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(54) Title: NUCLEIC ACID MOLECULES TARGETING SUPEROXIDE DISMUTASE 1 (SOD1)

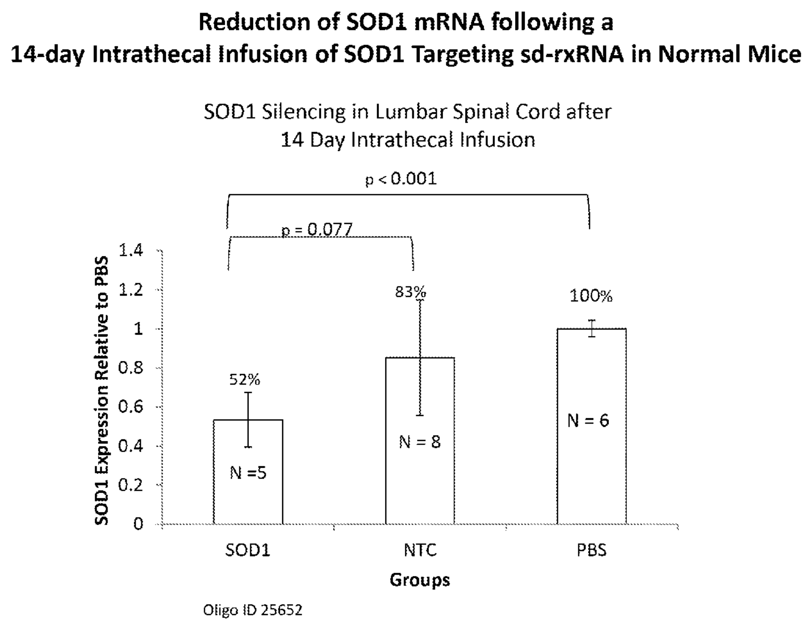

FIG. 6

(57) **Abstract**: Aspects of the invention relate to methods for treating ALS comprising administering to a subject in need thereof a therapeutically effective amount of a nucleic acid molecule that is directed against a gene encoding superoxide dismutase 1 (SOD1).

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NUCLEIC ACID MOLECULES TARGETING SUPEROXIDE DISMUTASE 1 (SOD1)

RELATED APPLICATIONS

This application claims the benefit under 35 U.S.C. § 119(e) of U.S. Provisional Application Serial No. 62/189,050, filed on July 6, 2015, entitled "NUCLEIC ACID MOLECULES TARGETING SUPEROXIDE DISMUTASE 1 (SOD1)", the entire contents of which are incorporated herein by reference.

FIELD OF THE INVENTION

The disclosure relates, at least in part, to the use of nucleic acid molecules with improved *in vivo* delivery properties targeting SOD1 for the treatment of neurological disorders such as amyotrophic lateral sclerosis (ALS).

BACKGROUND

Complementary oligonucleotide sequences are promising therapeutic agents and useful research tools in elucidating gene functions. However, prior art oligonucleotide molecules suffer from several problems that may impede their clinical development, and frequently make it difficult to achieve intended efficient inhibition of gene expression (including protein synthesis) using such compositions *in vivo*.

A major problem has been the delivery of these compounds to cells and tissues. Conventional double-stranded RNAi compounds, 19-29 bases long, form a highly negatively-charged rigid helix of approximately 1.5 by 10-15 nm in size. This rod type molecule cannot get through the cell-membrane and as a result has very limited efficacy both *in vitro* and *in vivo*. As a result, all conventional RNAi compounds require some kind of delivery vehicle to promote their tissue distribution and cellular uptake. This is considered to be a major limitation of the RNAi technology.

There have been previous attempts to apply chemical modifications to oligonucleotides to improve their cellular uptake properties. One such modification was the attachment of a cholesterol molecule to the oligonucleotide. A first report on this approach was by Letsinger *et al.*, in 1989. Subsequently, ISIS Pharmaceuticals, Inc. (Carlsbad, CA) reported on more advanced techniques in attaching the cholesterol molecule to the oligonucleotide (Manoharan, 1992).

With the discovery of siRNAs in the late nineties, similar types of modifications were attempted on these molecules to enhance their delivery profiles. Cholesterol molecules conjugated to slightly modified (Soutschek, 2004) and heavily modified (Wolfrum, 2007) siRNAs appeared in the literature. Yamada *et al.*, 2008 also reported on the use of advanced linker chemistries which further improved cholesterol mediated uptake of siRNAs. In spite of all this effort, the uptake of these types of compounds impaired to be inhibited in the presence of biological fluids resulting in highly limited efficacy in gene silencing *in vivo*, limiting the applicability of these compounds in a clinical setting.

SUMMARY

In some aspects, the disclosure relates to an isolated double stranded nucleic acid molecule directed agains superoxide dismutase 1 (SOD1) comprising a guide strand and a passenger strand, wherein the isolated double stranded nucleic acid molecule includes a double stranded region and a single stranded region, wherein the region of the molecule that is double stranded is from 8-15 nucleotides long, wherein the guide strand contains a single stranded region that is 2-14 nucleotides long, wherein the guide strand contains 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21 or 22 phosphorothioate modifications, wherein the passenger strand is 8 to 15 nucleotides long, wherein the passenger strand contains 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13 or 14 phosphorothioate modifications, and wherein at least 40% of the nucleotides of the isolated double stranded nucleic acid molecule are modified.

In some embodiments, at least 60% of the nucleotides are modified. In some embodiments, at least one strand of the isolated double stranded nucleic acid molecule comprises a completely phosphorothioated backbone. In some embodiments, at least one strand of the isolated double stranded nucleic acid molecule is completely phosphorothioated, or is completely phosphorothioated with the exception of one residue. In some embodiments, at least one of the nucleotides of the isolated double stranded nucleic acid molecule that is modified comprises a 2'O-methyl or a 2'-fluoro modification.

In some embodiments, the isolated double stranded nucleic acid molecule is directed against superoxide dismutase 1 (SOD1). In some embodiments, the isolated double stranded nucleic acid molecule does not comprise the modification pattern of SEQ ID NO: 40 described in PCT Publication number WO2010/033247.

In some embodiments, a plurality of the U's and/or C's include a hydrophobic modification, selected from the group consisting of methyl, isobutyl, octyl, imidazole or thiophene, wherein the modifications are located on positions 4 or 5 of U's and/or C's.

In some embodiments, the isolated double stranded nucleic acid molecule comprises at least 12 contiguous nucleotides of a sequence selected from the sequences within Tables 1-8, including the modification pattern provided in Tables 1-8.

In some aspects, the disclosure relates to an isolated double stranded nucleic acid molecule that comprises at least 12 contiguous nucleotides of a sequence selected from the sequences within Tables 1-8, wherein if the isolated double stranded nucleic acid molecule comprises at least 12 contiguous nucleotides of a sequence selected from SEQ ID NOs: 70, 71, 72, 73, 79, 80, 81, or 84 in Table 2, then the guide strand contains more than 6 phosphorothioate modifications. In some embodiments, the isolated double stranded nucleic acid molecule further comprises a hydrophobic conjugate that is attached to the isolated double stranded nucleic acid molecule.

In some embodiments, the sense strand of the isolated nucleic acid molecule comprises at least 12 consecutive nucleotides of SEQ ID NO: 2, SEQ ID NO: 32, or SEQ ID NO: 122, and the guide strand comprises at least 12 consecutive nucleotides of SEQ ID NO: 61, SEQ ID NO: 91, or SEQ ID NO: 123. In some embodiments, the sense strand of the isolated nucleic acid molecule comprises SEQ ID NO: 2, SEQ ID NO: 32, or SEQ ID NO: 122, and the guide strand comprises SEQ ID NO: 61, SEQ ID NO: 91, or SEQ ID NO: 123.

In some embodiments, the sense strand of the isolated nucleic acid molecule comprises at least 12 consecutive nucleotides of SEQ ID NO: 4, SEQ ID NO: 34, or SEQ ID NO: 126, and the guide strand comprises at least 12 consecutive nucleotides of SEQ ID NO: 63 or SEQ ID NO: 93. In some embodiments, the sense strand of the isolated nucleic acid molecule comprises SEQ ID NO: 4, SEQ ID NO: 34, or SEQ ID NO: 126, and the guide strand comprises SEQ ID NO: 63 or SEQ ID NO: 93.

In some embodiments, the sense strand of the isolated nucleic acid molecule comprises at least 12 consecutive nucleotides of SEQ ID NO: 9, SEQ ID NO: 38, or SEQ ID NO:135, and the guide strand comprises at least 12 consecutive nucleotides of SEQ ID NO: 68, SEQ ID NO: 97, or SEQ ID NO: 136. In some embodiments, the sense strand of the isolated nucleic acid molecule comprises SEQ ID NO: 9, SEQ ID NO: 38, or SEQ ID NO:135, and the guide strand comprises SEQ ID NO: 68, SEQ ID NO: 97, or SEQ ID NO: 136.

In some embodiments, the sense strand of the isolated nucleic acid molecule comprises at least 12 consecutive nucleotides of SEQ ID NO: 10 or SEQ ID NO: 39, and the guide strand comprises at least 12 consecutive nucleotides of SEQ ID NO: 69 or SEQ ID NO: 98. In some embodiments, the sense strand of the isolated nucleic acid molecule comprises SEQ ID NO: 10 or SEQ ID NO: 39, and the guide strand comprises SEQ ID NO: 69 or SEQ ID NO: 98.

In some embodiments, the sense strand of the isolated double stranded nucleic acid molecule comprises at least 12 consecutive nucleotides of SEQ ID NO: 5, SEQ ID NO: 127 or SEQ ID NO: 137, and the guide strand comprises at least 12 consecutive nucleotides of SEQ ID NO: 64, SEQ ID NO: 128 or SEQ ID NO: 138. In some embodiments, the sense strand of the isolated nucleic acid molecule comprises SEQ ID NO: 5, SEQ ID NO: 127 or SEQ ID NO: 137, and the guide strand comprises SEQ ID NO: 64, SEQ ID NO: 128 or SEQ ID NO: 138.

In some embodiments, the isolated double stranded nucleic acid does not form a hairpin.

In some aspects, the disclosure relates to a composition comprising an isolated double stranded nucleic acid molecule as described by the disclosure. In some embodiments, the composition comprises an excipient (*e.g.*, a pharmaceutically acceptable carrier). In some embodiments, the composition comprises a second therapeutic agent, such as a nucleic acid (*e.g.*, sd-rxRNA, *etc.*), small molecule, peptide, or polypeptide (*e.g.*, antibody).

In some aspects, the disclosure relates to a method for treating ALS comprising administering to a subject in need thereof a therapeutically effective amount of a nucleic acid molecule that is directed against a gene encoding superoxide dismutase 1 (SOD1).

In some aspects, the disclosure relates to a method for treating ALS comprising administering to a subject in need thereof a therapeutically effective amount of an isolated double stranded nucleic acid molecule directed against superoxide dismutase 1 (SOD1) comprising a guide strand and a passenger strand, wherein the isolated double stranded nucleic acid molecule includes a double stranded region and a single stranded region, wherein the region of the molecule that is double stranded is from 8-15 nucleotides long, wherein the guide strand contains a single stranded region that is 2-14 nucleotides long, wherein the guide strand contains 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21 or 22 phosphorothioate modifications, wherein the passenger strand is 8 to 15 nucleotides long, wherein the passenger strand contains 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13 or 14 phosphorothioate modifications, wherein at least 40% of the nucleotides of the isolated double stranded nucleic acid molecule are modified, and wherein the isolated double stranded nucleic acid molecule comprises at least 12 contiguous nucleotides of a sequence selected from the sequences within Tables 1-8, including the modification pattern provided in Tables 1-8.

In some embodiments, the isolated double stranded nucleic acid molecule is completely phosphorothioated, or is completely phosphorothioated with the exception of one residue In some embodiments, at least one of the nucleotides of the isolated double stranded nucleic acid molecule that is modified comprises a 2'O-methyl or a 2'-fluoro modification.

In some embodiments, the isolated double stranded nucleic acid molecule further comprises a hydrophobic conjugate that is attached to the isolated double stranded nucleic acid molecule.

In some embodiments, the isolated double stranded nucleic acid molecule is formulated for delivery to the central nervous system.

In some embodiments, the isolated double stranded nucleic acid molecule is administered via intrathecal infusion and/or injection.

In some embodiments of methods described herein, the sense strand of the isolated nucleic acid molecule comprises at least 12 consecutive nucleotides of SEQ ID NO: 2, SEQ ID NO: 32, or SEQ ID NO: 122, and the guide strand comprises at least 12 consecutive nucleotides of SEQ ID NO: 61, SEQ ID NO: 91, or SEQ ID NO: 123.

In some embodiments of methods described herein, the sense strand of the isolated nucleic acid molecule comprises SEQ ID NO: 2, SEQ ID NO: 32, or SEQ ID NO: 122, and the guide strand comprises SEQ ID NO: 61, SEQ ID NO: 91, or SEQ ID NO: 123.

In some embodiments of methods described herein, the sense strand of the isolated nucleic acid molecule comprises at least 12 consecutive nucleotides of SEQ ID NO: 4, SEQ ID NO: 34, or SEQ ID NO: 126, and the guide strand comprises at least 12 consecutive nucleotides of SEQ ID NO: 63 or SEQ ID NO: 93.

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NO: 9, SEQ ID NO: 38, or SEQ ID NO:135, and the guide strand comprises at least 12 consecutive nucleotides of SEQ ID NO: 68, SEQ ID NO: 97, or SEQ ID NO: 136.

In some embodiments of methods described herein, the sense strand of the isolated nucleic acid molecule comprises SEQ ID NO: 9, SEQ ID NO: 38, or SEQ ID NO:135, and the guide strand comprises SEQ ID NO: 68, SEQ ID NO: 97, or SEQ ID NO: 136.

In some embodiments of methods described herein, the sense strand of the isolated nucleic acid molecule comprises at least 12 consecutive nucleotides of SEQ ID NO: 10 or SEQ ID NO: 39, and the guide strand comprises at least 12 consecutive nucleotides of SEQ ID NO: 69 or SEQ ID NO: 98.

In some embodiments of methods described herein, the sense strand of the isolated nucleic acid molecule comprises SEQ ID NO: 10 or SEQ ID NO: 39, and the guide strand comprises SEQ ID NO: 69 or SEQ ID NO: 98.

In some embodiments of methods described herein, the sense strand of the isolated double stranded nucleic acid molecule comprises at least 12 consecutive nucleotides of SEQ ID NO: 5, SEQ ID NO: 127 or SEQ ID NO: 137, and the guide strand comprises at least 12 consecutive nucleotides of SEQ ID NO: 64, SEQ ID NO: 128 or SEQ ID NO: 138.

In some embodiments of methods described herein, the sense strand of the isolated nucleic acid molecule comprises SEQ ID NO: 5, SEQ ID NO: 127 or SEQ ID NO: 137, and the guide strand comprises SEQ ID NO: 64, SEQ ID NO: 128 or SEQ ID NO: 138.

Each of the limitations of the invention can encompass various embodiments of the invention. It is, therefore, anticipated that each of the limitations of the invention involving any one element or combinations of elements can be included in each aspect of the invention. This invention is not limited in its application to the details of construction and the arrangement of components set forth in the following description or illustrated in the drawings. The invention is capable of other embodiments and of being practiced or of being carried out in various ways.

BRIEF DESCRIPTION OF THE DRAWINGS

FIG. 1. Reducing phosphorothioate content results in active ps-rxRNA variants with reduced cellular toxicity *in vitro*. The upper panel shows SOD1 silencing and the lower panel

shows cell viability. These data demonstrate that ps-rxRNA variants 25635 and 25637 are potent and have a more favorable cellular toxicity profile (*in vitro*) compared to 25600.

FIG. 2. Reducing phosphorothioate content results in active ps-rxRNA variants with reduced cellular toxicity *in vitro*. The upper panel shows SOD1 silencing and the lower panel shows cell viability. These data show that ps-rxRNA variant 25645 is potent and has a slightly more favorable cellular toxicity profile (*in vitro*) compared to 25600.

FIG. 3. Brain-cerebellum (IC injection in rat). Penetration increases with change in chemistry content; uptake 25635<25637<25645< 25652. * Frontal cortex, not cerebellum.

FIG. 4. Cervical spinal cord-transverse cut. 25637 and var 25645 are similar but both are less than 25652 in distribution.

FIG. 5. Cervical spinal cord-longitudinal cut. 25637 is less than 25652 in distribution into the tissue. Administration of original fl-ps-rxRNA compound results in full brain and spinal cord penetration. ps-rxRNA variant 1 (25635) in this particular assay was insufficient to achieve brain or spinal cord penetration. ps-rxRNA variants 2 and 3 (25637 and 25645, respectively) both result in uptake by cells of the brain and spinal cord but less than original fl-ps-rxRNA (25652).

FIG. 6. Reduction of SOD1 mRNA following a 14-day intrathecal infusion of SOD1 targeting sd-rxRNA in C57BL/6 normal (non-transgenic) mice using an implanted osmotic pump. 14 day silencing with Oligo ID 25652 in normal mice. 14 day osmotic pumps were filled with 100 µl of 10 mg/ml of each compound. Gene expression analysis by qPCR was normalized to the PPIB housekeeping gene and was plotted relative to SOD1 expression in the PBS group. Silencing was observed in the lumbar region of the spinal cord only (region of catheter placement). The pump flow rate was 0.25 µl/hr; therefore 60 µg of SOD1 targeting or non-targeting control (NTC) ps-rxRNA was delivered each day for 14 days (840 µg total). 24 C57BL/6J mice were used for this study including 8 for Oligo ID 25652: 8 for NTC ps-rxRNA; and 8 for PBS.

FIG. 7. Reduction of SOD1 mRNA following a 14-day intrathecal infusion of SOD1 targeting sd-rxRNA Variant 3 (Oligo ID 25645) in normal mice. 14 day intrathecal infusion was performed with implanted osmotic pump in C57BL/6 normal (non-transgenic) mice. 14 day osmotic pumps were filled with 100 µl of 10 mg/ml of each compound. 60 µg of SOD1 targeting or non-targeting control (NTC) sd-rxRNA variant were administered per day. Gene expression analysis by qPCR was performed to normalize gene expression to the PPIB housekeeping gene and plotted relative to SOD1 expression in the PBS group. Silencing was observed in the lumbar region of the spinal cord only (region of catheter placement). The

pump flow rate was 0.25 µl/hr; therefore 60 µg was delivered each day for 14 days (840 µg total). 30 C57BL/6J mice were used for this study, including 10 for Oligo ID 25645; 10 for NTC ps-rxRNA; and 10 for PBS.

FIG. 8 depicts data generated using SOD1 sd-rxRNA variant octyl modifications.

FIG. 9 depicts data generated using SOD1 sd-rxRNA variant octyl modifications.

FIG. 10 depicts data generated using SOD1 sd-rxRNA variant thiophene modifications.

FIG. 11 depicts data generated using SOD1 sd-rxRNA variant isobutyl modifications.

DETAILED DESCRIPTION

SOD1 (copper/zinc superoxide dismutase)

As used herein, "SOD1" refers to the Superoxide Dismutase 1 enzyme, which is one of three superoxide dismutases involved in converting harmful superoxide radicals to water. Approximately 10% of all ALS cases are dominantly inherited, and of these ~20% are due to defects in cytosolic superoxide dismutase 1 (SOD1). In addition, SOD1 has been implicated in non-familial (e.g. sporadic) forms of ALS. (Jones, C. T., Brock, D. J. H., Chancellor, A. M., Warlow, C. P., Swingler, R. J. *Cu/Zn superoxide dismutase (SOD1) mutations and sporadic amyotrophic lateral sclerosis.* Lancet 342: 1050-1051, 1993). Without wishing to be bound by any theory, several lines of investigation have suggested that the mutations in SOD1 do not cause ALS through loss of the dismutase activity of this enzyme. Rather, mutant SOD1 is neurotoxic through multiple alternate mechanisms, many of which entail conformational instability and aberrant binding and aggregation of the mutant protein. While the precise details of the toxicity of mutant SOD1 are not fully defined, it is abundantly clear that reduction of the burden of mutant SOD1 protein in animal models significantly delays death. This has been achieved using both antisense oligonucleotides (ASO) (Smith et al) and siRNA (Maxwell, Pasinelli et al. 2004) (Xia, Zhou et al. 2006) (Wang, Ghosh et al. 2008). These studies illustrate the principle that siRNA-based drugs represent a potentially significant therapeutic advance for the treatment of ALS and many other CNS disorders. In both cases, efficacy was achieved by delivery of high amounts of material over long periods of time; these studies illustrate the point that a major limitation of ASO and siRNA therapies in their present forms is the lack of optimally efficient and non-toxic *in vivo* delivery systems (Smith, Miller et al. 2006; Wang, Ghosh et al. 2008).

Amyotrophic lateral sclerosis (ALS)

ALS is a progressive neurodegenerative disease affecting motor neurons in the central nervous system. Degeneration of the motor neurons results in paralysis and eventual death, usually due to respiratory failure. In a subset of cases, ALS is caused by dominantly transmitted mutations in the gene encoding cytosolic superoxide dismutase (SOD1). Transgenic expression of mutant SOD1 causes ALS in mice.

Nucleic acid molecules

As used herein, "nucleic acid molecule" includes but is not limited to: sd-rxRNA, sd-rxRNA variant, rxRNAori, oligonucleotides, ASO, siRNA, shRNA, miRNA, ncRNA, cp-lasiRNA, aiRNA, single-stranded nucleic acid molecules, double-stranded nucleic acid molecules, RNA and DNA. In some embodiments, the nucleic acid molecule is a chemically modified nucleic acid molecule, such as a chemically modified oligonucleotide.

sd-rxRNA molecules

Aspects of the invention relate to sd-rxRNA molecules. As used herein, an "sd-rxRNA" or an "sd-rxRNA molecule" refers to a self-delivering RNA molecule such as those described in, and incorporated by reference from, US Patent No. 8,796,443, granted on August 5, 2014, entitled "REDUCED SIZE SELF-DELIVERING RNAI COMPOUNDS", US Patent No. 9,175,289, granted on November 3, 2015, entitled "REDUCED SIZE SELF-DELIVERING RNAI COMPOUNDS", and PCT Publication No. WO2010/033247 (Application No. PCT/US2009/005247), filed on September 22, 2009, and entitled "REDUCED SIZE SELF-DELIVERING RNAI COMPOUNDS." Briefly, an sd-rxRNA, (also referred to as an sd-rxRNA^{nano}) is an isolated asymmetric double stranded nucleic acid molecule comprising a guide strand, with a minimal length of 16 nucleotides, and a passenger strand of 8-18 nucleotides in length, wherein the double stranded nucleic acid molecule has a double stranded region and a single stranded region, the single stranded region having 4-12 nucleotides in length and having at least three nucleotide backbone modifications. In preferred embodiments, the double stranded nucleic acid molecule has one end that is blunt or includes a one or two nucleotide overhang. sd-rxRNA molecules can be optimized through chemical modification, and in some instances through attachment of hydrophobic conjugates. In some embodiments, the isolated double stranded nucleic acid molecule does not comprise the modification pattern of SEQ ID NO: 40 described in PCT Publication number WO2010/033247.

In some embodiments, an sd-rxRNA comprises an isolated double stranded nucleic acid molecule comprising a guide strand and a passenger strand, wherein the region of the molecule that is double stranded is from 8-15 nucleotides long, wherein the guide strand contains a single stranded region that is 4-12 nucleotides long, wherein the single stranded region of the guide strand contains 3, 4, 5, 6, 7, 8, 9, 10, 11 or 12 phosphorothioate modifications, and wherein at least 40% of the nucleotides of the double stranded nucleic acid are modified.

In some embodiments, an sd-rxRNA variant comprises an sd-rxRNA that contains an 18 to 23 nucleotide long guide strand (antisense strand) and a 10 to 15 nucleotide long passenger strand (sense strand). The guide strand and passenger strand can form an asymmetric duplex. In some embodiments, the guide strand contains between 6 to 22 backbone modifications, including phosphorothioate modifications. In some embodiments, the passenger strand contains between 2 to 14 backbone modifications, including phosphorothioate modifications. In some embodiments, the passenger strand is attached to a hydrophobic conjugate. The term "sd-rxRNA variant" is used interchangeably herein with "ps-rxRNA".

In some embodiments, the guide strand contains 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21 or 22 phosphorothioate modifications. In some embodiments, the guide strand is completely phosphorothioated. In some embodiments, the passenger strand contains 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13 or 14 phosphorothioate modifications. In some embodiments, the passenger strand is completely phosphorothioated. It was surprisingly found herein that high levels of phosphorothioate modifications can lead to increased delivery of isolated double stranded nucleic acid molecules.

Nucleic acid molecules associated with the invention are also referred to herein as polynucleotides, isolated double stranded or duplex nucleic acids, oligonucleotides, nano molecules, nano RNA, sd-rxRNA^{nano}, sd-rxRNA or RNA molecules of the invention.

sd-rxRNAs are much more effectively taken up by cells compared to conventional siRNAs. These molecules are highly efficient in silencing of target gene expression and offer significant advantages over previously described RNAi molecules including high activity in the presence of serum, efficient self-delivery, compatibility with a wide variety of linkers, and reduced presence or complete absence of chemical modifications that are associated with toxicity.

In contrast to single-stranded polynucleotides, duplex polynucleotides have traditionally been difficult to deliver to a cell as they have rigid structures and a large number of negative charges which makes membrane transfer difficult. sd-rxRNAs however, although partially double-stranded, are recognized *in vivo* as single-stranded and, as such, are capable of efficiently being delivered across cell membranes. As a result the polynucleotides of the invention are capable in many instances of self-delivery. Thus, the polynucleotides of the invention may be formulated in a manner similar to conventional RNAi agents or they may be delivered to the cell or subject alone (or with non-delivery type carriers) and allowed to self-deliver. In one embodiment of the present invention, self-delivering asymmetric double-stranded RNA molecules are provided in which one portion of the molecule resembles a conventional RNA duplex and a second portion of the molecule is single stranded.

The oligonucleotides of the invention in some aspects have a combination of asymmetric structures including a double stranded region and a single stranded region of 5 nucleotides or longer, specific chemical modification patterns and are conjugated to lipophilic or hydrophobic molecules. This class of RNAi like compounds have superior efficacy *in vitro* and *in vivo*. It is believed that the reduction in the size of the rigid duplex region in combination with phosphorothioate modifications applied to a single stranded region contribute to the observed superior efficacy.

In some embodiments an RNAi compound associated with the invention comprises an asymmetric compound comprising a duplex region (required for efficient RISC entry of 8-15 bases long) and single stranded region of 4-12 nucleotides long. In some embodiments, the duplex region is 13 or 14 nucleotides long. A 6 or 7 nucleotide single stranded region is preferred in some embodiments. In some embodiments, the RNAi compound comprises 2-12 phosphorothioate internucleotide linkages (referred to as phosphorothioate modifications). 6-8 phosphorothioate internucleotide linkages are preferred in some embodiments. In some embodiments, the RNAi compounds include a unique chemical modification pattern, which provides stability and is compatible with RISC entry. The combination of these elements has resulted in unexpected properties which are highly useful for delivery of RNAi reagents *in vitro* and *in vivo*.

The chemical modification pattern, which provides stability and is compatible with RISC entry includes modifications to the sense, or passenger, strand as well as the antisense, or guide, strand. For instance the passenger strand can be modified with any chemical entities which confirm stability and do not interfere with activity. Such modifications include 2' ribo modifications (O-methyl, 2' F, 2 deoxy and others) and backbone modification like phosphorothioate modifications. A preferred chemical modification pattern in the passenger strand includes O-methyl modification of C and U nucleotides within the passenger strand or alternatively the passenger strand may be completely O-methyl modified.

The guide strand, for example, may also be modified by any chemical modification which confirms stability without interfering with RISC entry. A preferred chemical modification pattern in the guide strand includes the majority of C and U nucleotides being 2' F modified and the 5' end being phosphorylated. Another preferred chemical modification pattern in the guide strand includes 2'O-methyl modification of position 1 and C/U in positions 11-18 and 5' end chemical phosphorylation. Yet another preferred chemical modification pattern in the guide strand includes 2'O-methyl modification of position 1 and C/U in positions 11-18 and 5' end chemical phosphorylation and 2'F modification of C/U in positions 2-10. In some embodiments the passenger strand and/or the guide strand contains at least one 5-methyl C or U modifications.

In some embodiments, at least 30% of the nucleotides in the sd-rxRNA are modified. For example, at least 30%, 31%, 32%, 33%, 34%, 35%, 36%, 37%, 38%, 39%, 40%, 41%, 42%, 43%, 44%, 45%, 46%, 47%, 48%, 49%, 50%, 51%, 52%, 53%, 54%, 55%, 56%, 57%, 58%, 59%, 60%, 61%, 62%, 63%, 64%, 65%, 66%, 67%, 68%, 69%, 70%, 71%, 72%, 73%, 74%, 75%, 76%, 77%, 78%, 79%, 80%, 81%, 82%, 83%, 84%, 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98% or 99% of the nucleotides in the sd-rxRNA are modified. In some embodiments, 100% of the nucleotides in the sd-rxRNA are modified.

The above-described chemical modification patterns of the oligonucleotides of the invention are well tolerated and actually improved efficacy of asymmetric RNAi compounds. In some embodiments, elimination of any of the described components (Guide strand stabilization, phosphorothioate stretch, sense strand stabilization and hydrophobic conjugate) or increase in size in some instances results in sub-optimal efficacy and in some instances complete loss of efficacy. The combination of elements results in development of a compound, which is fully active following passive delivery to cells such as HeLa cells.

The sd-rxRNA can be further improved in some instances by improving the hydrophobicity of compounds using of novel types of chemistries. For example, one chemistry is related to use of hydrophobic base modifications. Any base in any position might be modified, as long as modification results in an increase of the partition coefficient of the base. The preferred locations for modification chemistries are positions 4 and 5 of the pyrimidines. The major advantage of these positions is (a) ease of synthesis and (b) lack of interference with base-pairing and A form helix formation, which are essential for RISC complex loading and target recognition. A version of sd-rxRNA compounds where multiple deoxy Uridines are present without interfering with overall compound efficacy was used. In addition major improvement in tissue distribution and cellular uptake might be obtained by optimizing the structure of the hydrophobic conjugate. In some of the preferred embodiment the structure of sterol is modified to alter (increase/ decrease) C17 attached chain. This type of modification results in significant increase in cellular uptake and improvement of tissue uptake prosperities *in vivo*.

dsRNA formulated according to the invention also includes rxRNAori. rxRNAori refers to a class of RNA molecules described in and incorporated by reference from PCT Publication No. WO2009/102427 (Application No. PCT/US2009/000852), filed on February 11, 2009, and entitled, "MODIFIED RNAI POLYNUCLEOTIDES AND USES THEREOF" and US Patent Publication No. US 2011-0039914 entitled "MODIFIED RNAI POLYNUCLEOTIDES AND USES THEREOF."

