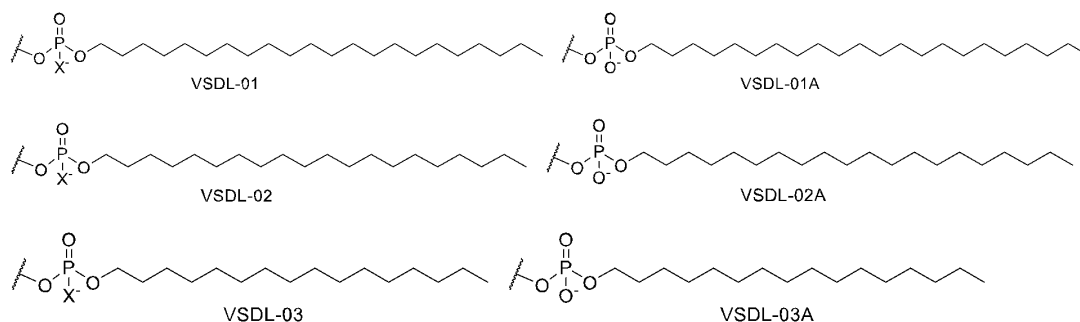


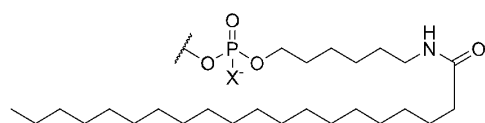
[0186] In some embodiments, the ligand is hexadecyloxy. For example, a C16 ligand may conjugate to a uridine nucleotide at 2' position, which is abbreviated as "C16U" herein and has a



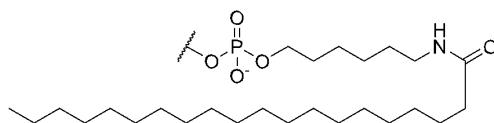
structure of

[0187] In some embodiments, the ligand is VSDL-01. In some embodiments, the ligand is VSDL-01A. In some embodiments, the ligand is VSDL-02. In some embodiments, the ligand is VSDL-02A. In some embodiments, the ligand is VSDL-03. In some embodiments, the ligand is VSDL-03A. In some embodiments, the ligand is VSDL-04. In some embodiments, the ligand is VSDL-04A. In some embodiments, the ligand is VSDL-05. In some embodiments, the ligand is VSDL-05A. In some embodiments, the ligand is VSDL-06. In some embodiments, the ligand is VSDL-06A. In some embodiments, the ligand is VSDL-07. In some embodiments, the ligand is VSDL-07A. In some embodiments, the ligand is VSDL-08. In some embodiments, the ligand is VSDL-08A. In some embodiments, the ligand is VSDL-09. In some embodiments, the ligand is VSDL-10. In some embodiments, the ligand is VSDL-11. In some embodiments, the ligand is VSDL-12. In some embodiments, the ligand is VSDL-13. In some embodiments, the ligand is VSDL-14. VSDL-01, VSDL-01A, VSDL-02, VSDL-02A, VSDL-03, VSDL-03A, VSDL-04, VSDL-04A, VSDL-05, VSDL-05A, VSDL-06, VSDL-06A, VSDL-07, VSDL-07A, VSDL-08, VSDL-08A, VSDL-09, VSDL-10, VSDL-11, VSDL-12, VSDL-13 or VSDL-14 having the following structures, wherein X is S or O.

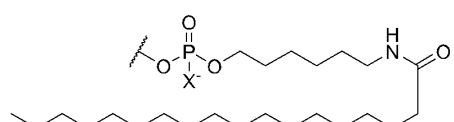




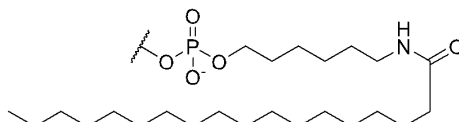
VSDL-04



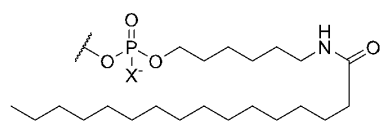
VSDL-04A



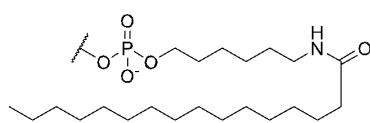
VSDL-05



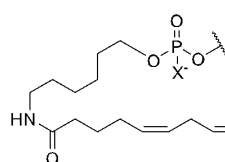
VSDL-05A



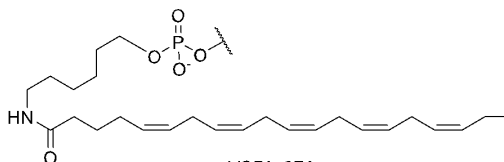
VSDL-06



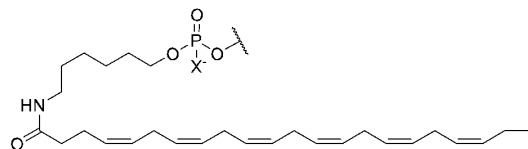
VSDL-06A



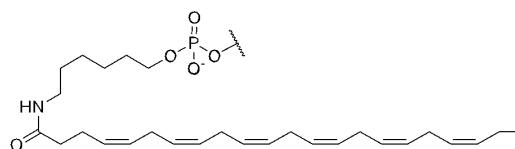
VSDL-07



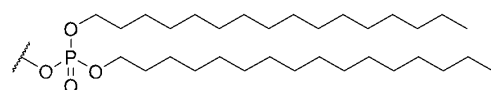
VSDL-07A



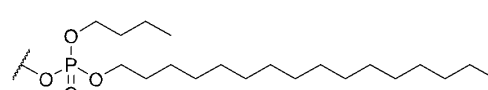
VSDL-08



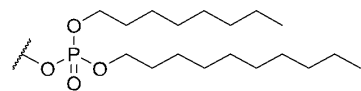
VSDL-08A



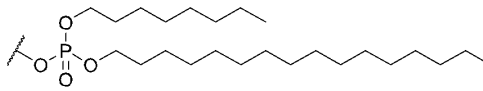
VSDL-09



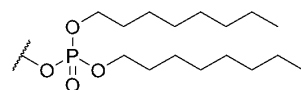
VSDL-10



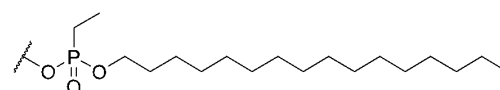
VSDL-11



VSDL-12

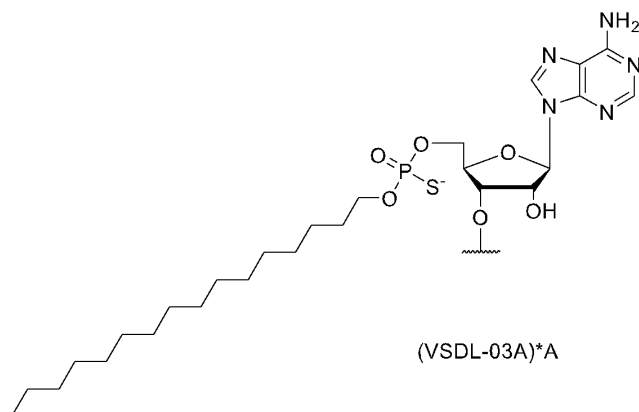


VSDL-13



VSDL-14

For example, “5'-(VSDL-03A)*A-3' ” having a structure of



[0188] h. Target Gene

[0189] In some embodiments, the double-stranded oligonucleotide agent provided herein is capable of inhibiting expression of a target gene. In certain embodiments, the target gene is selected from the group consisting of AGT, complement factor B, DGAT2, DUX, ANGPTL8, APOC3, F12, INHBE, PNPLA3, Serpinc1, APP, SOD1, TMPRSS6, KHK, PCSK9, VEGFA, ANGPTL3, ANGPTL4, C3, C5, TTR, IGF-1R, VEGFR, ANG2, GIPR, GPR75, ActRII, NUDT21, PLN, HSD17b13, CNOT6L, PTP1B, CFHR, ATX, CIDEA, mARC1, TSHR, CB1 and LPA.

[0190] In some embodiments, the targeting region contained in the first fragment provided herein is sufficiently complementary to a part of an mRNA encoding the target gene. In some embodiments, the targeting region is at least 80%, 85%, 90% or 95% complementary to a part of an mRNA encoding the target gene. In some embodiments, the targeting region is 100% complementary (perfect complementary) to a part of an mRNA encoding the target gene.

[0191] II. Novel Blocking Group and Novel Ligand

[0192] Also provided herein is a double-stranded oligonucleotide agent comprising a double-stranded oligonucleotide operably linked to a blocking group, wherein the blocking group comprises M03 or M06.

[0193] In certain embodiments, the double-strand oligonucleotide can be any suitable siRNA or RNAi agent.

[0194] Also provided herein is a double-stranded oligonucleotide agent comprising a double-stranded oligonucleotide operably linked to a ligand, where the ligand comprises a chemical

structure selected from the group consisting of: VSDL-01, VSDL-01A, VSDL-02, VSDL-02A, VSDL-03, VSDL-03A, VSDL-04, VSDL-04A, VSDL-05, VSDL-05A, VSDL-06, VSDL-06A, VSDL-07, VSDL-07A, VSDL-08, VSDL-08A, VSDL-09, VSDL-10, VSDL-11, VSDL-12, VSDL-13, and VSDL-14. In certain embodiments, the double-strand oligonucleotide can be any suitable siRNA or RNAi agent.

[0195] In some embodiments, the double-stranded oligonucleotide agent operably linked to a blocking group or a ligand comprising a sense strand and an anti-sense strand. In some embodiments, the double-stranded oligonucleotide agent operably linked to a blocking group or a ligand comprising a blocking group conjugated to the 5'end of the anti-sense strand. In some embodiments, the double-stranded oligonucleotide agent operably linked to a blocking group or a ligand comprising a ligand conjugated to the 5'end of the anti-sense strand, 5'end of the sense strand or 3'end of the sense strand. In some embodiments, the double-stranded oligonucleotide agent operably linked to a blocking group or a ligand is operably linked to the blocking group at one end, and is operably linked to the ligand at another end. In some embodiments, the double-stranded oligonucleotide agent operably linked to a blocking group or a ligand is capable of inhibiting expression of a target gene via RNA interference.

[0196] In some embodiments, the double-stranded oligonucleotide agent operably linked to a blocking group or a ligand comprises a sense strand and an anti-sense strand, wherein each strand has 14 to 30 nucleotides.

[0197] In some embodiments, the anti-sense strand of the double-stranded oligonucleotide agent operably linked to a blocking group or a ligand is sufficiently complementary to part of a target RNA or an mRNA encoding a target gene, and the sense strand of the double-stranded oligonucleotide agent operably linked to a blocking group or a ligand is sufficiently complementary to the targeting region of the anti-sense strand.

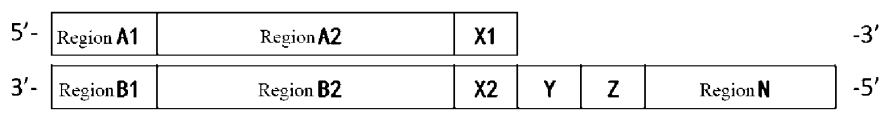
[0198] In some embodiments, the blocking group is conjugated to the 5'end of the anti-sense strand.

[0199] In some embodiments, the ligand is conjugated to the 5'end of the anti-sense strand, 5'end of the sense strand or 3'end of the sense strand.

[0200] In some embodiments, the anti-sense strand of the double-stranded oligonucleotide agent operably linked to a blocking group or a ligand is operably linked to the blocking group at one end, and is operably linked to the ligand at another end, and/or

[0201] In some embodiments, the anti-sense strand of the double-stranded oligonucleotide agent operably linked to a blocking group or a ligand capable of silencing a target RNA or inhibiting expression of a target gene via RNA interference.

[0202] In one aspect, disclosed herein is a double-stranded RNA (dsRNA), comprising a sense strand and an anti-sense strand, of a structure represented by Formula (I):



wherein

the sense strand consists of region A1, region A2 and X1 (from 5' to 3'),

the anti-sense strand consists of region N, Z, Y, X2, region B2 and region B1 (from 5' to 3'),

the region A1 is 0-15 nucleotides,

the region B1 is 0-15 nucleotides,

the region A2 and region B2 has the same length and is at least 1 nucleotide,

the region A2 and region B2 comprise a complementary double-stranded region,

each of X1, X2, Y and Z is independently 1 nucleotide, wherein X1 and X2 can form a hydrogen bond,

the region N is at least 1 nucleotide,

the total length of the sense strand is 17-23 nt,

the total length of the anti-sense strand is at least 26 nt,

the dsRNA comprises no more than 23 complementary base pairs, and

each of the nucleotides of the dsRNA is independently optionally modified.

[0203] In some embodiments, the sense strand consists of region A1, region A2 and X1 (from 5' to 3'), the anti-sense strand consists of region N, Z, Y, X2, region B2 and region B1 (from 5' to 3'), the region A1 is 1-6 nucleotides, the region B1 is 0-6 nucleotides, the region A2 and region B2 has the same length and is at least 1 nucleotide, the region A2 and region B2 comprise a complementary double-stranded region, each of X1, X2, Y and Z is independently 1 nucleotide, wherein X1 and X2 are complementary, the region N is at least 1 nucleotide, the total length of the sense strand is 17-23 nt, the total length of the anti-sense strand is at least 26 nt, the dsRNA comprises no more than 23 complementary base pairs, and each of the nucleotides of the dsRNA is independently optionally modified.

[0204] In some embodiments, each of X2 and Y is independently A, dA, U or dU, and Z is G or dG.

[0205] In some embodiments, each of X2 and Y is independently A, dA, U or dU, Z is G or dG, and each of the nucleotides of the dsRNA is independently modified.

[0206] In some embodiments, X2 is A or U, and Z is G.

[0207] In some embodiments, X2 is A or U, Z is G, and each of the nucleotides of the dsRNA is independently modified.

[0208] In some embodiments, Y is A or U.

[0209] In some embodiments, Y is A or U, and each of the nucleotides of the dsRNA is independently modified.

[0210] In some embodiments, X2 is A or U and Y is A or U.

[0211] In some embodiments, X2 is A or U, Y is A or U, and each of the nucleotides of the dsRNA is independently modified.

[0212] In some embodiments, Y is A.

[0213] In some embodiments, Y is A, and each of the nucleotides of the dsRNA is independently modified.

[0214] In some embodiments, X1 is A or U.

[0215] In some embodiments, X1 is A, U or I.

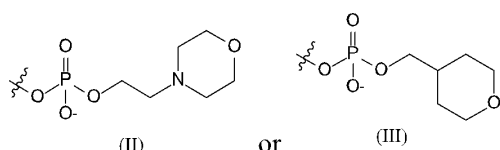
[0216] In some embodiments, X1 is A, U, G or I.

[0217] In some embodiments, X1 is A, U or I, and each of the nucleotides of the dsRNA is independently modified.

[0218] In some embodiments, at least 1 nucleotide of Y, Z and region N is modified.

[0219] In some embodiments, the region N consists of 1 nucleotide and group X, group X is nucleotide, blocking group or a combination of nucleotide and blocking group, wherein the blocking group is a structure resisting nuclease decomposition, the blocking group is linked to the region N at 5' end, and the blocking group is independently optionally modified.

[0220] In some embodiments, the blocking group is selected from inverted abasic



deoxyribonucleotide or RX, wherein RX is

[0221] In some embodiments, the region N consists of 1 nucleotide and 1 blocking group.

[0222] In some embodiments, the 1 nucleotide and 1 blocking group of the region N are linked via phosphorothioate linkage.

[0223] In some embodiments, the region N consists of 2 nucleotides and 1 blocking group.

[0224] In some embodiments, the 2 nucleotides and 1 blocking group of the region N are linked via phosphorothioate linkage.

[0225] In some embodiments, the region N consists of 1 nucleotide and 2 blocking groups.

[0226] In some embodiments, the 1 nucleotide and 2 blocking groups of the region N are linked via phosphorothioate linkage.

[0227] In some embodiments, the region N consists of 1 nucleotide and 2 blocking groups.

[0228] In some embodiments, the 1 nucleotide and 2 blocking groups of the region N are linked via a linkage, wherein the linkage is via a single bond.

[0229] In some embodiments, the 1 nucleotide and 2 blocking groups of the region N are linked via a linkage, wherein the linkage is via a phosphodiester, phosphorothioate or phosphorodithioate linkage.

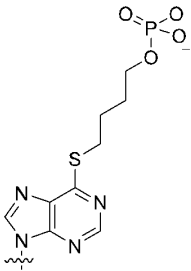
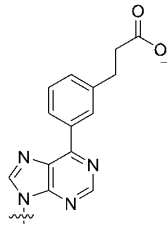
[0230] In some embodiments, all nucleotides of the dsRNA are independently modified.

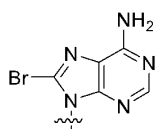
[0231] In some embodiments, the nucleotide modification comprises 1, 2 or more selected from the group consisting of base modifications, sugar ring modifications, phosphate skeleton

modifications, and end modifications. Base modifications include substitutions with a stabilized base, a destabilized base, or a base paired with extended partner libraries; base removal (an abasic nucleotide), or a conjugated base. Sugar modifications include modifications at the 2' position, 3' position, or 4' position or sugar substitutions. Phosphate skeleton modifications comprise modifications or replacement of a phosphodiester linkage. End modifications comprise a 5' end modifications (phosphorylation, conjugation, inverted linkage, etc.) and 3' end modifications (conjugation, DNA nucleotide, inverted linkage, etc.).

[0232] In some embodiments, the nucleotide modification comprises 1, 2 or more selected from the group consisting of 2'-OMe modification, 2'-F modification, 2'-deoxy modification, VP modification, 5'-MP modification, PS modification, PS2 modification, MP modification, MOP modification, invAB modification, LNA modification, UNA modification and a modification enhancing the affinity of dsRNA and ARGO protein.

[0233] In some embodiments, the modification enhancing the affinity of dsRNA and ARGO

protein includes replacement of a nucleotide with ,  or



[0234] In some embodiments, the nucleotide modification comprises 1, 2 or more selected from the group consisting of 2'-OMe, 2'-F, 2'-deoxy, VP, 5'-MP, PS, PS2, MP, MOP, invAB and a modification enhancing the affinity of dsRNA and ARGO protein.

[0235] In some embodiments, the modification enhancing the affinity of dsRNA and ARGO protein is on X2.

[0236] In some embodiments, the region A1 and the region B1 comprise a complementary double-strand region.