In some embodiments, an rxRNAori molecule comprises a double-stranded RNA (dsRNA) construct of 12-35 nucleotides in length, for inhibiting expression of a target gene, comprising: a sense strand having a 5'-end and a 3'-end, wherein the sense strand is highly modified with 2'-modified ribose sugars, and wherein 3-6 nucleotides in the central portion of the sense strand are not modified with 2'-modified ribose sugars and, an antisense strand having a 5'-end and a 3'-end, which hybridizes to the sense strand and to mRNA of the target gene, wherein the dsRNA inhibits expression of the target gene in a sequence-dependent manner.

rxRNAori can contain any of the modifications described herein. In some embodiments, at least 30% of the nucleotides in the rxRNAori are modified. For example, at least 30%, 31%, 32%, 33%, 34%, 35%, 36%, 37%, 38%, 39%, 40%, 41%, 42%, 43%, 44%, 45%, 46%, 47%, 48%, 49%, 50%, 51%, 52%, 53%, 54%, 55%, 56%, 57%, 58%, 59%, 60%, 61%, 62%, 63%, 64%, 65%, 66%, 67%, 68%, 69%, 70%, 71%, 72%, 73%, 74%, 75%, 76%, 77%, 78%, 79%, 80%, 81%, 82%, 83%, 84%, 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98% or 99% of the nucleotides in the rxRNAori are modified. In some embodiments, 100% of the nucleotides in the sd-rxRNA are modified. In some embodiments, only the passenger strand of the rxRNAori contains modifications.

This invention is not limited in its application to the details of construction and the arrangement of components set forth in the following description or illustrated in the drawings. The invention is capable of other embodiments and of being practiced or of being carried out in various ways. Also, the phraseology and terminology used herein is for the purpose of description and should not be regarded as limiting. The use of "including," "comprising," or "having," "containing," "involving," and variations thereof herein, is meant to encompass the items listed thereafter and equivalents thereof as well as additional items.

Aspects of the invention relate to isolated double stranded nucleic acid molecules comprising a guide (antisense) strand and a passenger (sense) strand. As used herein, the term "double-stranded" refers to one or more nucleic acid molecules in which at least a portion of the nucleomonomers are complementary and hydrogen bond to form a double-stranded region. In some embodiments, the length of the guide strand ranges from 16-29 nucleotides long. In certain embodiments, the guide strand is 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, or 29 nucleotides long. The guide strand has complementarity to a target gene. Complementarity between the guide strand and the target gene may exist over any portion of the guide strand. Complementarity as used herein may be perfect complementarity or less than perfect complementarity as long as the guide strand is sufficiently complementary to the target that it mediates RNAi. In some embodiments complementarity refers to less than 25%, 20%, 15%, 10%, 5%, 4%, 3%, 2%, or 1% mismatch between the guide strand and the target. Perfect complementarity refers to 100% complementarity. In some embodiments, siRNA sequences with insertions, deletions, and single point mutations relative to the target sequence have also been found to be effective for inhibition. Moreover, not all positions of a siRNA contribute equally to target recognition. Mismatches in the center of the siRNA are most critical and essentially abolish target RNA cleavage. Mismatches upstream of the center or upstream of the cleavage site referencing the antisense strand are tolerated but significantly reduce target RNA cleavage. Mismatches downstream of the center or cleavage site referencing the antisense strand, preferably located near the 3' end of the antisense strand, e.g. 1, 2, 3, 4, 5 or 6 nucleotides from the 3' end of the antisense strand, are tolerated and reduce target RNA cleavage only slightly.

While not wishing to be bound by any particular theory, in some embodiments, the guide strand is at least 16 nucleotides in length and anchors the Argonaute protein in RISC. In some embodiments, when the guide strand loads into RISC it has a defined seed region and target mRNA cleavage takes place across from position 10-11 of the guide strand. In some embodiments, the 5' end of the guide strand is or is able to be phosphorylated. The nucleic acid molecules described herein may be referred to as minimum trigger RNA.

In some embodiments, the length of the passenger strand ranges from 8-15 nucleotides long. In certain embodiments, the passenger strand is 8, 9, 10, 11, 12, 13, 14 or 15 nucleotides long. The passenger strand has complementarity to the guide strand.

Complementarity between the passenger strand and the guide strand can exist over any portion of the passenger or guide strand. In some embodiments, there is 100% complementarity between the guide and passenger strands within the double stranded region of the molecule.

Aspects of the invention relate to double stranded nucleic acid molecules with minimal double stranded regions. In some embodiments the region of the molecule that is double stranded ranges from 8-15 nucleotides long. In certain embodiments, the region of the molecule that is double stranded is 8, 9, 10, 11, 12, 13, 14 or 15 nucleotides long. In certain embodiments the double stranded region is 13 or 14 nucleotides long. There can be 100% complementarity between the guide and passenger strands, or there may be one or more mismatches between the guide and passenger strands. In some embodiments, on one end of the double stranded molecule, the molecule is either blunt-ended or has a one-nucleotide overhang. The single stranded region of the molecule is in some embodiments between 4-12 nucleotides long. For example the single stranded region can be 4, 5, 6, 7, 8, 9, 10, 11 or 12 nucleotides long. However, in certain embodiments, the single stranded region can also be less than 4 or greater than 12 nucleotides long. In certain embodiments, the single stranded region is at least 6 or at least 7 nucleotides long.

RNAi constructs associated with the invention can have a thermodynamic stability (ΔG) of less than -13 kkal/mol. In some embodiments, the thermodynamic stability (ΔG) is less than -20 kkal/mol. In some embodiments there is a loss of efficacy when (ΔG) goes below -21 kkal/mol. In some embodiments a (ΔG) value higher than -13 kkal/mol is compatible with aspects of the invention. Without wishing to be bound by any theory, in some embodiments a molecule with a relatively higher (ΔG) value may become active at a relatively higher concentration, while a molecule with a relatively lower (ΔG) value may become active at a relatively lower concentration. In some embodiments, the (ΔG) value may be higher than -9 kkcal/mol. The gene silencing effects mediated by the RNAi constructs associated with the invention, containing minimal double stranded regions, are unexpected because molecules of almost identical design but lower thermodynamic stability have been demonstrated to be inactive (Rana et al 2004).

Without wishing to be bound by any theory, a stretch of 8-10 bp of dsRNA or dsDNA may be structurally recognized by protein components of RISC or co-factors of RISC. Additionally, there is a free energy requirement for the triggering compound that it may be either sensed by the protein components and/or stable enough to interact with such components so that it may be loaded into the Argonaute protein. If optimal thermodynamics are present and there is a double stranded portion that is preferably at least 8 nucleotides then the duplex will be recognized and loaded into the RNAi machinery.

In some embodiments, thermodynamic stability is increased through the use of LNA bases. In some embodiments, additional chemical modifications are introduced . Several non-limiting examples of chemical modifications include: 5' Phosphate, 2'-O-methyl, 2'-O-ethyl, 2'-fluoro, ribothymidine, C-5 propynyl-dC (pdC) and C-5 propynyl-dU (pdU); C-5 propynyl-C (pC) and C-5 propynyl-U (pU); 5-methyl C, 5-methyl U, 5-methyl dC, 5-methyl dU methoxy, (2,6-diaminopurine), 5'-Dimethoxytrityl-N4-ethyl-2'-deoxyCytidine and MGB (minor groove binder). It should be appreciated that more than one chemical modification can be combined within the same molecule.

Molecules associated with the invention are optimized for increased potency and/or reduced toxicity. For example, nucleotide length of the guide and/or passenger strand, and/or the number of phosphorothioate modifications in the guide and/or passenger strand, can in some aspects influence potency of the RNA molecule, while replacing 2'-fluoro (2'F) modifications with 2'-O-methyl (2'OMe) modifications can in some aspects influence toxicity of the molecule. Specifically, reduction in 2'F content of a molecule is predicted to reduce toxicity of the molecule. Furthermore, the number of phosphorothioate modifications in an RNA molecule can influence the uptake of the molecule into a cell, for example the efficiency of passive uptake of the molecule into a cell. Preferred embodiments of molecules described herein have no 2'F modification and yet are characterized by equal efficacy in cellular uptake and tissue penetration. Such molecules represent a significant improvement over prior art, such as molecules described by Accell and Wolfrum, which are heavily modified with extensive use of 2'F.

In some embodiments, a guide strand is approximately 18-19 nucleotides in length and has approximately 2-14 phosphate modifications. For example, a guide strand can contain 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14 or more than 14 nucleotides that are phosphate-modified. The guide strand may contain one or more modifications that confer increased stability without interfering with RISC entry. The phosphate modified nucleotides, such as phosphorothioate modified nucleotides, can be at the 3' end, 5' end or spread throughout the guide strand. In some embodiments, the 3' terminal 10 nucleotides of the guide strand contains 1, 2, 3, 4, 5, 6, 7, 8, 9 or 10 phosphorothioate modified nucleotides. The guide strand can also contain 2'F and/or 2'OMe modifications, which can be located throughout the molecule. In some embodiments, the nucleotide in position one of the guide strand (the nucleotide in the most 5' position of the guide strand) is 2'OMe modified and/or phosphorylated. C and U nucleotides within the guide strand can be 2'F modified. For example, C and U nucleotides in positions 2-10 of a 19 nt guide strand (or corresponding positions in a guide strand of a different length) can be 2'F modified. C and U nucleotides within the guide strand can also be 2'OMe modified. For example, C and U nucleotides in positions 11-18 of a 19 nt guide strand (or corresponding positions in a guide strand of a different length) can be 2'OMe modified. In some embodiments, the nucleotide at the most 3' end of the guide strand is unmodified. In certain embodiments, the majority of Cs and Us within the guide strand are 2'F modified and the 5' end of the guide strand is phosphorylated. In other embodiments, position 1 and the Cs or Us in positions 11-18 are 2'OMe modified and the 5' end of the guide strand is phosphorylated. In other embodiments, position 1 and the Cs or Us in positions 11-18 are 2'OMe modified, the 5' end of the guide strand is phosphorylated, and the Cs or Us in position 2-10 are 2'F modified.

In some aspects, an optimal passenger strand is approximately 11-14 nucleotides in length. The passenger strand may contain modifications that confer increased stability. One or more nucleotides in the passenger strand can be 2'OMe modified. In some embodiments, one or more of the C and/or U nucleotides in the passenger strand is 2'OMe modified, or all of the C and U nucleotides in the passenger strand are 2'OMe modified. In certain embodiments, all of the nucleotides in the passenger strand are 2'OMe modified. One or more of the nucleotides on the passenger strand can also be phosphate-modified such as phosphorothioate modified. The passenger strand can also contain 2' ribo, 2'F and 2 deoxy modifications or any combination of the above. Chemical modification patterns on both the guide and passenger strand can be well tolerated and a combination of chemical modifications can lead to increased efficacy and self-delivery of RNA molecules.

Aspects of the invention relate to RNAi constructs that have extended single-stranded regions relative to double stranded regions, as compared to molecules that have been used previously for RNAi. The single stranded region of the molecules may be modified to promote cellular uptake or gene silencing. In some embodiments, phosphorothioate modification of the single stranded region influences cellular uptake and/or gene silencing. The region of the guide strand that is phosphorothioate modified can include nucleotides within both the single stranded and double stranded regions of the molecule. In some embodiments, the single stranded region includes 2-12 phosphorothioate modifications. For example, the single stranded region can include 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, or 12 phosphorothioate modifications. In some instances, the single stranded region contains 6-8 phosphorothioate modifications.

Molecules associated with the invention are also optimized for cellular uptake. In RNA molecules described herein, the guide and/or passenger strands can be attached to a conjugate. In certain embodiments the conjugate is hydrophobic. The hydrophobic conjugate can be a small molecule with a partition coefficient that is higher than 10. The conjugate can be a sterol-type molecule such as cholesterol, or a molecule with an increased length polycarbon chain attached to C17, and the presence of a conjugate can influence the ability of an RNA molecule to be taken into a cell with or without a lipid transfection reagent. The conjugate can be attached to the passenger or guide strand through a hydrophobic linker. In some embodiments, a hydrophobic linker is 5-12C in length, and/or is hydroxypyrrolidine-based. In some embodiments, a hydrophobic conjugate is attached to the passenger strand and the CU residues of either the passenger and/or guide strand are modified. In some embodiments, at least 50%, 55%, 60%, 65%, 70%, 75%, 80%, 85%, 90% or 95% of the CU residues on the passenger strand and/or the guide strand are modified. In some aspects, molecules associated with the invention are self-delivering (sd). As used herein, "self-delivery" refers to the ability of a molecule to be delivered into a cell without the need for an additional delivery vehicle such as a transfection reagent.

Aspects of the invention relate to selecting molecules for use in RNAi. In some embodiments, molecules that have a double stranded region of 8-15 nucleotides can be selected for use in RNAi. In some embodiments, molecules are selected based on their thermodynamic stability (ΔG). In some embodiments, molecules will be selected that have a (ΔG) of less than -13 kkal/mol. For example, the (ΔG) value may be -13, -14, -15, -16, -17, -18, -19, -21, -22 or less than -22 kkal/mol. In other embodiments, the (ΔG) value may be higher than -13 kkal/mol. For example, the (ΔG) value may be -12, -11, -10, -9, -8, -7 or more than -7 kkal/mol. It should be appreciated that ΔG can be calculated using any method known in the art. In some embodiments ΔG is calculated using Mfold, available through the Mfold internet site (mfold.bioinfo.rpi.edu/cgi-bin/rna-form1.cgi). Methods for calculating ΔG are described in, and are incorporated by reference from, the following references: Zuker, M. (2003) Nucleic Acids Res., 31(13):3406-15; Mathews, D. H., Sabina, J., Zuker, M. and Turner, D. H. (1999) J. Mol. Biol. 288:911-940; Mathews, D. H., Disney, M. D., Childs, J. L., Schroeder, S. J., Zuker, M., and Turner, D. H. (2004) Proc. Natl. Acad. Sci. 101:7287-

7292; Duan, S., Mathews, D. H., and Turner, D. H. (2006) Biochemistry 45:9819-9832; Wuchty, S., Fontana, W., Hofacker, I. L., and Schuster, P. (1999) Biopolymers 49:145-165.

In certain embodiments, the polynucleotide contains 5'- and/or 3'-end overhangs. The number and/or sequence of nucleotides overhang on one end of the polynucleotide may be the same or different from the other end of the polynucleotide. In certain embodiments, one or more of the overhang nucleotides may contain chemical modification(s), such as phosphorothioate or 2'-OMe modification.

In certain embodiments, the polynucleotide is unmodified. In other embodiments, at least one nucleotide is modified. In further embodiments, the modification includes a 2'-H or 2'-modified ribose sugar at the 2nd nucleotide from the 5'-end of the guide sequence. The "2nd nucleotide" is defined as the second nucleotide from the 5'-end of the polynucleotide.

As used herein, "2'-modified ribose sugar" includes those ribose sugars that do not have a 2'-OH group. "2'-modified ribose sugar" does not include 2'-deoxyribose (found in unmodified canonical DNA nucleotides). For example, the 2'-modified ribose sugar may be 2'-O-alkyl nucleotides, 2′-deoxy-2′-fluoro nucleotides, 2′-deoxy nucleotides, or combination thereof.

In certain embodiments, the 2'-modified nucleotides are pyrimidine nucleotides (*e.g.*, C /U). Examples of 2'-O-alkyl nucleotides include 2'-O-methyl nucleotides, or 2'-O-allyl nucleotides.

In certain embodiments, the sd-rxRNA polynucleotide of the invention with the above-referenced 5'-end modification exhibits significantly (*e.g.*, at least about 25%, 30%, 35%, 40%, 45%, 50%, 55%, 60%, 65%, 70%, 75%, 80%, 85%, 90% or more) less "off-target" gene silencing when compared to similar constructs without the specified 5'-end modification, thus greatly improving the overall specificity of the RNAi reagent or therapeutics.

As used herein, "off-target" gene silencing refers to unintended gene silencing due to, for example, spurious sequence homology between the antisense (guide) sequence and the unintended target mRNA sequence.

According to this aspect of the invention, certain guide strand modifications further increase nuclease stability, and/or lower interferon induction, without significantly decreasing RNAi activity (or no decrease in RNAi activity at all).

Certain combinations of modifications may result in further unexpected advantages, as partly manifested by enhanced ability to inhibit target gene expression, enhanced serum stability, and/or increased target specificity, *etc.*

In certain embodiments, the guide strand comprises a 2'-O-methyl modified nucleotide at the 2nd nucleotide on the 5'-end of the guide strand and no other modified nucleotides.

In other aspects, the sd-rxRNA structures of the present invention mediates sequence-dependent gene silencing by a microRNA mechanism. As used herein, the term "microRNA" ("miRNA"), also referred to in the art as "small temporal RNAs" ("stRNAs"), refers to a small (10-50 nucleotide) RNA which are genetically encoded (e.g., by viral, mammalian, or plant genomes) and are capable of directing or mediating RNA silencing. An "miRNA disorder" shall refer to a disease or disorder characterized by an aberrant expression or activity of an miRNA.

microRNAs are involved in down-regulating target genes in critical pathways, such as development and cancer, in mice, worms and mammals. Gene silencing through a microRNA mechanism is achieved by specific yet imperfect base-pairing of the miRNA and its target messenger RNA (mRNA). Various mechanisms may be used in microRNA-mediated down-regulation of target mRNA expression.

miRNAs are noncoding RNAs of approximately 22 nucleotides which can regulate gene expression at the post transcriptional or translational level during plant and animal development. One common feature of miRNAs is that they are all excised from an approximately 70 nucleotide precursor RNA stem-loop termed pre-miRNA, probably by Dicer, an RNase III-type enzyme, or a homolog thereof. Naturally-occurring miRNAs are expressed by endogenous genes *in vivo* and are processed from a hairpin or stem-loop precursor (pre-miRNA or pri-miRNAs) by Dicer or other RNAses. miRNAs can exist transiently *in vivo* as a double-stranded duplex but only one strand is taken up by the RISC complex to direct gene silencing.

In some embodiments a version of sd-rxRNA compounds, which are effective in cellular uptake and inhibiting of miRNA activity are described. Essentially the compounds are similar to RISC entering version but large strand chemical modification patterns are optimized in the way to block cleavage and act as an effective inhibitor of the RISC action. For example, the compound might be completely or mostly O-methyl modified with the phosphorothioate content described previously. For these types of compounds the 5' phosphorylation is not necessary in some embodiments. The presence of double stranded region is preferred as it is promotes cellular uptake and efficient RISC loading.

Another pathway that uses small RNAs as sequence-specific regulators is the RNA interference (RNAi) pathway, which is an evolutionarily conserved response to the presence of double-stranded RNA (dsRNA) in the cell. The dsRNAs are cleaved into ~20-base pair (bp) duplexes of small-interfering RNAs (siRNAs) by Dicer. These small RNAs get assembled into multiprotein effector complexes called RNA-induced silencing complexes (RISCs). The siRNAs then guide the cleavage of target mRNAs with perfect complementarity.

Some aspects of biogenesis, protein complexes, and function are shared between the siRNA pathway and the miRNA pathway. Single-stranded polynucleotides may mimic the dsRNA in the siRNA mechanism, or the microRNA in the miRNA mechanism.

In certain embodiments, the modified RNAi constructs may have improved stability in serum and/or cerebral spinal fluid compared to an unmodified RNAi constructs having the same sequence.

In certain embodiments, the structure of the RNAi construct does not induce interferon response in primary cells, such as mammalian primary cells, including primary cells from human, mouse and other rodents, and other non-human mammals. In certain embodiments, the RNAi construct may also be used to inhibit expression of a target gene in an invertebrate organism.

To further increase the stability of the subject constructs *in vivo*, the 3'-end of the structure may be blocked by protective group(s). For example, protective groups such as inverted nucleotides, inverted abasic moieties, or amino-end modified nucleotides may be used. Inverted nucleotides may comprise an inverted deoxynucleotide. Inverted abasic moieties may comprise an inverted deoxyabasic moiety, such as a 3',3'-linked or 5',5'-linked deoxyabasic moiety.

The RNAi constructs of the invention are capable of inhibiting the synthesis of any target protein encoded by target gene(s). The invention includes methods to inhibit expression of a target gene either in a cell *in vitro*, or *in vivo*. As such, the RNAi constructs of the invention are useful for treating a patient with a disease characterized by the overexpression of a target gene.

The target gene can be endogenous or exogenous (*e.g.*, introduced into a cell by a virus or using recombinant DNA technology) to a cell. Such methods may include introduction of RNA into a cell in an amount sufficient to inhibit expression of the target gene. By way of example, such an RNA molecule may have a guide strand that is complementary to the nucleotide sequence of the target gene, such that the composition inhibits expression of the target gene.

The invention also relates to vectors expressing the nucleic acids of the invention, and cells comprising such vectors or the nucleic acids. The cell may be a mammalian cell *in vivo* or in culture, such as a human cell.

The invention further relates to compositions comprising the subject RNAi constructs, and a pharmaceutically acceptable carrier or diluent.

The method may be carried out *in vitro, ex vivo,* or *in vivo*, in, for example, mammalian cells in culture, such as a human cell in culture.

The target cells (*e.g.*, mammalian cell) may be contacted in the presence of a delivery reagent, such as a lipid (*e.g.*, a cationic lipid) or a liposome.

Another aspect of the invention provides a method for inhibiting the expression of a target gene in a mammalian cell, comprising contacting the mammalian cell with a vector expressing the subject RNAi constructs.

In one aspect of the invention, a longer duplex polynucleotide is provided, including a first polynucleotide that ranges in size from about 16 to about 30 nucleotides; a second polynucleotide that ranges in size from about 26 to about 46 nucleotides, wherein the first polynucleotide (the antisense strand) is complementary to both the second polynucleotide (the sense strand) and a target gene, and wherein both polynucleotides form a duplex and wherein the first polynucleotide contains a single stranded region longer than 6 bases in length and is modified with alternative chemical modification pattern, and/or includes a conjugate moiety that facilitates cellular delivery. In this embodiment, between about 40% to about 90% of the nucleotides of the passenger strand between about 40% to about 90% of the nucleotides of the guide strand, and between about 40% to about 90% of the nucleotides of the single stranded region of the first polynucleotide are chemically modified nucleotides.

In an embodiment, the chemically modified nucleotide in the polynucleotide duplex may be any chemically modified nucleotide known in the art, such as those discussed in detail above. In a particular embodiment, the chemically modified nucleotide is selected from the group consisting of 2' F modified nucleotides, 2'-O-methyl modified and 2'deoxy nucleotides. In another particular embodiment, the chemically modified nucleotides results from "hydrophobic modifications" of the nucleotide base. In another particular embodiment, the chemically modified nucleotides are phosphorothioates. In an additional particular embodiment, chemically modified nucleotides are combination of phosphorothioates, 2'-O-methyl, 2'deoxy, hydrophobic modifications and phosphorothioates. As these groups of modifications refer to modification of the ribose ring, back bone and nucleotide, it is feasible that some modified nucleotides will carry a combination of all three modification types.

In another embodiment, the chemical modification is not the same across the various regions of the duplex. In a particular embodiment, the first polynucleotide (the passenger strand), has a large number of diverse chemical modifications in various positions. For this polynucleotide up to 90% of nucleotides might be chemically modified and/or have mismatches introduced.

In another embodiment, chemical modifications of the first or second polynucleotide include, but not limited to, 5' position modification of Uridine and Cytosine (4-pyridyl, 2-pyridyl, indolyl, phenyl (C_6H_5OH); tryptophanyl (C8H6N)CH2CH(NH2)CO), isobutyl, butyl, aminobenzyl; phenyl; naphthyl, etc.), where the chemical modification might alter base pairing capabilities of a nucleotide. For the guide strand an important feature of this aspect of the invention is the position of the chemical modification relative to the 5' end of the antisense and sequence. For example, chemical phosphorylation of the 5' end of the guide strand is usually beneficial for efficacy. O-methyl modifications in the seed region of the sense strand (position 2-7 relative to the 5' end) are not generally well tolerated, whereas 2'F and deoxy are well tolerated. The mid part of the guide strand and the 3' end of the guide strand are more permissive in a type of chemical modifications applied. Deoxy modifications are not tolerated at the 3' end of the guide strand.

A unique feature of this aspect of the invention involves the use of hydrophobic modification on the bases. In one embodiment, the hydrophobic modifications are preferably positioned near the 5' end of the guide strand, in other embodiments, they localized in the middle of the guides strand, in other embodiment they localized at the 3' end of the guide strand and yet in another embodiment they are distributed thought the whole length of the polynucleotide. The same type of patterns is applicable to the passenger strand of the duplex.

The other part of the molecule is a single stranded region. In some embodiments, the single stranded region is expected to range from 7 to 40 nucleotides.

In one embodiment, the single stranded region of the first polynucleotide contains modifications selected from the group consisting of between 40% and 90% hydrophobic base modifications, between 40%-90% phosphorothioates, between 40% -90% modification of the ribose moiety, and any combination of the preceding.

Efficiency of guide strand (first polynucleotide) loading into the RISC complex might be altered for heavily modified polynucleotides, so in one embodiment, the duplex polynucleotide includes a mismatch between nucleotide 9, 11, 12, 13, or 14 on the guide strand (first polynucleotide) and the opposite nucleotide on the sense strand (second polynucleotide) to promote efficient guide strand loading.

More detailed aspects of the invention are described in the sections below.

Duplex Characteristics

Double-stranded oligonucleotides of the invention may be formed by two separate complementary nucleic acid strands. Duplex formation can occur either inside or outside the cell containing the target gene.

As used herein, the term "duplex" includes the region of the double-stranded nucleic acid molecule(s) that is (are) hydrogen bonded to a complementary sequence. Double-stranded oligonucleotides of the invention may comprise a nucleotide sequence that is sense to a target gene and a complementary sequence that is antisense to the target gene. The sense and antisense nucleotide sequences correspond to the target gene sequence, *e.g.*, are identical or are sufficiently identical to effect target gene inhibition (*e.g.*, are about at least about 98% identical, 96% identical, 94%, 90% identical, 85% identical, or 80% identical) to the target gene sequence.

In certain embodiments, the double-stranded oligonucleotide of the invention is double-stranded over its entire length, *i.e.*, with no overhanging single-stranded sequence at either end of the molecule, *i.e.*, is blunt-ended. In other embodiments, the individual nucleic acid molecules can be of different lengths. In other words, a double-stranded oligonucleotide of the invention is not double-stranded over its entire length. For instance, when two separate nucleic acid molecules are used, one of the molecules, *e.g.*, the first molecule comprising an antisense sequence, can be longer than the second molecule hybridizing thereto (leaving a portion of the molecule single-stranded). Likewise, when a single nucleic acid molecule is used a portion of the molecule at either end can remain single-stranded.

In one embodiment, a double-stranded oligonucleotide of the invention contains mismatches and/or loops or bulges, but is double-stranded over at least about 70% of the length of the oligonucleotide. In another embodiment, a double-stranded oligonucleotide of the invention is double-stranded over at least about 80% of the length of the oligonucleotide. In another embodiment, a double-stranded oligonucleotide of the invention is double-stranded over at least about 90%-95% of the length of the oligonucleotide. In another embodiment, a double-stranded oligonucleotide of the invention is double-stranded over at least about 96%-98% of the length of the oligonucleotide. In certain embodiments, the double-stranded oligonucleotide of the invention contains at least or up to 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, or 15 mismatches.

Modifications

The nucleotides of the invention may be modified at various locations, including the sugar moiety, the phosphodiester linkage, and/or the base.

In some embodiments, the base moiety of a nucleoside may be modified. For example, a pyrimidine base may be modified at the 2, 3, 4, 5, and/or 6 position of the pyrimidine ring. In some embodiments, the exocyclic amine of cytosine may be modified. A purine base may also be modified. For example, a purine base may be modified at the 1, 2, 3, 6, 7, or 8 position. In some embodiments, the exocyclic amine of adenine may be modified. In some cases, a nitrogen atom in a ring of a base moiety may be substituted with another atom, such as carbon. A modification to a base moiety may be any suitable modification. Examples of modifications are known to those of ordinary skill in the art. In some embodiments, the base modifications include alkylated purines or pyrimidines, acylated purines or pyrimidines, or other heterocycles.

In some embodiments, a pyrimidine may be modified at the 5 position. For example, the 5 position of a pyrimidine may be modified with an alkyl group, an alkynyl group, an alkenyl group, an acyl group, or substituted derivatives thereof. In other examples, the 5 position of a pyrimidine may be modified with a hydroxyl group or an alkoxyl group or substituted derivative thereof. Also, the N^4 position of a pyrimidine may be alkylated. In still further examples, the pyrimidine 5-6 bond may be saturated, a nitrogen atom within the pyrimidine ring may be substituted with a carbon atom, and/or the O^2 and O^4 atoms may be substituted with sulfur atoms. It should be understood that other modifications are possible as well.

In other examples, the N^7 position and/or N^2 and/or N^3 position of a purine may be modified with an alkyl group or substituted derivative thereof. In further examples, a third ring may be fused to the purine bicyclic ring system and/or a nitrogen atom within the purine ring system may be substituted with a carbon atom. It should be understood that other modifications are possible as well.

Non-limiting examples of pyrimidines modified at the 5 position are disclosed in U.S. Patent 5,591,843, U.S. Patent 7,205,297, U.S. Patent 6,432,963, and U.S. Patent 6,020,483;

non-limiting examples of pyrimidines modified at the N^4 position are disclosed in U.S Patent 5,580,731; non-limiting examples of purines modified at the 8 position are disclosed in U.S. Patent 6,355,787 and U.S. Patent 5,580,972; non-limiting examples of purines modified at the N^6 position are disclosed in U.S. Patent 4,853,386, U.S. Patent 5,789,416, and U.S. Patent 7,041,824; and non-limiting examples of purines modified at the 2 position are disclosed in U.S. Patent 4,201,860 and U.S. Patent 5,587,469, all of which are incorporated herein by reference.

Non-limiting examples of modified bases include N^4,N^4-ethanocytosine, 7-deazaxanthosine, 7-deazaguanosine, 8-oxo-N^6-methyladenine, 4-acetylcytosine, 5-(carboxyhydroxylmethyl) uracil, 5-fluorouracil, 5-bromouracil, 5-carboxymethylaminomethyl-2-thiouracil, 5-carboxymethylaminomethyl uracil, dihydrouracil, inosine, N^6-isopentenyl-adenine, 1-methyladenine, 1-methylpseudouracil, 1-methylguanine, 1-methylinosine, 2,2-dimethylguanine, 2-methyladenine, 2-methylguanine, 3-methylcytosine, 5-methylcytosine, N^6 -methyladenine, 7-methylguanine, 5-methylaminomethyl uracil, 5-methoxy aminomethyl-2-thiouracil, 5-methoxyuracil, 2-methylthio-N^6-isopentenyladenine, pseudouracil, 5-methyl-2-thiouracil, 2-thiouracil, 4-thiouracil, 5-methyluracil, 2-thiocytosine, and 2,6-diaminopurine. In some embodiments, the base moiety may be a heterocyclic base other than a purine or pyrimidine. The heterocyclic base may be optionally modified and/or substituted.

Sugar moieties include natural, unmodified sugars, *e.g.*, monosaccharide (such as pentose, *e.g.*, ribose, deoxyribose), modified sugars and sugar analogs. In general, possible modifications of nucleomonomers, particularly of a sugar moiety, include, for example, replacement of one or more of the hydroxyl groups with a halogen, a heteroatom, an aliphatic group, or the functionalization of the hydroxyl group as an ether, an amine, a thiol, or the like.

One particularly useful group of modified nucleomonomers are 2'-O-methyl nucleotides. Such 2'-O-methyl nucleotides may be referred to as "methylated," and the corresponding nucleotides may be made from unmethylated nucleotides followed by alkylation or directly from methylated nucleotide reagents. Modified nucleomonomers may be used in combination with unmodified nucleomonomers. For example, an oligonucleotide of the invention may contain both methylated and unmethylated nucleomonomers.

Some exemplary modified nucleomonomers include sugar- or backbone-modified ribonucleotides. Modified ribonucleotides may contain a non-naturally occurring base (instead of a naturally occurring base), such as uridines or cytidines modified at the 5'-

position, *e.g.*, 5'-(2-amino)propyl uridine and 5'-bromo uridine; adenosines and guanosines modified at the 8-position, *e.g.*, 8-bromo guanosine; deaza nucleotides, *e.g.*, 7-deaza-adenosine; and N-alkylated nucleotides, *e.g.*, N6-methyl adenosine. Also, sugar-modified ribonucleotides may have the 2'-OH group replaced by a H, alxoxy (or OR), R or alkyl, halogen, SH, SR, amino (such as NH_2, NHR, NR_2), or CN group, wherein R is lower alkyl, alkenyl, or alkynyl.

Modified ribonucleotides may also have the phosphodiester group connecting to adjacent ribonucleotides replaced by a modified group, *e.g.*, of phosphorothioate group. More generally, the various nucleotide modifications may be combined.

Although the antisense (guide) strand may be substantially identical to at least a portion of the target gene (or genes), at least with respect to the base pairing properties, the sequence need not be perfectly identical to be useful, *e.g.*, to inhibit expression of a target gene's phenotype. Generally, higher homology can be used to compensate for the use of a shorter antisense gene. In some cases, the antisense strand generally will be substantially identical (although in antisense orientation) to the target gene.

The use of 2′-O-methyl modified RNA may also be beneficial in circumstances in which it is desirable to minimize cellular stress responses. RNA having 2′-O-methyl nucleomonomers may not be recognized by cellular machinery that is thought to recognize unmodified RNA. The use of 2′-O-methylated or partially 2′-O-methylated RNA may avoid the interferon response to double-stranded nucleic acids, while maintaining target RNA inhibition. This may be useful, for example, for avoiding the interferon or other cellular stress responses, both in short RNAi (*e.g.*, siRNA) sequences that induce the interferon response, and in longer RNAi sequences that may induce the interferon response.

Overall, modified sugars may include D-ribose, 2′-O-alkyl (including 2′-O-methyl and 2′-O-ethyl), *i.e.*, 2′-alkoxy, 2′-amino, 2′-S-alkyl, 2′-halo (including 2′-fluoro), 2′-methoxyethoxy, 2′-allyloxy ($-OCH_2CH=CH_2$), 2′-propargyl, 2′-propyl, ethynyl, ethenyl, propenyl, and cyano and the like. In one embodiment, the sugar moiety can be a hexose and incorporated into an oligonucleotide as described (Augustyns, K., *et al.*, *Nucl. Acids. Res.* 18:4711 (1992)). Exemplary nucleomonomers can be found, *e.g.*, in U.S. Pat. No. 5,849,902, incorporated by reference herein.

Definitions of specific functional groups and chemical terms are described in more detail below. For purposes of this invention, the chemical elements are identified in accordance with the Periodic Table of the Elements, CAS version, *Handbook of Chemistry and Physics, 75th Ed.*, inside cover, and specific functional groups are generally defined as described therein. Additionally, general principles of organic chemistry, as well as specific functional moieties and reactivity, are described in *Organic Chemistry*, Thomas Sorrell, University Science Books, Sausalito: 1999, the entire contents of which are incorporated herein by reference.

Certain compounds of the present invention may exist in particular geometric or stereoisomeric forms. The present invention contemplates all such compounds, including *cis*- and *trans*-isomers, *R*- and *S*-enantiomers, diastereomers, (D)-isomers, (L)-isomers, the racemic mixtures thereof, and other mixtures thereof, as falling within the scope of the invention. Additional asymmetric carbon atoms may be present in a substituent such as an alkyl group. All such isomers, as well as mixtures thereof, are intended to be included in this invention.

Isomeric mixtures containing any of a variety of isomer ratios may be utilized in accordance with the present invention. For example, where only two isomers are combined, mixtures containing 50:50, 60:40, 70:30, 80:20, 90:10, 95:5, 96:4, 97:3, 98:2, 99:1, or 100:0 isomer ratios are all contemplated by the present invention. Those of ordinary skill in the art will readily appreciate that analogous ratios are contemplated for more complex isomer mixtures.

If, for instance, a particular enantiomer of a compound of the present invention is desired, it may be prepared by asymmetric synthesis, or by derivation with a chiral auxiliary, where the resulting diastereomeric mixture is separated and the auxiliary group cleaved to provide the pure desired enantiomers. Alternatively, where the molecule contains a basic functional group, such as amino, or an acidic functional group, such as carboxyl, diastereomeric salts are formed with an appropriate optically-active acid or base, followed by resolution of the diastereomers thus formed by fractional crystallization or chromatographic means well known in the art, and subsequent recovery of the pure enantiomers.

In certain embodiments, oligonucleotides of the invention comprise 3′ and 5′ termini (except for circular oligonucleotides). In one embodiment, the 3′ and 5′ termini of an oligonucleotide can be substantially protected from nucleases *e.g.*, by modifying the 3′ or 5′ linkages (*e.g.*, U.S. Pat. No. 5,849,902 and WO 98/13526). For example, oligonucleotides can be made resistant by the inclusion of a "blocking group." The term "blocking group" as used herein refers to substituents (*e.g.*, other than OH groups) that can be attached to oligonucleotides or nucleomonomers, either as protecting groups or coupling groups for synthesis (*e.g.*, FITC, propyl (CH_2-CH_2-CH_3), glycol (-O-CH_2-CH_2-O-) phosphate (PO_3^{2-}), hydrogen phosphonate, or phosphoramidite). "Blocking groups" also include "end blocking groups" or "exonuclease blocking groups" which protect the 5′ and 3′ termini of the oligonucleotide, including modified nucleotides and non-nucleotide exonuclease resistant structures.

Exemplary end-blocking groups include cap structures (*e.g.*, a 7-methylguanosine cap), inverted nucleomonomers, *e.g.*, with 3′-3′ or 5′-5′ end inversions (see, *e.g.*, Ortiagao *et al.* 1992. *Antisense Res. Dev.* 2:129), methylphosphonate, phosphoramidite, non-nucleotide groups (*e.g.*, non-nucleotide linkers, amino linkers, conjugates) and the like. The 3′ terminal nucleomonomer can comprise a modified sugar moiety. The 3′ terminal nucleomonomer comprises a 3′-O that can optionally be substituted by a blocking group that prevents 3′-exonuclease degradation of the oligonucleotide. For example, the 3′-hydroxyl can be esterified to a nucleotide through a 3′→3′ internucleotide linkage. For example, the alkyloxy radical can be methoxy, ethoxy, or isopropoxy, and preferably, ethoxy. Optionally, the 3′→3′linked nucleotide at the 3′ terminus can be linked by a substitute linkage. To reduce nuclease degradation, the 5′ most 3′→5′ linkage can be a modified linkage, *e.g.*, a phosphorothioate or a P-alkyloxyphosphotriester linkage. Preferably, the two 5′ most 3′→5′ linkages are modified linkages. Optionally, the 5′ terminal hydroxy moiety can be esterified with a phosphorus containing moiety, *e.g.*, phosphate, phosphorothioate, or P-ethoxyphosphate.

One of ordinary skill in the art will appreciate that the synthetic methods, as described herein, utilize a variety of protecting groups. By the term "protecting group," as used herein, it is meant that a particular functional moiety, *e.g.*, O, S, or N, is temporarily blocked so that a reaction can be carried out selectively at another reactive site in a multifunctional compound. In certain embodiments, a protecting group reacts selectively in good yield to give a protected substrate that is stable to the projected reactions; the protecting group should be selectively removable in good yield by readily available, preferably non-toxic reagents that do not attack the other functional groups; the protecting group forms an easily separable derivative (more preferably without the generation of new stereogenic centers); and the protecting group has a minimum of additional functionality to avoid further sites of reaction. As detailed herein, oxygen, sulfur, nitrogen, and carbon protecting groups may be utilized. Hydroxyl protecting groups include methyl, methoxylmethyl (MOM), methylthiomethyl (MTM), *t*-butylthiomethyl, (phenyldimethylsilyl)methoxymethyl (SMOM), benzyloxymethyl (BOM),

p-methoxybenzyloxymethyl (PMBM), (4-methoxyphenoxy)methyl (*p*-AOM), guaiacolmethyl (GUM), *t*-butoxymethyl, 4-pentenyloxymethyl (POM), siloxymethyl, 2-

methoxyethoxymethyl (MEM), 2,2,2-trichloroethoxymethyl, bis(2-chloroethoxy)methyl, 2-(trimethylsilyl)ethoxymethyl (SEMOR), tetrahydropyranyl (THP), 3-bromotetrahydropyranyl, tetrahydrothiopyranyl, 1-methoxycyclohexyl, 4-methoxytetrahydropyranyl (MTHP), 4-methoxytetrahydrothiopyranyl, 4-methoxytetrahydrothiopyranyl S,S-dioxide, 1-[(2-chloro-4-methyl)phenyl]-4-methoxypiperidin-4-yl (CTMP), 1,4-dioxan-2-yl, tetrahydrofuranyl, tetrahydrothiofuranyl, 2,3,3a,4,5,6,7,7a-octahydro-7,8,8-trimethyl-4,7-methanobenzofuran-2-yl, 1-ethoxyethyl, 1-(2-chloroethoxy)ethyl, 1-methyl-1-methoxyethyl, 1-methyl-1-benzyloxyethyl, 1-methyl-1-benzyloxy-2-fluoroethyl, 2,2,2-trichloroethyl, 2-trimethylsilylethyl, 2-(phenylselenyl)ethyl, *t*-butyl, allyl, *p*-chlorophenyl, *p*-methoxyphenyl, 2,4-dinitrophenyl, benzyl, *p*-methoxybenzyl, 3,4-dimethoxybenzyl, *o*-nitrobenzyl, *p*-nitrobenzyl, *p*-halobenzyl, 2,6-dichlorobenzyl, *p*-cyanobenzyl, *p*-phenylbenzyl, 2-picolyl, 4-picolyl, 3-methyl-2-picolyl *N*-oxido, diphenylmethyl, *p,p*′-dinitrobenzhydryl, 5-dibenzosuberyl, triphenylmethyl, α-naphthyldiphenylmethyl, *p*-methoxyphenyldiphenylmethyl, di(*p*-methoxyphenyl)phenylmethyl, tri(*p*-methoxyphenyl)methyl, 4-(4′-bromophenacyloxyphenyl)diphenylmethyl, 4,4′,4″-tris(4,5-dichlorophthalimidophenyl)methyl, 4,4′,4″-tris(levulinoyloxyphenyl)methyl, 4,4′,4″-tris(benzoyloxyphenyl)methyl, 3-(imidazol-1-yl)bis(4′,4″-dimethoxyphenyl)methyl, 1,1-bis(4-methoxyphenyl)-1′-pyrenylmethyl, 9-anthryl, 9-(9-phenyl)xanthenyl, 9-(9-phenyl-10-oxo)anthryl, 1,3-benzodithiolan-2-yl, benzisothiazolyl S,S-dioxido, trimethylsilyl (TMS), triethylsilyl (TES), triisopropylsilyl (TIPS), dimethylisopropylsilyl (IPDMS), diethylisopropylsilyl (DEIPS), dimethylthexylsilyl, *t*-butyldimethylsilyl (TBDMS), *t*-butyldiphenylsilyl (TBDPS), tribenzylsilyl, tri-*p*-xylylsilyl, triphenylsilyl, diphenylmethylsilyl (DPMS), *t*-butylmethoxyphenylsilyl (TBMPS), formate, benzoylformate, acetate, chloroacetate, dichloroacetate, trichloroacetate, trifluoroacetate, methoxyacetate, triphenylmethoxyacetate, phenoxyacetate, *p*-chlorophenoxyacetate, 3-phenylpropionate, 4-oxopentanoate (levulinate), 4,4-(ethylenedithio)pentanoate (levulinoyldithioacetal), pivaloate, adamantoate, crotonate, 4-methoxycrotonate, benzoate, *p*-phenylbenzoate, 2,4,6-trimethylbenzoate (mesitoate), alkyl methyl carbonate, 9-fluorenylmethyl carbonate (Fmoc), alkyl ethyl carbonate, alkyl 2,2,2-trichloroethyl carbonate (Troc), 2-(trimethylsilyl)ethyl carbonate (TMSEC), 2-(phenylsulfonyl) ethyl carbonate (Psec), 2-(triphenylphosphonio) ethyl carbonate (Peoc), alkyl isobutyl carbonate, alkyl vinyl carbonate alkyl allyl carbonate, alkyl *p*-nitrophenyl carbonate, alkyl benzyl carbonate, alkyl *p*-methoxybenzyl carbonate, alkyl 3,4-dimethoxybenzyl carbonate, alkyl *o*-nitrobenzyl carbonate, alkyl *p*-nitrobenzyl carbonate, alkyl *S*-benzyl thiocarbonate, 4-ethoxy-1-napththyl carbonate, methyl dithiocarbonate, 2-iodobenzoate, 4-azidobutyrate, 4-nitro-4-methylpentanoate, *o*-(dibromomethyl)benzoate, 2-formylbenzenesulfonate, 2-(methylthiomethoxy)ethyl, 4-(methylthiomethoxy)butyrate, 2-(methylthiomethoxymethyl)benzoate, 2,6-dichloro-4-methylphenoxyacetate, 2,6-dichloro-4-(1,1,3,3-tetramethylbutyl)phenoxyacetate, 2,4-bis(1,1-dimethylpropyl)phenoxyacetate, chlorodiphenylacetate, isobutyrate, monosuccinoate, (*E*)-2-methyl-2-butenoate, *o*-(methoxycarbonyl)benzoate, α-naphthoate, nitrate, alkyl *N,N,N',N'*-tetramethylphosphorodiamidate, alkyl *N*-phenylcarbamate, borate, dimethylphosphinothioyl, alkyl 2,4-dinitrophenylsulfenate, sulfate, methanesulfonate (mesylate), benzylsulfonate, and tosylate (Ts). For protecting 1,2- or 1,3-diols, the protecting groups include methylene acetal, ethylidene acetal, 1-*t*-butylethylidene ketal, 1-phenylethylidene ketal, (4-methoxyphenyl)ethylidene acetal, 2,2,2-trichloroethylidene acetal, acetonide, cyclopentylidene ketal, cyclohexylidene ketal, cycloheptylidene ketal, benzylidene acetal, *p*-methoxybenzylidene acetal, 2,4-dimethoxybenzylidene ketal, 3,4-dimethoxybenzylidene acetal, 2-nitrobenzylidene acetal, methoxymethylene acetal, ethoxymethylene acetal, dimethoxymethylene ortho ester, 1-methoxyethylidene ortho ester, 1-ethoxyethylidine ortho ester, 1,2-dimethoxyethylidene ortho ester, α-methoxybenzylidene ortho ester, 1-(*N,N*-dimethylamino)ethylidene derivative, α-(*N,N'*-dimethylamino)benzylidene derivative, 2-oxacyclopentylidene ortho ester, di-*t*-butylsilylene group (DTBS), 1,3-(1,1,3,3-tetraisopropyldisiloxanylidene) derivative (TIPDS), tetra-*t*-butoxydisiloxane-1,3-diylidene derivative (TBDS), cyclic carbonates, cyclic boronates, ethyl boronate, and phenyl boronate. Amino-protecting groups include methyl carbamate, ethyl carbamante, 9-fluorenylmethyl carbamate (Fmoc), 9-(2-sulfo)fluorenylmethyl carbamate, 9-(2,7-dibromo)fluoroenylmethyl carbamate, 2,7-di-*t*-butyl-[9-(10,10-dioxo-10,10,10,10-tetrahydrothioxanthyl)]methyl carbamate (DBD-Tmoc), 4-methoxyphenacyl carbamate (Phenoc), 2,2,2-trichloroethyl carbamate (Troc), 2-trimethylsilylethyl carbamate (Teoc), 2-phenylethyl carbamate (hZ), 1-(1-adamantyl)-1-methylethyl carbamate (Adpoc), 1,1-dimethyl-2-haloethyl carbamate, 1,1-dimethyl-2,2-dibromoethyl carbamate (DB-*t*-BOC), 1,1-dimethyl-2,2,2-trichloroethyl carbamate (TCBOC), 1-methyl-1-(4-biphenylyl)ethyl carbamate (Bpoc), 1-(3,5-di-*t*-butylphenyl)-1-methylethyl carbamate (*t*-Bumeoc), 2-(2′- and 4′-pyridyl)ethyl carbamate (Pyoc), 2-(*N,N*-dicyclohexylcarboxamido)ethyl carbamate, *t*-butyl carbamate (BOC), 1-adamantyl carbamate (Adoc), vinyl carbamate (Voc), allyl carbamate (Alloc), 1-isopropylallyl carbamate (Ipaoc), cinnamyl carbamate (Coc), 4-nitrocinnamyl carbamate (Noc), 8-quinolyl carbamate, *N*-hydroxypiperidinyl carbamate, alkyldithio carbamate, benzyl carbamate (Cbz), *p*-methoxybenzyl carbamate (Moz), *p*-nitobenzyl carbamate, *p*-bromobenzyl carbamate, *p*-chlorobenzyl carbamate, 2,4-dichlorobenzyl carbamate, 4-methylsulfinylbenzyl carbamate (Msz), 9-anthrylmethyl carbamate, diphenylmethyl carbamate, 2-methylthioethyl carbamate, 2-methylsulfonylethyl carbamate, 2-(*p*-toluenesulfonyl)ethyl carbamate, [2-(1,3-dithianyl)]methyl carbamate (Dmoc), 4-methylthiophenyl carbamate (Mtpc), 2,4-dimethylthiophenyl carbamate (Bmpc), 2-phosphonioethyl carbamate (Peoc), 2-triphenylphosphonioisopropyl carbamate (Ppoc), 1,1-dimethyl-2-cyanoethyl carbamate, *m*-chloro-*p*-acyloxybenzyl carbamate, *p*-(dihydroxyboryl)benzyl carbamate, 5-benzisoxazolylmethyl carbamate, 2-(trifluoromethyl)-6-chromonylmethyl carbamate (Tcroc), *m*-nitrophenyl carbamate, 3,5-dimethoxybenzyl carbamate, *o*-nitrobenzyl carbamate, 3,4-dimethoxy-6-nitrobenzyl carbamate, phenyl(*o*-nitrophenyl)methyl carbamate, phenothiazinyl-(10)-carbonyl derivative, *N'*-*p*-toluenesulfonylaminocarbonyl derivative, *N'*-phenylaminothiocarbonyl derivative, *t*-amyl carbamate, *S*-benzyl thiocarbamate, *p*-cyanobenzyl carbamate, cyclobutyl carbamate, cyclohexyl carbamate, cyclopentyl carbamate, cyclopropylmethyl carbamate, *p*-decyloxybenzyl carbamate, 2,2-dimethoxycarbonylvinyl carbamate, *o*-(*N,N*-dimethylcarboxamido)benzyl carbamate, 1,1-dimethyl-3-(*N,N*-dimethylcarboxamido)propyl carbamate, 1,1-dimethylpropynyl carbamate, di(2-pyridyl)methyl carbamate, 2-furanylmethyl carbamate, 2-iodoethyl carbamate, isoborynl carbamate, isobutyl carbamate, isonicotinyl carbamate, *p*-(*p'*-methoxyphenylazo)benzyl carbamate, 1-methylcyclobutyl carbamate, 1-methylcyclohexyl carbamate, 1-methyl-1-cyclopropylmethyl carbamate, 1-methyl-1-(3,5-dimethoxyphenyl)ethyl carbamate, 1-methyl-1-(*p*-phenylazophenyl)ethyl carbamate, 1-methyl-1-phenylethyl carbamate, 1-methyl-1-(4-pyridyl)ethyl carbamate, phenyl carbamate, *p*-(phenylazo)benzyl carbamate, 2,4,6-tri-*t*-butylphenyl carbamate, 4-(trimethylammonium)benzyl carbamate, 2,4,6-trimethylbenzyl carbamate, formamide, acetamide, chloroacetamide, trichloroacetamide, trifluoroacetamide, phenylacetamide, 3-phenylpropanamide, picolinamide, 3-pyridylcarboxamide, *N*-benzoylphenylalanyl derivative, benzamide, *p*-phenylbenzamide, *o*-nitophenylacetamide, *o*-nitrophenoxyacetamide, acetoacetamide, (*N'*-dithiobenzyloxycarbonylamino)acetamide, 3-(*p*-hydroxyphenyl)propanamide, 3-(*o*-nitrophenyl)propanamide, 2-methyl-2-(*o*-nitrophenoxy)propanamide, 2-methyl-2-(*o*-phenylazophenoxy)propanamide, 4-chlorobutanamide, 3-methyl-3-nitrobutanamide, *o*-nitrocinnamide, *N*-acetylmethionine derivative, *o*-nitrobenzamide, *o*-(benzoyloxymethyl)benzamide, 4,5-diphenyl-3-oxazolin-2-one, *N*-phthalimide, *N*-dithiasuccinimide (Dts), *N*-2,3-diphenylmaleimide, *N*-2,5-

dimethylpyrrole, *N*-1,1,4,4-tetramethyldisilylazacyclopentane adduct (STABASE), 5-substituted 1,3-dimethyl-1,3,5-triazacyclohexan-2-one, 5-substituted 1,3-dibenzyl-1,3,5-triazacyclohexan-2-one, 1-substituted 3,5-dinitro-4-pyridone, *N*-methylamine, *N*-allylamine, *N*-[2-(trimethylsilyl)ethoxy]methylamine (SEM), *N*-3-acetoxypropylamine, *N*-(1-isopropyl-4-nitro-2-oxo-3-pyroolin-3-yl)amine, quaternary ammonium salts, *N*-benzylamine, *N*-di(4-methoxyphenyl)methylamine, *N*-5-dibenzosuberylamine, *N*-triphenylmethylamine (Tr), *N*-[(4-methoxyphenyl)diphenylmethyl]amine (MMTr), *N*-9-phenylfluorenylamine (PhF), *N*-2,7-dichloro-9-fluorenylmethyleneamine, *N*-ferrocenylmethylamino (Fcm), *N*-2-picolylamino *N'*-oxide, *N*-1,1-dimethylthiomethyleneamine, *N*-benzylideneamine, *N*-*p*-methoxybenzylideneamine, *N*-diphenylmethyleneamine, *N*-[(2-pyridyl)mesityl]methyleneamine, *N*-(*N'*,*N'*-dimethylaminomethylene)amine, *N,N'*-isopropylidenediamine, *N*-*p*-nitrobenzylideneamine, *N*-salicylideneamine, *N*-5-chlorosalicylideneamine, *N*-(5-chloro-2-hydroxyphenyl)phenylmethyleneamine, *N*-cyclohexylideneamine, *N*-(5,5-dimethyl-3-oxo-1-cyclohexenyl)amine, *N*-borane derivative, *N*-diphenylborinic acid derivative, *N*-[phenyl(pentacarbonylchromium- or tungsten)carbonyl]amine, *N*-copper chelate, *N*-zinc chelate, *N*-nitroamine, *N*-nitrosoamine, amine *N*-oxide, diphenylphosphinamide (Dpp), dimethylthiophosphinamide (Mpt), diphenylthiophosphinamide (Ppt), dialkyl phosphoramidates, dibenzyl phosphoramidate, diphenyl phosphoramidate, benzenesulfenamide, *o*-nitrobenzenesulfenamide (Nps), 2,4-dinitrobenzenesulfenamide, pentachlorobenzenesulfenamide, 2-nitro-4-methoxybenzenesulfenamide, triphenylmethylsulfenamide, 3-nitropyridinesulfenamide (Npys), *p*-toluenesulfonamide (Ts), benzenesulfonamide, 2,3,6,-trimethyl-4-methoxybenzenesulfonamide (Mtr), 2,4,6-trimethoxybenzenesulfonamide (Mtb), 2,6-dimethyl-4-methoxybenzenesulfonamide (Pme), 2,3,5,6-tetramethyl-4-methoxybenzenesulfonamide (Mte), 4-methoxybenzenesulfonamide (Mbs), 2,4,6-trimethylbenzenesulfonamide (Mts), 2,6-dimethoxy-4-methylbenzenesulfonamide (iMds), 2,2,5,7,8-pentamethylchroman-6-sulfonamide (Pmc), methanesulfonamide (Ms), β-trimethylsilylethanesulfonamide (SES), 9-anthracenesulfonamide, 4-(4′,8′-dimethoxynaphthylmethyl)benzenesulfonamide (DNMBS), benzylsulfonamide, trifluoromethylsulfonamide, and phenacylsulfonamide. Exemplary protecting groups are detailed herein. However, it will be appreciated that the present invention is not intended to be limited to these protecting groups; rather, a variety of additional equivalent protecting groups can be readily identified using the above criteria and utilized in the method of the present invention. Additionally, a variety of protecting groups are described in *Protective Groups in Organic Synthesis,* Third Ed. Greene, T.W. and Wuts, P.G., Eds., John Wiley & Sons, New York: 1999, the entire contents of which are hereby incorporated by reference.

It will be appreciated that the compounds, as described herein, may be substituted with any number of substituents or functional moieties. In general, the term "substituted" whether preceded by the term "optionally" or not, and substituents contained in formulas of this invention, refer to the replacement of hydrogen radicals in a given structure with the radical of a specified substituent. When more than one position in any given structure may be substituted with more than one substituent selected from a specified group, the substituent may be either the same or different at every position. As used herein, the term "substituted" is contemplated to include all permissible substituents of organic compounds. In a broad aspect, the permissible substituents include acyclic and cyclic, branched and unbranched, carbocyclic and heterocyclic, aromatic and nonaromatic substituents of organic compounds. Heteroatoms such as nitrogen may have hydrogen substituents and/or any permissible substituents of organic compounds described herein which satisfy the valencies of the heteroatoms. Furthermore, this invention is not intended to be limited in any manner by the permissible substituents of organic compounds. Combinations of substituents and variables envisioned by this invention are preferably those that result in the formation of stable compounds useful in the treatment, for example, of infectious diseases or proliferative disorders. The term "stable", as used herein, preferably refers to compounds which possess stability sufficient to allow manufacture and which maintain the integrity of the compound for a sufficient period of time to be detected and preferably for a sufficient period of time to be useful for the purposes detailed herein.

The term "aliphatic," as used herein, includes both saturated and unsaturated, straight chain (*i.e.*, unbranched), branched, acyclic, cyclic, or polycyclic aliphatic hydrocarbons, which are optionally substituted with one or more functional groups. As will be appreciated by one of ordinary skill in the art, "aliphatic" is intended herein to include, but is not limited to, alkyl, alkenyl, alkynyl, cycloalkyl, cycloalkenyl, and cycloalkynyl moieties. Thus, as used herein, the term "alkyl" includes straight, branched and cyclic alkyl groups. An analogous convention applies to other generic terms such as "alkenyl," "alkynyl," and the like. Furthermore, as used herein, the terms "alkyl," "alkenyl," "alkynyl," and the like encompass both substituted and unsubstituted groups. In certain embodiments, as used herein, "lower alkyl" is used to indicate those alkyl groups (cyclic, acyclic, substituted, unsubstituted, branched, or unbranched) having 1-6 carbon atoms.

In certain embodiments, the alkyl, alkenyl, and alkynyl groups employed in the invention contain 1-20 aliphatic carbon atoms. In certain other embodiments, the alkyl, alkenyl, and alkynyl groups employed in the invention contain 1-10 aliphatic carbon atoms. In yet other embodiments, the alkyl, alkenyl, and alkynyl groups employed in the invention contain 1-8 aliphatic carbon atoms. In still other embodiments, the alkyl, alkenyl, and alkynyl groups employed in the invention contain 1-6 aliphatic carbon atoms. In yet other embodiments, the alkyl, alkenyl, and alkynyl groups employed in the invention contain 1-4 carbon atoms. Illustrative aliphatic groups thus include, but are not limited to, for example, methyl, ethyl, *n*-propyl, isopropyl, cyclopropyl, -CH_2-cyclopropyl, vinyl, allyl, *n*-butyl, *sec*-butyl, isobutyl, *tert*-butyl, cyclobutyl, -CH_2-cyclobutyl, *n*-pentyl, sec-pentyl, isopentyl, *tert*-pentyl, cyclopentyl, -CH_2-cyclopentyl, *n*-hexyl, *sec*-hexyl, cyclohexyl, -CH_2-cyclohexyl moieties and the like, which again, may bear one or more substituents. Alkenyl groups include, but are not limited to, for example, ethenyl, propenyl, butenyl, 1-methyl-2-buten-1-yl, and the like. Representative alkynyl groups include, but are not limited to, ethynyl, 2-propynyl (propargyl), 1-propynyl, and the like.

Some examples of substituents of the above-described aliphatic (and other) moieties of compounds of the invention include, but are not limited to aliphatic; heteroaliphatic; aryl; heteroaryl; arylalkyl; heteroarylalkyl; alkoxy; aryloxy; heteroalkoxy; heteroaryloxy; alkylthio; arylthio; heteroalkylthio; heteroarylthio; -F; -Cl; -Br; -I; -OH; -NO_2; -CN; -CF_3; -CH_2CF_3; -$CHCl_2$; -CH_2OH; -CH_2CH_2OH; -CH_2NH_2; -$CH_2SO_2CH_3$; -C(O)R_x; -$CO_2(R_x)$; -CON$(R_x)_2$; -OC(O)R_x; -OCO_2R_x; -OCON$(R_x)_2$; -N$(R_x)_2$; -S$(O)_2R_x$; -NR_x(CO)R_x wherein each occurrence of R_x independently includes, but is not limited to, aliphatic, heteroaliphatic, aryl, heteroaryl, arylalkyl, or heteroarylalkyl, wherein any of the aliphatic, heteroaliphatic, arylalkyl, or heteroarylalkyl substituents described above and herein may be substituted or unsubstituted, branched or unbranched, cyclic or acyclic, and wherein any of the aryl or heteroaryl substituents described above and herein may be substituted or unsubstituted. Additional examples of generally applicable substituents are illustrated by the specific embodiments described herein.