- [0237] In some embodiments, the region A1 is 1 nucleotide, and the region B1 is 3 nucleotides.
- [0238] In some embodiments, the length of region A2 or B2 is 20 nt.
- [0239] In some embodiments, the region A1 is 0 nucleotide, and the region B1 is 2 nucleotides.
- [0240] In some embodiments, the total length of the sense strand is 17-21 nt. In some embodiments, the total length of the sense strand is 18-21 nt.
- [0241] In some embodiments, the total length of the anti-sense strand is 26-35 nt. In some embodiments, the total length of the anti-sense strand is 26-32 nt. In some embodiments, the total length of the anti-sense strand is 26-28 nt. In some embodiments, the total length of the anti-sense strand is 26-27 nt. In some embodiments, the total length of the anti-sense strand is 26 nt, 27 nt, 28 nt, 29 nt or 30 nt.
- [0242] In some embodiments, the region A1 is 1-4 nucleotides.
- [0243] In some embodiments, the total length of the anti-sense strand is 26-27 nt, and the region A1 is 1-4 nucleotides.
- [0244] In some embodiments, the region N comprises inverted abasic deoxyribonucleotide (invAB).
- [0245] In some embodiments, the total length of the anti-sense strand is 26-27 nt, the region A1 is 1-4 nucleotides, and the region N comprises inverted abasic deoxyribonucleotide (invAB).
- [0246] In some embodiments, Z and the region N is linked via a phosphorothioate linkage.
- [0247] In some embodiments, X2 and the region B2 is linked via a phosphorothioate linkage.
- [0248] In some embodiments, Z and the region N is linked via a phosphorothioate linkage, and X2 and the region B2 is linked via a phosphorothioate linkage.
- [0249] In some embodiments, the total length of the anti-sense strand is 26-27 nt, the region A1 is 1-4 nucleotides, and Z and the region N is linked via a phosphorothioate linkage.
- [0250] In some embodiments, the total length of the anti-sense strand is 26-27 nt, the region A1 is 1-4 nucleotides, and X2 and the region B2 is linked via a phosphorothioate linkage.
- [0251] In some embodiments, the total length of the anti-sense strand is 26-27 nt, the region A1 is 1-4 nucleotides, Z and the region N is linked via a phosphorothioate linkage, and X2 and the region B2 is linked via a phosphorothioate linkage.
- [0252] In some embodiments, the total length of the anti-sense strand is 26-27 nt, the region A1 is 1-4 nucleotides, the region N comprises inverted abasic deoxyribonucleotide (invAB), and Z and

the region N is linked via a phosphorothioate linkage.

[0253] In some embodiments, the total length of the anti-sense strand is 26-27 nt, the region A1 is 1-4 nucleotides, the region N comprises inverted abasic deoxyribonucleotide (invAB), and X2 and the region B2 is linked via a phosphorothioate linkage.

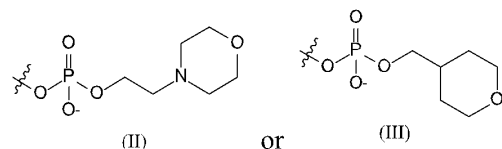
[0254] In some embodiments, the total length of the anti-sense strand is 26-27 nt, the region A1 is 1-4 nucleotides, the region N comprises inverted abasic deoxyribonucleotide (invAB), Z and the region N is linked via a phosphorothioate linkage, and X2 and the region B2 is linked via a phosphorothioate linkage.

[0255] In some embodiments, the total length of the sense strand is 17-21 nt, the total length of the anti-sense strand is 26-27 nt, and the region A1 is 1-4 nucleotides.

[0256] In some embodiments, the total length of the sense strand is 17-21 nt, and the region N comprises inverted abasic deoxyribonucleotide (invAB).

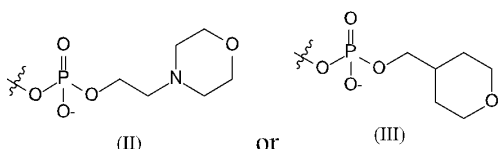
[0257] In some embodiments, the total length of the sense strand is 17-21 nt, the region A1 is 1-4 nucleotides, and the region N comprises inverted abasic deoxyribonucleotide (invAB).

[0258] In some embodiments, the total length of the anti-sense strand is 26-27 nt, the region A1 is 1-4 nucleotides, position 1 counting from its 5' end of the region N is replaced with RX, wherein



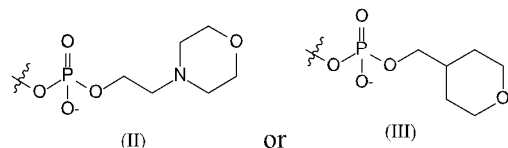
the RX is (II) or (III), and Z and the region N is linked via a phosphorothioate linkage.

[0259] In some embodiments, the total length of the anti-sense strand is 26-27 nt, the region A1 is 1-4 nucleotides, position 1 counting from its 5' end of the region N is replaced with RX, wherein



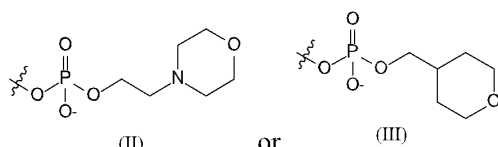
the RX is (II) or (III), and X2 and the region B2 is linked via a phosphorothioate linkage.

[0260] In some embodiments, the total length of the anti-sense strand is 26-27 nt, the region A1 is 1-4 nucleotides, position 1 counting from its 5' end of the region N is replaced with RX, wherein



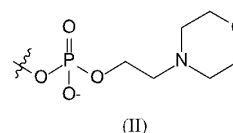
the RX is (II) or (III), Z and the region N is linked via a phosphorothioate linkage, and X2 and the region B2 is linked via a phosphorothioate linkage.

[0261] In some embodiments, the total length of the sense strand is 17-21 nt, the region A1 is 1-4 nucleotides, and position 1 counting from its 5' end of the region N is replaced with RX, wherein

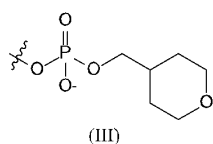


the RX is (II) or (III).

[0262] In some embodiments, the total length of the sense strand is 17-21 nt, the total length of the anti-sense strand is 26-27 nt, the region A1 is 1-4 nucleotides, and position 1 counting from its



5' end of the region N is replaced with RX, wherein the RX is (II) or



[0263] In some embodiments, the total length of the sense strand is 17-21 nt, and Z and the region N is linked via a phosphorothioate linkage.

[0264] In some embodiments, the total length of the sense strand is 17-21 nt, and X2 and the region B2 is linked via a phosphorothioate linkage.

[0265] In some embodiments, the total length of the sense strand is 17-21 nt, Z and the region N is linked via a phosphorothioate linkage, and X2 and the region B2 is linked via a phosphorothioate linkage.

[0266] In some embodiments, the total length of the sense strand is 17-21 nt, the total length of the anti-sense strand is 26-27 nt, the region A1 is 1-4 nucleotides, and Z and the region N is linked via a phosphorothioate linkage.

[0267] In some embodiments, the total length of the sense strand is 17-21 nt, the total length of

the anti-sense strand is 26-27 nt, the region A1 is 1-4 nucleotides, and X2 and the region B2 is linked via a phosphorothioate linkage.

[0268] In some embodiments, the total length of the sense strand is 17-21 nt, the total length of the anti-sense strand is 26-27 nt, the region A1 is 1-4 nucleotides, Z and the region N is linked via a phosphorothioate linkage, and X2 and the region B2 is linked via a phosphorothioate linkage.

[0269] In some embodiments, the total length of the sense strand is 17-21 nt, the total length of the anti-sense strand is 26-27 nt, the region A1 is 1-4 nucleotides, the region N comprises inverted abasic deoxyribonucleotide (invAB), and Z and the region N is linked via a phosphorothioate linkage.

[0270] In some embodiments, the total length of the sense strand is 17-21 nt, the total length of the anti-sense strand is 26-27 nt, the region A1 is 1-4 nucleotides, the region N comprises inverted abasic deoxyribonucleotide (invAB), and X2 and the region B2 is linked via a phosphorothioate linkage.

[0271] In some embodiments, the total length of the sense strand is 17-21 nt, the total length of the anti-sense strand is 26-27 nt, the region A1 is 1-4 nucleotides, the region N comprises inverted abasic deoxyribonucleotide (invAB), Z and the region N is linked via a phosphorothioate linkage, and X2 and the region B2 is linked via a phosphorothioate linkage.

[0272] In some embodiments, positions 1 and 2 and positions 2 and 3 counting from its 5' end and 3' end of the sense strand and the anti-sense strand are independently optionally linked via a phosphorothioate linkage.

[0273] In some embodiments, positions 1 and 2 and positions 2 and 3 counting from its 5' end and 3' end of the sense strand and the anti-sense strand are linked via a phosphorothioate linkage.

[0274] In one aspect, disclosed herein is a dsRNA conjugate, comprising a dsRNA disclosed herein and a delivery system, wherein the delivery system is capable to deliver the dsRNA to a target RNA and thereby provide RNA interference effect, the delivery system is conjugated with the dsRNA, and the dsRNA conjugate comprises the delivery system is 1 or more.

[0275] In some embodiments, a delivery system is selected from the group consisting of a liposome, lipid nanoparticle (LNP), lipid complex, lipid polymeric complex (LPP), mannose delivery system, N-acetylgalactosamine (GalNAc) conjugated delivery system, Apelin receptor targeting delivery system, integrin receptor targeting delivery system, peptide, antibody and any

combination thereof.

[0276] In some embodiments, the delivery system is conjugated at the 3' end of the sense strand.

[0277] In some embodiments, the delivery system is conjugated at the 5' end of the sense strand.

[0278] In some embodiments, the delivery system is conjugated at the 3' end of the anti-sense strand.

[0279] In some embodiments, the delivery system is conjugated at the 5' end of the anti-sense strand.

[0280] In some embodiments, the delivery system is conjugated at a nucleotide which is not at an end of the dsRNA.

[0281] In one aspect, disclosed herein is a composition comprising a dsRNA disclosed herein and a pharmaceutically acceptable carrier or excipient.

[0282] In one aspect, disclosed herein is use of the dsRNA, dsRNA conjugate or composition disclosed herein in the manufacturing of a medicament for treatment of a disease or disorder associated with RNA interference of a gene expression.

[0283] In some embodiments, the gene is selected from the group consisting of AGT, complement factor B, DGAT2, DUX, ANGPTL8, APOC3, F12, INHBE, PNPLA3, Serpine1, APP, SOD1, TMPRSS6, KHK, PCSK9, VEGFA, ANGPTL3, ANGPTL4, C3, C5, TTR, IGF-1R, VEGFR, ANG2, GIPR, GPR75, ActRII, NUDT21, PLN, HSD17b13, CNOT6L, PTP1B, CFHR, ATX, CIDEB, mARC1, TSHR, CB1 and LPA.

[0284] In some embodiments, the gene is selected from the group consisting of AGT, complement factor B, DGAT2, SOD1, KHK, APP, DUX, ANGPTL3, ANGPTL4 and ANGPTL8.

[0285] In one aspect, disclosed herein is a dsRNA, dsRNA conjugate or composition disclosed herein, for use of treating a disease or disorder associated with RNA interference of a gene expression.

[0286] Embodiment I-1. A double-stranded RNA (dsRNA), comprising a sense strand and an anti-sense strand, of a structure represented by Formula (I):

5'-	Region A1	Region A2	X1				-3'
3'-	Region B1	Region B2	X2	Y	Z	Region N	-5'

wherein

the sense strand consists of region A1, region A2 and X1 (from 5' to 3'),
the anti-sense strand consists of region N, Z, Y, X2, region B2 and region B1 (from 5' to 3'),
the region A1 is 0-15 nucleotides,
the region B1 is 0-15 nucleotides,
the region A2 and region B2 are of equal length which is at least 1 nucleotide,
the region A2 and region B2 comprise a double-stranded region that forms reverse complements,
each of X1, X2, Y and Z is independently 1 nucleotide, wherein X1 and X2 can associate with a hydrogen bond,
the region N is at least 1 nucleotide,
the total length of the sense strand is 17-23 nt,
the total length of the anti-sense strand is at least 26 nt,
the dsRNA comprises no more than 23 complementary base pairs, and
each of the nucleotides of the dsRNA is independently optionally modified.

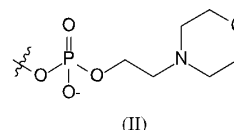
[0287] Embodiment I-2. The dsRNA of Embodiment I-1, wherein X2 is A or U, and Z is G.

[0288] Embodiment I-3. The dsRNA of Embodiment I-2, wherein Y is A or U.

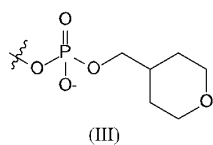
[0289] Embodiment I-4. The dsRNA of Embodiment I-3, wherein Y is A.

[0290] Embodiment I-5. The dsRNA of any one of Embodiments I-1 to I-4, wherein the region N consists of 1 nucleotide and group X, wherein the group X is nucleotide, blocking group or a combination of nucleotide and blocking group, wherein the blocking group is a structure capable of resisting nuclease degradation, the blocking group is linked to the region N at 5' end, and the blocking group is independently optionally modified.

[0291] Embodiment I-6. The dsRNA of Embodiment I-5, wherein the blocking group is selected



or



from inverted abasic deoxyribonucleotide or RX, wherein RX is

[0292] Embodiment I-7. The dsRNA of Embodiment I-6, wherein the region N consists of 1 nucleotide and 1 blocking group.

[0293] Embodiment I-8. The dsRNA of Embodiment I-7, wherein the 1 nucleotide and 1 blocking group of the region N are linked via phosphorothioate linkage.

[0294] Embodiment I-9. The dsRNA of any one of Embodiments I-1 to I-8, wherein all nucleotides of the dsRNA are independently modified.

[0295] Embodiment I-10. The dsRNA of any one of Embodiments I-1 to I-8, wherein the nucleotide modification comprises 1, 2 or more selected from the group consisting of 2'-OMe, 2'-F, 2'-deoxy, VP, 5'-MP, PS, PS2, MP, MOP, invAB and a modification enhancing the affinity of dsRNA and ARGO protein.

[0296] Embodiment I-11. The dsRNA of Embodiment I-10, wherein the modification enhancing the affinity of dsRNA and ARGO protein is on X2.

[0297] Embodiment I-12. The dsRNA of any one of Embodiments I-1 to I-11, wherein the total length of the sense strand is 17-21 nt; further, the total length of the sense strand is 18-21 nt.

[0298] Embodiment I-13. The dsRNA of any one of Embodiments I-1 to I-11, wherein the total length of the anti-sense strand is 26-35 nt; further, the total length of the anti-sense strand is 26-27 nt.

[0299] Embodiment I-14. The dsRNA of any one of Embodiments I-1 to I-11, wherein the region A1 and the region B1 comprise a complementary double-strand region.

[0300] Embodiment I-15. The dsRNA of Embodiment I-14, wherein the region A1 is 1 nucleotide, and the region B1 is 3 nucleotides.

[0301] Embodiment I-16. The dsRNA of Embodiment I-15, wherein the length of region A2 or B2 is 20 nt.

[0302] Embodiment I-17. The dsRNA of any one of Embodiments I-1 to I-11, wherein the region A1 is 0 nucleotide, and the region B1 is 2 nucleotides.

[0303] Embodiment I-18. Use of the dsRNA of any one of Embodiments I-1 to I-17 or a pharmaceutical composition thereof in the manufacturing of a medicament for treatment of a disease or disorder associated with RNA interference of a gene expression; further, the gene is selected from the group consisting of AGT, complement factor B, DGAT2, DUX, ANGPTL8, APOC3, F12, INHBE, PNPLA3, Serpinc1, APP, SOD1, TMPRSS6, KHK, PCSK9, VEGFA,

ANGPTL3, ANGPTL4, C3, C5, TTR, IGF-1R, VEGFR, ANG2, GIPR, GPR75, ActRII, NUDT21, PLN, HSD17b13 and LPA.

Methods of Treatment

[0304] Provide herein is a method of modulating the expression of a target gene in a subject in need thereof, comprising administering to the subject a pharmaceutically effective amount of a double-stranded oligonucleotide agent disclosed herein or a pharmaceutical composition disclosed herein.

[0305] Provide herein is a method of inhibiting the expression of a target gene in a subject in need thereof, comprising administering to the subject a pharmaceutically effective amount of a double-stranded oligonucleotide agent disclosed herein or a pharmaceutical composition disclosed herein.

[0306] Provide herein is a method of treating a disease or disorder in a subject in need thereof, by modulating (*e.g.*, inhibiting) the expression of a target gene, for example comprising administering to the subject a pharmaceutically effective amount of a double-stranded oligonucleotide agent disclosed herein or a pharmaceutical composition disclosed herein.

[0307] Provide herein is a method of treating a disease or disorder in a subject in need thereof, comprising administering to the subject a pharmaceutically effective amount of a double-stranded oligonucleotide agent disclosed herein or a pharmaceutical composition disclosed herein. In some embodiments, the disease or disorder is associated with the target gene. In some embodiments the disease or disorder is associated with the overexpression or activation of the target gene.

[0308] In some embodiments, the target gene includes, but not limited to, AGT, CFB, DGAT2, DUX, ANGPTL8, APOC3, F12, INHBE, PNPLA3, Serpinc1, APP, SOD1, TMPRSS6, KHK, PCSK9, VEGFA, ANGPTL3, ANGPTL4, C3, C5, TTR, IGF-1R, VEGFR, ANG2, GIPR, GPR75, ActRII, NUDT21, PLN, HSD17b13, CNOT6L, PTP1B, CFHR, ATX, CIDEB, mARC1, TSHR, CB1 and LPA.

[0309] The double-stranded oligonucleotide agent disclosed herein or a pharmaceutical composition disclosed herein can be administered to a subject by a variety of routes, depending upon whether local or systemic treatment is desired and upon the area to be treated. Administration may be topical (including ophthalmic, vaginal, rectal, intranasal, transdermal), oral or parenteral. Parenteral administration includes intravenous drip, subcutaneous, intraperitoneal or intramuscular injection, or intrathecal or intraventricular administration.

[0310] The route and site of administration may be chosen to enhance targeting. For example, to target muscle cells, intramuscular injection into the muscles of interest would be a logical choice. Lung cells might be targeted by administering the oligonucleotide in aerosol form.

[0311] The defined amount can be an amount effective to treat or prevent a disease or disorder, *e.g.*, a disease or disorder associated with the target RNA. The unit dose, for example, can be administered by injection (*e.g.*, intravenous, subcutaneous or intramuscular), an inhaled dose, or a topical application.