The term "heteroaliphatic," as used herein, refers to aliphatic moieties that contain one or more oxygen, sulfur, nitrogen, phosphorus, or silicon atoms, *e.g.*, in place of carbon atoms. Heteroaliphatic moieties may be branched, unbranched, cyclic or acyclic and include saturated and unsaturated heterocycles such as morpholino, pyrrolidinyl, *etc.* In certain embodiments, heteroaliphatic moieties are substituted by independent replacement of one or more of the hydrogen atoms thereon with one or more moieties including, but not limited to aliphatic; heteroaliphatic; aryl; heteroaryl; arylalkyl; heteroarylalkyl; alkoxy; aryloxy; heteroalkoxy; heteroaryloxy; alkylthio; arylthio; heteroalkylthio; heteroarylthio; -F; -Cl; -Br; -I; -OH; $-NO_2$; -CN; $-CF_3$; $-CH_2CF_3$; $-CHCl_2$; $-CH_2OH$; $-CH_2CH_2OH$; $-CH_2NH_2$; $-CH_2SO_2CH_3$; $-C(O)R_x$; $-CO_2(R_x)$; $-CON(R_x)_2$; $-OC(O)R_x$; $-OCO_2R_x$; $-OCON(R_x)_2$; $-N(R_x)_2$; $-S(O)_2R_x$; $-NR_x(CO)R_x$, wherein each occurrence of R_x independently includes, but is not limited to, aliphatic, heteroaliphatic, aryl, heteroaryl, arylalkyl, or heteroarylalkyl, wherein any of the aliphatic, heteroaliphatic, arylalkyl, or heteroarylalkyl substituents described above and herein may be substituted or unsubstituted, branched or unbranched, cyclic or acyclic, and wherein any of the aryl or heteroaryl substituents described above and herein may be substituted or unsubstituted. Additional examples of generally applicable substitutents are illustrated by the specific embodiments described herein.

The terms “halo” and “halogen” as used herein refer to an atom selected from fluorine, chlorine, bromine, and iodine.

The term “alkyl” includes saturated aliphatic groups, including straight-chain alkyl groups (*e.g.*, methyl, ethyl, propyl, butyl, pentyl, hexyl, heptyl, octyl, nonyl, decyl, *etc.*), branched-chain alkyl groups (isopropyl, tert-butyl, isobutyl, *etc.*), cycloalkyl (alicyclic) groups (cyclopropyl, cyclopentyl, cyclohexyl, cycloheptyl, cyclooctyl), alkyl substituted cycloalkyl groups, and cycloalkyl substituted alkyl groups. In certain embodiments, a straight chain or branched chain alkyl has 6 or fewer carbon atoms in its backbone (*e.g.*, C_1-C_6 for straight chain, C_3-C_6 for branched chain), and more preferably 4 or fewer. Likewise, preferred cycloalkyls have from 3-8 carbon atoms in their ring structure, and more preferably have 5 or 6 carbons in the ring structure. The term C_1-C_6 includes alkyl groups containing 1 to 6 carbon atoms.

Moreover, unless otherwise specified, the term alkyl includes both “unsubstituted alkyls” and “substituted alkyls,” the latter of which refers to alkyl moieties having independently selected substituents replacing a hydrogen on one or more carbons of the hydrocarbon backbone. Such substituents can include, for example, alkenyl, alkynyl, halogen, hydroxyl, alkylcarbonyloxy, arylcarbonyloxy, alkoxycarbonyloxy, aryloxycarbonyloxy, carboxylate, alkylcarbonyl, arylcarbonyl, alkoxycarbonyl, aminocarbonyl, alkylaminocarbonyl, dialkylaminocarbonyl, alkylthiocarbonyl, alkoxyl, phosphate, phosphonato, phosphinato, cyano, amino (including alkyl amino, dialkylamino, arylamino, diarylamino, and alkylarylamino), acylamino (including alkylcarbonylamino, arylcarbonylamino, carbamoyl and ureido), amidino, imino, sulfhydryl, alkylthio, arylthio, thiocarboxylate, sulfates, alkylsulfinyl, sulfonato, sulfamoyl, sulfonamido, nitro, trifluoromethyl, cyano, azido, heterocyclyl, alkylaryl, or an aromatic or heteroaromatic moiety. Cycloalkyls can be further substituted, *e.g.*, with the substituents described above. An "alkylaryl" or an "arylalkyl" moiety is an alkyl substituted with an aryl (*e.g.*, phenylmethyl (benzyl)). The term "alkyl" also includes the side chains of natural and unnatural amino acids. The term "n-alkyl" means a straight chain (*i.e.*, unbranched) unsubstituted alkyl group.

The term "alkenyl" includes unsaturated aliphatic groups analogous in length and possible substitution to the alkyls described above, but that contain at least one double bond. For example, the term "alkenyl" includes straight-chain alkenyl groups (*e.g.*, ethylenyl, propenyl, butenyl, pentenyl, hexenyl, heptenyl, octenyl, nonenyl, decenyl, *etc.*), branched-chain alkenyl groups, cycloalkenyl (alicyclic) groups (cyclopropenyl, cyclopentenyl, cyclohexenyl, cycloheptenyl, cyclooctenyl), alkyl or alkenyl substituted cycloalkenyl groups, and cycloalkyl or cycloalkenyl substituted alkenyl groups. In certain embodiments, a straight chain or branched chain alkenyl group has 6 or fewer carbon atoms in its backbone (*e.g.*, C_2-C_6 for straight chain, C_3-C_6 for branched chain). Likewise, cycloalkenyl groups may have from 3-8 carbon atoms in their ring structure, and more preferably have 5 or 6 carbons in the ring structure. The term C_2-C_6 includes alkenyl groups containing 2 to 6 carbon atoms.

Moreover, unless otherwise specified, the term alkenyl includes both "unsubstituted alkenyls" and "substituted alkenyls," the latter of which refers to alkenyl moieties having independently selected substituents replacing a hydrogen on one or more carbons of the hydrocarbon backbone. Such substituents can include, for example, alkyl groups, alkynyl groups, halogens, hydroxyl, alkylcarbonyloxy, arylcarbonyloxy, alkoxycarbonyloxy, aryloxycarbonyloxy, carboxylate, alkylcarbonyl, arylcarbonyl, alkoxycarbonyl, aminocarbonyl, alkylaminocarbonyl, dialkylaminocarbonyl, alkylthiocarbonyl, alkoxyl, phosphate, phosphonato, phosphinato, cyano, amino (including alkyl amino, dialkylamino, arylamino, diarylamino, and alkylarylamino), acylamino (including alkylcarbonylamino, arylcarbonylamino, carbamoyl and ureido), amidino, imino, sulfhydryl, alkylthio, arylthio, thiocarboxylate, sulfates, alkylsulfinyl, sulfonato, sulfamoyl, sulfonamido, nitro, trifluoromethyl, cyano, azido, heterocyclyl, alkylaryl, or an aromatic or heteroaromatic moiety.

The term "alkynyl" includes unsaturated aliphatic groups analogous in length and possible substitution to the alkyls described above, but which contain at least one triple bond. For example, the term "alkynyl" includes straight-chain alkynyl groups (*e.g.*, ethynyl, propynyl, butynyl, pentynyl, hexynyl, heptynyl, octynyl, nonynyl, decynyl, *etc.*), branched-chain alkynyl groups, and cycloalkyl or cycloalkenyl substituted alkynyl groups. In certain embodiments, a straight chain or branched chain alkynyl group has 6 or fewer carbon atoms in its backbone (*e.g.*, C_2-C_6 for straight chain, C_3-C_6 for branched chain). The term C_2-C_6 includes alkynyl groups containing 2 to 6 carbon atoms.

Moreover, unless otherwise specified, the term alkynyl includes both "unsubstituted alkynyls" and "substituted alkynyls," the latter of which refers to alkynyl moieties having independently selected substituents replacing a hydrogen on one or more carbons of the hydrocarbon backbone. Such substituents can include, for example, alkyl groups, alkynyl groups, halogens, hydroxyl, alkylcarbonyloxy, arylcarbonyloxy, alkoxycarbonyloxy, aryloxycarbonyloxy, carboxylate, alkylcarbonyl, arylcarbonyl, alkoxycarbonyl, aminocarbonyl, alkylaminocarbonyl, dialkylaminocarbonyl, alkylthiocarbonyl, alkoxyl, phosphate, phosphonato, phosphinato, cyano, amino (including alkyl amino, dialkylamino, arylamino, diarylamino, and alkylarylamino), acylamino (including alkylcarbonylamino, arylcarbonylamino, carbamoyl and ureido), amidino, imino, sulfhydryl, alkylthio, arylthio, thiocarboxylate, sulfates, alkylsulfinyl, sulfonato, sulfamoyl, sulfonamido, nitro, trifluoromethyl, cyano, azido, heterocyclyl, alkylaryl, or an aromatic or heteroaromatic moiety.

Unless the number of carbons is otherwise specified, "lower alkyl" as used herein means an alkyl group, as defined above, but having from one to five carbon atoms in its backbone structure. "Lower alkenyl" and "lower alkynyl" have chain lengths of, for example, 2-5 carbon atoms.

The term "alkoxy" includes substituted and unsubstituted alkyl, alkenyl, and alkynyl groups covalently linked to an oxygen atom. Examples of alkoxy groups include methoxy, ethoxy, isopropyloxy, propoxy, butoxy, and pentoxy groups. Examples of substituted alkoxy groups include halogenated alkoxy groups. The alkoxy groups can be substituted with independently selected groups such as alkenyl, alkynyl, halogen, hydroxyl, alkylcarbonyloxy, arylcarbonyloxy, alkoxycarbonyloxy, aryloxycarbonyloxy, carboxylate, alkylcarbonyl, arylcarbonyl, alkoxycarbonyl, aminocarbonyl, alkylaminocarbonyl, dialkylaminocarbonyl, alkylthiocarbonyl, alkoxyl, phosphate, phosphonato, phosphinato, cyano, amino (including alkyl amino, dialkylamino, arylamino, diarylamino, and alkylarylamino), acylamino (including alkylcarbonylamino, arylcarbonylamino, carbamoyl and ureido), amidino, imino, sulffiydryl, alkylthio, arylthio, thiocarboxylate, sulfates, alkylsulfmyl, sulfonato, sulfamoyl, sulfonamido, nitro, trifluoromethyl, cyano, azido, heterocyclyl, alkylaryl, or an aromatic or heteroaromatic moieties. Examples of halogen substituted alkoxy groups include, but are not limited to, fluoromethoxy, difluoromethoxy, trifluoromethoxy, chloromethoxy, dichloromethoxy, trichloromethoxy, *etc*.

The term "heteroatom" includes atoms of any element other than carbon or hydrogen. Preferred heteroatoms are nitrogen, oxygen, sulfur and phosphorus.

The term "hydroxy" or "hydroxyl" includes groups with an -OH or $-O^-$ (with an appropriate counterion).

The term "halogen" includes fluorine, bromine, chlorine, iodine, *etc*. The term "perhalogenated" generally refers to a moiety wherein all hydrogens are replaced by halogen atoms.

The term "substituted" includes independently selected substituents which can be placed on the moiety and which allow the molecule to perform its intended function. Examples of substituents include alkyl, alkenyl, alkynyl, aryl, $(CR'R'')_{0-3}NR'R''$, $(CR'R'')_{0-3}CN$, NO_2, halogen, $(CR'R'')_{0-3}C(halogen)_3$, $(CR'R'')_{0-3}CH(halogen)_2$, $(CR'R'')_{0-3}CH_2(halogen)$, $(CR'R'')_{0-3}CONR'R''$, $(CR'R'')_{0-3}S(O)_{1-2}NR'R''$, $(CR'R'')_{0-3}CHO$, $(CR'R'')_{0-3}O(CR'R'')_{0-3}H$, $(CR'R'')_{0-3}S(O)_{0-2}R'$, $(CR'R'')_{0-3}O(CR'R'')_{0-3}H$, $(CR'R'')_{0-3}COR'$, $(CR'R'')_{0-3}CO_2R'$, or $(CR'R'')_{0-3}OR'$ groups; wherein each R′ and R″ are each independently hydrogen, a C_1-C_5 alkyl, C_2-C_5 alkenyl, C_2-C_5 alkynyl, or aryl group, or R′ and R″ taken together are a benzylidene group or a —$(CH_2)_2O(CH_2)_2$- group.

The term "amine" or "amino" includes compounds or moieties in which a nitrogen atom is covalently bonded to at least one carbon or heteroatom. The term "alkyl amino" includes groups and compounds wherein the nitrogen is bound to at least one additional alkyl group. The term "dialkyl amino" includes groups wherein the nitrogen atom is bound to at least two additional alkyl groups.

The term "ether" includes compounds or moieties which contain an oxygen bonded to two different carbon atoms or heteroatoms. For example, the term includes "alkoxyalkyl," which refers to an alkyl, alkenyl, or alkynyl group covalently bonded to an oxygen atom which is covalently bonded to another alkyl group.

The terms "polynucleotide," "nucleotide sequence," "nucleic acid," "nucleic acid molecule," "nucleic acid sequence," and "oligonucleotide" refer to a polymer of two or more nucleotides. The polynucleotides can be DNA, RNA, or derivatives or modified versions thereof. The polynucleotide may be single-stranded or double-stranded. The polynucleotide can be modified at the base moiety, sugar moiety, or phosphate backbone, for example, to improve stability of the molecule, its hybridization parameters, *etc*. The polynucleotide may comprise a modified base moiety which is selected from the group including but not limited to 5-fluorouracil, 5-bromouracil, 5-chlorouracil, 5-iodouracil, hypoxanthine, xanthine, 4-acetylcytosine, 5-(carboxyhydroxylmethyl) uracil, 5-carboxymethylaminomethyl-2-thiouridine, 5- carboxymethylaminomethyluracil, dihydrouracil, beta-D-galactosylqueosine, inosine, N6-isopentenyladenine, 1-methylguanine, 1-methylinosine, 2,2- dimethylguanine, 2-methyladenine, 2-methylguanine, 3-methylcytosine, 5- methylcytosine, N6-adenine, 7-methylguanine, 5-methylaminomethyluracil, 5- methoxyaminomethyl-2-thiouracil, beta-D-mannosylqueosine, 5'- methoxycarboxymethyluracil, 5-methoxyuracil, 2-methylthio-N6-isopentenyladenine, wybutoxosine, pseudouracil, queosine, 2-thiocytosine, 5-methyl-2-thiouracil, 2-thiouracil, 4-thiouracil, 5-methyluracil, uracil- 5-oxyacetic acid methylester, uracil-5-oxyacetic acid, 5-methyl-2- thiouracil, 3-(3-amino-3-N-2-carboxypropyl) uracil, and 2,6-diaminopurine. The olynucleotide may compirse a modified sugar moiety (*e.g.*, 2′-fluororibose, ribose, 2′-deoxyribose, 2′-O-methylcytidine, arabinose, and hexose), and/or a modified phosphate moiety (*e.g.*, phosphorothioates and 5′ -N-phosphoramidite linkages). A nucleotide sequence typically carries genetic information, including the information used by cellular machinery to make proteins and enzymes. These terms include double- or single-stranded genomic and cDNA, RNA, any synthetic and genetically manipulated polynucleotide, and both sense and antisense polynucleotides. This includes single- and double-stranded molecules, *i.e.*, DNA-DNA, DNA-RNA, and RNA-RNA hybrids, as well as "protein nucleic acids" (PNA) formed by conjugating bases to an amino acid backbone.

The term "base" includes the known purine and pyrimidine heterocyclic bases, deazapurines, and analogs (including heterocyclic substituted analogs, *e.g.*, aminoethyoxy phenoxazine), derivatives (*e.g.*, 1-alkyl-, 1-alkenyl-, heteroaromatic- and 1-alkynyl derivatives) and tautomers thereof. Examples of purines include adenine, guanine, inosine, diaminopurine, and xanthine and analogs (*e.g.*, 8-oxo-N^6-methyladenine or 7-diazaxanthine) and derivatives thereof. Pyrimidines include, for example, thymine, uracil, and cytosine, and their analogs (*e.g.*, 5-methylcytosine, 5-methyluracil, 5-(1-propynyl)uracil, 5-(1-propynyl)cytosine and 4,4-ethanocytosine). Other examples of suitable bases include non-purinyl and non-pyrimidinyl bases such as 2-aminopyridine and triazines.

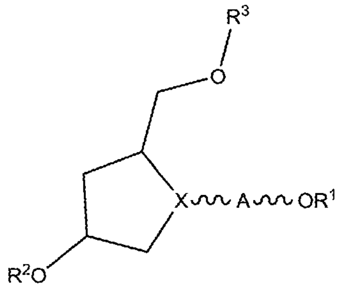
In a preferred embodiment, the nucleomonomers of an oligonucleotide of the invention are RNA nucleotides. In another preferred embodiment, the nucleomonomers of an oligonucleotide of the invention are modified RNA nucleotides. Thus, the oligonucleotides contain modified RNA nucleotides.

The term "nucleoside" includes bases which are covalently attached to a sugar moiety, preferably ribose or deoxyribose. Examples of preferred nucleosides include ribonucleosides and deoxyribonucleosides. Nucleosides also include bases linked to amino acids or amino acid analogs which may comprise free carboxyl groups, free amino groups, or protecting groups. Suitable protecting groups are well known in the art (see P. G. M. Wuts and T. W. Greene, "Protective Groups in Organic Synthesis", 2nd Ed., Wiley-Interscience, New York, 1999).

The term "nucleotide" includes nucleosides which further comprise a phosphate group or a phosphate analog.

The nucleic acid molecules may be associated with a hydrophobic moiety for targeting and/or delivery of the molecule to a cell. In certain embodiments, the hydrophobic moiety is associated with the nucleic acid molecule through a linker. In certain embodiments, the association is through non-covalent interactions. In other embodiments, the association is through a covalent bond. Any linker known in the art may be used to associate the nucleic acid with the hydrophobic moiety. Linkers known in the art are described in published international PCT applications, WO 92/03464, WO 95/23162, WO 2008/021157, WO 2009/021157, WO 2009/134487, WO 2009/126933, U.S. Patent Application Publication 2005/0107325, U.S. Patent 5,414,077, U.S. Patent 5,419,966, U.S. Patent 5,512,667, U.S. Patent 5,646,126, and U.S. Patent 5,652,359, which are incorporated herein by reference. The linker may be as simple as a covalent bond to a multi-atom linker. The linker may be cyclic or acyclic. The linker may be optionally substituted. In certain embodiments, the linker is capable of being cleaved from the nucleic acid. In certain embodiments, the linker is capable of being hydrolyzed under physiological conditions. In certain embodiments, the linker is capable of being cleaved by an enzyme (*e.g.*, an esterase or phosphodiesterase). In certain embodiments, the linker comprises a spacer element to separate the nucleic acid from the hydrophobic moiety. The spacer element may include one to thirty carbon or heteroatoms. In certain embodiments, the linker and/or spacer element comprises protonatable functional groups. Such protonatable functional groups may promote the endosomal escape of the nucleic acid molecule. The protonatable functional groups may also aid in the delivery of the nucleic acid to a cell, for example, neutralizing the overall charge of the molecule. In other embodiments, the linker and/or spacer element is biologically inert (that is, it does not impart biological activity or function to the resulting nucleic acid molecule).

In certain embodiments, the nucleic acid molecule with a linker and hydrophobic moiety is of the formulae described herein. In certain embodiments, the nucleic acid molecule is of the formula:



wherein

X is N or CH;

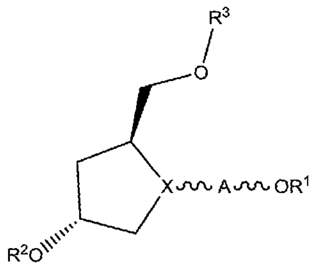
A is a bond; substituted or unsubstituted, cyclic or acyclic, branched or unbranched aliphatic; or substituted or unsubstituted, cyclic or acyclic, branched or unbranched heteroaliphatic;

R^1 is a hydrophobic moiety;

R^2 is hydrogen; an oxygen-protecting group; cyclic or acyclic, substituted or unsubstituted, branched or unbranched aliphatic; cyclic or acyclic, substituted or unsubstituted, branched or unbranched heteroaliphatic; substituted or unsubstituted, branched or unbranched acyl; substituted or unsubstituted, branched or unbranched aryl; substituted or unsubstituted, branched or unbranched heteroaryl; and

R^3 is a nucleic acid.

In certain embodiments, the molecule is of the formula:



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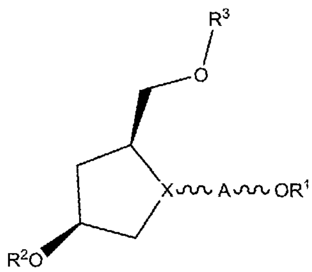
In certain embodiments, the molecule is of the formula:



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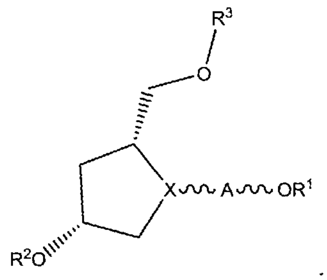
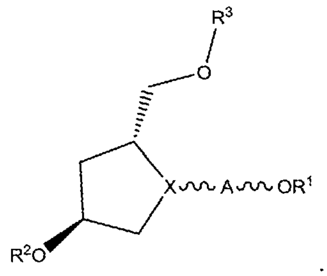
In certain embodiments, the molecule is of the formula:



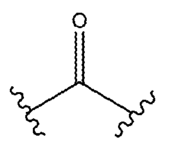
In certain embodiments, the molecule is of the formula:



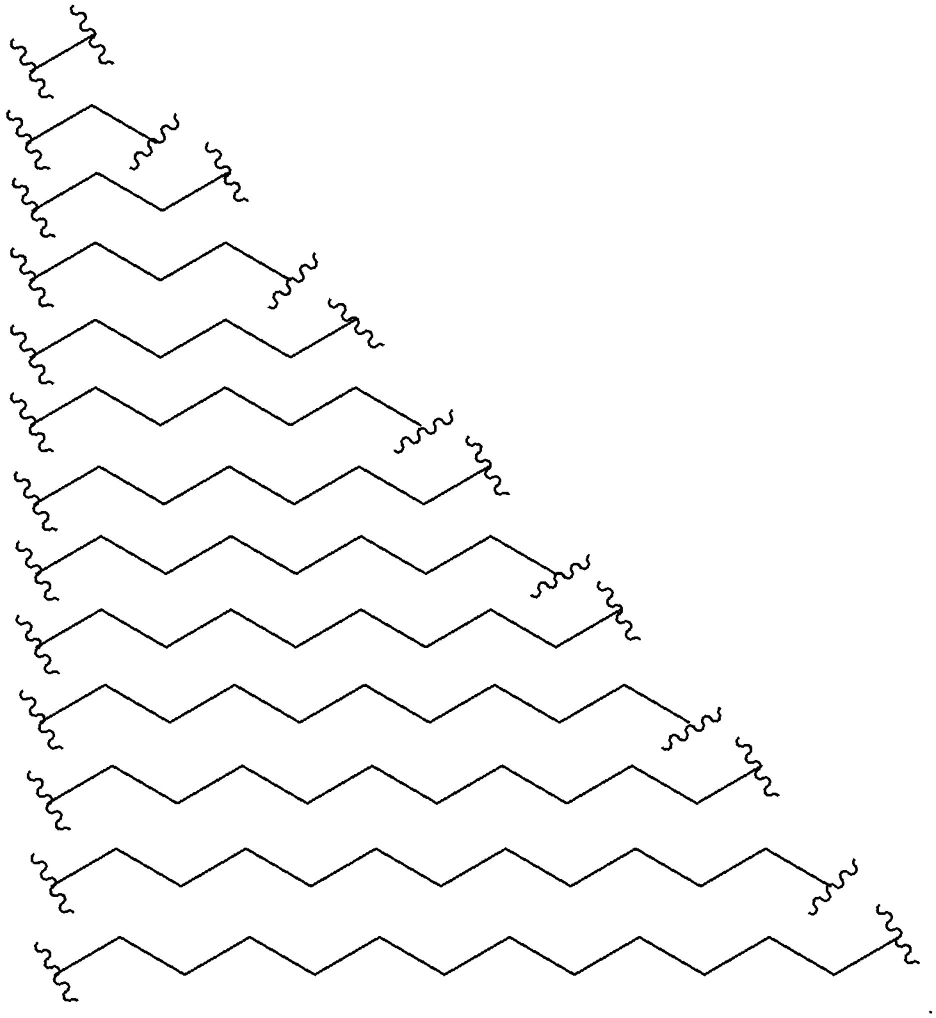
In certain embodiments, X is N. In certain embodiments, X is CH.

In certain embodiments, A is a bond. In certain embodiments, A is substituted or unsubstituted, cyclic or acyclic, branched or unbranched aliphatic. In certain embodiments, A is acyclic, substituted or unsubstituted, branched or unbranched aliphatic. In certain embodiments, A is acyclic, substituted, branched or unbranched aliphatic. In certain embodiments, A is acyclic, substituted, unbranched aliphatic. In certain embodiments, A is acyclic, substituted, unbranched alkyl. In certain embodiments, A is acyclic, substituted, unbranched C_{1-20} alkyl. In certain embodiments, A is acyclic, substituted, unbranched C_{1-12} alkyl. In certain embodiments, A is acyclic, substituted, unbranched C_{1-10} alkyl. In certain embodiments, A is acyclic, substituted, unbranched C_{1-8} alkyl. In certain embodiments, A is acyclic, substituted, unbranched C_{1-6} alkyl. In certain embodiments, A is substituted or unsubstituted, cyclic or acyclic, branched or unbranched heteroaliphatic. In certain embodiments, A is acyclic, substituted or unsubstituted, branched or unbranched heteroaliphatic. In certain embodiments, A is acyclic, substituted, branched or unbranched heteroaliphatic. In certain embodiments, A is acyclic, substituted, unbranched heteroaliphatic.

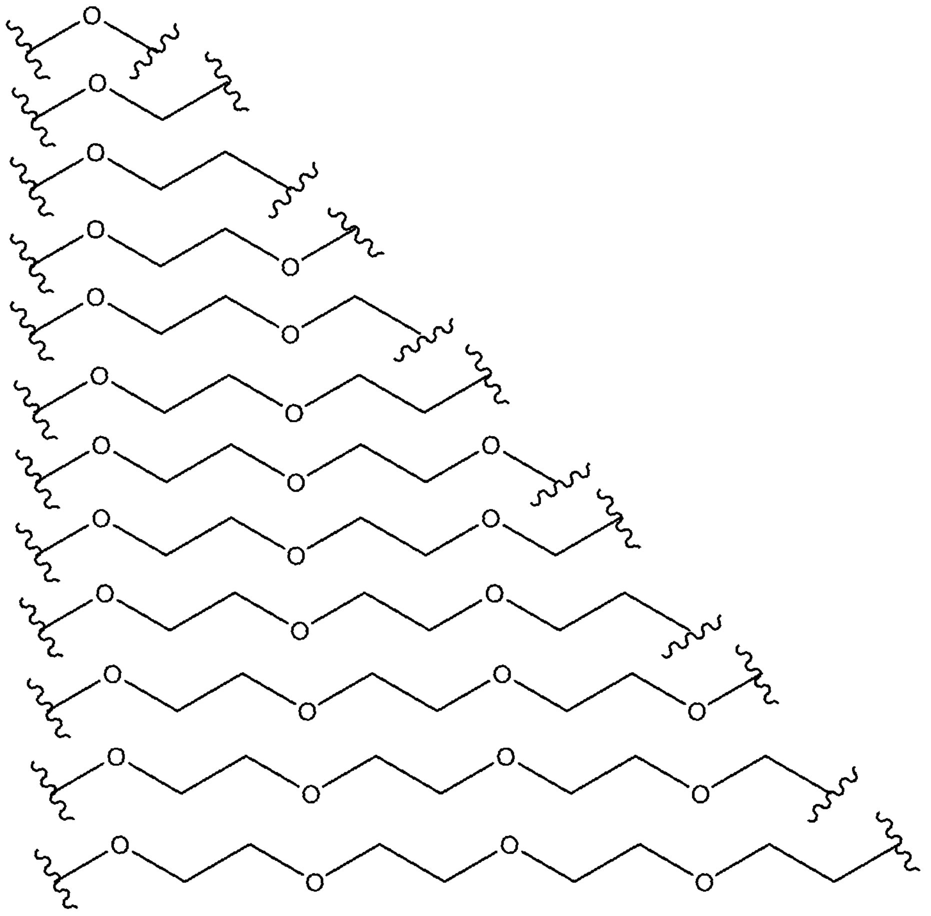
In certain embodiments, A is of the formula:



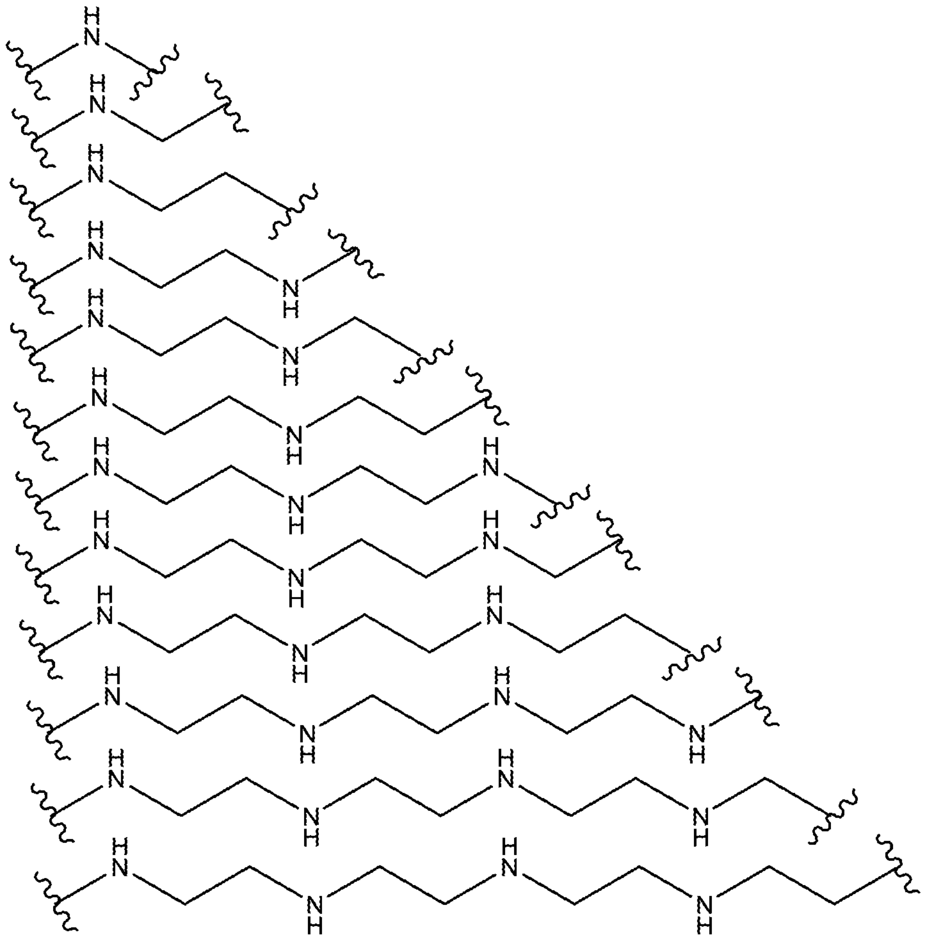
In certain embodiments, A is of one of the formulae:



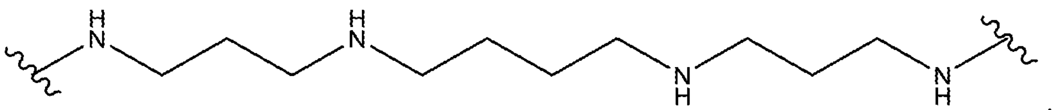
In certain embodiments, A is of one of the formulae:



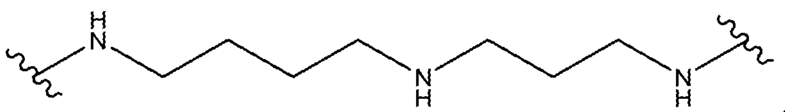
In certain embodiments, A is of one of the formulae:



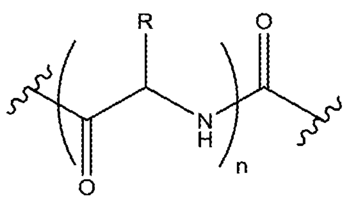
In certain embodiments, A is of the formula:



In certain embodiments, A is of the formula:



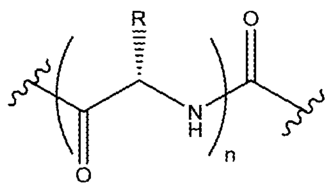
In certain embodiments, A is of the formula:



wherein

each occurrence of R is independently the side chain of a natural or unnatural amino acid; and

n is an integer between 1 and 20, inclusive. In certain embodiments, A is of the formula:



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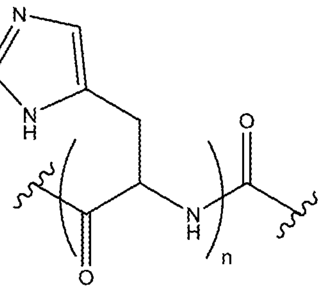
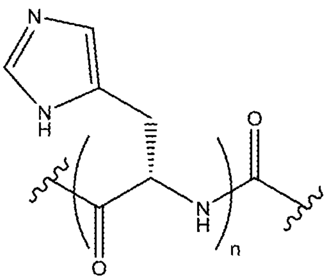
In certain embodiments, each occurrence of R is independently the side chain of a natural amino acid. In certain embodiments, n is an integer between 1 and 15, inclusive. In certain embodiments, n is an integer between 1 and 10, inclusive. In certain embodiments, n is an integer between 1 and 5, inclusive.

In certain embodiments, A is of the formula:



wherein n is an integer between 1 and 20, inclusive. In certain embodiments, A is of the formula:



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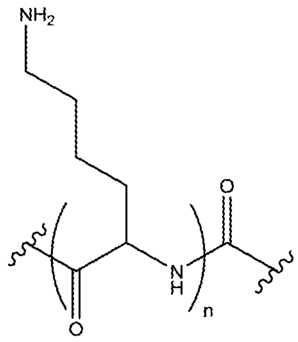
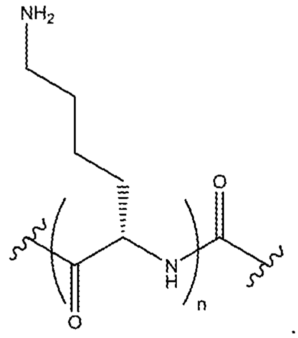
In certain embodiments, n is an integer between 1 and 15, inclusive. In certain embodiments, n is an integer between 1 and 10, inclusive. In certain embodiments, n is an integer between 1 and 5, inclusive.

In certain embodiments, A is of the formula:

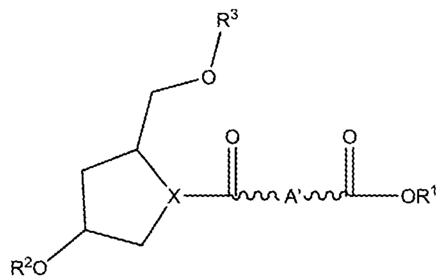


wherein n is an integer between 1 and 20, inclusive. In certain embodiments, A is of the formula:



In certain embodiments, n is an integer between 1 and 15, inclusive. In certain embodiments, n is an integer between 1 and 10, inclusive. In certain embodiments, n is an integer between 1 and 5, inclusive.

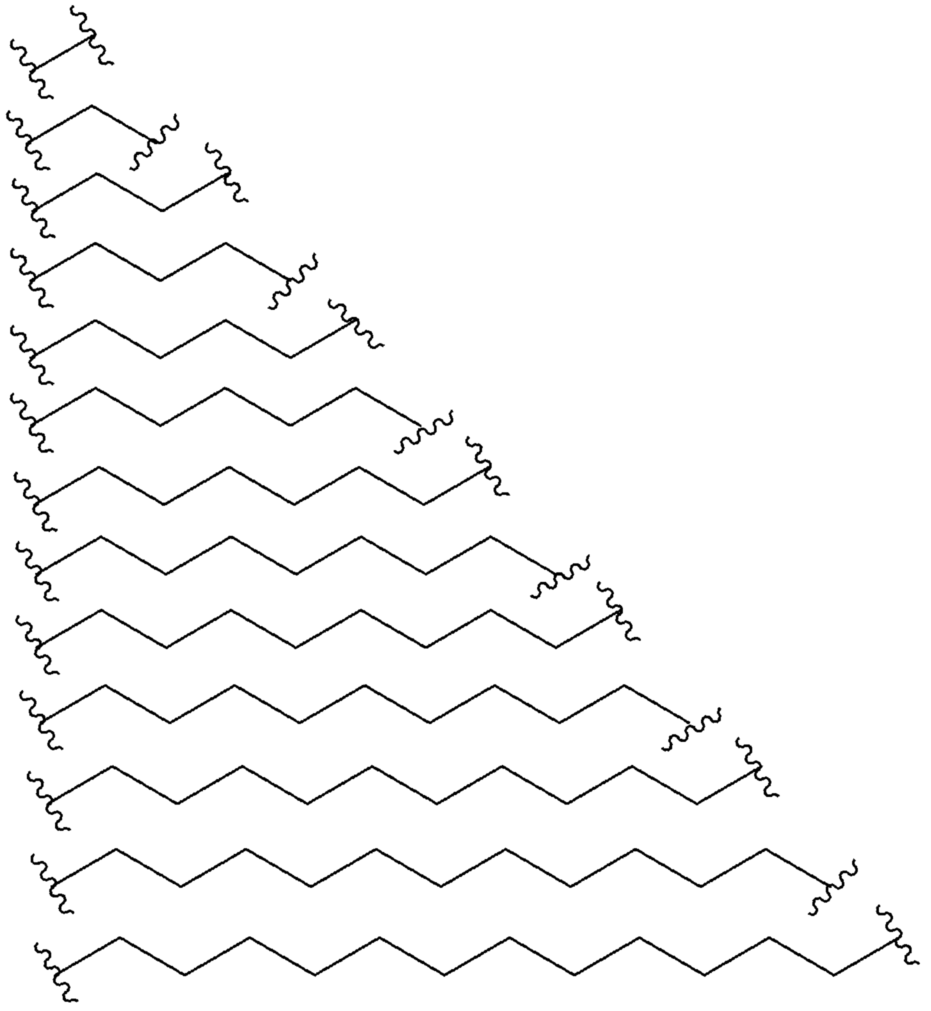
In certain embodiments, the molecule is of the formula:



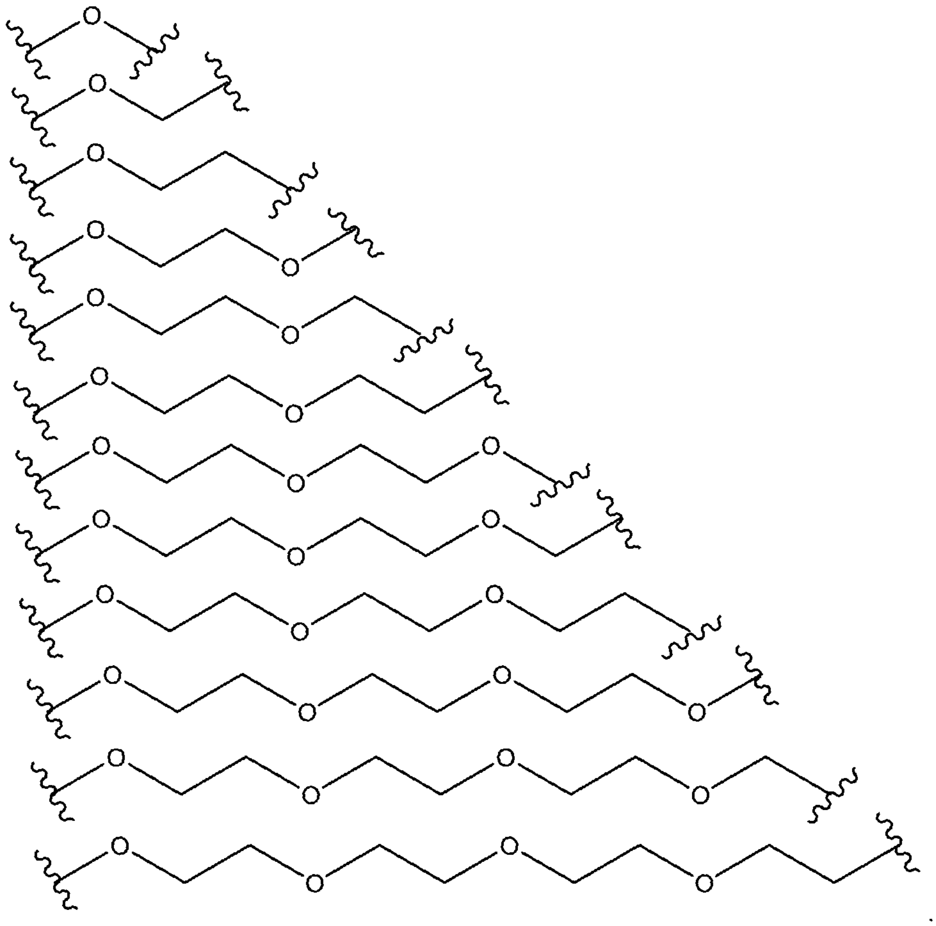
wherein X, R^1, R^2, and R^3 are as defined herein; and

A′ is substituted or unsubstituted, cyclic or acyclic, branched or unbranched aliphatic; or substituted or unsubstituted, cyclic or acyclic, branched or unbranched heteroaliphatic.

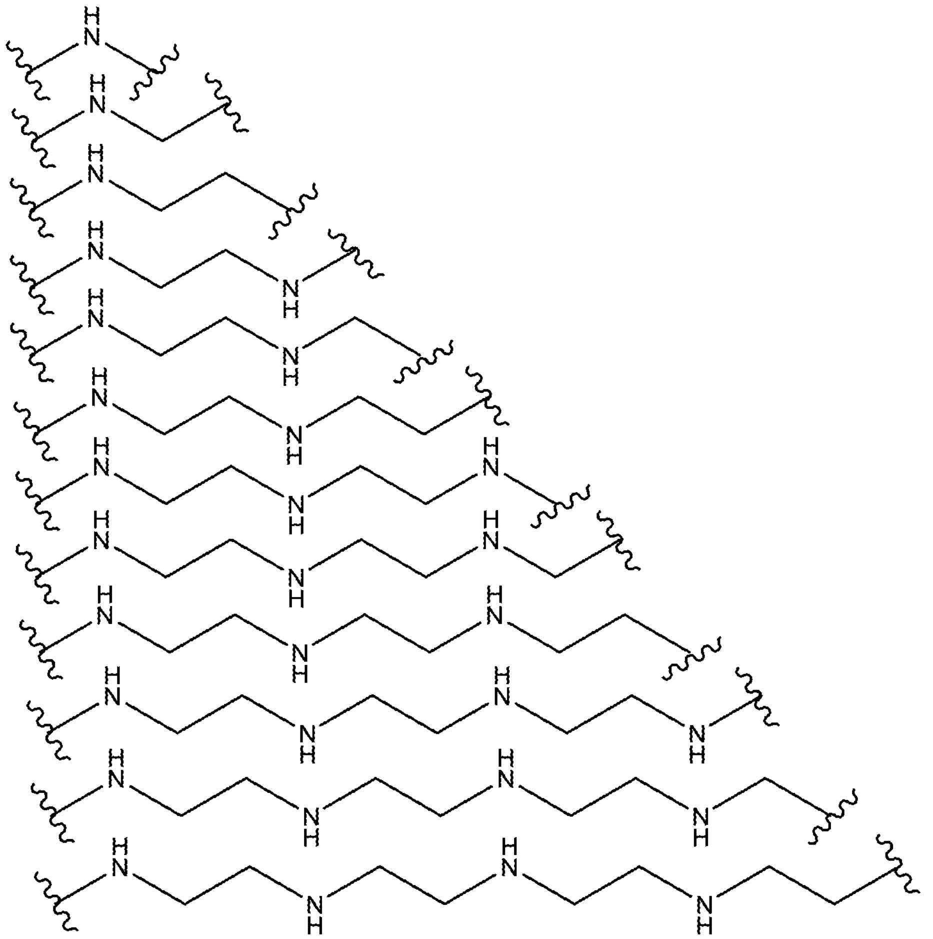
In certain embodiments, A′ is of one of the formulae:



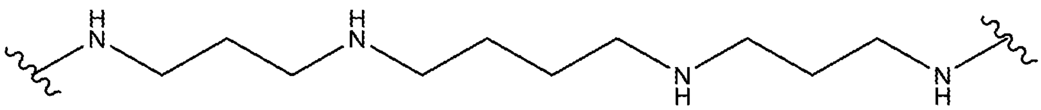
In certain embodiments, A is of one of the formulae:



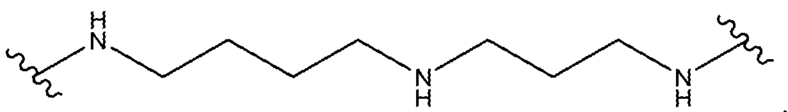
In certain embodiments, A is of one of the formulae:



In certain embodiments, A is of the formula:

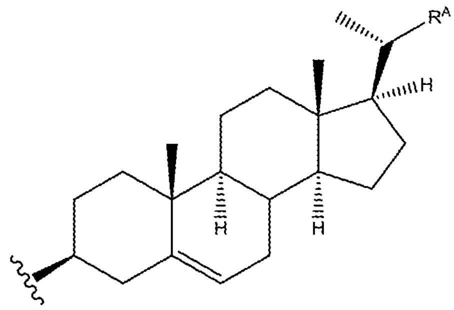


In certain embodiments, A is of the formula:



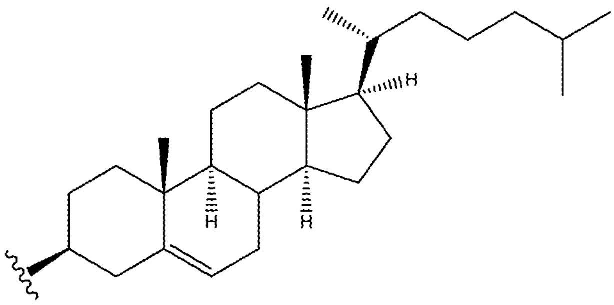
In certain embodiments, R^1 is a steroid. In certain embodiments, R^1 is a cholesterol. In certain embodiments, R^1 is a lipophilic vitamin. In certain embodiments, R^1 is a vitamin A. In certain embodiments, R^1 is a vitamin E.

In certain embodiments, R^1 is of the formula:



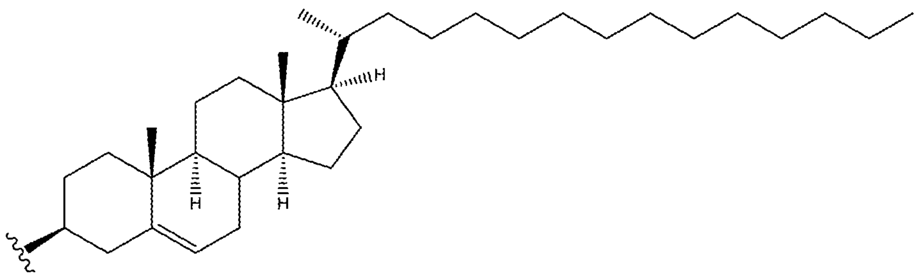
wherein R^A is substituted or unsubstituted, cyclic or acyclic, branched or unbranched aliphatic; or substituted or unsubstituted, cyclic or acyclic, branched or unbranched heteroaliphatic.

In certain embodiments, R^1 is of the formula:



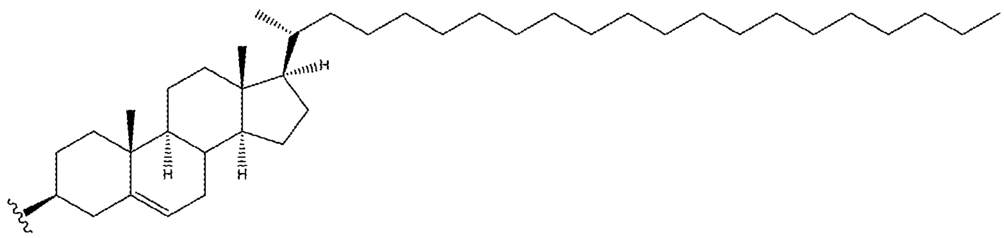
.

In certain embodiments, R^1 is of the formula:



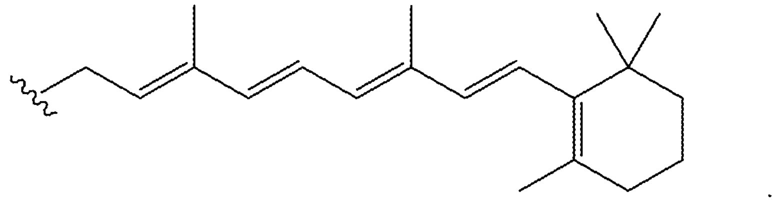
.

In certain embodiments, R^1 is of the formula:

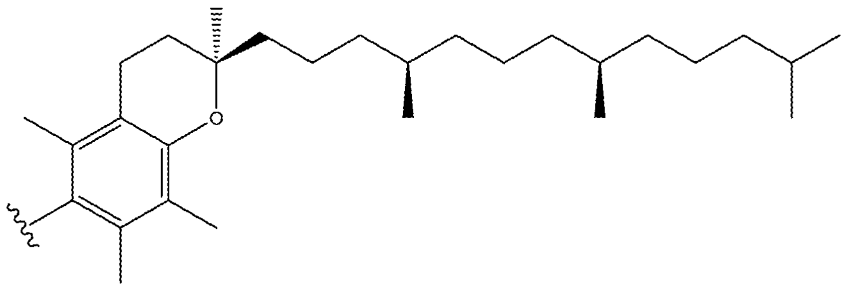


.

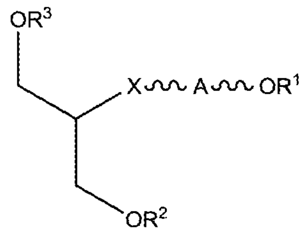
In certain embodiments, R^1 is of the formula:



In certain embodiments, R^1 is of the formula:



In certain embodiments, the nucleic acid molecule is of the formula:



wherein

X is N or CH;

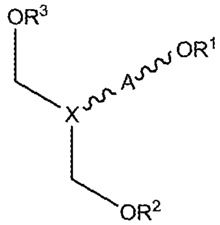
A is a bond; substituted or unsubstituted, cyclic or acyclic, branched or unbranched aliphatic; or substituted or unsubstituted, cyclic or acyclic, branched or unbranched heteroaliphatic;

R^1 is a hydrophobic moiety;

R^2 is hydrogen; an oxygen-protecting group; cyclic or acyclic, substituted or unsubstituted, branched or unbranched aliphatic; cyclic or acyclic, substituted or unsubstituted, branched or unbranched heteroaliphatic; substituted or unsubstituted, branched or unbranched acyl; substituted or unsubstituted, branched or unbranched aryl; substituted or unsubstituted, branched or unbranched heteroaryl; and

R^3 is a nucleic acid.

In certain embodiments, the nucleic acid molecule is of the formula:



wherein

X is N or CH;

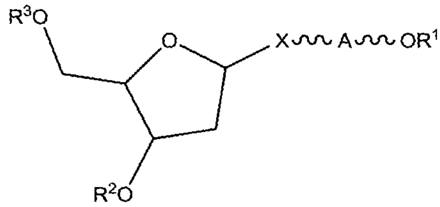
A is a bond; substituted or unsubstituted, cyclic or acyclic, branched or unbranched aliphatic; or substituted or unsubstituted, cyclic or acyclic, branched or unbranched heteroaliphatic;

R^1 is a hydrophobic moiety;

R^2 is hydrogen; an oxygen-protecting group; cyclic or acyclic, substituted or unsubstituted, branched or unbranched aliphatic; cyclic or acyclic, substituted or unsubstituted, branched or unbranched heteroaliphatic; substituted or unsubstituted, branched or unbranched acyl; substituted or unsubstituted, branched or unbranched aryl; substituted or unsubstituted, branched or unbranched heteroaryl; and

R^3 is a nucleic acid.

In certain embodiments, the nucleic acid molecule is of the formula:



wherein

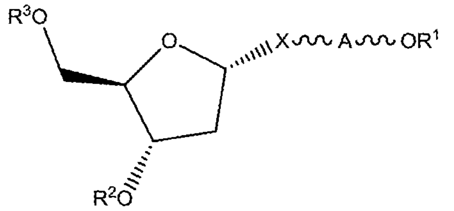
X is N or CH;

A is a bond; substituted or unsubstituted, cyclic or acyclic, branched or unbranched aliphatic; or substituted or unsubstituted, cyclic or acyclic, branched or unbranched heteroaliphatic;

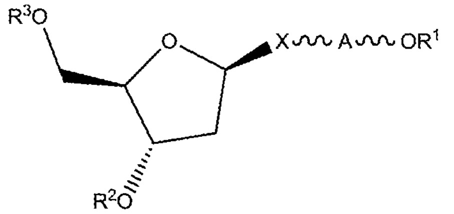
R^1 is a hydrophobic moiety;

R^2 is hydrogen; an oxygen-protecting group; cyclic or acyclic, substituted or unsubstituted, branched or unbranched aliphatic; cyclic or acyclic, substituted or unsubstituted, branched or unbranched heteroaliphatic; substituted or unsubstituted, branched or unbranched acyl; substituted or unsubstituted, branched or unbranched aryl; substituted or unsubstituted, branched or unbranched heteroaryl; and

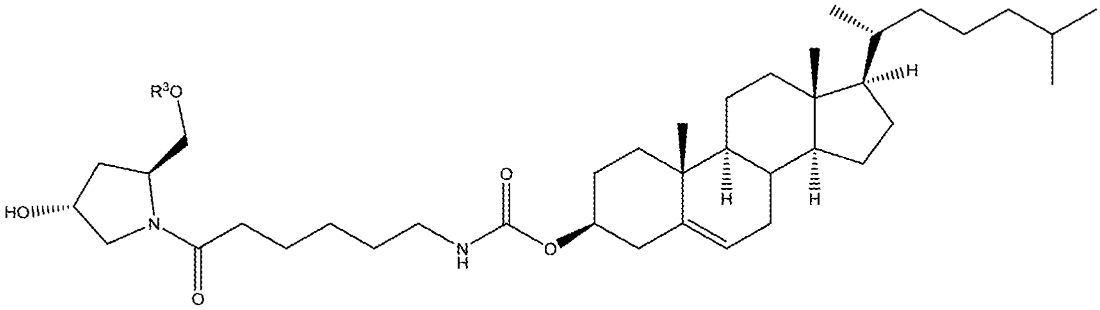
R^3 is a nucleic acid. In certain embodiments, the nucleic acid molecule is of the formula:



In certain embodiments, the nucleic acid molecule is of the formula:

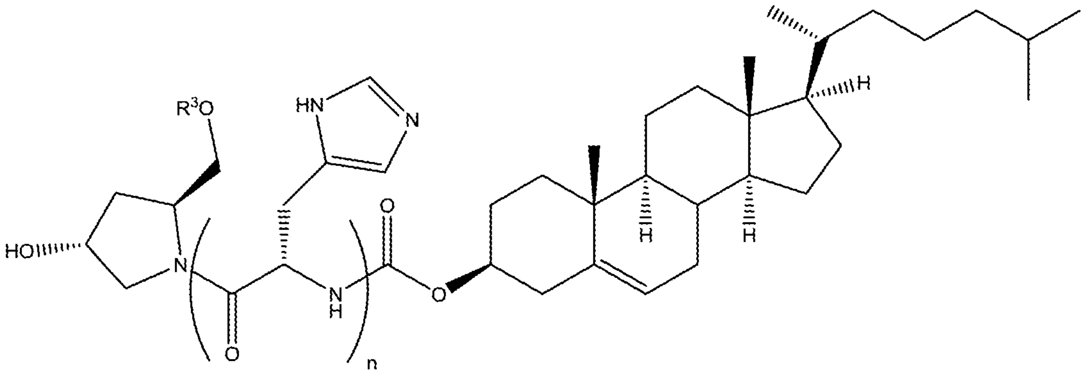


In certain embodiments, the nucleic acid molecule is of the formula:



wherein R^3 is a nucleic acid.

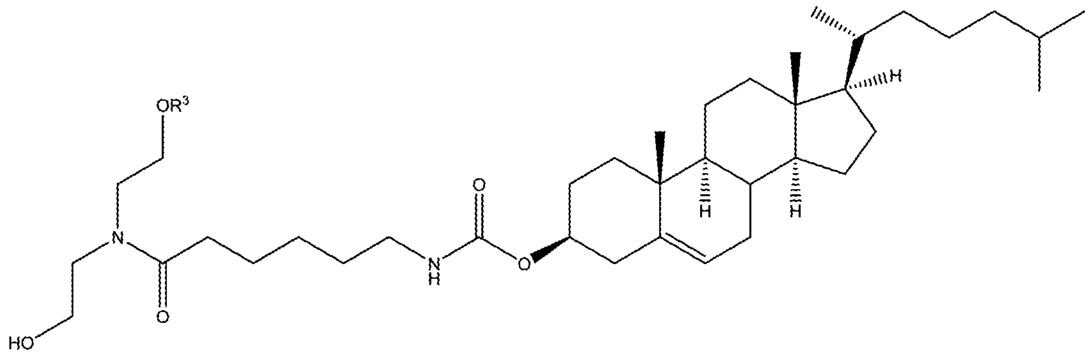
In certain embodiments, the nucleic acid molecule is of the formula:



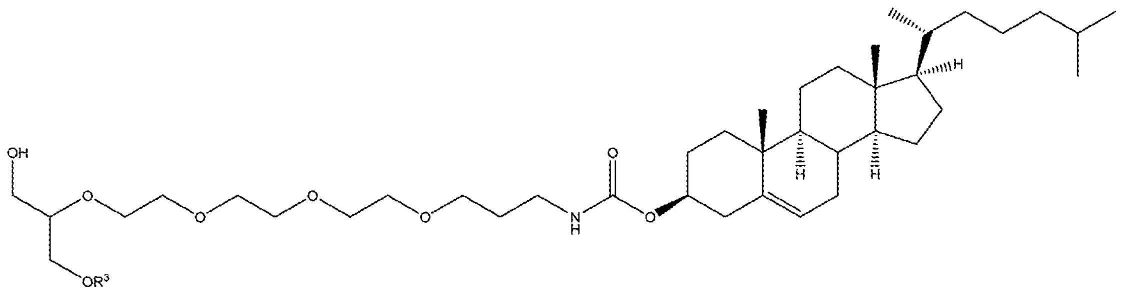
wherein R^3 is a nucleic acid; and

n is an integer between 1 and 20, inclusive.