[0312] In some embodiments, the unit dose is administered less frequently than once a day, *e.g.*, less than every 2, 4, 8 or 30 days. In another embodiment, the unit dose is not administered with a frequency (*e.g.*, not a regular frequency). For example, the unit dose may be administered a single time.

[0313] In one embodiment, the effective dose is administered with other traditional therapeutic modalities. The effective dose can be administered in a single dose or in two or more doses, as desired or considered appropriate under the specific circumstances. If desired to facilitate repeated or frequent infusions, implantation of a delivery device, *e.g.*, a pump, semi-permanent stent (*e.g.*, intravenous, intraperitoneal, intracisternal or intracapsular), or reservoir may be advisable.

Pharmaceutical Composition

[0314] The oligonucleotide agents disclosed herein may be formulated for pharmaceutical use. Pharmaceutically acceptable compositions comprise the oligonucleotide agent (*e.g.*, a pharmaceutically effective amount of the oligonucleotide agent) disclosed herein, taken alone or formulated together with one or more pharmaceutically acceptable carriers (additives), excipient and/or diluents.

[0315] The pharmaceutical compositions may be specially formulated for administration in solid or liquid form, including those adapted for the following: (1) oral administration, for example, drenches (aqueous or non-aqueous solutions or suspensions), tablets, *e.g.*, those targeted for buccal, sublingual, and systemic absorption, boluses, powders, granules, pastes for application to the tongue; (2) parenteral administration, for example, by subcutaneous, intramuscular, intravenous or epidural injection as, for example, a sterile solution or suspension, or sustained-release formulation; (3) topical application, for example, as a cream, ointment, or a controlled-release patch or spray applied to the skin; (4) intravaginally or intrarectally, for example, as a pessary, cream or foam;

(5) sublingually; (6) ocularly; (7) transdermally; or (8) nasally. Delivery using subcutaneous or intravenous methods can be particularly advantageous.

[0316] The pharmaceutically-acceptable carrier can be a composition or vehicle, such as a liquid or solid filler, diluent, excipient, manufacturing aid (*e.g.*, lubricant, talc magnesium, calcium or zinc stearate, or steric acid), or solvent encapsulating material, involved in carrying or transporting the subject compound from one organ, or portion of the body, to another organ, or portion of the body. Each carrier must be “acceptable” in the sense of being compatible with the other ingredients of the formulation and not injurious to the patient. Some examples of materials which can serve as pharmaceutically-acceptable carriers include: (1) sugars, such as lactose, glucose and sucrose; (2) starches, such as corn starch and potato starch; (3) cellulose, and its derivatives, such as sodium carboxymethyl cellulose, ethyl cellulose and cellulose acetate; (4) powdered tragacanth; (5) malt; (6) gelatin; (7) lubricating agents, such as magnesium stearate, sodium lauryl sulfate and talc; (8) excipients, such as cocoa butter and suppository waxes; (9) oils, such as peanut oil, cottonseed oil, safflower oil, sesame oil, olive oil, corn oil and soybean oil; (10) glycols, such as propylene glycol; (11) polyols, such as glycerin, sorbitol, mannitol and polyethylene glycol; (12) esters, such as ethyl oleate and ethyl laurate; (13) agar; (14) buffering agents, such as magnesium hydroxide and aluminum hydroxide; (15) alginic acid; (16) pyrogen-free water; (17) isotonic saline; (18) Ringer's solution; (19) ethyl alcohol; (20) pH buffered solutions; (21) polyesters, polycarbonates and/or polyanhydrides; (22) bulking agents, such as polypeptides and amino acids (23) serum component, such as serum albumin, HDL and LDL; and (22) other non-toxic compatible substances employed in pharmaceutical formulations.

[0317] The formulations may conveniently be presented in unit dosage form and may be prepared by any methods well known in the art of pharmacy. The amount of active ingredient which can be combined with a carrier material to produce a single dosage form will vary depending upon the host being treated, the particular mode of administration. The amount of active ingredient which can be combined with a carrier material to produce a single dosage form will generally be that amount of the compound which produces a therapeutic effect. Generally, out of one hundred percent, this amount will range from about 0.1 percent to about ninety-nine percent of active ingredient, preferably from about 5 percent to about 70 percent, most preferably from about 10 percent to about 30 percent.

[0318] In certain embodiments, a formulation of the present invention comprises an excipient selected from the group consisting of cyclodextrins, celluloses, liposomes, micelle forming agents, *e.g.*, bile acids, and polymeric carriers, *e.g.*, polyesters and polyanhydrides; and an oligonucleotide disclosed herein. In certain embodiments, a formulation renders orally bioavailable for an oligonucleotide agent disclosed herein.

[0319] The double-stranded oligonucleotide agents can be formulated in combination with another agent, *e.g.*, another therapeutic agent or a stabilizing agent, *e.g.*, a protein that complexes with double-stranded oligonucleotide agent. Still other agents include chelators, *e.g.*, EDTA (*e.g.*, to remove divalent cations such as Mg^{2+}), salts, RNase inhibitors (*e.g.*, a broad specificity RNase inhibitor such as RNasin) and so forth.

[0320] Methods of preparing these formulations or compositions include the step of bringing into association an oligonucleotide agent disclosed herein with the carrier and, optionally, one or more accessory ingredients. In general, the formulations are prepared by uniformly and intimately bringing into association an oligonucleotide disclosed herein with liquid carriers, or finely divided solid carriers, or both, and then, if necessary, shaping the product.

[0321] In some cases, in order to prolong the effect of a drug, it is desirable to slow the absorption of the drug from subcutaneous or intramuscular injection. This may be accomplished by the use of a liquid suspension of crystalline or amorphous material having poor water solubility. The rate of absorption of the drug then depends upon its rate of dissolution which, in turn, may depend upon crystal size and crystalline form. Alternatively, delayed absorption of a parenterally-administered drug form is accomplished by dissolving or suspending the drug in an oil vehicle.

[0322] An oligonucleotide agent disclosed herein may be formulated for administration in any convenient way for use in human or veterinary medicine, by analogy with other pharmaceuticals.

Examples

[0323] *Abbreviations*

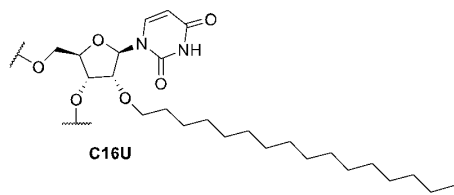
[0324] As used herein, the term “nucleotide” encompasses a ribonucleotide and deoxyribonucleotide. As used herein, “G”, “C”, “A”, “T” and “U” refer to a nucleotide containing guanine, cytosine, adenine, thymine and uracil, respectively. When “d” is marked before A, U, C, G or T, it stands for a 2'-deoxyribonucleotide (DNA), and when no “d” is marked, it stands for a ribonucleotide (RNA). For example, “dA” stands for deoxyadenosine, “dG” stands for

deoxyguanosine, “dT” stands for deoxythymidine, “dU” stands for deoxyuridine, and “dC” stands for deoxycytidine; “A” stands for adenosine, “G” stands for guanosine, “T” stands for ribothymidine or 5-methyluridine, “U” stands for uridine, and “C” stands for cytidine.

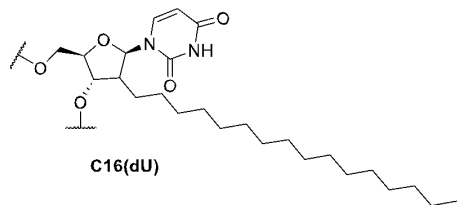
[0325] As used herein, when “f” is marked after A, U, C, G, and T, it stands for a nucleotide modified with 2'-fluoro. For example, “Af” stands for 2'-fluoroadenosine, “Gf” stands for 2'-fluoroguanosine, “Tf” stands for 2'-fluororibothymidine or 5-methyl-2'-fluorouridine, “Uf” stands for 2'-fluorouridine, and “Cf” stands for 2'-fluorocytidine.

[0326] As used herein, a lowercase letter such as “a”, “u”, “c”, “g”, “t” and “i” stands for 2'-methoxy modified A, U, C, G, T, and I, respectively. For example, “a” stands for 2'-methoxyadenosine, “g” stands for 2'-methoxyguanosine, “t” stands for 2'-methoxyribothymidine or 5-methyl-2'-methoxyuridine, “u” stands for 2'-methoxyuridine, and “c” stands for 2'-methoxycytidine.

[0327] As used herein, “C16” or “C16 modification” refers to 2'-O-C16 modification or to 2'-C16 modification. When “C16” marked before A, U, C, G or T, it stands for a nucleotide modified

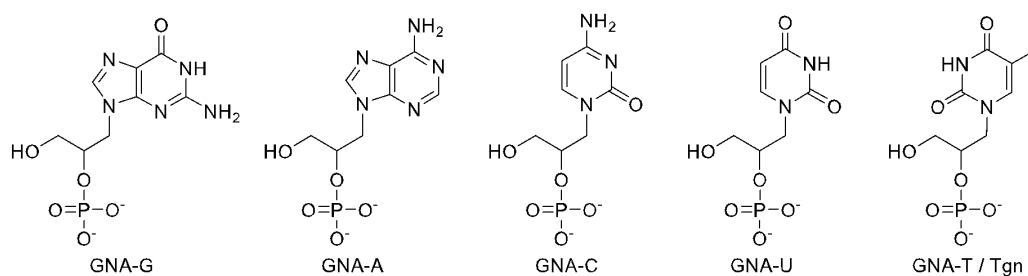


with 2'-hexadecyloxy. For example, “C16U” stands for



For example, “C16(dU)” stands for

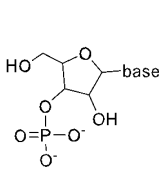
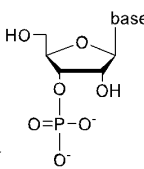
[0328] As used herein, when “GNA-” is marked before A, U, C, G, and T, it stands for a nucleotide modified with Glycol nucleic acid. “Tgn” is another name of “GNA-T”, “Tgn” is equal to “GNA-T”. For example, GNA-G, GNA-A, GNA-C, GNA-U, and GNA-T have the following structures.

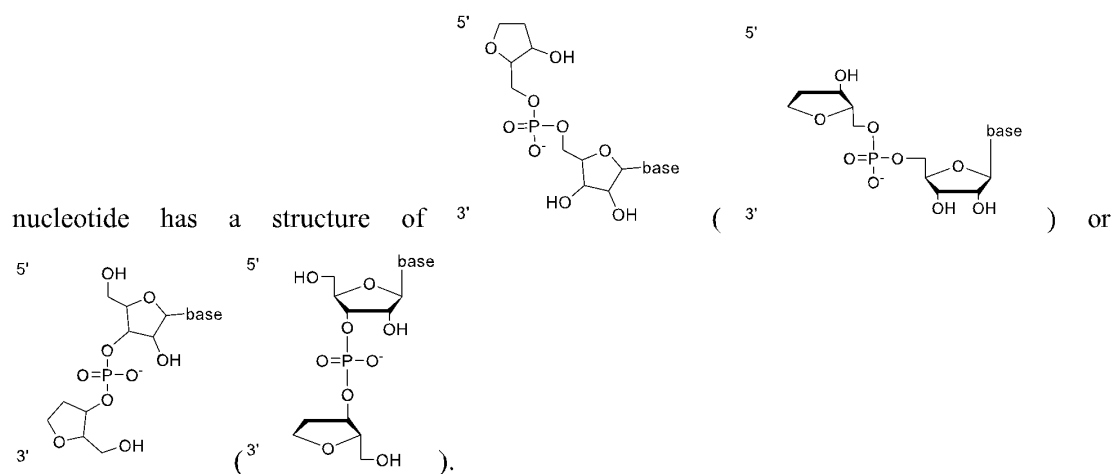


[0329] As used herein, “*” between two nucleotides such as A, U, C, G and T stands for a phosphorothioate internucleotide linkage, *i.e.*, the two nucleotides are linked via a phosphorothioate linkage. As used herein, the absence of “*” between two nucleotides such as A, U, C, G and T stands for an unmodified internucleotide linkage (*i.e.*, phosphodiester linkage).

[0330] For example, a sequence of “5’-AdUgCf*T-3’” stands for a sequence, wherein starting from the 5’ end, position 1 is adenosine, position 2 is deoxyuridine, position 3 is 2’-methoxyguanosine, position 4 is 2’-fluorocytidine, position 5 is ribothymidine, the position 4 and position 5 are linked via a phosphorothioate linkage and the other adjacent positions are linked via a phosphodiester linkage.

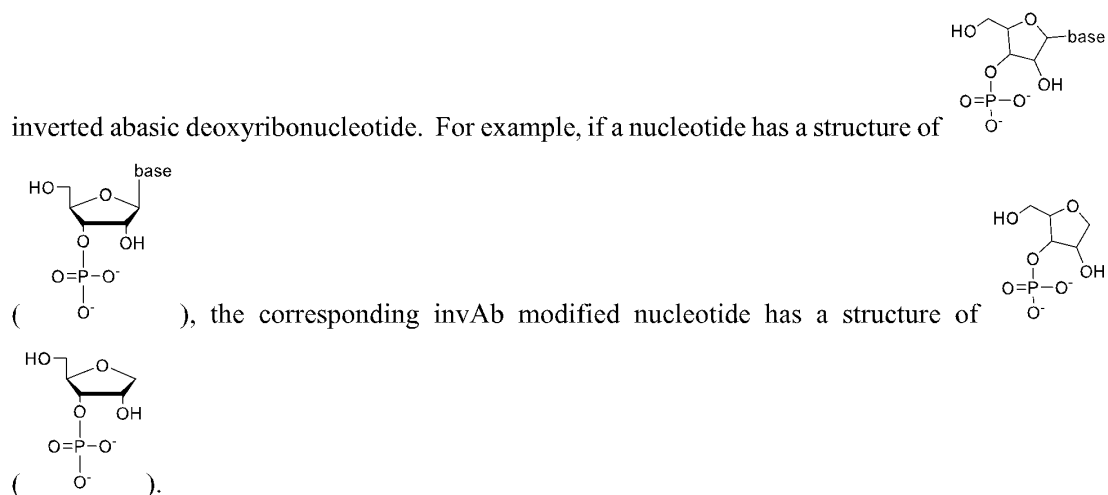
[0331] As used herein, “invAB” or “invAB modification” refers to a nucleotide conjugated (*e.g.*, conjugated at 5’ end or 3’ end) with an inverted abasic deoxyribonucleotide. For example, if a

nucleotide has a structure of  (), the corresponding invAB modified

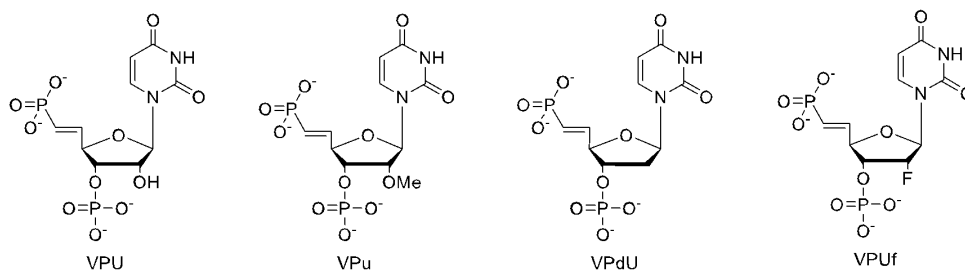


[0332] As used herein, “invAb” or “invAb modification” refers to replace a nucleotide with an

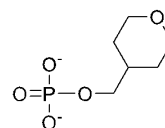
inverted abasic deoxyribonucleotide. For example, if a nucleotide has a structure of



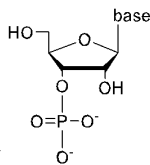
[0333] As used herein, “VP” or “VP modification” refers to a nucleotide with (E)-vinylphosphate modification (e.g., 5’-(E)-vinylphosphate modification). For example, VP modified U, u, dU and Uf have the following structures.



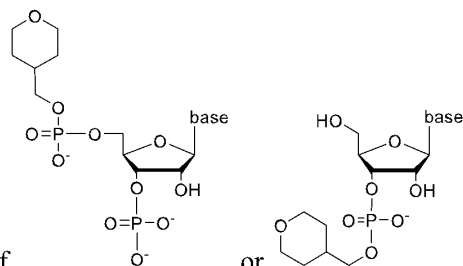
[0334] As used herein, “M03” or “M03 modification” refers to a nucleotide conjugated (*e.g.*,



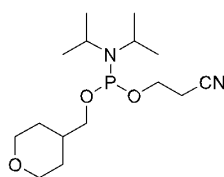
conjugated at 5' end or 3' end) with a molecule having a structure of . For example,



if a nucleotide has a structure of , the corresponding M03 modified nucleotide has a

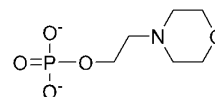


structure of or . In some embodiments, the conjugation with

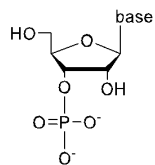


M03 is by the reaction of a nucleotide and .

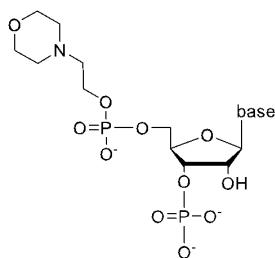
[0335] As used herein, “M06” or “M06 modification” refers to a nucleotide conjugated (*e.g.*,



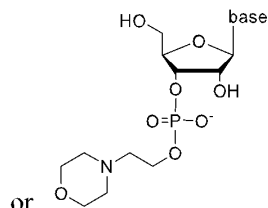
conjugated at 5' end or 3' end) with a molecule having a structure of . For



example, if a nucleotide has a structure of , the corresponding M06 modified

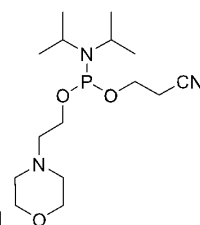


nucleotide has a structure of



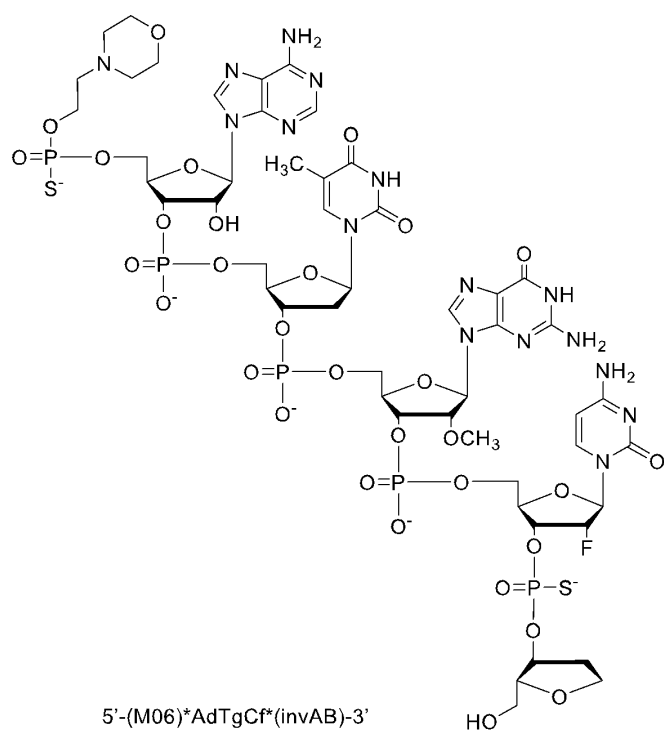
or

. In some

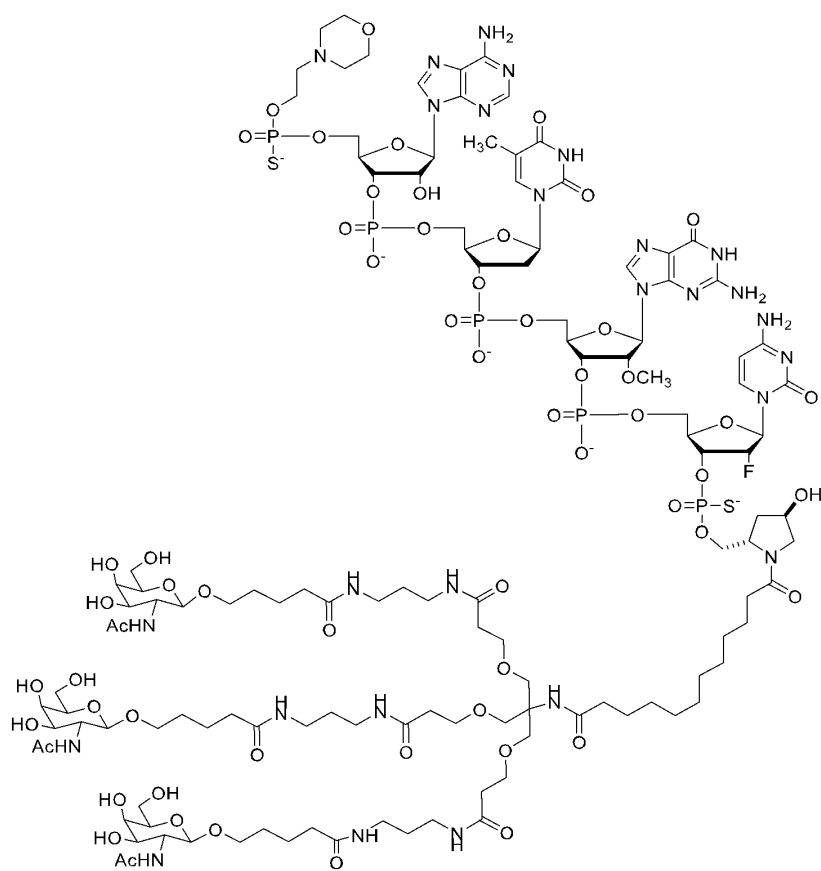


embodiments, the conjugation with M06 is by the reaction of a nucleotide and

[0336] For Example, a sequence of “5’-(M06)*AdTgCf*(invAB)-3’” stands for an oligonucleotide having a structure of



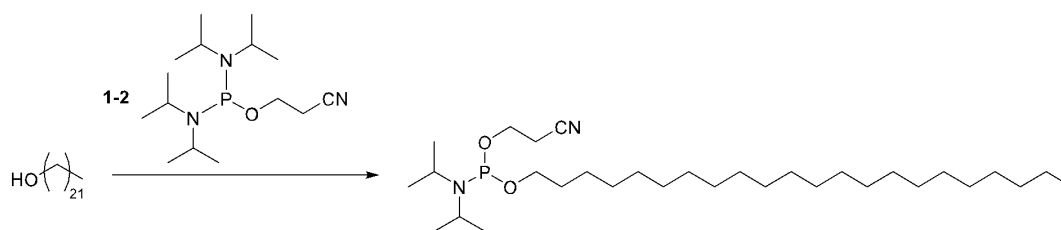
[0337] For Example, a sequence of “5’-(M06)*AdTgCf*[L96]-3’” stands for an oligonucleotide having a structure of



5'-(M06)*AdTgCf*[L96]-3'

[0338] Example 1 Synthesis of Precursors of Ligands

[0339] Example 1.1 Synthesis of Precursor of VSDL-01 (2-cyanoethyl docosyl diisopropylphosphoramidite)



1-1

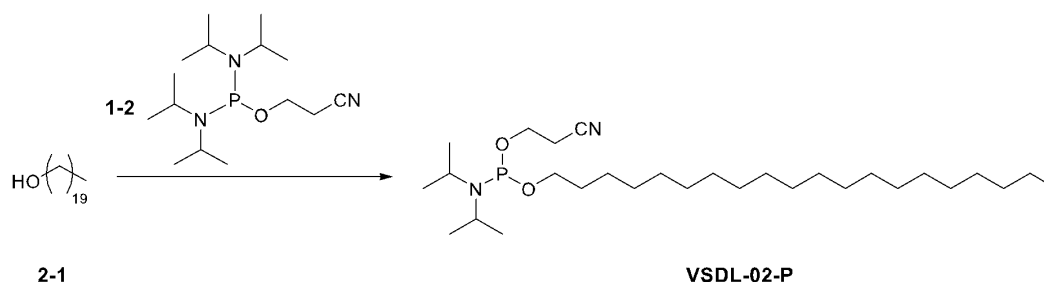
VSDL-01-P

[0340] To a solution of compound **1-1** (10.00 g, 30.62 mmol) in anhydrous DCM (100 mL) was added 1H-imidazole-4,5-dicarbonitrile (1.81 g, 15.31 mmol), and then a solution of compound **1-2** (9.23 g, 30.62 mmol) in anhydrous DCM (20 mL) was added dropwise to the mixture. The reaction mixture was stirred at 25°C for 16 hours under N₂. The mixture was concentrated in vacuo to remove DCM. The residue was purified by silica gel chromatography (eluting with hexane: EtOAc = 5:1, containing 1% TEA) to give Precursor of **VSDL-01**.

[0341] ¹H NMR (400 MHz, CDCl₃) δ 3.91-3.72 (m, 2H), 3.68 - 3.51 (m, 4H), 2.63 (t, *J* = 6.8 Hz, 2H), 1.58 (p, *J* = 6.8 Hz, 2H), 1.30-1.23 (m, 38H), 1.17 (dd, *J* = 6.8, 4.2 Hz, 12H), 0.87 (t, *J* = 6.8 Hz, 3H).

[0342] ³¹P NMR: (400 MHz, CDCl₃) δ 147.17.

[0343] **Example 1.2 Synthesis of Precursor of VSDL-02 (2-cyanoethyl icosyl diisopropylphosphoramidite)**

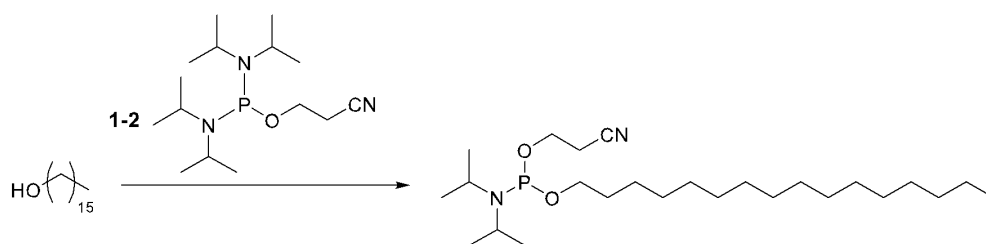


[0344] Similar to the synthesis method of Example 1.1.

[0345] ¹H NMR (400 MHz, CDCl₃) δ 3.92-3.72 (m, 2H), 3.66 - 3.50 (m, 4H), 2.62 (t, *J* = 6.8 Hz, 2H), 1.60 (p, *J* = 6.8 Hz, 2H), 1.28-1.24 (m, 34H), 1.17 (dd, *J* = 6.8, 4.2 Hz, 12H), 0.88 (t, *J* = 6.8 Hz, 3H).

[0346] ³¹P NMR: (400 MHz, CDCl₃) δ 147.32.

[0347] **Example 1.3 Synthesis of Precursor of VSDL-03 (2-cyanoethyl hexadecyl diisopropylphosphoramidite)**



3-1

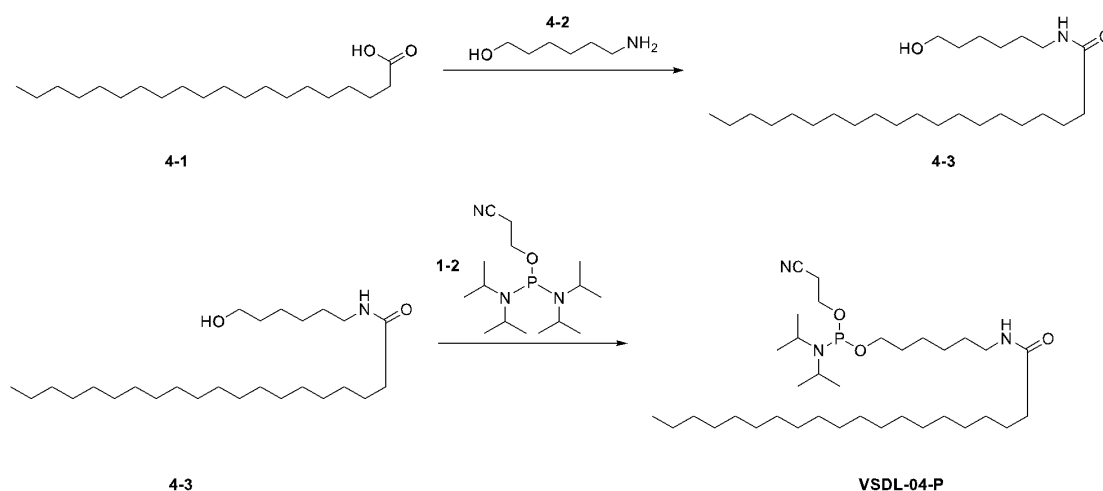
VSDL-03-P

[0348] Similar to the synthesis method of Example 1.1.

[0349] ¹H NMR (400 MHz, CDCl₃) δ 3.90-3.72 (m, 2H), 3.68 - 3.52 (m, 4H), 2.63 (t, *J* = 6.8 Hz, 2H), 1.59 (p, *J* = 6.8 Hz, 2H), 1.28-1.24 (m, 26H), 1.17 (d, *J* = 6.8, 4.2 Hz, 12H), 0.87 (t, *J* = 6.8 Hz, 3H).

[0350] ³¹P NMR: (400 MHz, CDCl₃) δ 147.24.

[0351] Example 1.4 Synthesis of Precursor of VSDL-04 (2-cyanoethyl (6-icosanamido)hexyl) diisopropylphosphoramidite)



[0352] To a solution of compound 4-1 (3.12g, 10 mmol) and TEA (3.04g, 30 mmol) in anhydrous DCM (200 mL) was added dropwise pentafluorophenyl trifluoroacetate (4.2g, 15 mmol). After stirring for 15 min, compound 4-2 (1.4g, 12 mmol) was added to the mixture. The reaction mixture was stirred at 25°C for 16 hours. The mixture was concentrated under vacuum to

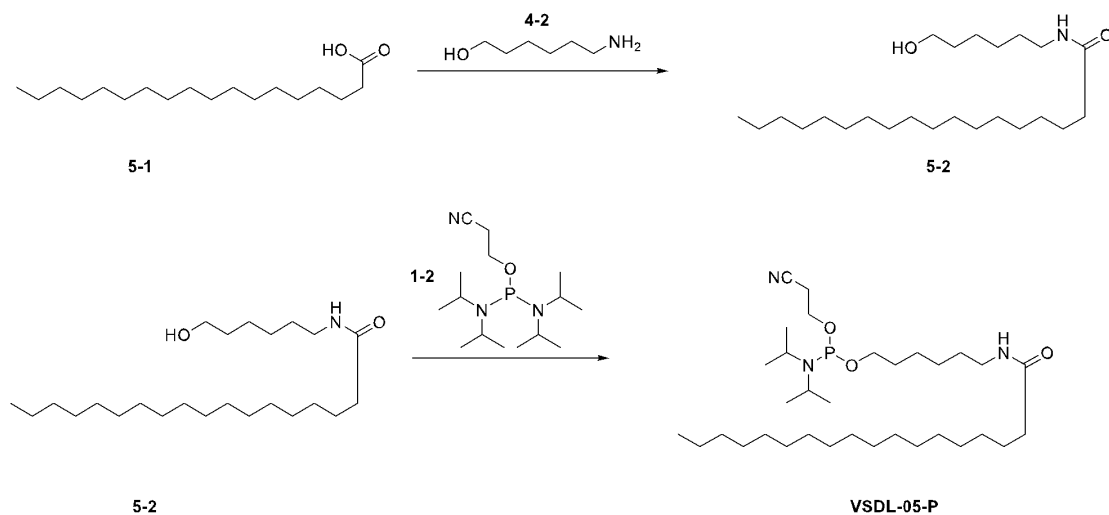
remove DCM. The residue was purified by silica gel chromatography (eluting with hexane: EtOAc = 5:1) to give compound **4-3** (3.82 g).

[0353] To a solution of compound **4-3** (3.63 g, 8.82 mmol) in anhydrous DMF (60 mL) was added 1H-imidazole-4,5-dicarbonitrile (0.52 g, 4.41 mmol), and then a solution of compound **1-2** (2.65 g, 8.82 mmol) in anhydrous DMF (5 mL) was added dropwise to the mixture. The reaction mixture was stirred at 25°C for 16 hours under N₂. The mixture was diluted with sat. aq. NaHCO₃ (200 mL), extracted by EtOAc (3 x 50 mL), the organic phase was combined, dried over anhydrous Na₂SO₄, concentrated under vacuum. The residue was purified by silica gel chromatography (eluting with hexane: EtOAc = 5:1, containing 1% TEA) to give Precursor of **VSDL-04**.

[0354] ¹H NMR (400 MHz, CDCl₃) δ 5.45 (t, *J* = 5.6 Hz, 1H), 3.92-3.75 (m, 2H), 3.70-3.54 (m, 4H), 3.23 (q, *J* = 6.8 Hz, 2H), 2.64 (t, *J* = 6.4 Hz, 2H), 2.14 (t, *J* = 7.6 Hz, 2H), 1.63-1.57 (m, 4H), 1.53-1.56 (m, 2H), 1.28-1.24 (m, 36H), 1.17 (dd, *J* = 6.8, 4.2 Hz, 12H), 0.87 (t, *J* = 6.8 Hz, 3H).

[0355] ³¹P NMR: (400 MHz, CDCl₃) δ 147.32.

[0356] Example 1.5 Synthesis of Precursor of **VSDL-05** (2-cyanoethyl (6-stearamido)hexyl) diisopropylphosphoramidite)

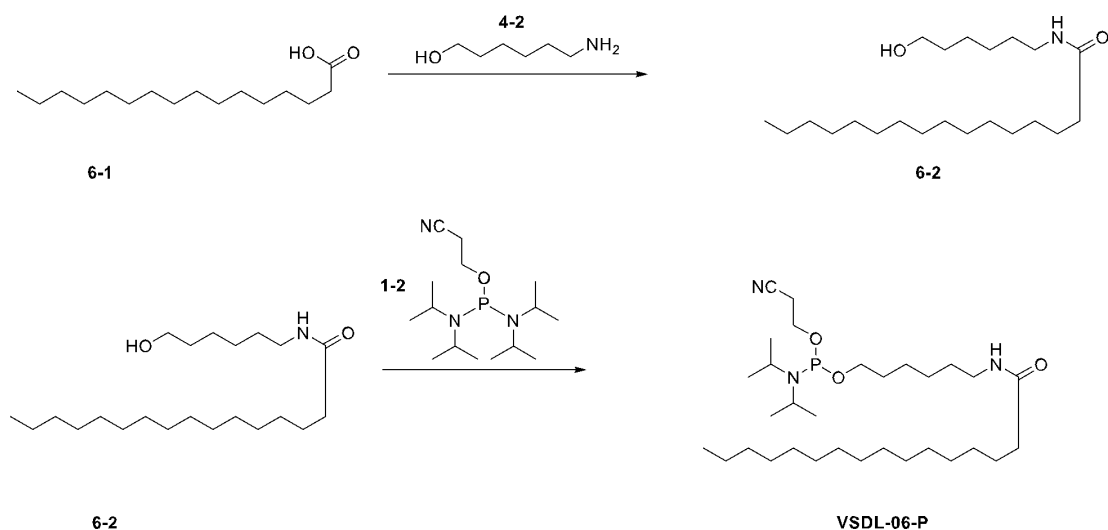


[0357] Similar to the synthesis method of Example 1.4.

[0358] ^1H NMR (400 MHz, CDCl_3) δ 5.47 (t, $J = 5.6$ Hz, 1H), 3.89-3.75 (m, 2H), 3.63-3.67 (m, 4H), 3.23 (q, $J = 6.8$ Hz, 2H), 2.64 (t, $J = 6.4$ Hz, 2H), 2.14 (t, $J = 7.6$ Hz, 2H), 1.66-1.57 (m, 4H), 1.51-1.46 (m, 2H), 1.27-1.23 (m, 32H), 1.17 (dd, $J = 6.8, 4.2$ Hz, 12H), 0.87 (t, $J = 6.8$ Hz, 3H).

[0359] ^{31}P NMR: (400 MHz, CDCl_3) δ 147.20.