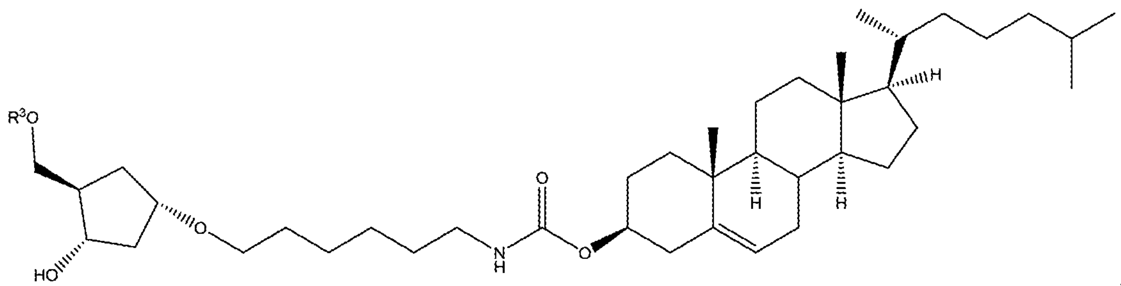
In certain embodiments, the nucleic acid molecule is of the formula:



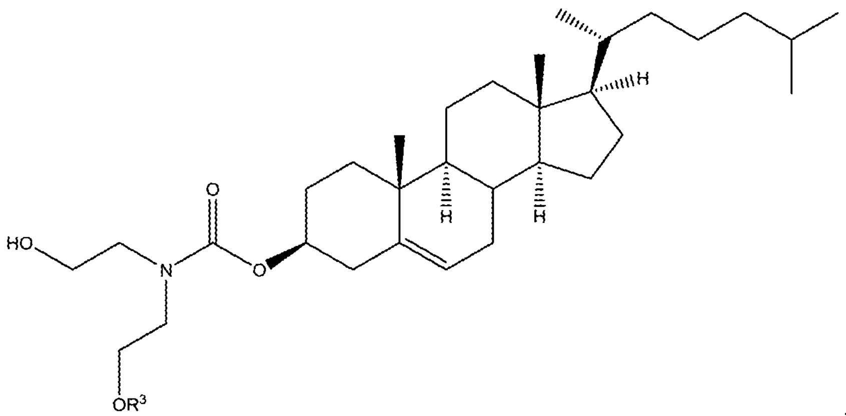
In certain embodiments, the nucleic acid molecule is of the formula:



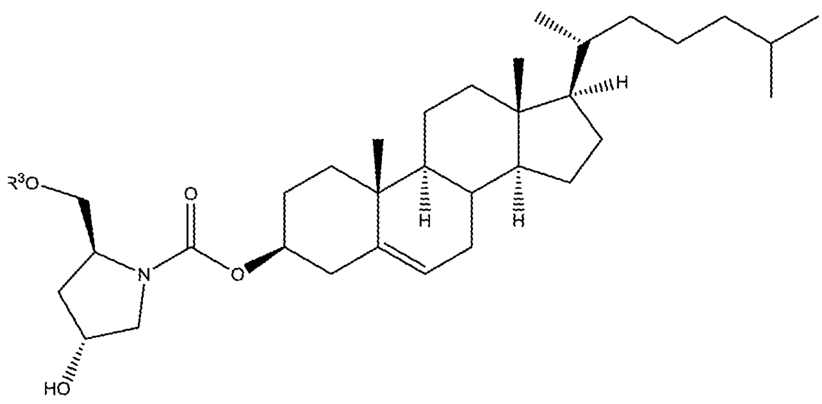
In certain embodiments, the nucleic acid molecule is of the formula:



In certain embodiments, the nucleic acid molecule is of the formula:



In certain embodiments, the nucleic acid molecule is of the formula:



As used herein, the term "linkage" includes a naturally occurring, unmodified phosphodiester moiety (-O-(PO^{2-})-O-) that covalently couples adjacent nucleomonomers. As used herein, the term "substitute linkage" includes any analog or derivative of the native phosphodiester group that covalently couples adjacent nucleomonomers. Substitute linkages include phosphodiester analogs, *e.g.*, phosphorothioate, phosphorodithioate, and P-ethyoxyphosphodiester, P-ethoxyphosphodiester, P-alkyloxyphosphotriester, methylphosphonate, and nonphosphorus containing linkages, *e.g.*, acetals and amides. Such substitute linkages are known in the art (*e.g.*, Bjergarde *et al.* 1991. Nucleic Acids Res. 19:5843; Caruthers *et al.* 1991. Nucleosides Nucleotides. 10:47). In certain embodiments, non-hydrolizable linkages are preferred, such as phosphorothiate linkages.

In certain embodiments, oligonucleotides of the invention comprise hydrophobically modified nucleotides or "hydrophobic modifications." As used herein "hydrophobic modifications" refers to bases that are modified such that (1) overall hydrophobicity of the base is significantly increased, and/or (2) the base is still capable of forming close to regular Watson –Crick interaction. Several non-limiting examples of base modifications include 5-position uridine and cytidine modifications such as phenyl, 4-pyridyl, 2-pyridyl, indolyl, and isobutyl, phenyl (C6H5OH); tryptophanyl (C8H6N)CH2CH(NH2)CO), Isobutyl, butyl, aminobenzyl; phenyl; and naphthyl.

Another type of conjugates that can be attached to the end (3' or 5' end), the loop region, or any other parts of the sd-rxRNA might include a sterol, sterol type molecule, peptide, small molecule, protein, etc. In some embodiments, a sd-rxRNA may contain more than one conjugates (same or different chemical nature). In some embodiments, the conjugate is cholesterol.

Another way to increase target gene specificity, or to reduce off-target silencing effect, is to introduce a 2'-modification (such as the 2'-O methyl modification) at a position corresponding to the second 5'-end nucleotide of the guide sequence. Antisense (guide) sequences of the invention can be "chimeric oligonucleotides" which comprise an RNA-like and a DNA-like region.

The language "RNase H activating region" includes a region of an oligonucleotide, *e.g.*, a chimeric oligonucleotide, that is capable of recruiting RNase H to cleave the target RNA strand to which the oligonucleotide binds. Typically, the RNase activating region contains a minimal core (of at least about 3-5, typically between about 3-12, more typically, between about 5-12, and more preferably between about 5-10 contiguous nucleomonomers) of DNA or DNA-like nucleomonomers. (See, *e.g.*, U.S. Pat. No. 5,849,902). Preferably, the RNase H activating region comprises about nine contiguous deoxyribose containing nucleomonomers.

The language "non-activating region" includes a region of an antisense sequence, *e.g.*, a chimeric oligonucleotide, that does not recruit or activate RNase H. Preferably, a non-activating region does not comprise phosphorothioate DNA. The oligonucleotides of the invention comprise at least one non-activating region. In one embodiment, the non-activating region can be stabilized against nucleases or can provide specificity for the target by being complementary to the target and forming hydrogen bonds with the target nucleic acid molecule, which is to be bound by the oligonucleotide.

In one embodiment, at least a portion of the contiguous polynucleotides are linked by a substitute linkage, *e.g.*, a phosphorothioate linkage.

In certain embodiments, most or all of the nucleotides beyond the guide sequence (2'-modified or not) are linked by phosphorothioate linkages. Such constructs tend to have improved pharmacokinetics due to their higher affinity for serum proteins. The phosphorothioate linkages in the non-guide sequence portion of the polynucleotide generally do not interfere with guide strand activity, once the latter is loaded into RISC. It is surprisingly demonstrated herein that high levels of phosphorothioate modification can lead to improved delivery. In some embodiments, the guide and/or passenger strand is completely phosphorothioated.

Antisense (guide) sequences of the present invention may include "morpholino oligonucleotides." Morpholino oligonucleotides are non-ionic and function by an RNase H-independent mechanism. Each of the 4 genetic bases (Adenine, Cytosine, Guanine, and Thymine/Uracil) of the morpholino oligonucleotides is linked to a 6-membered morpholine ring. Morpholino oligonucleotides are made by joining the 4 different subunit types by, *e.g.*, non-ionic phosphorodiamidate inter-subunit linkages. Morpholino oligonucleotides have many advantages including: complete resistance to nucleases (Antisense & Nucl. Acid Drug Dev. 1996. 6:267); predictable targeting (Biochemica Biophysica Acta. 1999. 1489:141); reliable activity in cells (Antisense & Nucl. Acid Drug Dev. 1997. 7:63); excellent sequence specificity (Antisense & Nucl. Acid Drug Dev. 1997. 7:151); minimal non-antisense activity (Biochemica Biophysica Acta. 1999. 1489:141); and simple osmotic or scrape delivery (Antisense & Nucl. Acid Drug Dev. 1997. 7:291). Morpholino oligonucleotides are also preferred because of their non-toxicity at high doses. A discussion of the preparation of morpholino oligonucleotides can be found in Antisense & Nucl. Acid Drug Dev. 1997. 7:187.

The chemical modifications described herein are believed, based on the data described herein, to promote single stranded polynucleotide loading into the RISC. Single stranded polynucleotides have been shown to be active in loading into RISC and inducing gene silencing. However, the level of activity for single stranded polynucleotides appears to be 2 to 4 orders of magnitude lower when compared to a duplex polynucleotide.

The present invention provides a description of the chemical modification patterns, which may (a) significantly increase stability of the single stranded polynucleotide (b) promote efficient loading of the polynucleotide into the RISC complex and (c) improve uptake of the single stranded nucleotide by the cell. The chemical modification patterns may include combination of ribose, backbone, hydrophobic nucleoside and conjugate type of modifications. In addition, in some of the embodiments, the 5' end of the single polynucleotide may be chemically phosphorylated.

In yet another embodiment, the present invention provides a description of the chemical modifications patterns, which improve functionality of RISC inhibiting polynucleotides. Single stranded polynucleotides have been shown to inhibit activity of a preloaded RISC complex through the substrate competition mechanism. For these types of molecules, conventionally called antagomers, the activity usually requires high concentration and *in vivo* delivery is not very effective. The present invention provides a description of the chemical modification patterns, which may (a) significantly increase stability of the single stranded polynucleotide (b) promote efficient recognition of the polynucleotide by the RISC as a substrate and/or (c) improve uptake of the single stranded nucleotide by the cell. The chemical modification patterns may include combination of ribose, backbone, hydrophobic nucleoside and conjugate type of modifications.

The modifications provided by the present invention are applicable to all polynucleotides. This includes single stranded RISC entering polynucleotides, single stranded RISC inhibiting polynucleotides, conventional duplexed polynucleotides of variable length (15- 40 bp), asymmetric duplexed polynucleotides, and the like. Polynucleotides may be modified with wide variety of chemical modification patterns, including 5' end, ribose, backbone and hydrophobic nucleoside modifications.

Aspects of the invention relate to nucleic acid molecules that are highly modified with phosphorothioate backbone modifications. Completely phosphorothioated compounds (21552) that are highly active were disclosed in PCT Publication No. WO2011/119852, incorporated by reference herein. Interestingly, compounds that contained a 21 mer guide strand that was completely phosphorothioate modified were active, however, reducing the guide strand length by two nucleotides, e.g., 19 mer guide strand (21550), resulted in reduced activity. Without wishing to be bound by any theory, increasing the guide strand from 19 to 21 nucleotides (fully phosphorothioated guide strands) may increase the melting temperature between the guide strand and mRNA, resulting in enhanced silencing activity. Varying phosphorothioate content on the 13 mer passenger strand such that it was either completely phosphorothioate modified or contained 6 phosphorothioate modifications did not result in altered activity (21551 vs 21556 as disclosed in PCT Publication No. WO2011/119852).

Several past groups have tried to develop completely phosphorothioated RNAi compounds. Completely phosphorothioated duplexes, lacking single stranded regions, have been designed and tested before, however, these compounds did not demonstrate acceptable pharmacokinetic profiles. Completely phosphorothioated single stranded RNAi compounds have also been designed previously, however, these compounds did not efficiently enter RISC. PCT Publication No. WO2011/119852, incorporated by reference herein in its entirety, disclosed hybrid RNAi compounds that efficiently enter RISC and contain complete phosphorothioated backbones.

In some aspects, the disclosure relates to the discovery of isolated double stranded nucleic acid molecules having highly phosphorothioated backbones (*e.g.*, having at least one strand with a completely phosphorothioated backbone, or almost completely phosphorothioated backbone (*e.g.*, having one un-phosphorothioated residue)). It was surprisingly found herein that high levels of phosphorothioate modifications mediate increased levels of cellular uptake of the isolated double stranded nucleic acid molecule in the central nervous system (CNS) relative to isolated double stranded nucleic acid molecules having less phosphorothioate modifications (*e.g.* not having at least one strand that is fully phosphorothioated or almost completely phosphorothioated).

Synthesis

Oligonucleotides of the invention can be synthesized by any method known in the art, *e.g.*, using enzymatic synthesis and/or chemical synthesis. The oligonucleotides can be synthesized *in vitro* (*e.g.*, using enzymatic synthesis and chemical synthesis) or *in vivo* (using recombinant DNA technology well known in the art).

In a preferred embodiment, chemical synthesis is used for modified polynucleotides. Chemical synthesis of linear oligonucleotides is well known in the art and can be achieved by solution or solid phase techniques. Preferably, synthesis is by solid phase methods. Oligonucleotides can be made by any of several different synthetic procedures including the phosphoramidite, phosphite triester, H-phosphonate, and phosphotriester methods, typically by automated synthesis methods.

Oligonucleotide synthesis protocols are well known in the art and can be found, *e.g.*, in U.S. Pat. No. 5,830,653; WO 98/13526; Stec *et al.* 1984. *J. Am. Chem. Soc.* 106:6077; Stec *et al.* 1985. *J. Org. Chem.* 50:3908; Stec *et al.* J. Chromatog. 1985. 326:263; LaPlanche *et al.* 1986. *Nucl. Acid. Res.* 1986. 14:9081; Fasman G. D., 1989. Practical Handbook of Biochemistry and Molecular Biology. 1989. CRC Press, Boca Raton, Fla.; Lamone. 1993. *Biochem. Soc. Trans.* 21:1; U.S. Pat. No. 5,013,830; U.S. Pat. No. 5,214,135; U.S. Pat. No. 5,525,719; Kawasaki *et al.* 1993. *J. Med. Chem.* 36:831; WO 92/03568; U.S. Pat. No. 5,276,019; and U.S. Pat. No. 5,264,423.

The synthesis method selected can depend on the length of the desired oligonucleotide and such choice is within the skill of the ordinary artisan. For example, the phosphoramidite and phosphite triester method can produce oligonucleotides having 175 or more nucleotides, while the H-phosphonate method works well for oligonucleotides of less than 100 nucleotides. If modified bases are incorporated into the oligonucleotide, and particularly if modified phosphodiester linkages are used, then the synthetic procedures are altered as needed according to known procedures. In this regard, Uhlmann *et al.* (1990, *Chemical Reviews* 90:543-584) provide references and outline procedures for making oligonucleotides with modified bases and modified phosphodiester linkages. Other exemplary methods for making oligonucleotides are taught in Sonveaux. 1994. "Protecting Groups in Oligonucleotide Synthesis"; Agrawal. *Methods in Molecular Biology* 26:1. Exemplary synthesis methods are also taught in "Oligonucleotide Synthesis - A Practical Approach" (Gait, M. J. IRL Press at Oxford University Press. 1984). Moreover, linear oligonucleotides of defined sequence, including some sequences with modified nucleotides, are readily available from several commercial sources.

The oligonucleotides may be purified by polyacrylamide gel electrophoresis, or by any of a number of chromatographic methods, including gel chromatography and high pressure liquid chromatography. To confirm a nucleotide sequence, especially unmodified nucleotide sequences, oligonucleotides may be subjected to DNA sequencing by any of the known procedures, including Maxam and Gilbert sequencing, Sanger sequencing, capillary electrophoresis sequencing, the wandering spot sequencing procedure or by using selective chemical degradation of oligonucleotides bound to Hybond paper. Sequences of short oligonucleotides can also be analyzed by laser desorption mass spectroscopy or by fast atom bombardment (McNeal, *et al.*, 1982, *J. Am. Chem. Soc.* 104:976; Viari, *et al.*, 1987, *Biomed. Environ. Mass Spectrom.* 14:83; Grotjahn *et al.*, 1982, *Nuc. Acid Res.* 10:4671). Sequencing methods are also available for RNA oligonucleotides.

The quality of oligonucleotides synthesized can be verified by testing the oligonucleotide by capillary electrophoresis and denaturing strong anion HPLC (SAX-HPLC) using, *e.g.*, the method of Bergot and Egan. 1992. *J. Chrom.* 599:35.

Other exemplary synthesis techniques are well known in the art (see, *e.g.*, Sambrook *et al.*, Molecular Cloning: a Laboratory Manual, Second Edition (1989); DNA Cloning, Volumes I and II (DN Glover Ed. 1985); Oligonucleotide Synthesis (M J Gait Ed, 1984; Nucleic Acid Hybridisation (B D Hames and S J Higgins eds. 1984); A Practical Guide to Molecular Cloning (1984); or the series, Methods in Enzymology (Academic Press, Inc.)).

In certain embodiments, the subject RNAi constructs or at least portions thereof are transcribed from expression vectors encoding the subject constructs. Any art recognized vectors may be use for this purpose. The transcribed RNAi constructs may be isolated and purified, before desired modifications (such as replacing an unmodified sense strand with a modified one, *etc.*) are carried out.

Uptake of Oligonucleotides by Cells

Oligonucleotides and oligonucleotide compositions are contacted with (*i.e.*, brought into contact with, also referred to herein as administered or delivered to) and taken up by one or more cells or a cell lysate. The term "cells" includes prokaryotic and eukaryotic cells, preferably vertebrate cells, and, more preferably, mammalian cells. In a preferred embodiment, the oligonucleotide compositions of the invention are contacted with human cells.

Oligonucleotide compositions of the invention can be contacted with cells *in vitro*, *e.g.*, in a test tube or culture dish, (and may or may not be introduced into a subject) or *in vivo*, *e.g.*, in a subject such as a mammalian subject. In some embodiments, Oligonucleotides are administered topically or through electroporation. Oligonucleotides are taken up by cells at a slow rate by endocytosis, but endocytosed oligonucleotides are generally sequestered and not available, *e.g.*, for hybridization to a target nucleic acid molecule. In one embodiment, cellular uptake can be facilitated by electroporation or calcium phosphate precipitation. However, these procedures are only useful for *in vitro* or *ex vivo* embodiments, are not convenient and, in some cases, are associated with cell toxicity.

In another embodiment, delivery of oligonucleotides into cells can be enhanced by suitable art recognized methods including calcium phosphate, DMSO, glycerol or dextran, electroporation, or by transfection, *e.g.*, using cationic, anionic, or neutral lipid compositions or liposomes using methods known in the art (see *e.g.*, WO 90/14074; WO 91/16024; WO 91/17424; U.S. Pat. No. 4,897,355; Bergan *et al.* 1993. *Nucleic Acids Research.* 21:3567). Enhanced delivery of oligonucleotides can also be mediated by the use of vectors (See *e.g.*, Shi, Y. 2003. Trends Genet 2003 Jan. 19:9; Reichhart J M *et al.* Genesis. 2002. 34(1-2):1604, Yu *et al.* 2002. Proc. Natl. Acad Sci. USA 99:6047; Sui *et al.* 2002. Proc. Natl. Acad Sci. USA 99:5515) viruses, polyamine or polycation conjugates using compounds such as polylysine, protamine, or Ni, N12-bis (ethyl) spermine (see, *e.g.*, Bartzatt, R. *et al.*1989. *Biotechnol. Appl. Biochem.* 11:133; Wagner E. *et al.* 1992. *Proc. Natl. Acad. Sci.* 88:4255).

In certain embodiments, the sd-rxRNA of the invention may be delivered by using various beta-glucan containing particles, referred to as GeRPs (glucan encapsulated RNA loaded particle), described in, and incorporated by reference from, US Provisional Application No. 61/310,611, filed on March 4, 2010 and entitled "Formulations and Methods for Targeted Delivery to Phagocyte Cells." Such particles are also described in, and incorporated by reference from US Patent Publications US 2005/0281781 A1, and US 2010/0040656, US Patent No. 8,815,818 and in PCT publications WO 2006/007372, and WO 2007/050643. The sd-rxRNA molecule may be hydrophobically modified and optionally may be associated with a lipid and/or amphiphilic peptide. In certain embodiments, the beta-glucan particle is derived from yeast. In certain embodiments, the payload trapping molecule is a polymer, such as those with a molecular weight of at least about 1000 Da, 10,000 Da, 50,000 Da, 100 kDa, 500 kDa, etc. Preferred polymers include (without limitation) cationic polymers, chitosans, or PEI (polyethylenimine), etc.

Glucan particles can be derived from insoluble components of fungal cell walls such as yeast cell walls. In some embodiments, the yeast is Baker's yeast. Yeast-derived glucan molecules can include one or more of ß-(1,3)-Glucan, ß-(1,6)-Glucan, mannan and chitin. In some embodiments, a glucan particle comprises a hollow yeast cell wall whereby the particle maintains a three dimensional structure resembling a cell, within which it can complex with or encapsulate a molecule such as an RNA molecule. Some of the advantages associated with the use of yeast cell wall particles are availability of the components, their biodegradable nature, and their ability to be targeted to phagocytic cells.

In some embodiments, glucan particles can be prepared by extraction of insoluble components from cell walls, for example by extracting Baker's yeast (Fleischmann's) with 1M NaOH/pH 4.0 H2O, followed by washing and drying. Methods of preparing yeast cell wall particles are discussed in, and incorporated by reference from U.S. Patents 4,810,646, 4,992,540, 5,082,936, 5,028,703, 5,032,401, 5,322,841, 5,401,727, 5,504,079, 5,607,677, 5,968,811, 6,242,594, 6,444,448, 6,476,003, US Patent Publications 2003/0216346, 2004/0014715 and 2010/0040656, and PCT published application WO02/12348.

Protocols for preparing glucan particles are also described in, and incorporated by reference from, the following references: Soto and Ostroff (2008), "Characterization of multilayered nanoparticles encapsulated in yeast cell wall particles for DNA delivery." *Bioconjug Chem* 19(4):840-8; Soto and Ostroff (2007), "Oral Macrophage Mediated Gene Delivery System," *Nanotech*, Volume 2, Chapter 5 ("Drug Delivery"), pages 378-381; and Li et al. (2007), "Yeast glucan particles activate murine resident macrophages to secrete proinflammatory cytokines via MyD88-and Syk kinase-dependent pathways." *Clinical Immunology* 124(2):170-181.

Glucan containing particles such as yeast cell wall particles can also be obtained commercially. Several non-limiting examples include: Nutricell MOS 55 from Biorigin (Sao Paolo, Brazil), SAF-Mannan (SAF Agri, Minneapolis, Minn.), Nutrex (Sensient Technologies, Milwaukee, Wis.), alkali-extracted particles such as those produced by Nutricepts (Nutricepts Inc., Burnsville, Minn.) and ASA Biotech, acid-extracted WGP particles from Biopolymer Engineering, and organic solvent-extracted particles such as

Adjuvax^{TM} from Alpha-beta Technology, Inc. (Worcester, Mass.) and microparticulate glucan from Novogen (Stamford, Conn.).

Glucan particles such as yeast cell wall particles can have varying levels of purity depending on the method of production and/or extraction. In some instances, particles are alkali-extracted, acid-extracted or organic solvent-extracted to remove intracellular components and/or the outer mannoprotein layer of the cell wall. Such protocols can produce particles that have a glucan (w/w) content in the range of 50% - 90%. In some instances, a particle of lower purity, meaning lower glucan w/w content may be preferred, while in other embodiments, a particle of higher purity, meaning higher glucan w/w content may be preferred.

Glucan particles, such as yeast cell wall particles, can have a natural lipid content. For example, the particles can contain 1%, 2%, 3%, 4%, 5%, 6%, 7%, 8%, 9%, 10%, 11%, 12%, 13%, 14%, 15%, 16%, 17%, 18%, 19%, 20% or more than 20% w/w lipid. In the Examples section, the effectiveness of two glucan particle batches are tested: YGP SAF and YGP SAF + L (containing natural lipids). In some instances, the presence of natural lipids may assist in complexation or capture of RNA molecules.

Glucan containing particles typically have a diameter of approximately 2-4 microns, although particles with a diameter of less than 2 microns or greater than 4 microns are also compatible with aspects of the invention.

The RNA molecule(s) to be delivered are complexed or "trapped" within the shell of the glucan particle. The shell or RNA component of the particle can be labeled for visualization, as described in, and incorporated by reference from, Soto and Ostroff (2008) *Bioconjug Chem* 19:840. Methods of loading GeRPs are discussed further below.

The optimal protocol for uptake of oligonucleotides will depend upon a number of factors, the most crucial being the type of cells that are being used. Other factors that are important in uptake include, but are not limited to, the nature and concentration of the oligonucleotide, the confluence of the cells, the type of culture the cells are in (*e.g.*, a suspension culture or plated) and the type of media in which the cells are grown.

Encapsulating Agents

Encapsulating agents entrap oligonucleotides within vesicles. In another embodiment of the invention, an oligonucleotide may be associated with a carrier or vehicle, *e.g.*, liposomes or micelles, although other carriers could be used, as would be appreciated by one skilled in the art. Liposomes are vesicles made of a lipid bilayer having a structure similar to biological membranes. Such carriers are used to facilitate the cellular uptake or targeting of the oligonucleotide, or improve the oligonucleotide's pharmacokinetic or toxicologic properties.

For example, the oligonucleotides of the present invention may also be administered encapsulated in liposomes, pharmaceutical compositions wherein the active ingredient is contained either dispersed or variously present in corpuscles consisting of aqueous concentric layers adherent to lipidic layers. The oligonucleotides, depending upon solubility, may be present both in the aqueous layer and in the lipidic layer, or in what is generally termed a liposomic suspension. The hydrophobic layer, generally but not exclusively, comprises phopholipids such as lecithin and sphingomyelin, steroids such as cholesterol, more or less ionic surfactants such as diacetylphosphate, stearylamine, or phosphatidic acid, or other materials of a hydrophobic nature. The diameters of the liposomes generally range from about 15 nm to about 5 microns.

The use of liposomes as drug delivery vehicles offers several advantages. Liposomes increase intracellular stability, increase uptake efficiency and improve biological activity. Liposomes are hollow spherical vesicles composed of lipids arranged in a similar fashion as those lipids which make up the cell membrane. They have an internal aqueous space for entrapping water soluble compounds and range in size from 0.05 to several microns in diameter. Several studies have shown that liposomes can deliver nucleic acids to cells and that the nucleic acids remain biologically active. For example, a lipid delivery vehicle originally designed as a research tool, such as Lipofectin or LIPOFECTAMINE™ 2000, can deliver intact nucleic acid molecules to cells.

Specific advantages of using liposomes include the following: they are non-toxic and biodegradable in composition; they display long circulation half-lives; and recognition molecules can be readily attached to their surface for targeting to tissues. Finally, cost-effective manufacture of liposome-based pharmaceuticals, either in a liquid suspension or lyophilized product, has demonstrated the viability of this technology as an acceptable drug delivery system.

In some aspects, formulations associated with the invention might be selected for a class of naturally occurring or chemically synthesized or modified saturated and unsaturated fatty acid residues. Fatty acids might exist in a form of triglycerides, diglycerides or individual fatty acids. In another embodiment, the use of well-validated mixtures of fatty acids and/or fat emulsions currently used in pharmacology for parenteral nutrition may be utilized.

Liposome based formulations are widely used for oligonucleotide delivery. However, most of commercially available lipid or liposome formulations contain at least one positively charged lipid (cationic lipids). The presence of this positively charged lipid is believed to be essential for obtaining a high degree of oligonucleotide loading and for enhancing liposome fusogenic properties. Several methods have been performed and published to identify optimal positively charged lipid chemistries. However, the commercially available liposome formulations containing cationic lipids are characterized by a high level of toxicity. *In vivo* limited therapeutic indexes have revealed that liposome formulations containing positive charged lipids are associated with toxicity (i.e. elevation in liver enzymes) at concentrations only slightly higher than concentration required to achieve RNA silencing.

Nucleic acids associated with the invention can be hydrophobically modified and can be encompassed within neutral nanotransporters. Further description of neutral nanotransporters is incorporated by reference from PCT Application PCT/US2009/005251, filed on September 22, 2009, and entitled "Neutral Nanotransporters." Such particles enable quantitative oligonucleotide incorporation into non-charged lipid mixtures. The lack of toxic levels of cationic lipids in such neutral nanotransporter compositions is an important feature.

As demonstrated in PCT/US2009/005251, oligonucleotides can effectively be incorporated into a lipid mixture that is free of cationic lipids and such a composition can effectively deliver a therapeutic oligonucleotide to a cell in a manner that it is functional. For example, a high level of activity was observed when the fatty mixture was composed of a phosphatidylcholine base fatty acid and a sterol such as a cholesterol. For instance, one preferred formulation of neutral fatty mixture is composed of at least 20% of DOPC or DSPC and at least 20% of sterol such as cholesterol. Even as low as 1:5 lipid to oligonucleotide ratio was shown to be sufficient to get complete encapsulation of the oligonucleotide in a non-charged formulation.

The neutral nanotransporters compositions enable efficient loading of oligonucleotide into neutral fat formulation. The composition includes an oligonucleotide that is modified in a manner such that the hydrophobicity of the molecule is increased (for example a hydrophobic molecule is attached (covalently or no-covalently) to a hydrophobic molecule on the oligonucleotide terminus or a non-terminal nucleotide, base, sugar, or backbone), the modified oligonucleotide being mixed with a neutral fat formulation (for example containing at least 25 % of cholesterol and 25% of DOPC or analogs thereof). A cargo molecule, such as another lipid can also be included in the composition. This composition, where part of the formulation is built into the oligonucleotide itself, enables efficient encapsulation of oligonucleotide in neutral lipid particles.

In some aspects, stable particles ranging in size from 50 to 140 nm can be formed upon complexing of hydrophobic oligonucleotides with preferred formulations. It is interesting to mention that the formulation by itself typically does not form small particles, but rather, forms agglomerates, which are transformed into stable 50-120 nm particles upon addition of the hydrophobic modified oligonucleotide.

The neutral nanotransporter compositions of the invention include a hydrophobic modified polynucleotide, a neutral fatty mixture, and optionally a cargo molecule. A "hydrophobic modified polynucleotide" as used herein is a polynucleotide of the invention (i.e. sd-rxRNA) that has at least one modification that renders the polynucleotide more hydrophobic than the polynucleotide was prior to modification. The modification may be achieved by attaching (covalently or non-covalently) a hydrophobic molecule to the polynucleotide. In some instances the hydrophobic molecule is or includes a lipophilic group.

The term "lipophilic group" means a group that has a higher affinity for lipids than its affinity for water. Examples of lipophilic groups include, but are not limited to, cholesterol, a cholesteryl or modified cholesteryl residue, adamantine, dihydrotesterone, long chain alkyl, long chain alkenyl, long chain alkynyl, olely-lithocholic, cholenic, oleoyl-cholenic, palmityl, heptadecyl, myrisityl, bile acids, cholic acid or taurocholic acid, deoxycholate, oleyl litocholic acid, oleoyl cholenic acid, glycolipids, phospholipids, sphingolipids, isoprenoids, such as steroids, vitamins, such as vitamin E, fatty acids either saturated or unsaturated, fatty acid esters, such as triglycerides, pyrenes, porphyrines, Texaphyrine, adamantane, acridines, biotin, coumarin, fluorescein, rhodamine, Texas-Red, digoxygenin, dimethoxytrityl, t-butyldimethylsilyl, t-butyldiphenylsilyl, cyanine dyes (e.g. Cy3 or Cy5), Hoechst 33258 dye, psoralen, or ibuprofen. The cholesterol moiety may be reduced (e.g. as in cholestan) or may be substituted (e.g. by halogen). A combination of different lipophilic groups in one molecule is also possible.

The hydrophobic molecule may be attached at various positions of the polynucleotide. As described above, the hydrophobic molecule may be linked to the terminal residue of the polynucleotide such as the 3' of 5'-end of the polynucleotide. Alternatively, it may be linked to an internal nucleotide or a nucleotide on a branch of the polynucleotide. The hydrophobic molecule may be attached, for instance to a 2'-position of the nucleotide. The hydrophobic molecule may also be linked to the heterocyclic base, the sugar or the backbone of a nucleotide of the polynucleotide.

The hydrophobic molecule may be connected to the polynucleotide by a linker moiety. Optionally the linker moiety is a non-nucleotidic linker moiety. Non-nucleotidic linkers are e.g. abasic residues (dSpacer), oligoethyleneglycol, such as triethyleneglycol (spacer 9) or hexaethylenegylcol (spacer 18), or alkane-diol, such as butanediol. The spacer units are preferably linked by phosphodiester or phosphorothioate bonds. The linker units may appear just once in the molecule or may be incorporated several times, e.g. via phosphodiester, phosphorothioate, methylphosphonate, or amide linkages.