[0360] Example 1.6 Synthesis of Precursor of VSDL-06 (2-cyanoethyl (6-palmitamido)hexyl diisopropylphosphoramidite)

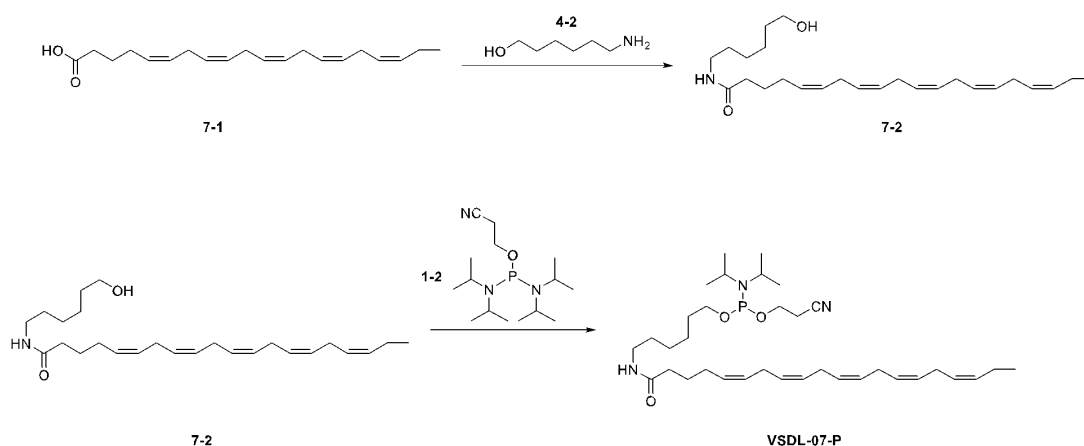


[0361] Similar to the synthesis method of Example 1.4.

[0362] ^1H NMR (400 MHz, CDCl_3) δ 5.69 (t, $J = 5.6$ Hz, 1H), 3.90-3.75 (m, 2H), 3.69-3.54 (m, 4H), 3.26-3.21 (m, 2H), 2.65 (t, $J = 6.4$ Hz, 2H), 2.15 (t, $J = 7.6$ Hz, 2H), 1.65-1.58 (m, 4H), 1.54-1.47 (m, 2H), 1.28-1.24 (m, 28H), 1.18 (dd, $J = 6.8, 4.2$ Hz, 12H), 0.88 (t, $J = 6.8$ Hz, 3H).

[0363] ^{31}P NMR: (400 MHz, CDCl_3) δ 147.25.

[0364] Example 1.7 Synthesis of Precursor of VSDL-07 (2-cyanoethyl (6-((5Z,8Z,11Z,14Z,17Z)-icosa-5,8,11,14,17-pentaenamido)hexyl) diisopropylphosphoramidite)

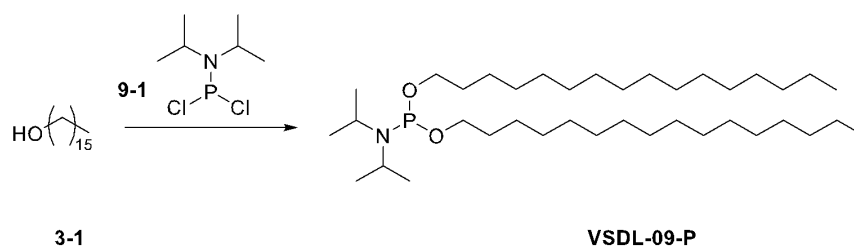


[0365] Similar to the synthesis method of Example 1.4.

[0366] ^1H NMR (400 MHz, CDCl_3) δ 5.52-5.24 (m, 11H), 3.89-3.74 (m, 2H), 3.69-3.53 (m, 4H), 3.25-3.20 (m, 2H), 2.85-2.78 (m, 8H), 2.63 (t, $J = 6.5$ Hz, 2H), 2.17-2.03 (m, 6H), 1.74-1.68 (m, 2H), 1.64-1.57 (m, 2H), 1.53-1.46 (m, 2H), 1.42-1.31 (m, 4H), 1.17 (dd, $J = 6.8, 4.2$ Hz, 12H), 0.97 (t, $J = 7.6$ Hz, 3H).

[0367] ^{31}P NMR: (400 MHz, CDCl_3) δ 147.32.

[0368] Example 1.9 Synthesis of Precursor of VSDL-09 (dihexadecyl diisopropylphosphoramidite)

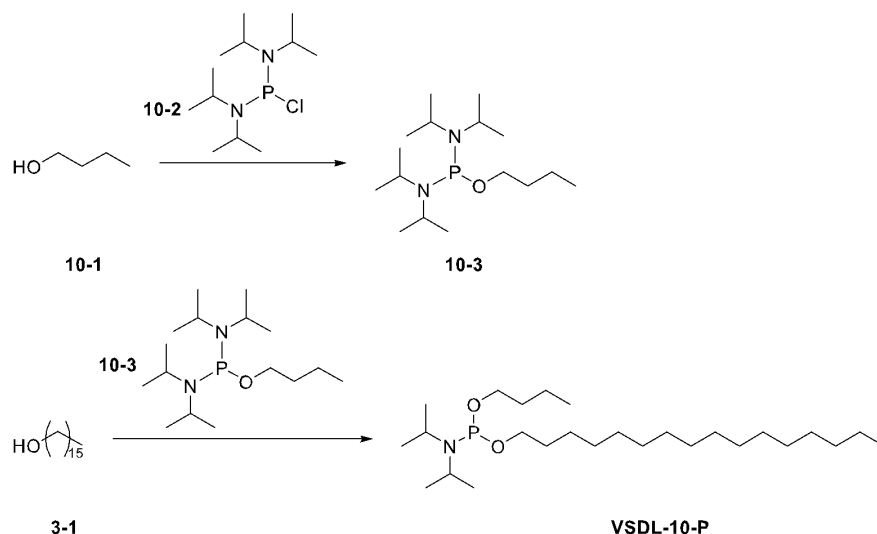


[0369] To a solution of compound **9-1** (1 g, 4.949 mmol) in anhydrous THF (5 mL) was added TEA (1.376 mL, 9.898 mmol). The reaction mixture was cooled to 0°C by ice bath, and then compound **3-1** (2.934 mL, 9.898 mmol) was added dropwise under nitrogen protection. The system temperature was kept around 0°C during addition. After addition, a suspension containing white solid was obtained, the ice bath was removed and restored to room temperature, the stirring was continued at room temperature for 3 hours. The mixture was filtered, the filter was washed by THF, the filtrate was collected, concentrated under vacuum to give Precursor of **VSDL-09**.

[0370] ^1H NMR (400 MHz, CDCl_3) δ 3.68-3.52 (m, 6H), 1.64-1.57 (m, 4H), 1.36-1.25 (m, 52H), 1.18 (d, $J = 6.8$ Hz, 12H), 0.88 (t, $J = 6.8$ Hz, 6H).

[0371] ^{31}P NMR: (400 MHz, CDCl_3) δ 144.91.

[0372] **Example 1.10 Synthesis of Precursor of VSDL-10 (butyl hexadecyl diisopropylphosphoramidite)**



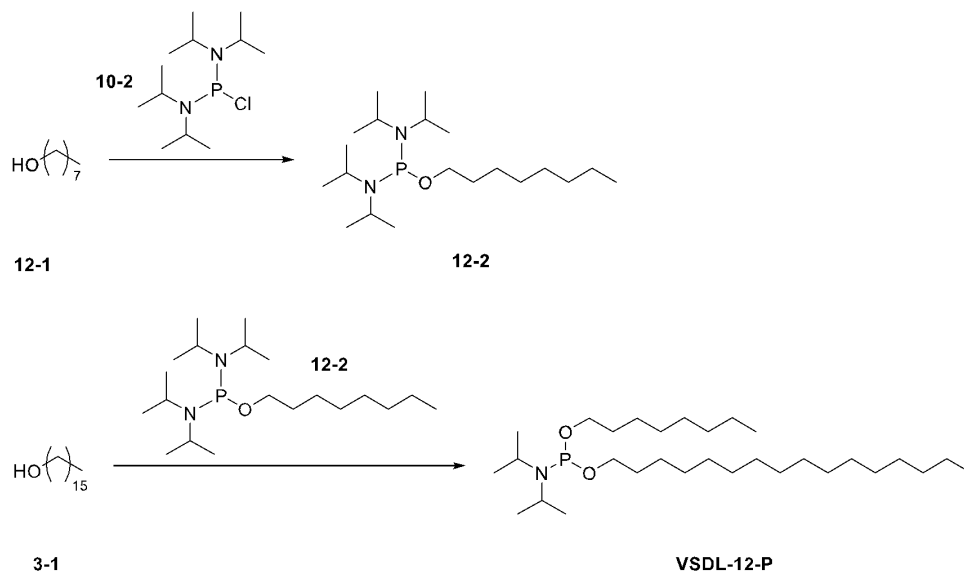
[0373] To a solution of compound **10-2** (4 g, 15 mmol) in anhydrous THF (10 mL) was added TEA (3.13 mL, 22.49 mmol). The reaction mixture was cooled to 0°C by ice bath, and then compound **10-1** (1.11 g, 15 mmol) was added dropwise under nitrogen protection. The system temperature was kept around 0°C during addition. After addition, the stirring was continued at room temperature for 3 hours. The mixture was concentrated under vacuum to give compound **10-3** which was used directly in next step without further purification.

[0374] To a solution of compound **3-1** (1.33g, 5.47 mmol) in anhydrous DCM (10 mL) was added 1H-imidazole-4,5-dicarbonitrile (0.32 g, 2.74 mmol), and then a solution of compound **10-3** (2.5 g, 8.21 mmol) in anhydrous DCM (5 mL) was added dropwise to the mixture. The reaction mixture was stirred at 25°C for 16 hours under N_2 . The mixture was concentrated under vacuum. The residue was purified by silica gel chromatography (eluting with hexane: EtOAc = 5:1, containing 1% TEA) to give Precursor of **VSDL-10**.

[0375] ^1H NMR (400 MHz, CDCl_3) δ 3.69-3.51 (m, 6H), 1.64-1.55 (m, 4H), 1.44-1.22 (m, 28H), 1.17 (d, $J = 6.8$ Hz, 12H), 0.90 (dt, $J = 14.0, 7.2$ Hz, 6H).

[0376] ^{31}P NMR: (400 MHz, CDCl_3) δ 145.00.

[0377] **Example 1.12 Synthesis of Precursor of VSDL-12 (hexadecyl octyl diisopropylphosphoramidite)**



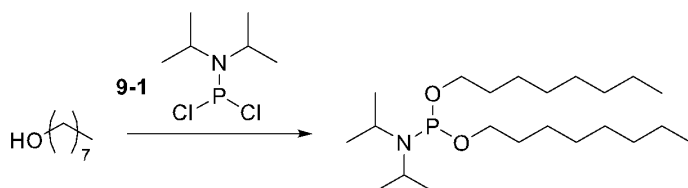
[0378] To a solution of compound **10-2** (4 g, 15 mmol) in anhydrous THF (10 mL) was added TEA (3.13 mL, 22.49 mmol). The reaction mixture was cooled to 0°C by ice bath, and then compound **12-1** (1.95 g, 15 mmol) was added dropwise under nitrogen protection. The system temperature was kept around 0°C during addition. After addition, the stirring was continued at room temperature for 3 hours. The mixture was concentrated under vacuum to give compound **12-2** which was used directly in next step without further purification.

[0379] To a solution of compound **3-1** (1.61 g, 6.66 mmol) in anhydrous DCM (10 mL) was added 1H-imidazole-4,5-dicarbonitrile (0.39 g, 3.33 mmol), and then a solution of compound **12-2** (3.6 g, 9.98 mmol) in anhydrous DCM (5 mL) was added dropwise to the mixture. The reaction mixture was stirred at 25°C for 16 hours under N_2 . The mixture was concentrated under vacuum. The residue was purified by silica gel chromatography (eluting with hexane: EtOAc = 5:1, containing 1% TEA) to give Precursor of **VSDL-12**.

[0380] ^1H NMR (400 MHz, CDCl_3) δ 3.69-3.52(m, 6H), 1.64-1.56 (m, 4H), 1.37-1.25 (m, 36H), 1.17 (d, $J = 6.8$ Hz, 12H), 0.90-0.86 (m, 6H).

[0381] ^{31}P NMR: (400 MHz, CDCl_3) δ 144.97.

[0382] **Example 1.13 Synthesis of Precursor of VSDL-13 (dioctyl diisopropylphosphoramidite)**



12-1

VSDL-13-P

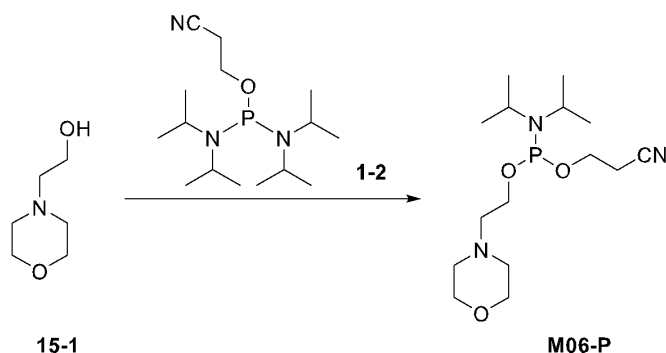
[0383] Similar to the synthesis method of Example 1.9.

[0384] ^1H NMR (400 MHz, CDCl_3) δ 3.67-3.51 (m, 6H), 1.64-1.57 (m, 4H), 1.37-1.26 (m, 20H), 1.18 (d, $J = 6.8$ Hz, 12H), 0.89-0.85 (m, 6H).

[0385] ^{31}P NMR: (400 MHz, CDCl_3) δ 145.00.

[0386] **Example 2 Synthesis of Precursors of Blocking Groups**

[0387] **Example 2.1 Synthesis of Precursor of M06 (2-cyanoethyl (2-morpholinoethyl) diisopropylphosphoramidite)**



15-1

M06-P

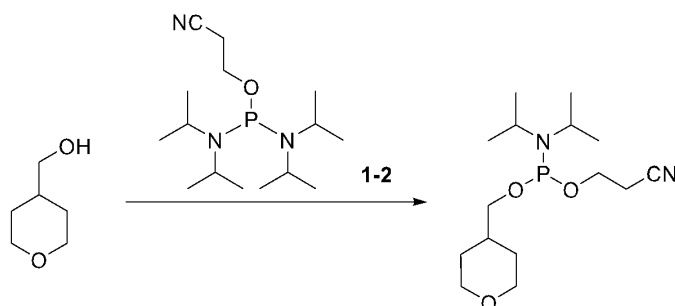
[0388] To a solution of compound **15-1** (10.88 g, 83.05 mmol) in anhydrous DCM (100 mL) was added 1H-imidazole-4,5-dicarbonitrile (4.9 g, 41.53 mmol). The reaction mixture was cooled to 0 °C, and then compound **1-2** (25 g, 83.05 mmol) in DCM (30 mL) was added to the mixture. The mixture was stirred at 25°C for 16 hours under N_2 . The mixture was filtered and

concentrated to remove DCM. The residue was diluted with hexane and purified by silica gel chromatography (eluting with hexane) to give Precursor of **M06**.

[0389] ^1H NMR: (400 MHz, CDCl_3) δ 3.87 - 3.75 (m, 3H), 3.71 - 3.65 (m, 5H), 3.62-3.53 (m, 2H), 2.65 - 2.57 (m, 4H), 2.54-2.45 (m, 4H), 1.17 (dd, J = 8.0, 4.0 Hz, 12H).

[0390] ^{31}P NMR: (160 MHz, CDCl_3) δ 148.02.

[0391] **Example 2.2 Synthesis of Precursor of M03 (2-cyanoethyl ((tetrahydro-2H-pyran-4-yl)methyl) diisopropylphosphoramidite)**



[0392] **16-1**

M03-P

[0393] To a solution of compound **16-1** (1.16 g, 10 mmol) in anhydrous DCM (20 mL) was added 1H-imidazole-4,5-dicarbonitrile (0.6 g, 5 mmol). The reaction mixture was cooled to 0 °C, and then compound **1-2** (3 g, 10 mmol) in DCM (5 mL) was added to the mixture. The mixture was stirred at 25°C for 16 hours under N_2 . The mixture was filtered and concentrated to remove DCM. The residue was diluted with hexane and purified by silica gel chromatography (eluting with hexane) to give Precursor of **M03**.

[0394] ^1H NMR: (400 MHz, CDCl_3) δ 3.98-3.94 (m, 2H), 3.88-3.73 (m, 2H), 3.61-3.34 (m, 6H), 2.62 (t, J = 8.0 Hz, 2H), 1.88-1.78 (m, 1H), 1.66-1.59 (m, 2H), 1.39-1.31 (m, 2H), 1.17 (dd, J = 8.0, 4.0Hz, 12H).

[0395] ^{31}P NMR: (160 MHz, CDCl_3) δ 147.82.

[0396] **Example 3 Preparation of Double-Stranded Oligonucleotides**

[0397] Oligonucleotide synthesis was performed on a MerMade 12 synthesizer using standard solid-phase synthesis protocols. All phosphoramidites were purchased from Hongene Biotech, including 2'-OMe-rA(Bz), 2'-OMe-rC(Ac), 2'-OMe-rU, 2'-OMe-rG(iBu), and 2'-F-rA(Bz), 2'-F-rC(Ac), 2'-F-rU, 2'-F-rG(iBu). 2'-OMe-rU was dissolved in a mixed solvent of DMF/acetonitrile

(1:4, v/v), while all other phosphoramidites were dissolved in acetonitrile and molecular sieves (3 Å) were added. Unylinker CPG was used as the solid support unless otherwise noted. The synthesis cycle consists of four individual steps, detritylation, coupling, oxidation (or sulfurization), and capping. 5-Ethylthio-1H-tetrazole (ETT, 0.25 M in acetonitrile) was used as activator solution. A 0.05 M solution of 3-((N,N-dimethylaminomethylidene)amino)-3H-1,2,4-dithiazole-5-thione (DDTT) in pyridine was employed to introduce phosphorothioate linkages. Upon completion of solid-phase synthesis, the phosphate protecting group (2-cyanoethyl group) was removed by a 20% solution of diethylamine (DEA) in acetonitrile for 1 h. Cleavage from solid support and nucleobase deprotection (C&D) were performed in NH_4OH at 65 °C for 5 h. The crude oligonucleotide solution was concentrated by centrifugal evaporation under elevated temperature (45 °C) and reduced pressure (5.6 Torr) for 8 h to give crude oligo as a solid. The resulting solid was subjected to prep-HPLC purification. The appropriate fractions were pooled and lyophilized to give the purified product.

[0398] Annealing of single strand oligonucleotides to produce double-stranded oligonucleotides.

[0399] The single strand oligonucleotides to be annealed were prepared with sterile RNase Free H_2O .

[0400] The annealing reaction system: The mixture was placed in a 95 °C water bath for 10 minutes (for ≥ 100 nmol, 20 minutes), then it was quickly put into a 60 °C water bath and allowed to cool, then lyophilized to give double-stranded oligonucleotides and stored at low temperature.

[0401] Complementary double-stranded oligonucleotides were prepared by combining equimolar single-stranded oligonucleotides solutions.

[0402] **Example 4 Bioactivity Assays of Double-Stranded Oligonucleotides**

[0403] **Example 4.1 Huh7 cell transfection assay**

[0404] Dilute the cell suspension with 10% FBS DMEM to the final cell density 2×10^5 cell/mL. Add 20 μL /well siRNA-RNAiMAX complex to the 96-well plate and followed by addition of cell suspension (100 μL /well). The cells were cultured at 37°C and 5% CO_2 for 24 hours.

[0405] The intracellular RNA was isolated with RNeasy kit (Qiagen-74182) by Instruction Manual. The RNA was reversed transcribed using HiScript® III RT SuperMix for qPCR (+gDNA wiper) by Instruction Manual. The expression levels of the target genes were quantified using qPCR with gene-specific primers (*e.g.*, with commercially available TaqMan® Assays or primers shown

below). GAPDH was measured as housekeeping gene in parallel. The samples were processed in an Applied Biosystems Fast Real-Time PCR system for TaqMan cycling, cycle according to the thermal profiles below: 95°C for 10 min, then cycling at 95°C for 15 sec, 60°C for 1 min for 40 cycles.

[0406] Commercially Available TaqMan® Assays:

TaqMan® Assays	ID	Vendor
VEGFA	Thermo Hs00900055_m1	Thermo fisher
APOC3	Thermo Hs00163644_m1	Thermo fisher
PNPLA3	Thermo Hs00228747_m1	Thermo fisher
Antithrombin (Serpinc1)	Thermo Hs00166654_m1	Thermo fisher
TMPRSS6	Thermo Hs00542191_m1	Thermo fisher
AGT	Thermo Hs01586213_m1	Thermo fisher
PCSK9	Thermo Hs00545399_m1	Thermo fisher
ANGPTL3	Thermo Hs00205581_m1	Thermo fisher
C3	Thermo Hs00163811_m1	Thermo fisher
C5	Thermo Hs00156197_m1	Thermo fisher
TTR	Thermo Hs00174914_m1	Thermo fisher

[0407] Other Primers:

Name	Sequence (5'-3')
INHBE-F	(dT)(dC)(dA)(dC)(dA)(dG)(dA)(dC)(dT)(dC)(dC)(dA)(dC)(dT)(dT)(dC)(dA)(dG)(dC)(dC)
INHBE-R	(dA)(dA)(dA)(dG)(dA)(dG)(dT)(dG)(dC)(dC)(dA)(dG)(dG)(dA)(dA)(dG)(dG)(dG)(dT)
INHBE-Probe	(dC)(dA)(dG)(dC)(dC)(dA)(dC)(dA)(dG)(dG)(dC)(dG)(dG)(dG)(dC)(dA)(dT)(dG)(dG)(dT)(dA)(dC)(dA)(dG)(dG)
KHK-F	(dG)(dA)(dG)(dT)(dC)(dG)(dG)(dA)(dG)(dA)(dC)(dG)(dC)(dA)(dG)(dG)(dT)(dG)
KHK-R	(dT)(dT)(dT)(dT)(dA)(dA)(dA)(dG)(dC)(dG)(dC)(dG)(dG)(dC)(dT)(dA)(dA)(dG)(dC)(dG)
KHK-probe	(dG)(dG)(dA)(dG)(dA)(dG)(dT)(dG)(dC)(dG)(dG)(dG)(dG)(dC)(dA)(dA)(dG)(dT)(dA)(dG)(dC)(dG)
SOD1-F	(dC)(dC)(dT)(dA)(dG)(dC)(dG)(dA)(dG)(dT)(dT)(dA)(dT)(dG)(dG)(dC)(dG)(dA)(dC)(dG)

SOD1-R	(dC)(dC)(dA)(dC)(dA)(dC)(dC)(dT)(dT)(dC)(dA)(dC)(dT)(dG)(dG)(dT)(dC)(dC)(dA)(dT)
APP-F	(dC)(dG)(dC)(dA)(dA)(dG)(dC)(dA)(dG)(dT)(dG)(dC)(dA)(dA)(dG)(dA)(dC)(dC)
APP-R	(dT)(dC)(dA)(dG)(dG)(dA)(dA)(dC)(dG)(dA)(dG)(dA)(dA)(dG)(dG)(dC)(dA)(dT)(dC)
APP-Probe	(dT)(dC)(dC)(dC)(dC)(dA)(dC)(dT)(dT)(dT)(dG)(dT)(dG)(dA)(dT)(dT)(dC)(dC)(dC)(dT)(dA)(dC)(dC)(dG)
ANGPTL4-1F	(dG)(dC)(dT)(dG)(dG)(dG)(dT)(dC)(dT)(dG)(dG)(dA)(dG)(dA)(dA)(dG)(dG)(dT)
ANGPTL4-1R	(dG)(dA)(dG)(dA)(dA)(dC)(dT)(dG)(dC)(dA)(dG)(dC)(dA)(dA)(dC)(dT)(dC)(dG)(dG)

Table 2: Test Compounds knock-down activity in Huh 7 transfection assays

Compound	IC ₅₀ (nM)	Target	SS (5'-3')	AS (5'-3')
ds1	<0.0024	APOC3	ACGGGACAGUAUUCUCAGUGA	ACGAUCACUGAGAAUACUGUCCCGUUU
ds2	0.060	INHBE	ACCAGUCGUCCAGAAUAACU	CUGAAGUUAUUCUGGGACGACUGGUCU
ds3	0.303	PNPLA3	UGAGCAAGAUUUGCAACUUGA	ACGAUCAAGUUGCAAAUUCUUGCUAUG
ds4	0.049	Antithrombin (Serpinc1)	GGUUAACACCAUUUACUUCAA	CCGAUUGAAGUAAAUGGUGUUAACCG
ds5	0.032	APP	GGCUACGAAAAUCCAACCUGA	UCGAUUAGGUUGGAUUUUCGUAGCCGU
ds6	0.057	SOD1	CACUUUAAUCCUCUAUCCAGA	AGGAUCUGGAUAGAGGAUUAAAAGUGAG
ds7	0.023	TMPRSS6	CCUUUGGAAUAAAGCUGCCUU	CCGUAAGGCAGCUUUUUAUCCAAAGGGC
ds8	0.090	KHK	GCAGGAAGCACUGAGAUUCGU	CCGAACGAAUCUCAGUGCUUCCUGCAC
ds9	<0.0024	PCSK9	CUAGACCUGUUUUGCUUUUGU	UGGAACAAAAGCAAAACAGGUCUAGAA
ds10	0.047	VEGF-A	AUGCAGAUUUGCGGAUCAA	ACGAUUUGAUCCGCAUAAUCUGCAUGG
ds11	0.603	ANGPTL3	GCUCAACAUAUUUGAUCAGUA	CCGAUACUGAUCAAAUAUGUUGAGCUU
ds12	0.003	C3	GAGCCGUUCUCUACAAUUACU	GCGAAGUAAUUGUAGAGAACGGCUCGG
ds13	0.432	C5	AAGCAAGAUUUUUUAUAAUA	ACGUUAAUUAUUUUUAUUCUUGCUUUU
ds14	0.002	TTR	UGGGAUUUCAUGUAACCAAGA	CCGAUCUUGGUUACAUGAAAUCCCAUC

Table 3: Test Compounds knock-down activity in Huh 7 transfection assay

Target gene: AGT					
AS(5'-3'): (invAB)*cZY(X2)*Gf*uac(Tgn)cucauugUfgGfaugac*g*a					
SS(5'-3'): g*u*caucCfaCfAfAfugagagUfa*c*(X1)					
Compound	IC ₅₀ (nM)	X1	X2	Y	Z
ds15	0.04	a	u	u	u
ds16	0.02	a	u	u	a
ds17	0.06	a	u	u	c
ds18	0.02	a	u	u	g
ds19	0.04	a	u	a	u
ds20	0.02	a	u	a	a
ds21	0.05	a	u	a	c
ds22	0.01	a	u	a	g
ds23	0.02	u	a	u	u
ds24	0.02	u	a	u	a
ds25	0.03	u	a	u	c
ds26	0.01	u	a	u	g
ds27	0.03	u	a	a	u
ds28	0.02	u	a	a	a
ds29	0.05	u	a	a	c
ds30	0.01	u	a	a	g

Table 4: Test Compounds knock-down activity in Huh 7 transfection assay

Target gene: ANGPTL3					
AS(5-3): (invAB)*cZY(X2)*A*c*UfgAfuCfaAfaUfaUfgUfuGfaGfca*a					
SS(5-3): (invAB)*gcucaacaUfAfUfuugaucagu(X1)					
Compound	IC ₅₀ (nM)	X1	X2	Y	Z
ds47	0.27	a	u	u	u

ds48	0.15	a	u	u	a
ds49	0.26	a	u	u	c
ds50	0.10	a	u	u	g
ds51	0.26	a	u	a	u
ds52	0.19	a	u	a	a
ds53	0.30	a	u	a	c
ds54	0.14	a	u	a	g
ds55	0.18	u	a	u	u
ds56	0.16	u	a	u	a
ds57	0.29	u	a	u	c
ds58	0.10	u	a	u	g
ds59	0.23	u	a	a	u
ds60	0.13	u	a	a	a
ds61	0.20	u	a	a	c
ds62	0.08	u	a	a	g

Table 5: Test Compounds knock-down activity in Huh 7 transfection assay

Target gene: AGT			
AS(5-3): (M06)* NZY(X2) *Gfuac(Tgn)cucauugugGfaugac*g*a			
SS(5-3): g*u*caucCfaCfAfAfugagagua*c*(X1)			
Compound	Relative IC ₅₀ to ds67	X1	NZY(X2)
ds64	1.14	u	cgaa
ds65	1.31	u	cgua
ds66	1.09	u	cgga
ds67	1.00	u	cgca
ds68	1.08	u	ggaa
ds69	2.59	u	ugaa
ds70	5.65	g	cgac

ds71	5.75	c	cgag
ds72	1.30	u	ccgccgaa

$$\text{Relative IC}_{50} = (\text{ds X}) / \text{ds67}$$

Table 6: Test Compounds knock-down activity in Huh 7 transfection assay

Target gene: PCSK9			
AS(5-3): NZY(X2)CfaAfAfAfgCfaAfaAfcAfgGfuCfuag*a*a			
SS(5-3): c*u*agacCfuGfu(dT)uugcuuuu*g*(X1)			
Compound	Relative IC ₅₀ to ds74	X1	NZY(X2)
ds74	1.00	u	(M06)*cgaa*
ds75	2.04	u	(M06)*cgua*
ds76	1.09	u	(M06)*cgga*
ds77	1.74	u	(M06)*cgca*
ds78	1.64	u	(M06)*agaa*
ds79	2.79	u	(M06)*ggua*
ds80	5.43	g	(M06)*cgac*
ds81	5.83	c	(M06)*cgag*
ds82	1.23	u	(M06)*gcuucgaa*

$$\text{Relative IC}_{50} = (\text{ds X}) / \text{ds74}$$

[0408] As shown in above data, the dsRNA agent disclosed herein displayed potent target gene expression inhibitory activity. Crossing dsRNA agents targeting different genes, dsRNA agents with a purine at Z displayed better inhibitory activities than a pyrimidine, more preferably, guanine at Z. It can be seen that dsRNA agents with adenine or uracil at X1/X2 displayed potent inhibitory activity, while dsRNA agents with cytosine or guanine at X1/X2 displayed relatively modest activity.

[0409] Surprisingly, the dsRNA agents with blocking groups (*e.g.*, M03, M06 or invAB) or ligand (*e.g.*, VSDL-03A) at 5' end of the anti-sense strand, displayed inhibitory activity against expression of the target gene, while other reported dsRNA agents would significantly or completely lose their activities when the 5' end of the anti-sense strand is linked to a blocking

group or ligand. While not wishing to be bound by theory, one of the rational explanation might be that the blocking group or ligand is cleaved together with the cleavage region of the dsRNA disclosed herein before provide RNAi effect.

[0410] Example 4.2 PHH free uptake KD assay

[0411] The primary human hepatocytes (PHH) mixed with the appropriate medium, and then adjusted the cell suspension to the final cell density of 6×10^5 cells/mL. The dsRNA was diluted and added 10 μ L/well to the collagen-I coated 96-well plate, and followed by addition of cell suspension (90 μ L/well). The cells were cultured at 37°C and 5% CO₂ for 48 hours.

[0412] The intracellular RNA was isolated with RNeasy kit (Qiagen-74182) by Instruction Manual. The RNA was reversed transcribed using HiScript® III RT SuperMix for qPCR (+gDNA wiper) by Instruction Manual. The expression levels of the target genes were quantified using qPCR with gene-specific primers (*e.g.*, with commercially available TaqMan® Assays or primers shown below). GAPDH was measured as housekeeping gene in parallel. The samples were processed in an Applied Biosystems Fast Real-Time PCR system for TaqMan cycling, cycle according to the thermal profiles below: 95°C for 10 min, then cycling at 95°C for 15 sec, 60°C for 1 min for 40 cycles.

[0413] Commercially Available TaqMan® Assays:

TaqMan® Assays	ID	Vendor
AGT	Thermo Hs01586213_m1	Thermo fisher
ANGPTL3	Thermo Hs00205581_m1	Thermo fisher
Antithrombin (Serpinc1)	Thermo Hs00166654_m1	Thermo fisher
PCSK9	Thermo Hs00545399_m1	Thermo fisher

[0414] Other Primers:

Name	Sequence (5'-3')
C3-F	(dG)(dT)(dG)(dC)(dT)(dG)(dC)(dC)(dC)(dA)(dG)(dT)(dT)(dT)(dC)(dG)(dA)(dG)(dG)(dT)(dC)(dA)(dT)(dA)
C3-R	(dT)(dC)(dC)(dC)(dG)(dA)(dA)(dG)(dA)(dT)(dG)(dA)(dC)(dA)(dA)(dA)(dG)(dG)(dC)(dA)(dG)(dT)(dT)
C3-probe	(dT)(dC)(dC)(dA)(dC)(dT)(dT)(dT)(dC)(dT)(dT)(dC)(dC)(dC)(dG)(dT)(dA)(dG)(dA)(dG)(dG)(dA)(dA)(dC)(dC)(dT)
SOD1-F	(dC)(dC)(dT)(dA)(dG)(dC)(dG)(dA)(dG)(dT)(dT)(dA)(dT)(dG)(dG)(dC)(dG)(dA)(dC)(dG)

SOD1-R	(dC)(dC)(dA)(dC)(dA)(dC)(dC)(dT)(dT)(dC)(dA)(dC)(dT)(dG)(dG)(dT)(dC)(dC)(dA)(dT)
INHBE-F	(dT)(dC)(dA)(dC)(dA)(dG)(dA)(dC)(dT)(dC)(dC)(dA)(dC)(dT)(dT)(dC)(dA)(dG)(dC)(dC)
INHBE-R	(dA)(dA)(dA)(dG)(dA)(dG)(dT)(dG)(dC)(dC)(dA)(dG)(dG)(dA)(dA)(dG)(dG)(dG)(dT)
INHBE-Probe	(dC)(dA)(dG)(dC)(dC)(dA)(dC)(dA)(dG)(dG)(dC)(dG)(dG)(dG)(dC)(dA)(dT)(dG)(dG)(dT)(dA)(dC)(dA)(dG)(dG)
APP-F	(dC)(dG)(dC)(dA)(dA)(dG)(dC)(dA)(dG)(dT)(dG)(dC)(dA)(dA)(dG)(dA)(dC)(dC)
APP-R	(dT)(dC)(dA)(dG)(dG)(dA)(dA)(dC)(dG)(dA)(dG)(dA)(dA)(dG)(dG)(dC)(dA)(dT)(dC)
APP-Probe	(dT)(dC)(dC)(dC)(dC)(dA)(dC)(dT)(dT)(dT)(dG)(dT)(dG)(dA)(dT)(dT)(dC)(dC)(dC)(dT)(dA)(dC)(dC)(dG)

Table 7: Test Compounds knock-down activity in primary human hepatocyte free-uptake assay

Compound	SS(5'-3')	AS(5'-3')	AGT EC ₅₀ (pM)
ds83	g*u*caucCfaCfAfAfugagagUfacu[L96]	(invAB)*cgaa*Gfuac(Tgn)cucauugUfgGfaugac*g*a	564
ds84	g*u*caucCfaCfAfAfugagagUfacu[L96]	(invAB)*CGAa*Gfuac(Tgn)cucauugUfgGfaugac*g*a	50.4
ds85	g*u*caucCfaCfAfAfugagagUfacu[L96]	(invAB)*CfGfAfa*Gfuac(Tgn)cucauugUfgGfaugac*g*a	56.2
ds86	g*u*caucCfaCfAfAfugagagUfacu[L96]	(invAB)*CfGfaa*Gfuac(Tgn)cucauugUfgGfaugac*g*a	52.3
ds87	g*u*caucCfaCfAfAfugagagUfacu[L96]	(invAB)*CfGaa*Gfuac(Tgn)cucauugUfgGfaugac*g*a	74.3
ds88	g*u*caucCfaCfAfAfugagagUfacu[L96]	(invAB)*CfGfAa*Gfuac(Tgn)cucauugUfgGfaugac*g*a	76.1
ds89	g*u*caucCfaCfAfAfugagagUfacu[L96]	(invAB)*CfGf(dA)a*Gfuac(Tgn)cucauugUfgGfaugac*g*a	70
ds90	g*u*caucCfaCfAfAfugagagUfacu[L96]	(invAB)*cgAa*Gfuac(Tgn)cucauugUfgGfaugac*g*a	126.1
ds91	g*u*caucCfaCfAfAfugagagUfacu[L96]	(invAB)*cg(dA)a*Gfuac(Tgn)cucauugUfgGfaugac*g*a	636.9
ds95	g*u*caucCfaCfAfAfugagagUfacu[L96]	c*g*aaGfuac(Tgn)cucauugUfgGfaugac*g*a	Inactive
ds96	g*u*caucCfaCfAfAfugagagUfacu[L96]	c*Gf*AfaGfuac(Tgn)cucauugUfgGfaugac*g*a	1400
ds97	g*u*caucCfaCfAfAfugagagUfacu[L96]	cgaa*Gf*uac(Tgn)cucauugUfgGfaugac*g*a	130
ds99	g*u*caucCfaCfAfAfugagagUfacu[L96]	(M06)*CfGfaa*Gfuac(Tgn)cucauugUfgGfaugac*g*a	10.5
ds100	g*u*caucCfaCfAfAfugagagUfacu[L96]	(M03)*CfGfaa*Gfuac(Tgn)cucauugUfgGfaugac*g*a	21.3
ds101	g*u*caucCfaCfAfAfugagagUfacu[L96]	u*Gf*uac(Tgn)cucauugUfgGfaugac*g*a	99.5

[0415] As shown in the table above, a cleavage region with both 2'-F and 2'-OMe displayed potent inhibitory activity. In preferred embodiments, X2 and Y are 2'-OMe modified, and Z and N1 are 2'-F modified.