Typical conjugation protocols involve the synthesis of polynucleotides bearing an aminolinker at one or more positions of the sequence, however, a linker is not required. The amino group is then reacted with the molecule being conjugated using appropriate coupling or activating reagents. The conjugation reaction may be performed either with the polynucleotide still bound to a solid support or following cleavage of the polynucleotide in solution phase. Purification of the modified polynucleotide by HPLC typically results in a pure material.

In some embodiments the hydrophobic molecule is a sterol type conjugate, a PhytoSterol conjugate, cholesterol conjugate, sterol type conjugate with altered side chain length, fatty acid conjugate, any other hydrophobic group conjugate, and/or hydrophobic modifications of the internal nucleoside, which provide sufficient hydrophobicity to be incorporated into micelles.

For purposes of the present invention, the term "sterols", refers or steroid alcohols are a subgroup of steroids with a hydroxyl group at the 3-position of the A-ring. They are amphipathic lipids synthesized from acetyl-coenzyme A via the HMG-CoA reductase pathway. The overall molecule is quite flat. The hydroxyl group on the A ring is polar. The rest of the aliphatic chain is non-polar. Usually sterols are considered to have an 8 carbon chain at position 17.

For purposes of the present invention, the term "sterol type molecules", refers to steroid alcohols, which are similar in structure to sterols. The main difference is the structure of the ring and number of carbons in a position 21 attached side chain.

For purposes of the present invention, the term "PhytoSterols" (also called plant sterols) are a group of steroid alcohols, phytochemicals naturally occurring in plants. There are more than 200 different known PhytoSterols

For purposes of the present invention, the term "Sterol side chain" refers to a chemical composition of a side chain attached at the position 17 of sterol-type molecule. In a standard definition sterols are limited to a 4 ring structure carrying a 8 carbon chain at position 17. In this invention, the sterol type molecules with side chain longer and shorter than conventional are described. The side chain may branched or contain double back bones.

Thus, sterols useful in the invention, for example, include cholesterols, as well as unique sterols in which position 17 has attached side chain of 2-7 or longer than 9 carbons. In a particular embodiment, the length of the polycarbon tail is varied between 5 and 9 carbons. Such conjugates may have significantly better *in vivo* efficacy, in particular delivery to liver. These types of molecules are expected to work at concentrations 5 to 9 fold lower then oligonucleotides conjugated to conventional cholesterols.

Alternatively the polynucleotide may be bound to a protein, peptide or positively charged chemical that functions as the hydrophobic molecule. The proteins may be selected from the group consisting of protamine, dsRNA binding domain, and arginine rich peptides. Exemplary positively charged chemicals include spermine, spermidine, cadaverine, and putrescine.

In another embodiment hydrophobic molecule conjugates may demonstrate even higher efficacy when it is combined with optimal chemical modification patterns of the polynucleotide (as described herein in detail), containing but not limited to hydrophobic modifications, phosphorothioate modifications, and 2' ribo modifications.

In another embodiment the sterol type molecule may be a naturally occurring PhytoSterols. The polycarbon chain may be longer than 9 and may be linear, branched and/or contain double bonds. Some PhytoSterol containing polynucleotide conjugates may be significantly more potent and active in delivery of polynucleotides to various tissues. Some PhytoSterols may demonstrate tissue preference and thus be used as a way to delivery RNAi specifically to particular tissues.

The hydrophobic modified polynucleotide is mixed with a neutral fatty mixture to form a micelle. The neutral fatty acid mixture is a mixture of fats that has a net neutral or slightly net negative charge at or around physiological pH that can form a micelle with the hydrophobic modified polynucleotide. For purposes of the present invention, the term "micelle" refers to a small nanoparticle formed by a mixture of non-charged fatty acids and phospholipids. The neutral fatty mixture may include cationic lipids as long as they are present in an amount that does not cause toxicity. In preferred embodiments the neutral fatty mixture is free of cationic lipids. A mixture that is free of cationic lipids is one that has less than 1% and preferably 0% of the total lipid being cationic lipid. The term "cationic lipid" includes lipids and synthetic lipids having a net positive charge at or around physiological pH. The term "anionic lipid" includes lipids and synthetic lipids having a net negative charge at or around physiological pH.

The neutral fats bind to the oligonucleotides of the invention by a strong but non-covalent attraction (*e.g.*, an electrostatic, van der Waals, pi-stacking, *etc.* interaction).

The neutral fat mixture may include formulations selected from a class of naturally occurring or chemically synthesized or modified saturated and unsaturated fatty acid residues. Fatty acids might exist in a form of triglycerides, diglycerides or individual fatty acids. In another embodiment the use of well-validated mixtures of fatty acids and/or fat emulsions currently used in pharmacology for parenteral nutrition may be utilized.

The neutral fatty mixture is preferably a mixture of a choline based fatty acid and a sterol. Choline based fatty acids include for instance, synthetic phosphocholine derivatives such as DDPC, DLPC, DMPC, DPPC, DSPC, DOPC, POPC, and DEPC. DOPC (chemical registry number 4235-95-4) is dioleoylphosphatidylcholine (also known as dielaidoylphosphatidylcholine, dioleoyl-PC, dioleoylphosphocholine, dioleoyl-sn-glycero-3-phosphocholine, dioleylphosphatidylcholine). DSPC (chemical registry number 816-94-4) is distearoylphosphatidylcholine (also known as 1,2-Distearoyl-sn-Glycero-3-phosphocholine).

The sterol in the neutral fatty mixture may be for instance cholesterol. The neutral fatty mixture may be made up completely of a choline based fatty acid and a sterol or it may optionally include a cargo molecule. For instance, the neutral fatty mixture may have at least 20% or 25% fatty acid and 20% or 25% sterol.

For purposes of the present invention, the term "Fatty acids" relates to conventional description of fatty acid. They may exist as individual entities or in a form of two-and triglycerides. For purposes of the present invention, the term "fat emulsions" refers to safe fat formulations given intravenously to subjects who are unable to get enough fat in their diet. It is an emulsion of soy bean oil (or other naturally occurring oils) and egg phospholipids. Fat emulsions are being used for formulation of some insoluble anesthetics. In this disclosure, fat emulsions might be part of commercially available preparations like Intralipid, Liposyn, Nutrilipid, modified commercial preparations, where they are enriched with particular fatty acids or fully de novo- formulated combinations of fatty acids and phospholipids.

In one embodiment, the cells to be contacted with an oligonucleotide composition of the invention are contacted with a mixture comprising the oligonucleotide and a mixture comprising a lipid, *e.g.*, one of the lipids or lipid compositions described supra for between about 12 hours to about 24 hours. In another embodiment, the cells to be contacted with an oligonucleotide composition are contacted with a mixture comprising the oligonucleotide and a mixture comprising a lipid, *e.g.*, one of the lipids or lipid compositions described supra for between about 1 and about five days. In one embodiment, the cells are contacted with a mixture comprising a lipid and the oligonucleotide for between about three days to as long as about 30 days. In another embodiment, a mixture comprising a lipid is left in contact with the cells for at least about five to about 20 days. In another embodiment, a mixture comprising a lipid is left in contact with the cells for at least about seven to about 15 days.

50%-60% of the formulation can optionally be any other lipid or molecule. Such a lipid or molecule is referred to herein as a cargo lipid or cargo molecule. Cargo molecules include but are not limited to intralipid, small molecules, fusogenic peptides or lipids or other small molecules might be added to alter cellular uptake, endosomal release or tissue distribution properties. The ability to tolerate cargo molecules is important for modulation of properties of these particles, if such properties are desirable. For instance the presence of some tissue specific metabolites might drastically alter tissue distribution profiles. For example use of Intralipid type formulation enriched in shorter or longer fatty chains with various degrees of saturation affects tissue distribution profiles of these type of formulations (and their loads).

An example of a cargo lipid useful according to the invention is a fusogenic lipid. For instance, the zwiterionic lipid DOPE (chemical registry number 4004-5-1, 1,2-Dioleoyl-sn-Glycero-3-phosphoethanolamine) is a preferred cargo lipid.

Intralipid may be comprised of the following composition: 1 000 mL contain: purified soybean oil 90 g, purified egg phospholipids 12 g, glycerol anhydrous 22 g, water for injection q.s. ad 1 000 mL. pH is adjusted with sodium hydroxide to pH approximately 8. Energy content/L: 4.6 MJ (190 kcal). Osmolality (approx.): 300 mOsm/kg water. In another embodiment fat emulsion is Liposyn that contains 5% safflower oil, 5% soybean oil, up to 1.2% egg phosphatides added as an emulsifier and 2.5% glycerin in water for injection. It may also contain sodium hydroxide for pH adjustment. pH 8.0 (6.0 - 9.0). Liposyn has an osmolarity of 276 m Osmol/liter (actual).

Variation in the identity, amounts and ratios of cargo lipids affects the cellular uptake and tissue distribution characteristics of these compounds. For example, the length of lipid tails and level of saturability will affect differential uptake to liver, lung, fat and cardiomyocytes. Addition of special hydrophobic molecules like vitamins or different forms of sterols can favor distribution to special tissues which are involved in the metabolism of particular compounds. In some embodiments, vitamin A or E is used. Complexes are formed at different oligonucleotide concentrations, with higher concentrations favoring more efficient complex formation.

In another embodiment, the fat emulsion is based on a mixture of lipids. Such lipids may include natural compounds, chemically synthesized compounds, purified fatty acids or any other lipids. In yet another embodiment the composition of fat emulsion is entirely artificial. In a particular embodiment, the fat emulsion is more than 70% linoleic acid. In yet another particular embodiment the fat emulsion is at least 1% of cardiolipin. Linoleic acid (LA) is an unsaturated omega-6 fatty acid. It is a colorless liquid made of a carboxylic acid with an 18-carbon chain and two cis double bonds.

In yet another embodiment of the present invention, the alteration of the composition of the fat emulsion is used as a way to alter tissue distribution of hydrophobicly modified polynucleotides. This methodology provides for the specific delivery of the polynucleotides to particular tissues.

In another embodiment the fat emulsions of the cargo molecule contain more than 70% of Linoleic acid ($C_{18}H_{32}O_2$) and/or cardiolipin.

Fat emulsions, like intralipid have been used before as a delivery formulation for some non-water soluble drugs (such as Propofol, re-formulated as Diprivan). Unique features of the present invention include (a) the concept of combining modified polynucleotides with the hydrophobic compound(s), so it can be incorporated in the fat micelles and (b) mixing it with the fat emulsions to provide a reversible carrier. After injection into a blood stream, micelles usually bind to serum proteins, including albumin, HDL, LDL and other. This binding is reversible and eventually the fat is absorbed by cells. The polynucleotide, incorporated as a part of the micelle will then be delivered closely to the surface of the cells. After that cellular uptake might be happening though variable mechanisms, including but not limited to sterol type delivery.

Complexing Agents

Complexing agents bind to the oligonucleotides of the invention by a strong but non-covalent attraction (*e.g.*, an electrostatic, van der Waals, pi-stacking, *etc.* interaction). In one embodiment, oligonucleotides of the invention can be complexed with a complexing agent to increase cellular uptake of oligonucleotides. An example of a complexing agent includes cationic lipids. Cationic lipids can be used to deliver oligonucleotides to cells. However, as discussed above, formulations free in cationic lipids are preferred in some embodiments.

The term "cationic lipid" includes lipids and synthetic lipids having both polar and non-polar domains and which are capable of being positively charged at or around physiological pH and which bind to polyanions, such as nucleic acids, and facilitate the delivery of nucleic acids into cells. In general cationic lipids include saturated and unsaturated alkyl and alicyclic ethers and esters of amines, amides, or derivatives thereof. Straight-chain and branched alkyl and alkenyl groups of cationic lipids can contain, *e.g.*, from 1 to about 25 carbon atoms. Preferred straight chain or branched alkyl or alkene groups have six or more carbon atoms. Alicyclic groups include cholesterol and other steroid groups. Cationic lipids can be prepared with a variety of counterions (anions) including, *e.g.*, Cl^-, Br^-, I^-, F^-, acetate, trifluoroacetate, sulfate, nitrite, and nitrate.

Examples of cationic lipids include polyethylenimine, polyamidoamine (PAMAM) starburst dendrimers, Lipofectin (a combination of DOTMA and DOPE), Lipofectase, LIPOFECTAMINE™ (*e.g.*, LIPOFECTAMINE™ 2000), DOPE, Cytofectin (Gilead Sciences, Foster City, Calif.), and Eufectins (JBL, San Luis Obispo, Calif.). Exemplary cationic liposomes can be made from N-[1-(2,3-dioleoloxy)-propyl]-N,N,N-trimethylammonium chloride (DOTMA), N-[1 -(2,3-dioleoloxy)-propyl]-N,N,N-trimethylammonium methylsulfate (DOTAP), 3β-[N-(N',N'-dimethylaminoethane)carbamoyl]cholesterol (DC-Chol), 2,3,-dioleyloxy-N-[2(sperminecarboxamido)ethyl]-N,N-dimethyl-1-propanaminium trifluoroacetate (DOSPA), 1,2-dimyristyloxypropyl-3-dimethyl-hydroxyethyl ammonium bromide; and dimethyldioctadecylammonium bromide (DDAB). The cationic lipid N-(1-(2,3-dioleyloxy)propyl)-N,N,N-trimethylammonium chloride (DOTMA), for example, was found to increase 1000-fold the antisense effect of a phosphorothioate oligonucleotide. (Vlassov *et al.*, 1994, Biochimica et Biophysica Acta 1197:95-108). Oligonucleotides can also be complexed with, *e.g.*, poly (L-lysine) or avidin and lipids may, or may not, be included in this mixture, *e.g.*, steryl-poly (L-lysine).

Cationic lipids have been used in the art to deliver oligonucleotides to cells (see, *e.g.*, U.S. Pat. Nos. 5,855,910; 5,851,548; 5,830,430; 5,780,053; 5,767,099; Lewis *et al.* 1996. *Proc. Natl. Acad. Sci. USA* 93:3176; Hope *et al.* 1998. *Molecular Membrane Biology* 15:1). Other lipid compositions which can be used to facilitate uptake of the instant oligonucleotides can be used in connection with the claimed methods. In addition to those listed supra, other lipid compositions are also known in the art and include, *e.g.*, those taught in U.S. Pat. No. 4,235,871; U.S. Pat. Nos. 4,501,728; 4,837,028; 4,737,323.

In one embodiment lipid compositions can further comprise agents, *e.g.*, viral proteins to enhance lipid-mediated transfections of oligonucleotides (Kamata, *et al*., 1994. *Nucl. Acids. Res.* 22:536). In another embodiment, oligonucleotides are contacted with cells as part of a composition comprising an oligonucleotide, a peptide, and a lipid as taught, *e.g.*, in U.S. patent 5,736,392. Improved lipids have also been described which are serum resistant (Lewis, *et al*., 1996. *Proc. Natl. Acad. Sci.* 93:3176). Cationic lipids and other complexing agents act to increase the number of oligonucleotides carried into the cell through endocytosis.

In another embodiment N-substituted glycine oligonucleotides (peptoids) can be used to optimize uptake of oligonucleotides. Peptoids have been used to create cationic lipid-like compounds for transfection (Murphy, *et al*., 1998. *Proc. Natl. Acad. Sci.* 95:1517). Peptoids can be synthesized using standard methods (*e.g.*, Zuckermann, R. N., *et al*. 1992. *J. Am. Chem. Soc.* 114:10646; Zuckermann, R. N., *et al*. 1992. *Int. J. Peptide Protein Res.* 40:497). Combinations of cationic lipids and peptoids, liptoids, can also be used to optimize uptake of the subject oligonucleotides (Hunag, *et al*., 1998. *Chemistry and Biology.* 5:345). Liptoids can be synthesized by elaborating peptoid oligonucleotides and coupling the amino terminal submonomer to a lipid via its amino group (Hunag, *et al*., 1998. *Chemistry and Biology.* 5:345).

It is known in the art that positively charged amino acids can be used for creating highly active cationic lipids (Lewis *et al*. 1996. *Proc. Natl. Acad. Sci. US.A.* 93:3176). In one embodiment, a composition for delivering oligonucleotides of the invention comprises a number of arginine, lysine, histidine or ornithine residues linked to a lipophilic moiety (see *e.g.*, U.S. Pat. No. 5,777,153).

In another embodiment, a composition for delivering oligonucleotides of the invention comprises a peptide having from between about one to about four basic residues. These basic residues can be located, *e.g.*, on the amino terminal, C-terminal, or internal region of the peptide. Families of amino acid residues having similar side chains have been defined in the art. These families include amino acids with basic side chains (*e.g.*, lysine, arginine, histidine), acidic side chains (*e.g.*, aspartic acid, glutamic acid), uncharged polar side chains (*e.g.*, glycine (can also be considered non-polar), asparagine, glutamine, serine, threonine, tyrosine, cysteine), nonpolar side chains (*e.g.*, alanine, valine, leucine, isoleucine, proline, phenylalanine, methionine, tryptophan), beta-branched side chains (*e.g.*, threonine, valine, isoleucine) and aromatic side chains (*e.g.*, tyrosine, phenylalanine, tryptophan, histidine). Apart from the basic amino acids, a majority or all of the other residues of the peptide can be selected from the non-basic amino acids, *e.g.*, amino acids other than lysine, arginine, or histidine. Preferably a preponderance of neutral amino acids with long neutral side chains are used.

In one embodiment, a composition for delivering oligonucleotides of the invention comprises a natural or synthetic polypeptide having one or more gamma carboxyglutamic acid residues, or γ-Gla residues. These gamma carboxyglutamic acid residues may enable the polypeptide to bind to each other and to membrane surfaces. In other words, a polypeptide having a series of γ-Gla may be used as a general delivery modality that helps an RNAi construct to stick to whatever membrane to which it comes in contact. This may at least slow RNAi constructs from being cleared from the blood stream and enhance their chance of homing to the target.

The gamma carboxyglutamic acid residues may exist in natural proteins (for example, prothrombin has 10 γ-Gla residues). Alternatively, they can be introduced into the purified, recombinantly produced, or chemically synthesized polypeptides by carboxylation using, for example, a vitamin K-dependent carboxylase. The gamma carboxyglutamic acid residues may be consecutive or non-consecutive, and the total number and location of such gamma carboxyglutamic acid residues in the polypeptide can be regulated / fine-tuned to achieve different levels of "stickiness" of the polypeptide.

In one embodiment, the cells to be contacted with an oligonucleotide composition of the invention are contacted with a mixture comprising the oligonucleotide and a mixture comprising a lipid, *e.g.*, one of the lipids or lipid compositions described supra for between about 12 hours to about 24 hours. In another embodiment, the cells to be contacted with an oligonucleotide composition are contacted with a mixture comprising the oligonucleotide and a mixture comprising a lipid, *e.g.*, one of the lipids or lipid compositions described supra for between about 1 and about five days. In one embodiment, the cells are contacted with a mixture comprising a lipid and the oligonucleotide for between about three days to as long as about 30 days. In another embodiment, a mixture comprising a lipid is left in contact with the cells for at least about five to about 20 days. In another embodiment, a mixture comprising a lipid is left in contact with the cells for at least about seven to about 15 days.

For example, in one embodiment, an oligonucleotide composition can be contacted with cells in the presence of a lipid such as cytofectin CS or GSV (available from Glen Research; Sterling, Va.), GS3815, GS2888 for prolonged incubation periods as described herein.

In one embodiment, the incubation of the cells with the mixture comprising a lipid and an oligonucleotide composition does not reduce the viability of the cells. Preferably, after the transfection period the cells are substantially viable. In one embodiment, after transfection, the cells are between at least about 70% and at least about 100% viable. In another embodiment, the cells are between at least about 80% and at least about 95% viable. In yet another embodiment, the cells are between at least about 85% and at least about 90% viable.

In one embodiment, oligonucleotides are modified by attaching a peptide sequence that transports the oligonucleotide into a cell, referred to herein as a "transporting peptide." In one embodiment, the composition includes an oligonucleotide which is complementary to a target nucleic acid molecule encoding the protein, and a covalently attached transporting peptide.

The language "transporting peptide" includes an amino acid sequence that facilitates the transport of an oligonucleotide into a cell. Exemplary peptides which facilitate the transport of the moieties to which they are linked into cells are known in the art, and include, *e.g.*, HIV TAT transcription factor, lactoferrin, Herpes VP22 protein, and fibroblast growth factor 2 (Pooga *et al.* 1998. *Nature Biotechnology*. 16:857; and Derossi *et al.* 1998. *Trends in Cell Biology*. 8:84; Elliott and O'Hare. 1997. Cell 88:223).

Oligonucleotides can be attached to the transporting peptide using known techniques, *e.g.*, (Prochiantz, A. 1996. *Curr. Opin. Neurobiol.* 6:629; Derossi *et al.* 1998. *Trends Cell Biol.* 8:84; Troy *et al.* 1996. *J. Neurosci.* 16:253), Vives *et al.* 1997. *J. Biol. Chem.* 272:16010). For example, in one embodiment, oligonucleotides bearing an activated thiol group are linked via that thiol group to a cysteine present in a transport peptide (*e.g.*, to the cysteine present in the β turn between the second and the third helix of the antennapedia homeodomain as taught, *e.g.*, in Derossi *et al.* 1998. *Trends Cell Biol.* 8:84; Prochiantz. 1996. *Current Opinion in Neurobiol.* 6:629; Allinquant *et al.* 1995. J Cell Biol. 128:919). In another embodiment, a Boc-Cys-(Npys)OH group can be coupled to the transport peptide as the last (N-terminal) amino acid and an oligonucleotide bearing an SH group can be coupled to the peptide (Troy *et al.* 1996. *J. Neurosci.* 16:253).

In one embodiment, a linking group can be attached to a nucleomonomer and the transporting peptide can be covalently attached to the linker. In one embodiment, a linker can function as both an attachment site for a transporting peptide and can provide stability against nucleases. Examples of suitable linkers include substituted or unsubstituted C_1-C_{20} alkyl chains, C_2-C_{20} alkenyl chains, C_2-C_{20} alkynyl chains, peptides, and heteroatoms (*e.g.*, S, O, NH, *etc.*). Other exemplary linkers include bifinctional crosslinking agents such as sulfosuccinimidyl-4-(maleimidophenyl)-butyrate (SMPB) (see, *e.g.*, Smith *et al.* Biochem J 1991.276: 417-2).

In one embodiment, oligonucleotides of the invention are synthesized as molecular conjugates which utilize receptor-mediated endocytotic mechanisms for delivering genes into cells (see, *e.g.*, Bunnell *et al.* 1992. *Somatic Cell and Molecular Genetics.* 18:559, and the references cited therein).

Targeting Agents

The delivery of oligonucleotides can also be improved by targeting the oligonucleotides to a cellular receptor. The targeting moieties can be conjugated to the oligonucleotides or attached to a carrier group (*i.e.*, poly(L-lysine) or liposomes) linked to the oligonucleotides. This method is well suited to cells that display specific receptor-mediated endocytosis.

For instance, oligonucleotide conjugates to 6-phosphomannosylated proteins are internalized 20-fold more efficiently by cells expressing mannose 6-phosphate specific receptors than free oligonucleotides. The oligonucleotides may also be coupled to a ligand for a cellular receptor using a biodegradable linker. In another example, the delivery construct is mannosylated streptavidin which forms a tight complex with biotinylated oligonucleotides. Mannosylated streptavidin was found to increase 20-fold the internalization of biotinylated oligonucleotides. (Vlassov *et al.* 1994. *Biochimica et Biophysica Acta* 1197:95-108).

In addition specific ligands can be conjugated to the polylysine component of polylysine-based delivery systems. For example, transferrin-polylysine, adenovirus-polylysine, and influenza virus hemagglutinin HA-2 N-terminal fusogenic peptides-polylysine conjugates greatly enhance receptor-mediated DNA delivery in eucaryotic cells. Mannosylated glycoprotein conjugated to poly(L-lysine) in aveolar macrophages has been employed to enhance the cellular uptake of oligonucleotides. Liang *et al.* 1999. *Pharmazie* 54:559-566.

Because malignant cells have an increased need for essential nutrients such as folic acid and transferrin, these nutrients can be used to target oligonucleotides to cancerous cells. For example, when folic acid is linked to poly(L-lysine) enhanced oligonucleotide uptake is seen in promyelocytic leukaemia (HL-60) cells and human melanoma (M-14) cells. Ginobbi *et al.* 1997. *Anticancer Res.* 17:29. In another example, liposomes coated with maleylated bovine serum albumin, folic acid, or ferric protoporphyrin IX, show enhanced cellular uptake of oligonucleotides in murine macrophages, KB cells, and 2.2.15 human hepatoma cells. Liang *et al.* 1999. *Pharmazie* 54:559-566.

Liposomes naturally accumulate in the liver, spleen, and reticuloendothelial system (so-called, passive targeting). By coupling liposomes to various ligands such as antibodies are protein A, they can be actively targeted to specific cell populations. For example, protein A-bearing liposomes may be pretreated with H-2K specific antibodies which are targeted to the mouse major histocompatibility complex-encoded H-2K protein expressed on L cells. (Vlassov *et al.* 1994. *Biochimica et Biophysica Acta* 1197:95-108).

Other *in vitro* and/or *in vivo* delivery of RNAi reagents are known in the art, and can be used to deliver the subject RNAi constructs. See, for example, U.S. patent application publications 20080152661, 20080112916, 20080107694, 20080038296, 20070231392, 20060240093, 20060178327, 20060008910, 20050265957, 20050064595, 20050042227, 20050037496, 20050026286, 20040162235, 20040072785, 20040063654, 20030157030, WO 2008/036825, WO04/065601, and AU2004206255B2, just to name a few (all incorporated by reference).

Administration

The optimal course of administration or delivery of the oligonucleotides may vary depending upon the desired result and/or on the subject to be treated. As used herein "administration" refers to contacting cells with oligonucleotides and can be performed *in vitro* or *in vivo*. The dosage of oligonucleotides may be adjusted to optimally reduce expression of a protein translated from a target nucleic acid molecule, *e.g.*, as measured by a readout of RNA stability or by a therapeutic response, without undue experimentation.

For example, expression of the protein encoded by the nucleic acid target can be measured to determine whether or not the dosage regimen needs to be adjusted accordingly. In addition, an increase or decrease in RNA or protein levels in a cell or produced by a cell can be measured using any art recognized technique. By determining whether transcription has been decreased, the effectiveness of the oligonucleotide in inducing the cleavage of a target RNA can be determined.

Any of the above-described oligonucleotide compositions can be used alone or in conjunction with a pharmaceutically acceptable carrier. As used herein, "pharmaceutically acceptable carrier" includes appropriate solvents, dispersion media, coatings, antibacterial and antifungal agents, isotonic and absorption delaying agents, and the like. The use of such media and agents for pharmaceutical active substances is well known in the art. Except insofar as any conventional media or agent is incompatible with the active ingredient, it can be used in the therapeutic compositions. Supplementary active ingredients can also be incorporated into the compositions.

In some embodiments, the disclosure relates to a composition (*e.g.*, pharmaceutical composition) comprising an oligonucleotide (*e.g.*, an isolated double stranded nucleic acid molecule). In some embodiments, the composition comprises an additional therapeutic agent. Non-limiting examples of additional therapeutic agents include but are not limited to nucleic acids (*e.g.*, sd-rxRNA, *etc.*), small molecules (*e.g.*, small molecules useful for treating cancer, neurodegenerative diseases, infectious diseases, autoimmune diseases, *etc.*), peptides (*e.g.*, peptides useful for treating cancer, neurodegenerative diseases, infectious diseases, autoimmune diseases, *etc.*), and polypeptides (*e.g.*, antibodies useful for treating cancer, neurodegenerative diseases, infectious diseases, autoimmune diseases, *etc.*). Compositions of the disclosure can have, in some embodiments, 2, 3, 4, 5, 6, 7, 8, 9, 10, or more additional therapeutic agents. In some embodiments, a composition comprises more than 10 additional therapeutic agents.

Oligonucleotides may be incorporated into liposomes or liposomes modified with polyethylene glycol or admixed with cationic lipids for parenteral administration. Incorporation of additional substances into the liposome, for example, antibodies reactive against membrane proteins found on specific target cells, can help target the oligonucleotides to specific cell types.

With respect to *in vivo* applications, the formulations of the present invention can be administered to a patient in a variety of forms adapted to the chosen route of administration, *e.g.*, parenterally, orally, or intraperitoneally, infusion, intrathecal delivery, parenchymal delivery, intravenous delivery or direct injection into the brain or spinal cord.

Aspects of the invention relate to delivery of nucleic acid molecules, such as sd-rxRNA or sd-rxRNA variants to the nervous system. For example, an sd-rxRNA or sd-rxRNA variant can be delivered to the brain or to the spinal cord. Any appropriate delivery mechanism for delivering an sd-rxRNA variant to the brain or spinal cord can be applied. In some embodiments, delivery to the brain or spinal cord occurs by infusion, intrathecal delivery, parenchymal delivery, intravenous delivery or direct injection into the brain or spinal cord. In some embodiments, an sd-rxRNA or an sd-rxRNA variant is delivered to a specific region of the brain. An sd-rxRNA or an sd-rxRNA variant can be modified or formulated appropriately to pass the blood-brain barrier. In other embodiments, an sd-rxRNA or an sd-rxRNA variant is administered in such a way that it does not need to cross the blood-brain barrier. In some embodiments, the sd-rxRNA or an sd-rxRNA variant is delivered by a pump or catheter system into the brain or spinal cord. Examples of such delivery are incorporated by reference from US Patent No. 6,093,180 (Elsberry). Techniques for infusing drugs into the brain are also incorporated by reference from US Patent No. 5,814014 (Elsberry et al.).

In some embodiments, nucleic acids are administered by parenteral administration, which includes administration by the following routes: intravenous; intramuscular; interstitially; intraarterially; subcutaneous; intra ocular; intrasynovial; trans epithelial, including transdermal; pulmonary via inhalation; ophthalmic; sublingual and buccal; topically, including ophthalmic; dermal; ocular; rectal; and nasal inhalation via insufflation. In some embodiments, the sd-rxRNA molecules are administered by intradermal injection or subcutaneously.

Pharmaceutical preparations for administration can include aqueous solutions of the active compounds in water-soluble or water-dispersible form. In addition, suspensions of the active compounds as appropriate oily injection suspensions may be administered. Suitable lipophilic solvents or vehicles include fatty oils, for example, sesame oil, or synthetic fatty acid esters, for example, ethyl oleate or triglycerides. Aqueous injection suspensions may contain substances which increase the viscosity of the suspension include, for example, sodium carboxymethyl cellulose, sorbitol, or dextran, optionally, the suspension may also contain stabilizers. The oligonucleotides of the invention can be formulated in liquid solutions, preferably in physiologically compatible buffers such as Hank's solution or Ringer's solution. In addition, the oligonucleotides may be formulated in solid form and redissolved or suspended immediately prior to use. Lyophilized forms are also included in the invention.