[0416] Also can be seen is that internucleotide linkage modification between X2 and Y or Y and Z would reduce the inhibitory activity of the dsRNA.

Table 8: Primary human hepatocyte free-uptake assay

Compound	SS(5'-3')	AS(5'-3')	EC ₅₀ (nM)
Target Gene: INHBE			
ds102	a*c*cagucgUfCfCfcagaauaacu[L96]	a*(dG)*uu(dA)u(dT)cugg(dG)aCfGacuggu*c*u	2.41
ds103	a*c*cagucgUfCfCfcagaauaacu[L96]	(invAB)*CfGfaa*(dG)uu(dA)u(dT)cugg(dG)aCfGacuggu*c*u	0.19
Target Gene: C5			
ds104	a*a*GfcAfaGfaUfAfUfuUfuUfuaAfaAfaa[L96]	u*Af*UfuAfuaAfaAfaaUfcUfuGfcuu*u*u	0.44
ds105	a*a*GfcAfaGfaUfAfUfuUfuUfuaAfaAfaa[L96]	(invAB)*CfGfau*AfUfuAfuaAfaAfaaUfcUfuGfcuu*u*u	0.18
Target Gene: Antithrombin(Serpinc1)			
ds106	Gf*g*UfuAfaCfaCfCfAfuUfuAfcUfuCfaAf[L96]	u*Uf*gAfaGfuAfaAfaaUfgUfuAfaCfc*a*g	1.2
ds107	Gf*g*UfuAfaCfaCfCfAfuUfuAfcUfuCfaAf[L96]	(invAB)*CfGfau*UfgAfaGfuAfaAfaaUfgUfuAfaCfc*a*g	0.33
Target Gene: C3			
ds108	g*a*gccgUfuCfUfCfuacaauaacu[L96]	a*Gf*uaaUfuGfUfagagAfaCfggcuc*g*g	1.5
ds109	g*a*gccgUfuCfUfCfuacaauaacu[L96]	(M06)*CfGfaa*GfuaaUfuGfUfagagAfaCfggcuc*g*g	0.68
Target Gene: APP			
ds116	g*g*cuacga(dA)a(dA)uccaaccuaa[L96]	u*Uf*aggu(Tgn)ggau(dT)uUfc(dG)uagcc*g*u	0.43
ds117	g*g*cuacga(dA)a(dA)uccaaccuaa[L96]	(invAB)*CfGfau*Ufaggu(Tgn)ggau(dT)uUfc(dG)uagcc*g*u	0.61

[0417] As shown in the table above, the dsRNA agents disclosed herein with a 5' extension on the anti-sense strand and a cleavage region displayed relatively more potent inhibitory activity against different genes and targets, in comparison with parent dsRNA agents without the 5' extension.

[0418] Example 4.3 In vivo mouse HDI model

[0419] Mice (BALB/C, 6-7 weeks, female) were dosed at Day 0 with vehicle or the test siRNA (5mg/kg). On Day 3, all mice were injected with plasmid DNA solution of 8% of their body weight through the tail vein within 5 seconds (injection volume (mL) = mouse body weight (g) ×8%). The mass of the plasmid injected into each mouse was 10 µg. All animals were sacrificed at Day 4. Liver tissues from all groups were taken for target mRNA analysis via qPCR method. The RNA

was reverse transcribed to cDNA with HiScript® III RT SuperMix for qPCR (+gDNA wiper) (Vazyme-R323) following the manual. The cDNA was quantified by qPCR. NEO (The sequence information is as follows in the table) mRNA were detected in parallel as an internal control.

Name	Primer sequences(5'-3')
NEO-F	(dG)(dA)(dT)(dG)(dG)(dA)(dT)(dT)(dG)(dC)(dA)(dC)(dG)(dC)(dA)(dG)(dG)(dT)(dT)(dC)(dT)(dC)
NEO-R	(dG)(dA)(dA)(dC)(dA)(dC)(dG)(dG)(dC)(dG)(dG)(dC)(dA)(dT)(dC)(dA)(dG)(dA)(dG)(dC)
NEO-Probe	(dC)(dG)(dC)(dT)(dT)(dG)(dG)(dT)(dG)(dG)(dA)(dG)(dA)(dG)(dG)(dC)(dT)(dA)(dT)(dT)(dC)(dG)(dG)(dC)(dT)(dA)(dT)(dG)(dA)

[0420] Test compounds:

Compound	SS (5'-3')	AS (5'-3')
ds100	g*u*caucCfaCfAfAfugagagUfacu[L96]	(M03)*CfGfaa*Gfuac(Tgn)cucauugUfgGfaugac*g*a
ds101	g*u*caucCfaCfAfAfugagagUfacu[L96]	u*Gf*uac(Tgn)cucauugUfgGfaugac*g*a

[0421] Results were shown in Fig. 1.

[0422] As shown in the in Fig. 1, the dsRNA agents disclosed herein with a 5' extension on the anti-sense strand and a cleavage region displayed significantly more potent inhibitory efficacy against target gene, in comparison with parent dsRNA agents without the 5' extension.

[0423] **Example 4.4 In vivo rat CNS target knock-down model (IT injection)**

[0424] Testing compounds were formulated at up to 20 mg/mL in 10 mM PBS (pH 7.4) and administered as 50 μL IT injections (0.9 mg/dose) by lumbar puncture. Animal tail flapping or tail tip shaking were used as the marker for successful operation. After dosing, remove the anesthesia maintenance device and put the animal back into the cage. Animals were sacrificed at Day14, and tissue samples were taken for qPCR analysis.

[0425] Measurement of SOD1 mRNA level by qPCR

[0426] RNAs from tissue samples were extracted by Automated Nucleic Acid Extraction Systems . Transfer the sample to a fresh RNase-free tube for cDNA synthesis and qPCR.

Primers	Sequences (5' -3')
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rSOD-1 F	(dC)(dT)(dC)(dA)(dG)(dG)(dA)(dG)(dA)(dG)(dC)(dA)(dT)(dT)(dC)(dC)(dA)(dT)(dC)(dA)(dT)(dT)(dG)
rSOD-1 R	(dC)(dA)(dA)(dT)(dC)(dA)(dC)(dA)(dC)(dC)(dA)(dC)(dA)(dA)(dG)(dC)(dC)(dA)(dA)(dG)(dC)

[0427] Test compounds:

Target gene: rat SOD-1		
Compound	SS (5'-3')	AS (5'-3')
ds110	c*a*uuu(C16U)AfaUfCfCfucacucua*a*a	{VPu}*Uf*UagAfgUfGfaggaUfuAfaaaug*a*g
ds111	c*a*uuuuAfaUfCfCfucacucua*a*u	(VSDL-03A)*CfGfaa*UfuagAfgUfGfaggaUfuAfaaaug*a*g
ds112	(VSDL-03A)*c*a*u*uuAfaUfCfCfucacucua*a*u	(M06)*CfGfaa*UfuagAfgUfGfaggaUfuAfaaaug*a*g
ds113	(VSDL-09)*c*a*u*uuAfaUfCfCfucacucua*a*u	(M06)*CfGfaa*UfuagAfgUfGfaggaUfuAfaaaug*a*g
ds114	c*a*uuu(C16U)AfaUfCfCfucacucua*a*a	u*Uf*UagAfgUfGfaggaUfuAfaaaug*a*g
ds115	c*a*uuu(C16U)AfaUfCfCfucacucua*a*u	(invAB)*CfGfaa*Uf*UagAfgUfGfaggaUfuAfaaaug*a*g

[0428] Results were shown in Fig. 2 and 3.

[0429] As shown in the in Fig. 2 and 3, the dsRNA agents disclosed herein with a 5' extension on the anti-sense strand and a cleavage region and VSDL ligands displayed comparable inhibitory efficacy against target gene, in comparison with parent dsRNA agents with the 5' VPu modification and C16U. However, the dsRNA agents disclosed herein displayed more tissue-specific target knockdown compared to dsRNA agents with the 5' VPu modification and C16U.

[0430] Example 4.5 In vivo mouse IVT model

[0431] A topical antibiotic (tobramycin) was applied to both eyes twice on the day before and twice on the day after IVT injection to mice. On Day 0, either 1×PBS or siRNA formulated in 1×PBS was administered in both eyes by IVT injection at 3 µg per dose. All animals were sacrificed on Day 7, and whole eye were sampled for qPCR analysis.

[0432] Test compounds:

Target gene: mTTR		
Compound	SS (5'-3')	AS (5'-3')

ds118	a*a*cag(C16U)GfuUfCfUfugcucuau*a*a	(VPu)*Uf*auaGfagcaagaAfcAfcuguu*u*u
ds119	a*a*caguGfuUfCfUfugcu*c*u*a*u*a*a	(VSDL-03A)*CfGfau*UfauaGfagcaagaAfcAfcuguu*u*u

[0433] Results were shown in Fig. 4.

[0434] As shown in the in Fig. 4, the dsRNA agents disclosed herein with a 5' extension on the anti-sense strand and a cleavage region and VSDL ligand displayed stronger inhibitory efficacy against target gene, in comparison with parent dsRNA agents with the 5' VPu modification and C16U.

[0435] Example 4.6 Rat liver homogenate processing reaction

[0436] Accurate 1.00mg of testing compound was dissolved in 0.961mL of water to obtain the working solutions at the corrected concentration of 1,000,000 ng/mL. The final sample with a concentration of 10,000 ng/mL were obtained by mix the working solution with 20% rat liver homogenate, followed by incubation at 37°C for 48h. The incubated samples were added with 50.0μL of IS working solution for extraction (Phenol/Chloroform/Isoamyl alcohol=25/24/1, v/v/v) for liquid-liquid extraction. After centrifugation, 300 μL of supernatant were transferred to solid-phase extraction. Then, transfer the samples into an equilibrated SPE plate, wash and elute the SPE plate. The collected eluent was evaporated. (LC-HRMA: Liquid Chromatography-High Resolution Mass Spectrometer; LC: Shimadzu,LC-30AD; HRMA: Q Exactive Plus or Q Exactive Focus (Thermo San Jose, CA))

[0437] Test compound:

Compound	SS (5'-3')	AS (5'-3')
ds99	g*u*caucCfaCfAfAfugagagUfacu[L96]	(M06)*CfGfaa*Gfuac(Tgn)cucauugUfgGfaugac*g*a

[0438] Results were shown in Fig. 5. After 48 h incubation, the observed products include unconsumed Test Compound (31%), Test Compound with one nucleotide cleaved at 3' end (15%), Test Compound with two nucleotides cleaved at 3' end and M06 cleaved (18%), Test Compound with three nucleotides cleaved at 5' end (15%+5%+10%) and other minor metabolites (5%).

[0439] In summary, (1) the only observed 5' cleavage product is Test Compound with three nucleotides cleaved at 5' end; (2) the observed 3' cleavage products include Test Compound with

one or two nucleotides cleaved at 3' end; and (3) the cleavage products of Test Compound with three nucleotides cleaved at 5' end is the major product among all cleavage products.

[0440] Example 4.7 NHP long-term plasma target know-down model

[0441] In vivo target knockdown effect of modified siRNA was evaluated in cynomolgus monkey. Animals (N=2 per group) received a single 2 mg/kg subcutaneous dose of dsRNA agent on day 1. Blood was obtained on Day -1, Day 2, Day 7, Day 14, Day 28, Day 42, Day 56 and Day 70 post-dose. Circulating AGT levels were quantified using an ELISA specific for human angiotensinogen (and cross-reactive with cynomolgus), according to manufacturer's protocol (Sino Biological KIT10994). Data were expressed as percent of baseline value and presented as mean plus/minus standard error of the mean.

[0442] Test compounds:

Compound	SS (5'-3')	AS (5'-3')
ds86	g*u*caucCfaCfAfAfugagagUfacu[L96]	(invAB)*CfGfaa*Gfuac(Tgn)cucauugUfgGfaugac*g*a
ds101	g*u*caucCfaCfAfAfugagagUfaca[L96]	u*Gf*uac(Tgn)cucauugUfgGfaugac*g*a

[0443] Results were shown in Fig. 6.

[0444] Example 4.8 Rat repeat-dose tolerability study

Repeated dose tolerability study was performed in rats. Testing compounds (ds86, ds99) were administered subcutaneously once weekly for 3 weeks (3 doses in total) at doses above 250 mg/kg. (300mg/kg*1, 250 mg/kg*2).

Compound	SS (5'-3')	AS (5'-3')
ds99	g*u*caucCfaCfAfAfugagagUfacu[L96]	(M06)*CfGfaa*Gfuac(Tgn)cucauugUfgGfaugac*g*a
ds86	g*u*caucCfaCfAfAfugagagUfacu[L96]	(invAB)*CfGfaa*Gfuac(Tgn)cucauugUfgGfaugac*g*a

[0445] Result: No abnormality was found in clinical observation and pathology plus gross anatomy.

[0446] Histopathology:

[0447] Kidney: Presence of mild basophilic granules and tubular cell vacuolation in both groups.

[0448] Liver: mild to modest vacuolation and pigmented hepatocytes and Kupffer cells in both groups.

[0449] Injection Site: Minimal to moderate mixed cell inflammation.

[0450] Conclusion: Findings consistent with GalNAc siRNA administration uptake and clearance and not considered adverse.

Claims

1. A double-stranded oligonucleotide agent comprising a sense strand and an anti-sense strand, wherein:

the sense strand and the anti-sense strand form a double-stranded portion of 15 to 27 base pairs in length and a 5' extension in the anti-sense strand;

the 5' extension has a length of at least three nucleotides and is cleavable from the most 3' nucleotide of the 5' extension, and the cleaved double-stranded oligonucleotide agent is capable of silencing a target RNA or inhibiting expression of a target gene via RNA interference.

2. The double-stranded oligonucleotide agent of claim 1, wherein the double-stranded oligonucleotide agent further comprises a 3' extension in the anti-sense strand.
3. The double-stranded oligonucleotide agent of any one of the preceding claims, wherein the double-stranded portion is formed by base pairing between a first fragment in the anti-sense strand and a second fragment in the sense strand, wherein the first fragment and the second fragment are of equal length.
4. The double-stranded oligonucleotide agent of any one of the preceding claims, wherein the double-stranded portion has a length of:
 - a) 15 to 25 nucleotide pairs, 15 to 24 nucleotide pairs, 15 to 23 nucleotide pairs, 16 to 24 nucleotide pairs, 16 to 23 nucleotide pairs, 16 to 22 nucleotide pairs, 16 to 21 nucleotide pairs, 16 to 20 nucleotide pairs, 17 to 23 nucleotide pairs, 17 to 22 nucleotide pairs, 17 to 21 nucleotide pairs, 17 to 20 nucleotide pairs, 18 to 23 nucleotide pairs, 18 to 22 nucleotide pairs, 18 to 21 nucleotide pairs, 18 to 20 nucleotide pairs, 19 to 23 nucleotide pairs, 19 to 22 nucleotide pairs, 19 to 21 nucleotide pairs; or

- b) 15 nucleotide pairs, 16 nucleotide pairs, 17 nucleotide pairs, 18 nucleotide pairs, 19 nucleotide pairs, 20 nucleotide pairs, 21 nucleotide pairs, 22 nucleotide pairs, or 23 nucleotide pairs.
5. The double-stranded oligonucleotide agent of any one of the preceding claims, wherein the sense strand has a length of 15 to 35, 16 to 35, 16 to 30, 16 to 27, 16 to 26, 16 to 25, 16 to 21, 17 to 35, 17 to 30, 17 to 25, 17 to 21, 18 to 35, 18 to 30, 18 to 25, 18 to 23, 18 to 21, 19 to 35, 19 to 30, 19 to 25, 19 to 21, 20 to 35, 20 to 30, 20 to 25, 20 to 23, 21 to 35, 21 to 30, 21 to 25, 21 to 23, such as 25, 24, 23, 22, or 21 nt.
6. The double-stranded oligonucleotide agent of any one of the preceding claims, wherein the 5' extension has a length of at least 3, 4, 5, 6, 7 or more nucleotides, and/or the 3' extension has a length of at least 1, 2, or more nucleotides.
7. The double-stranded oligonucleotide agent of any one of the preceding claims, wherein the first fragment comprises a targeting region which is sufficiently complementary to part of the target RNA or a target mRNA encoding the target gene.
8. The double-stranded oligonucleotide agent of any one of the preceding claims, wherein the 5' extension is enzymatically cleavable from the most 3' nucleotide of the 5' extension, optionally the 5' extension is specifically cleavable by an endonuclease, or optionally by a ribonuclease (RNase).
9. The double-stranded oligonucleotide agent of any one of the preceding claims, wherein the anti-sense strand comprises a cleavage region comprising a nucleotide sequence set forth in Formula A: (3'-5') X2-Y-Z, which is cleavable between X2 and Y, wherein the X2 is the most 5' nucleotide of the first fragment, and Y-Z are the two most 3' nucleotides of the 5' extension.
10. The double-stranded oligonucleotide agent of any one of the preceding claims, wherein the cleavage region further comprises a fourth nucleotide (N1) which is the third nucleotide from the 3' end of the 5' extension, and the cleavage region comprises a nucleotide

sequence set forth in Formula B: (3'-5') X2-Y-Z-N1, which is cleavable between X2 and Y.

11. The double-stranded oligonucleotide agent of any one of claims 1-9, wherein the cleavage region further comprises a third fragment (N), the third fragment comprises at least one nucleotide, wherein N1 is the most 3' nucleotide of the third fragment, and the cleavage region comprises a nucleotide sequence set forth in Formula B': (3'-5') X2-Y-Z-N, which is cleavable between X2 and Y, the length of N is 1-10 nucleotides, preferably the length of N is 1-5 nucleotides, further preferably the length of N is 1 nucleotides.
12. The double-stranded oligonucleotide agent of any one of claims 9-11, wherein Z is selected from G or A or a natural or non-natural analogue thereof.
13. The double-stranded oligonucleotide agent of any one of claims 9-12, wherein Z is selected from G or a natural or non-natural analogue thereof.
14. The double-stranded oligonucleotide agent of any one of claims 9-13, wherein X2 is selected from A or U, or a natural or non-natural analogue thereof.
15. The double-stranded oligonucleotide agent of any one of claims 9-11, wherein the Formula A has the nucleotide sequence from 3'-5' selected from the group consisting of: UUG, UAG, AUG, AAG, UUA, UAA, AUA, AAA, UCG, UGG, ACG, AGG, UCA, UGA, ACA, and AGA, or a natural or non-natural analogue thereof.
16. The double-stranded oligonucleotide agent of any one of claims 9-15, wherein the nucleotide Y is selected from A or U, or a natural or non-natural analogue thereof.
17. The double-stranded oligonucleotide agent of any one of claims 9-16, wherein the Formula A has the nucleotide sequence from 3'-5' selected from the group consisting of: UUG, UAG, AUG, AAG, UUA, UAA, AUA, and AAA.

18. The double-stranded oligonucleotide agent of any one of the preceding claims wherein at least one nucleotide in the cleavage region is a modified nucleotide, preferably all nucleotides in the cleavage region are modified nucleotides.
19. The double-stranded oligonucleotide agent of claim 18, wherein the modified nucleotide has a modified base, a modified sugar or a modified internucleotide linkage.
20. The double-stranded oligonucleotide agent of claim 18 or 19, wherein:
 - a) the modified base comprises hypoxanthine (I), xanthine, 7-methylguanine, 5, 6-dihydrouracil, 5-methylcytosine (m5C), 5-hydroxymethoylecytosine, N6-methyladenosine (m6A), 3-methyluridine (m3U), 5-methyluridine (m5U), pseudouridine, 2-thiouridine (s2U) and 5-propyluridine (5-pU),
 - b) the modified sugar comprises 2'-sugar modified, 3'-sugar modified and 5'-sugar modified, such as 2'-OMe (2'-O-methyl) modification, 2'-F (2'-deoxy-2'-fluoro) modification, 2'-O-MOE (2'-O-methoxyethyl) modification, 2'-deoxy (2'-d) modification, 5'-morpholine (5'-Mo) modification, unlocked nucleic acid (UNA) modification, glycol nucleic acid (GNA) modification, locked nucleic acid (LNA) modification, tricyclo-DNA (tcDNA) modification, (S)-constrained ethyl bridged nucleic acid ((S)-cEt-BNA) modification, 5'-(E)-vinylphosphate (VP) modificatio, 2'-O-C16 modification, conjugating with invert abasic nucleotide (invAB) at 5' end or 3' end, replacing with invert abasic nucleotide (invAb), replacing with 2,4-difluorotolyl ribonucleotide (rF), replacing with (S)-glycerol nucleic acid, replacing with inosine (I), conjugating with M03 at 5' end or 3' end, conjugating with M06 at 5' end or 3' end and conjugating with ligand such as a GalNAc ligand, a lipophilic ligand or other ligands targeting a receptor that could facilitate the endocytosis of siRNA conjugate, such as TfR targeting ligands, LDL-R targeting ligands, or integrin targeting ligands, and/or
 - c) the modified internucleotide linkage comprises methylphosphonate (MP), methoxypropyl methylphosphonate (MOP), phosphorothioate (PS), phosphorodithioate

(PS2), phosphoroselenoate, phosphorodiselenoate, phosphoroanilothioate, phosphoraniladate, phosphoramidate and PNA.

21. The double-stranded oligonucleotide agent of any one of claims 4-20, wherein:

- a) at least one of the internucleotide linkages between the nucleotides in the cleavage region is not phosphorothioate linkage;
- b) the internucleotide linkage between X2 and Y is not phosphorothioate linkage;
- c) the internucleotide linkage between Y and Z is not phosphorothioate linkage;
- d) none of the internucleotide linkages between the nucleotides in the cleavage region is phosphorothioate linkage;
- e) at least one of the internucleotide linkages between the nucleotides in the cleavage region is phosphodiester linkage;
- f) the internucleotide linkage between X2 and Y is phosphodiester linkage;
- g) the internucleotide linkage between Y and Z is phosphodiester linkage; and/or
- h) each of the internucleotide linkages between the nucleotides in the cleavage region is phosphodiester linkage.

22. The double-stranded oligonucleotide agent of any one of claims 4-21, wherein:

- a) at least one nucleotide in the cleavage region is 2'-OMe modified or 2'-F modified;
- b) each nucleotide in the cleavage region is either 2'-OMe modified or 2'-F modified;
- c) at least one nucleotide in the cleavage region is 2'-F modified;
- d) no more than two nucleotides in the cleavage region are 2'-F modified;

- e) Z in the Formula A or Formula B is 2'-F modified, and optionally X2 in the Formula A or Formula B is 2'-F modified;
 - f) N1 in the Formula A or Formula B is 2'-F modified;
 - g) both X2 and Y in the Formula A or Formula B are 2'-OMe modified, and both Z and N1 in the Formula A or Formula B are 2'-F modified; and/or
 - h) all of X2, Y and Z in the Formula A or Formula B are 2'-OMe modified, and N1 in the Formula A or Formula B is 2'-F modified.
23. The double-stranded oligonucleotide agent of any one of the preceding claims, wherein the double-stranded oligonucleotide agent further comprises at least one phosphorothioate or methylphosphonate internucleotide linkage.
24. The double-stranded oligonucleotide agent of any one of the preceding claims, wherein the double-stranded oligonucleotide agent further comprises:
- a) at least one phosphorothioate or methylphosphonate internucleotide linkage at position 1 and/or 2 of the first fragment (counting from its 5'-end); and/or
 - b) at least one phosphorothioate or methylphosphonate internucleotide linkage at position 1 and/or 2 of the first fragment (counting from its 3'-end).
25. The double-stranded oligonucleotide agent of any one of the preceding claims, wherein the double-stranded oligonucleotide agent further comprises:
- a) at least one phosphorothioate or methylphosphonate internucleotide linkage at positions 1 to 8, or at positions 1 to 6, or at position 1 and/or 2 of the second fragment (counting from its 5'-end); and/or
 - b) at least one phosphorothioate or methylphosphonate internucleotide linkage at positions 1 to 8, or at positions 1 to 6, or at position 1 and/or 2 of the second fragment (counting from its 3'-end).

26. The double-stranded oligonucleotide agent of any one of the preceding claims, wherein the anti-sense strand further comprises a blocking group at the 5' end, optionally the blocking group is linked to the 5' end of the 5' extension.
27. The double-stranded oligonucleotide agent of claim 26, wherein the blocking group comprises an abasic residue, inverted abasic residue, M03 or M06.
28. The double-stranded oligonucleotide agent of claim 26 or 27, wherein the blocking group is linked to the 5' end of the 5' extension via an internucleotide linkage, which is optionally modified or unmodified.
29. The double-stranded oligonucleotide agent of claim 28, wherein the blocking group is linked to the 5' end of the 5' extension via phosphorothioate linkage.
30. The double-stranded oligonucleotide agent of any one of the preceding claims, wherein the sense strand is complementary over its full length to the anti-sense strand.
31. The double-stranded oligonucleotide agent of any one of claims 9-30, wherein cleavage at the cleavage region generates a blunt end at the 5' end of the first fragment.
32. The double-stranded oligonucleotide agent of any one of the preceding claims, wherein:
- a) each nucleotide of the first fragment of the anti-sense strand is modified;
 - b) each nucleotide of the anti-sense strand is modified;
 - c) each nucleotide of the second fragment of the sense strand is modified; and/or
 - d) each nucleotide of the sense strand is modified.
33. The double-stranded oligonucleotide agent of any one of the preceding claims, wherein each nucleotide of the anti-sense strand is either 2'-F modified or 2'-OMe modified; and/or each nucleotide of the sense strand is either 2'-F modified or 2'-OMe modified.

34. The double-stranded oligonucleotide agent of any one of the preceding claims, wherein the sense strand further comprises:
- a) a motif of three consecutive 2'-F modified nucleotides, optionally the motif occurs at positions 9, 10 and 11 counting from 5' end nucleotide of the sense strand; and/or
 - b) 2'-F modified nucleotide at position 7 and/or 18 counting from 5' end nucleotide of the sense strand; and/or
 - c) an alternating motif from position 9 to 13 counting from 5' end, optionally 2'-F modification and 2'-OMe modification occur on alternating nucleotides in the motif.
35. The double-stranded oligonucleotide agent of any one of the preceding claims, wherein the first fragment of the anti-sense strand and/or the second fragment of the sense strand further comprises an alternating motif where modification occurs on alternating nucleotides in the motif, optionally 2'-F modification occurs on alternating nucleotides in the motif and/or 2'-OMe modification occurs on alternating nucleotides in the motif.
36. The double-stranded oligonucleotide agent of any one of the preceding claims, wherein the first fragment of the anti-sense strand and/or the second fragment of the sense strand comprises modification selected from the group consisting of 2'-OMe (2'-O-methyl) modification, 2'-F (2'-deoxy-2'-fluoro) modification, 2'-O-MOE (2'-O-methoxyethyl) modification, 2'-deoxy (2'-d) modification, 5'-morpholine (5'-Mo) modification, unlocked nucleic acid (UNA) modification, glycol nucleic acid (GNA) modification, locked nucleic acid (LNA) modification, tricyclo-DNA (tcDNA) modification, (S)-constrained ethyl bridged nucleic acid ((S)-cEt-BNA) modification, 5'-(E)-vinylphosphate (VP) modification, 2'-O-C16 modification, conjugating with invert abasic nucleotide (invAB) at 5' end or 3' end, replacing with invert abasic nucleotide (invAb), replacing with 2,4-difluorotolyl ribonucleotide (rF), replacing with (S)-glycerol nucleic acid and replacing with inosine (I).
37. A double-stranded oligonucleotide agent comprising a structure represented by Formula (C):

sense strand (5'→3'):	second fragment	
anti-sense strand (3'→5'):	first fragment	5' extension

Formula (C),

wherein the first fragment in the anti-sense strand and the second fragment in the sense strand form a double-stranded portion by base pairing, wherein the first fragment and the second fragment are of equal length;

wherein the 5' extension comprises at least three nucleotides in length;

wherein the anti-sense strand comprises a cleavage region comprising the most 5' nucleotide (X2) of the first fragment and the two most 3' nucleotides (Y-Z) of the 5' extension, and the cleavage region comprises a nucleotide sequence set forth in Formula A: (3'-5') X2-Y-Z, which is cleavable between X2 and Y, and the Formula A is as defined in any one of claims 9 to 36.

wherein the sense strand has a length of 15 to 35, 15 to 23, 15 to 22, or 15 to 21 nucleotides; and

wherein the anti-sense strand has a length of 25 to 35, 26 to 35, 26 to 30, 25 to 27, or 26 to 27 nucleotides, and optionally, the anti-sense strand further comprises a blocking group at the 5' end, optionally the blocking group is linked to the 5' end of the 5' extension.

38. The double-stranded oligonucleotide agent of claim 37, wherein the sense strand has a length of 17 to 23, 17 to 22, 21 to 23, or 17 to 21 nucleotides; and the anti-sense strand has a length of 25-30, 25 to 27, or 26 to 27 nucleotides.
39. The double-stranded oligonucleotide agent of claim 37, wherein the sense strand and the anti-sense strand have a length of a) 17nt and 20nt respectively; b) 18nt and 21nt,

respectively; c) 19nt and 22nt, respectively; d) 20nt and 23nt, respectively; or e) 21nt and 24nt, respectively.

40. A double-stranded oligonucleotide agent comprising a structure represented by Formula (D):

sense strand (5'→3'):		second fragment	
anti-sense strand (3'→5'):	3' extension	first fragment	5' extension

Formula (D),

wherein

the first fragment in the anti-sense strand and the second fragment in the sense strand form a double-stranded portion by base pairing, wherein the first fragment and the second fragment are of equal length;

the 5' extension comprises at least three nucleotides in length;

the anti-sense strand comprises a cleavage region comprising the most 5' nucleotide (X2) of the first fragment and the two most 3' nucleotides (Y-Z) of the 5' extension, and the cleavage region comprises a nucleotide sequence set forth in Formula A: (3'-5') X2-Y-Z, which is enzymatically cleavable between X2 and Y, and upon cleavage, the 5' extension is removed from its most 3' nucleotide (Y), and the Formula A is as defined in anyone of claims 9 to 36,

wherein the sense strand has a length of 15 to 35, 15 to 23, 15 to 22, 15 to 21, 16 to 25, 17 to 23, 18 to 23, 19 to 23, 19 to 21, 20 to 23, 20 to 21, 21 to 23, such as 17, 18, 19, 20, 21, 22, or 23 nucleotides; and

wherein the anti-sense strand has a length of 25 to 35, 25 to 30, 26 to 35, 26 to 30, 26 to 27, such as 25, 26, 27, 28, 29, or 30 nucleotides, and optionally, the anti-sense strand further

comprises a blocking group at the 5' end, optionally the blocking group is linked to the 5' end of the 5' extension.

41. The double-stranded oligonucleotide agent of claim 40, wherein the sense strand has a length of 17 to 23, 17 to 22, or 17 to 21 nucleotides; and wherein the anti-sense strand has a length of 22-28, 22 to 27, or 22 to 26 nucleotides.
42. The double-stranded oligonucleotide agent of claim 40, wherein the sense strand and the anti-sense strand have a length of a) 19 nt and 25 nt respectively; b) 20 nt and 25 nt, respectively; c) 21 nt and 25 nt, respectively; d) 19 nt and 26 nt, respectively; e) 20 nt and 26nt, respectively; f) 21 nt and 26 nt, respectively; or g) 21 nt and 27 nt, respectively.
43. The double-stranded oligonucleotide agent of any one of the preceding claims, wherein the double-stranded oligonucleotide agent further comprises a ligand operably linked to the sense strand or the anti-sense strand.
44. The double-stranded oligonucleotide agent of claim 43, wherein the ligand is operably linked to the 5' end of the sense strand or the 5' end of the anti-sense strand.
45. The double-stranded oligonucleotide agent of claim 43 or 44, wherein the ligand is linked to the double-stranded oligonucleotide agent with a phosphorothioate.
46. The double-stranded oligonucleotide agent of any one of claims 43-45, wherein the ligand is a GalNAc ligand, a lipophilic ligand or other ligands targeting a receptor that could facilitate the endocytosis of siRNA conjugate, such as TfR targeting ligands, LDL-R targeting ligands, or integrin targeting ligands.
47. The double-stranded oligonucleotide agent of any one of claims 44-46, wherein the ligand is selected from the group consisting of L96, C16U, C16G, C16A, C16C, VSDL-01, VSDL-01A, VSDL-02, VSDL-02A, VSDL-03, VSDL-03A, VSDL-04, VSDL-04A, VSDL-05, VSDL-05A, VSDL-06, VSDL-06A, VSDL-07, VSDL-07A, VSDL-08, VSDL-08A, VSDL-09, VSDL-10, VSDL-11, VSDL-12, VSDL-13, and VSDL-14.

48. The double-stranded oligonucleotide agent of any one of the preceding claims, wherein the target RNA encodes a target gene selected from the group consisting of AGT, CFB, DGAT2, DUX, ANGPTL8, APOC3, F12, INHBE, PNPLA3, Serpinc1, APP, SOD1, TMPRSS6, KHK, PCSK9, VEGFA, ANGPTL3, ANGPTL4, C3, C5, TTR, IGF-1R, VEGFR, ANG2, GIPR, GPR75, ActRII, NUDT21, PLN, HSD17b13, CNOT6L, PTP1B, CFHR, ATX, CIDEB, mARC1, TSHR, CB1 and LPA.
49. A double-stranded oligonucleotide agent comprising a double-stranded oligonucleotide operably linked to a blocking group, wherein the blocking group comprises M03 or M06.
50. A double-stranded oligonucleotide agent comprising a double-stranded oligonucleotide operably linked to a ligand, where the ligand comprises a chemical structure selected from the group consisting of: VSDL-01, VSDL-01A, VSDL-02, VSDL-02A, VSDL-03, VSDL-03A, VSDL-04, VSDL-04A, VSDL-05, VSDL-05A, VSDL-06, VSDL-06A, VSDL-07, VSDL-07A, VSDL-08, VSDL-08A, VSDL-09, VSDL-10, VSDL-11, VSDL-12, VSDL-13, and VSDL-14 .
51. The double-stranded oligonucleotide agent of claim 49 or 50, wherein:
- a) the double-stranded oligonucleotide comprises a sense strand and an anti-sense strand, wherein each strand has 14 to 30 nucleotides, and/or
 - b) the anti-sense strand is sufficiently complementary to part of a target RNA or an mRNA encoding a target gene, and the sense strand is sufficiently complementary to the targeting region of the anti-sense strand;
 - c) the blocking group is conjugated to the 5'end of the anti-sense strand; and/or
 - d) the ligand is conjugated to the 5'end of the anti-sense strand, 5'end of the sense strand or 3'end of the sense strand; and/or
 - e) the double-stranded oligonucleotide is operably linked to the blocking group at one end, and is operably linked to the ligand at another end, and/or

- f) the double-stranded oligonucleotide agent is capable of silencing a target RNA or inhibiting expression of a target gene via RNA interference.
52. A pharmaceutical composition comprising a double-stranded oligonucleotide agent of any one of the preceding claims, and a pharmaceutically acceptable carrier.
53. A method of inhibiting the expression of a target gene in a subject in need thereof, comprising administering to the subject a pharmaceutically effective amount of a double-stranded oligonucleotide agent of any one of claims 1-51 or a pharmaceutical composition of claim 52.
54. The method of claim 53, wherein the target gene is selected from the group consisting of AGT, CFB, DGAT2, DUX, ANGPTL8, APOC3, F12, INHBE, PNPLA3, Serpinc1, APP, SOD1, TMPRSS6, KHK, PCSK9, VEGFA, ANGPTL3, ANGPTL4, C3, C5, TTR, IGF-1R, VEGFR, ANG2, GIPR, GPR75, ActRII, NUDT21, PLN, HSD17b13, CNOT6L, PTP1B, CFHR, ATX, CIDEA, mARC1, TSHR, CB1 and LPA.
55. A method of treating a disease or a disorder in a subject in need thereof, comprising administering to the subject a pharmaceutically effective amount of a double-stranded oligonucleotide agent of any one of claims 1-51 or a pharmaceutical composition of claim 52.
56. The method of claim 55, wherein the disease or disorder is associated with the target gene.