Pharmaceutical preparations for administration can also include transdermal patches, ointments, lotions, creams, gels, drops, sprays, suppositories, liquids and powders. In addition, conventional pharmaceutical carriers, aqueous, powder or oily bases, or thickeners may be used in pharmaceutical preparations for topical administration.

Pharmaceutical preparations for oral administration can also include powders or granules, suspensions or solutions in water or non-aqueous media, capsules, sachets or tablets. In addition, thickeners, flavoring agents, diluents, emulsifiers, dispersing aids, or binders may be used in pharmaceutical preparations for oral administration.

For transmucosal or transdermal administration, penetrants appropriate to the barrier to be permeated are used in the formulation. Such penetrants are known in the art, and include, for example, for transmucosal administration bile salts and fusidic acid derivatives, and detergents. Transmucosal administration may be through nasal sprays or using suppositories. For oral administration, the oligonucleotides are formulated into conventional oral administration forms such as capsules, tablets, and tonics. For topical administration, the oligonucleotides of the invention are formulated into ointments, salves, gels, or creams as known in the art.

Drug delivery vehicles can be chosen *e.g.*, for *in vitro*, for systemic, or for topical administration. These vehicles can be designed to serve as a slow release reservoir or to deliver their contents directly to the target cell. An advantage of using some direct delivery drug vehicles is that multiple molecules are delivered per uptake. Such vehicles have been shown to increase the circulation half-life of drugs that would otherwise be rapidly cleared from the blood stream. Some examples of such specialized drug delivery vehicles which fall into this category are liposomes, hydrogels, cyclodextrins, biodegradable nanocapsules, and bioadhesive microspheres.

The described oligonucleotides may be administered systemically to a subject. Systemic absorption refers to the entry of drugs into the blood stream followed by distribution throughout the entire body. Administration routes which lead to systemic absorption include: intravenous, subcutaneous, intraperitoneal, and intranasal. Each of these administration routes delivers the oligonucleotide to accessible diseased cells. Following subcutaneous administration, the therapeutic agent drains into local lymph nodes and proceeds through the lymphatic network into the circulation. The rate of entry into the circulation has been shown to be a function of molecular weight or size. The use of a liposome or other drug carrier localizes the oligonucleotide at the lymph node. The oligonucleotide can be modified to diffuse into the cell, or the liposome can directly participate in the delivery of either the unmodified or modified oligonucleotide into the cell.

The chosen method of delivery will result in entry into cells. In some embodiments, preferred delivery methods include liposomes (10-400 nm), hydrogels, controlled-release polymers, and other pharmaceutically applicable vehicles, and microinjection or electroporation (for *ex vivo* treatments).

The pharmaceutical preparations of the present invention may be prepared and formulated as emulsions. Emulsions are usually heterogeneous systems of one liquid dispersed in another in the form of droplets usually exceeding 0.1 μm in diameter. The emulsions of the present invention may contain excipients such as emulsifiers, stabilizers, dyes, fats, oils, waxes, fatty acids, fatty alcohols, fatty esters, humectants, hydrophilic colloids, preservatives, and anti-oxidants may also be present in emulsions as needed. These excipients may be present as a solution in either the aqueous phase, oily phase or itself as a separate phase.

Examples of naturally occurring emulsifiers that may be used in emulsion formulations of the present invention include lanolin, beeswax, phosphatides, lecithin and acacia. Finely divided solids have also been used as good emulsifiers especially in combination with surfactants and in viscous preparations. Examples of finely divided solids that may be used as emulsifiers include polar inorganic solids, such as heavy metal hydroxides, nonswelling clays such as bentonite, attapulgite, hectorite, kaolin, montrnorillonite, colloidal aluminum silicate and colloidal magnesium aluminum silicate, pigments and nonpolar solids such as carbon or glyceryl tristearate.

Examples of preservatives that may be included in the emulsion formulations include methyl paraben, propyl paraben, quaternary ammonium salts, benzalkonium chloride, esters of p-hydroxybenzoic acid, and boric acid. Examples of antioxidants that may be included in the emulsion formulations include free radical scavengers such as tocopherols, alkyl gallates, butylated hydroxyanisole, butylated hydroxytoluene, or reducing agents such as ascorbic acid and sodium metabisulfite, and antioxidant synergists such as citric acid, tartaric acid, and lecithin.

In one embodiment, the compositions of oligonucleotides are formulated as microemulsions. A microemulsion is a system of water, oil and amphiphile which is a single optically isotropic and thermodynamically stable liquid solution. Typically microemulsions are prepared by first dispersing an oil in an aqueous surfactant solution and then adding a sufficient amount of a 4th component, generally an intermediate chain-length alcohol to form a transparent system.

Surfactants that may be used in the preparation of microemulsions include, but are not limited to, ionic surfactants, non-ionic surfactants, Brij 96, polyoxyethylene oleyl ethers, polyglycerol fatty acid esters, tetraglycerol monolaurate (ML310), tetraglycerol monooleate (MO310), hexaglycerol monooleate (PO310), hexaglycerol pentaoleate (PO500), decaglycerol monocaprate (MCA750), decaglycerol monooleate (MO750), decaglycerol sequioleate (S0750), decaglycerol decaoleate (DA0750), alone or in combination with cosurfactants. The cosurfactant, usually a short-chain alcohol such as ethanol, 1-propanol, and 1-butanol, serves to increase the interfacial fluidity by penetrating into the surfactant film and consequently creating a disordered film because of the void space generated among surfactant molecules.

Microemulsions may, however, be prepared without the use of cosurfactants and alcohol-free self-emulsifying microemulsion systems are known in the art. The aqueous phase may typically be, but is not limited to, water, an aqueous solution of the drug, glycerol, PEG300, PEG400, polyglycerols, propylene glycols, and derivatives of ethylene glycol. The oil phase may include, but is not limited to, materials such as Captex 300, Captex 355, Capmul MCM, fatty acid esters, medium chain (C_8-C_{12}) mono, di, and tri-glycerides, polyoxyethylated glyceryl fatty acid esters, fatty alcohols, polyglycolized glycerides, saturated polyglycolized C_8-C_{10} glycerides, vegetable oils and silicone oil.

Microemulsions are particularly of interest from the standpoint of drug solubilization and the enhanced absorption of drugs. Lipid based microemulsions (both oil/water and water/oil) have been proposed to enhance the oral bioavailability of drugs.

Microemulsions offer improved drug solubilization, protection of drug from enzymatic hydrolysis, possible enhancement of drug absorption due to surfactant-induced alterations in membrane fluidity and permeability, ease of preparation, ease of oral administration over solid dosage forms, improved clinical potency, and decreased toxicity (Constantinides *et al.*, Pharmaceutical Research, 1994, 11:1385; Ho *et al.*, J. Pharm. Sci., 1996, 85:138-143). Microemulsions have also been effective in the transdermal delivery of active components in both cosmetic and pharmaceutical applications. It is expected that the microemulsion compositions and formulations of the present invention will facilitate the increased systemic absorption of oligonucleotides from the gastrointestinal tract, as well as improve the local cellular uptake of oligonucleotides within the gastrointestinal tract, vagina, buccal cavity and other areas of administration.

In an embodiment, the present invention employs various penetration enhancers to affect the efficient delivery of nucleic acids, particularly oligonucleotides, to the skin of animals. Even non-lipophilic drugs may cross cell membranes if the membrane to be crossed is treated with a penetration enhancer. In addition to increasing the diffusion of non-lipophilic drugs across cell membranes, penetration enhancers also act to enhance the permeability of lipophilic drugs.

Five categories of penetration enhancers that may be used in the present invention include: surfactants, fatty acids, bile salts, chelating agents, and non-chelating non-surfactants. Other agents may be utilized to enhance the penetration of the administered oligonucleotides include: glycols such as ethylene glycol and propylene glycol, pyrrols such as 2-15 pyrrol, azones, and terpenes such as limonene, and menthone.

The oligonucleotides, especially in lipid formulations, can also be administered by coating a medical device, for example, a catheter, such as an angioplasty balloon catheter, with a cationic lipid formulation. Coating may be achieved, for example, by dipping the medical device into a lipid formulation or a mixture of a lipid formulation and a suitable solvent, for example, an aqueous-based buffer, an aqueous solvent, ethanol, methylene chloride, chloroform and the like. An amount of the formulation will naturally adhere to the surface of the device which is subsequently administered to a patient, as appropriate. Alternatively, a lyophilized mixture of a lipid formulation may be specifically bound to the surface of the device. Such binding techniques are described, for example, in K. Ishihara *et al.*, Journal of Biomedical Materials Research, Vol. 27, pp. 1309-1314 (1993), the disclosures of which are incorporated herein by reference in their entirety.

The useful dosage to be administered and the particular mode of administration will vary depending upon such factors as the cell type, or for *in vivo* use, the age, weight and the particular animal and region thereof to be treated, the particular oligonucleotide and delivery method used, the therapeutic or diagnostic use contemplated, and the form of the formulation, for example, suspension, emulsion, micelle or liposome, as will be readily apparent to those skilled in the art. Typically, dosage is administered at lower levels and increased until the desired effect is achieved. When lipids are used to deliver the oligonucleotides, the amount of lipid compound that is administered can vary and generally depends upon the amount of oligonucleotide agent being administered. For example, the weight ratio of lipid compound to oligonucleotide agent is preferably from about 1:1 to about 15:1, with a weight ratio of about 5:1 to about 10:1 being more preferred. Generally, the amount of cationic lipid compound which is administered will vary from between about 0.1 milligram (mg) to about 1 gram (g). By way of general guidance, typically between about 0.1 mg and about 10 mg of the particular oligonucleotide agent, and about 1 mg to about 100 mg of the lipid compositions, each per kilogram of patient body weight, is administered, although higher and lower amounts can be used.

The agents of the invention are administered to subjects or contacted with cells in a biologically compatible form suitable for pharmaceutical administration. By "biologically compatible form suitable for administration" is meant that the oligonucleotide is administered in a form in which any toxic effects are outweighed by the therapeutic effects of the oligonucleotide. In one embodiment, oligonucleotides can be administered to subjects. Examples of subjects include mammals, *e.g.*, humans and other primates; cows, pigs, horses, and farming (agricultural) animals; dogs, cats, and other domesticated pets; mice, rats, and transgenic non-human animals.

Administration of an active amount of an oligonucleotide of the present invention is defined as an amount effective, at dosages and for periods of time necessary to achieve the desired result. For example, an active amount of an oligonucleotide may vary according to factors such as the type of cell, the oligonucleotide used, and for *in vivo* uses the disease state, age, sex, and weight of the individual, and the ability of the oligonucleotide to elicit a desired response in the individual. Establishment of therapeutic levels of oligonucleotides within the cell is dependent upon the rates of uptake and efflux or degradation. Decreasing the degree of degradation prolongs the intracellular half-life of the oligonucleotide. Thus, chemically-modified oligonucleotides, *e.g.*, with modification of the phosphate backbone, may require different dosing.

The exact dosage of an oligonucleotide and number of doses administered will depend upon the data generated experimentally and in clinical trials. Several factors such as the desired effect, the delivery vehicle, disease indication, and the route of administration, will affect the dosage. Dosages can be readily determined by one of ordinary skill in the art and formulated into the subject pharmaceutical compositions. Preferably, the duration of treatment will extend at least through the course of the disease symptoms.

Dosage regimens may be adjusted to provide the optimum therapeutic response. For example, the oligonucleotide may be repeatedly administered, *e.g.*, several doses may be administered daily or the dose may be proportionally reduced as indicated by the exigencies of the therapeutic situation. One of ordinary skill in the art will readily be able to determine appropriate doses and schedules of administration of the subject oligonucleotides, whether the oligonucleotides are to be administered to cells or to subjects.

Administration of sd-rxRNAs can be optimized through testing of dosing regimens. In some embodiments, a single administration is sufficient. To further prolong the effect of the administered sd-rxRNA, the sd-rxRNA can be administered in a slow-release formulation or device, as would be familiar to one of ordinary skill in the art. The hydrophobic nature of sd-rxRNA compounds can enable use of a wide variety of polymers, some of which are not compatible with conventional oligonucleotide delivery.

In other embodiments, the sd-rxRNA is administered multiple times. In some instances it is administered daily, bi-weekly, weekly, every two weeks, every three weeks, monthly, every two months, every three months, every four months, every five months, every six months or less frequently than every six months. In some instances, it is administered multiple times per day, week, month and/or year. For example, it can be administered approximately every hour, 2 hours, 3 hours, 4 hours, 5 hours, 6 hours, 7 hours, 8 hours, 9 hours 10 hours, 12 hours or more than twelve hours. It can be administered 1, 2, 3, 4, 5, 6, 7, 8, 9, 10 or more than 10 times per day.

Aspects of the invention relate to administering sd-rxRNA molecules to a subject. In some instances the subject is a patient and administering the sd-rxRNA molecule involves administering the sd-rxRNA molecule in a doctor's office.

In some embodiments, more than one sd-rxRNA molecule is administered simultaneously. For example a composition may be administered that contains 1, 2, 3, 4, 5, 6, 7, 8, 9, 10 or more than 10 different sd-rxRNA molecules. In certain embodiments, a composition comprises 2 or 3 different sd-rxRNA molecules. When a composition comprises more than one sd-rxRNA, the sd-rxRNA molecules within the composition can be directed to the same gene or to different genes.

In some instances, the effective amount of sd-rxRNA that is delivered is at least approximately 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36, 37, 38, 39, 40, 41, 42, 43, 44, 45, 46, 47, 48, 49, 50, 51, 52, 53, 54, 55, 56, 57, 58, 59, 60, 61, 62, 63, 64, 65, 66, 67, 68, 69, 70, 71, 72, 73, 74, 75, 76, 77, 78, 79, 80, 81, 82, 83, 84, 85, 86, 87, 88, 89, 90, 91, 92, 93, 94, 95, 96, 97, 98, 99, 100 or more than 100 mg/kg including any intermediate values.

In some instances, the effective amount of sd-rxRNA that is delivered is at least approximately 1, 5, 10, 15, 20, 25, 30, 35, 40, 45, 50, 55, 60, 65, 70, 75, 80, 85, 90, 95, 100, 125, 150, 200, 250, 300, 350, 400, 450, 500, 550, 600, 650, 700, 750, 800, 850, 900, 950 or more than 950 μg including any intermediate values.

sd-rxRNA molecules administered through methods described herein can be effectively targeted to all the cell types in the nervous system.

Various modalities of introducing nucleic acids into a subject (*e.g.*, a cell of a subject) are contemplated by the disclosure. For example, nucleic acids (*e.g.*, a solution containing the nucleic acids) can be injected into a subject (*e.g.*, injected into a cell) or a subject (*e.g.*, a cell) can be bombarded by particles covered by the nucleic acids. In some embodiments, the cell or organism is soaked in a solution of the nucleic acid. In some embodiments, a nucleic acid is introduced into an organism or cell by electroporation of cell membranes in the presence of the nucleic acid. In some embodiments, a viral construct comprising the nucleic acid is packaged into a viral particle and accomplishes introduction of the nucleic acid into the cell and transcription of nucleic acid. Further examples of modalities for introducing nucleic acids into a subject (*e.g.*, a cell of a subject) include but are not limited to lipid-mediated carrier transport, chemical-mediated transport (*e.g.*, calcium phosphate), etc.

Nucleic acids can be introduced with additional components. For example, in some embodiments, the nucleic acid is introduced with a component that enhances nucleic acid uptake by the cell. In some embodiments, the nucleic acid is introduced with a component that inhibits annealing of single strands. In some embodiments, the nucleic acid is introduced with a component that stabilizes the nucleic acid molecule, or other-wise increases inhibition of the target gene.

Nucleic acid may be directly introduced into the cell (i.e., intracellularly); or introduced extracellularly into a cavity, interstitial space, into the circulation of an organism, introduced orally, or may be introduced by bathing a cell or organism in a solution containing the nucleic acid. Vascular or extravascular circulation, the blood or lymph system, and the cerebrospinal fluid are sites where the nucleic acid may be introduced.

In some embodiments, the cell with the target gene may be derived from any organism. In some embodiments, the cell with the target gene may be contained in (*e.g.*, housed by, or present within) any organism. For example, the organism may a plant, animal, protozoan, bacterium, virus, or fungus. The plant may be a monocot, dicot or gymnosperm; the animal may be a vertebrate or invertebrate. Preferred microbes are those used in agriculture or by industry, and those that are pathogenic for plants or animals.

Alternatively, vectors, e.g., transgenes encoding a siRNA of the invention can be engineered into a host cell or transgenic animal using art recognized techniques.

A further preferred use for the agents of the present invention (or vectors or transgenes encoding same) is a functional analysis to be carried out in eukaryotic cells, or eukaryotic non-human organisms, preferably mammalian cells or organisms and most preferably human cells, e.g. cell lines such as HeLa or 293 or rodents, e.g. rats and mice. By administering a suitable priming agent/RNAi agent which is sufficiently complementary to a target mRNA sequence to direct target-specific RNA interference, a specific knockout or knockdown phenotype can be obtained in a target cell, e.g. in cell culture or in a target organism.

Thus, a further subject matter of the invention is a eukaryotic cell or a eukaryotic non-human organism exhibiting a target gene-specific knockout or knockdown phenotype comprising a fully or at least partially deficient expression of at least one endogenous target gene wherein said cell or organism is transfected with at least one vector comprising DNA encoding an RNAi agent capable of inhibiting the expression of the target gene. It should be noted that the present invention allows a target-specific knockout or knockdown of several different endogenous genes due to the specificity of the RNAi agent.

Gene-specific knockout or knockdown phenotypes of cells or non-human organisms, particularly of human cells or non-human mammals may be used in analytic to procedures, e.g. in the functional and/or phenotypical analysis of complex physiological processes such as analysis of gene expression profiles and/or proteomes. Preferably the analysis is carried out by high throughput methods using oligonucleotide based chips.

Therapeutic use

By inhibiting the expression of a gene, the oligonucleotide compositions of the present invention can be used to treat any disease involving the expression of a protein, such as neurodegenerative disease.

Aspects of the invention relate to the use of nucleic acid molecules, such as sd-rxRNA or sd-rxRNA variants sd-rxRNA in treatment of disorders affecting the nervous system. In some embodiments, the sd-rxRNA is used to treat a neurodegenerative disorder. As used herein, the term "neurodegenerative disorder" refers to disorders, diseases or conditions that are caused by the deterioration of cell and tissue components of the nervous system. Some non-limiting examples of neurodegenerative disorders include stroke, Alzheimer's disease, Parkinson's disease, Huntington's disease, Peri ventricular leukomalacia (PVL), amyotrophic lateral sclerosis (ALS, "Lou Gehrig's disease"), ALS-Parkinson's-Dementia complex of

Guam, Friedrich's Ataxia, Wilson's disease, multiple sclerosis, cerebral palsy, progressive supranuclear palsy (Steel-Richardson syndrome), bulbar and pseudobulbar palsy, diabetic retinopathy, multi-infarct dementia, macular degeneration, Pick's disease, diffuse Lewy body disease, prion diseases such as Creutzfeldt-Jakob, Gerstmann-Straussler-Scheinker disease, Kuru and fatal familial insomnia,

primary lateral sclerosis, degenerative ataxias, Machado-Joseph disease/spinocerebellar ataxia type 3 and olivopontocerebellar degenerations, spinal and spinobulbar muscular atrophy (Kennedy's disease), familial spastic paraplegia, Wohlfart-Kugelberg-Welander disease, Tay-Sach's disease, multisystem degeneration (Shy-Drager syndrome), Gilles De La Tourette's disease, familial dysautonomia (Riley-Day syndrome), Kugelberg-Welander disease, subacute sclerosing panencephalitis, Werdnig-Hoffmann disease, synucleinopathies (including multiple system atrophy), Sandhoff disease, cortical basal degeneration, spastic paraparesis, primary progressive aphasia, progressive multifocal leukoencephalopathy, striatonigral degeneration, familial spastic disease, chronic epileptic conditions associated with neurodegeneration, Binswanger's disease, and dementia (including all underlying etiologies of dementia).

In some embodiments, the disorder is Parkinson's disease Huntington's or ALS. In certain embodiments, the disorder is ALS and the sd-rxRNA or sd-rxRNA variant targets SODl, a superoxide dismutase.

Neurodegenerative disorders may also be the result of a brain injury or trauma including that which is caused by a stroke, an injury to the head or spinal cord, or acute ischemic injury. Ischemic injuries refer to conditions that arise when the brain receives insufficient blood flow. In some embodiments, injury to the brain or nervous system can result from a traumatic injury, or could be the result of infection, radiation, chemical or toxic damage. Injury within the brain and nervous system, which may be diffuse or localized, includes an intracranial or intra vertebral lesion or hemorrhage, cerebral ischemia or infarction

including embolic occlusion and thrombotic occlusion, perinatal hypoxic-ischemic injury, whiplash, shaken infant syndrome, reperfusion following acute ischemia, or cardiac arrest. In one embodiment, *in vitro* treatment of cells with oligonucleotides can be used for *ex vivo* therapy of cells removed from a subject *(e.g.,* for treatment of leukemia or viral infection) or for treatment of cells which did not originate in the subject, but are to be administered to the subject *(e.g.,* to eliminate transplantation antigen expression on cells to be transplanted into a subject). In addition, *in vitro* treatment of cells can be used in nontherapeutic settings, *e.g.*, to evaluate gene function, to study gene regulation and protein synthesis or to evaluate improvements made to oligonucleotides designed to modulate gene expression or protein synthesis. *In vivo* treatment of cells can be useful in certain clinical settings where it is desirable to inhibit the expression of a protein. There are numerous medical conditions for which antisense therapy is reported to be suitable (see, *e.g.*, U.S. Pat. No. 5,830,653) as well as respiratory syncytial virus infection (WO 95/22,553) influenza virus (WO 94/23,028), and malignancies (WO 94/08,003). Other examples of clinical uses of antisense sequences are reviewed, *e.g.*, in Glaser. 1996. *Genetic Engineering News* 16:1. Exemplary targets for cleavage by oligonucleotides include, *e.g.*, protein kinase Ca, ICAM-1, c-raf kinase, p53, c-myb, and the bcr/abl fusion gene found in chronic myelogenous leukemia.

The subject nucleic acids can be used in RNAi-based therapy in any animal having RNAi pathway, such as human, non-human primate, non-human mammal, non-human vertebrates, rodents (mice, rats, hamsters, rabbits, etc.), domestic livestock animals, pets (cats, dogs, etc.), Xenopus, fish, insects (*Drosophila*, etc.), and worms (*C. elegans*), etc.

The invention provides methods for preventing in a subject, a disease or condition associated with an aberrant or unwanted target gene expression or activity, by administering to the subject a therapeutic agent (e.g., a RNAi agent or vector or transgene encoding same). If appropriate, subjects are first treated with a priming agent so as to be more responsive to the subsequent RNAi therapy. Subjects at risk for a disease which is caused or contributed to by aberrant or unwanted target gene expression or activity can be identified by, for example, any or a combination of diagnostic or prognostic assays as described herein. Administration of a prophylactic agent can occur prior to the manifestation of symptoms characteristic of the target gene aberrancy, such that a disease or disorder is prevented or, alternatively, delayed in its progression. Depending on the type of target gene aberrancy, for example, a target gene, target gene agonist or target gene antagonist agent can be used for treating the subject.

In another aspect, the invention pertains to methods of modulating target gene expression, protein expression or activity for therapeutic purposes. Accordingly, in an exemplary embodiment, the modulatory method of the invention involves contacting a cell capable of expressing target gene with a therapeutic agent of the invention that is specific for the target gene or protein (e.g., is specific for the mRNA encoded by said gene or specifying the amino acid sequence of said protein) such that expression or one or more of the activities of target protein is modulated. These modulatory methods can be performed *in vitro* (e.g., by culturing the cell with the agent), *in vivo* (e.g., by administering the agent to a subject), or *ex vivo*. Typically, subjects are first treated with a priming agent so as to be more responsive to the subsequent RNAi therapy. As such, the present invention provides methods of treating an individual afflicted with a disease or disorder characterized by aberrant or unwanted expression or activity of a target gene polypeptide or nucleic acid molecule. Inhibition of target gene activity is desirable in situations in which target gene is abnormally unregulated and/or in which decreased target gene activity is likely to have a beneficial effect.

The therapeutic agents of the invention can be administered to individuals to treat (prophylactically or therapeutically) disorders associated with aberrant or unwanted target gene activity. In conjunction with such treatment, pharmacogenomics (i.e., the study of the relationship between an individual's genotype and that individual's response to a foreign compound or drug) may be considered. Differences in metabolism of therapeutics can lead to severe toxicity or therapeutic failure by altering the relation between dose and blood concentration of the pharmacologically active drug. Thus, a physician or clinician may consider applying knowledge obtained in relevant pharmacogenomics studies in determining whether to administer a therapeutic agent as well as tailoring the dosage and/or therapeutic regimen of treatment with a therapeutic agent. Pharmacogenomics deals with clinically significant hereditary variations in the response to drugs due to altered drug disposition and abnormal action in affected persons. See, for example, Eichelbaum, M. et al. (1996) Clin. Exp. Pharmacol. Physiol. 23(10-11): 983-985 and Linder, M. W. et al. (1997) Clin. Chem. 43(2):254-266

The present invention is further illustrated by the following Examples, which in no way should be construed as further limiting. The entire contents of all of the references (including literature references, issued patents, published patent applications, and co pending patent applications) cited throughout this application are hereby expressly incorporated by reference.

EXAMPLES

Example 1: Identification of SOD1-Targeting sd-rxRNA variants

sd-rxRNA variants targeting SOD1 were designed, synthesized and screened *in vitro* to determine the ability of the sd-rxRNA variant to reduce target gene mRNA levels. The sd-rxRNA variants were tested for activity in HeLa cells (human cervical carcinoma cell line, 10,000 cells/well, 96 well plate). HeLa cells were treated with varying concentrations of a panel of SOD1-targeting sd-rxRNAs variants or non-targeting control in serum containing media. Concentrations tested were 5, 1 and 0.1 µM. The non-targeting control sd-rxRNA is of similar structure to the SOD1-targeting sd-rxRNA variants and contains similar stabilizing modifications throughout both strands. Forty eight hours post administration, cells were lysed and mRNA levels determined by the Quantigene branched DNA assay according to the manufacture's protocol using gene-specific probes (Affymetrix, Santa Clara, CA). Exemplary sense and antisense sequences are presented in Tables 1 and 2 below. FIGs. 1 and 2 demonstrate that the SOD1-targeting sd-rxRNA variants significantly reduce target gene mRNA levels *in vitro* in HeLa cells. Data were normalized to a house keeping gene (PPIB) and graphed with respect to the non-targeting control. Error bars represent the standard deviation from the mean of biological duplicates. Sequences corresponding to FIGs. 1 and 2 can be found in Tables 3 and 4, respectively.

Table 1. Exemplary sense oligonucleotides

Oligo Number	Gene symbol	Sense sequence	SEQ ID NO:	Sense Chemistry	Sense Backbone	Notes
25634	SOD1	GAGAGGCAUGUUA	1	DY547mm0m00m0m0mmm	ooooooooooosso	
25635	SOD1	GAGAGGCAUGUUA	2	DY547mm0m00m0m0mmm	ooooooooooosso	
25636	SOD1	GAGAGGCAUGUUA	3	DY547mm0m00m0m0mmm	ooooooooooosso	
25637	SOD1	GAGAGGCAUGUUA	4	DY547mm0m00m0m0mmm	ssssssssssssso	
25600	SOD1	GAGAGGCAUGUUA	5	mm0000m0m0mmm	ssssssssssssso	
25638	SOD1	GAGAGGCAUGUUA	6	DY547mm0m00m0m0mmm	ssssssssssssso	
25643	SOD1	GAGAGGCAUGUUA	7	DY547mm0m00m0m0mmm	ooooooooooosso	
25644	SOD1	GAGAGGCAUGUUA	8	DY547mm0m00m0m0mmm	ssssssssssssso	
25645	SOD1	GAGAGGCAUGUUA	9	DY547mm0m00m0m0mmm	ooooooooooosso	
25652	SOD1	GAGAGGCAUGUUA	10	DY547mm0000m0m0mmm	ssssssssssssso	
25568	SOD1	AGGZGGAAAZGAA	11	DY547mm0m00000m0mm	ssssssssssssso	Z= octyl, x = 5 methyl C, Y= 5 methyl U
25569	SOD1	AGGYGGAAAZGAA	12	DY547mm0m00000m0mm	ssssssssssssso	Z= octyl, x = 5 methyl C, Y= 5 methyl U

25570	SOD1	AGGZGGAAAYGAA	13	DY547mm0m00000m0mm	ssssssssssso	Z= octyl, x = 5 methyl C, Y= 5 methyl U
25571	SOD1	AGGYGGAAAYGAA	14	DY547mm0m00000m0mm	ssssssssssso	Z= octyl, x = 5 methyl C, Y= 5 methyl U
25572	SOD1	AGGUGGAAAUGAA	15	DY547mm0m00000m0mm	ssssssssssso	Z= octyl, x = 5 methyl C, Y= 5 methyl U
25573	SOD1	AGGUGGAAAUGAA	16	DY547mm0m00000m0mm	ssssssssssso	Z= octyl, x = 5 methyl C, Y= 5 methyl U
25574	SOD1	AGGUGGAAAUGAA	17	DY547mm0m00000m0mm	ssssssssssso	Z= octyl, x = 5 methyl C, Y= 5 methyl U
25575	SOD1	AGGUGGAAAUGAA	18	DY547mm0m00000m0mm	ssssssssssso	Z= octyl, x = 5 methyl C, Y= 5 methyl U
25576	SOD1	AGGUGGAAAUGAA	19	DY547mm0m00000m0mm	ssssssssssso	x = 5 methyl C, Y= 5 methyl U
25578	SOD1	AGGYGGAAAZGAA	20	DY547mm0m00000m0mm	ssssssssssso	Z= thiophene, x = 5 methyl C, Y= 5 methyl U
25579	SOD1	AGGZGGAAAYGAA	21	DY547mm0m00000m0mm	ssssssssssso	Z= thiophene, x = 5 methyl C, Y= 5 methyl U
25580	SOD1	AGGYGGAAAYGAA	22	DY547mm0m00000m0mm	ssssssssssso	Z= thiophene, x = 5 methyl C, Y= 5 methyl U
25584	SOD1	AGGUGGAAAUGAA	23	DY547mm0m00000m0mm	ssssssssssso	Z= thiophene, x = 5 methyl C, Y= 5 methyl U
25585	SOD1	AGGUGGAAAUGAA	24	DY547mm0m00000m0mm	ssssssssssso	Z= thiophene, x = 5 methyl C, Y= 5 methyl U
25586	SOD1	AGGZGGAAAZGAA	25	DY547mm0d00000d0mm	ssssssssssso	Z= isobutyl, x = 5 methyl C, Y= 5 methyl U
25587	SOD1	AGGYGGAAAZGAA	26	DY547mm0m00000d0mm	ssssssssssso	Z= isobutyl, x = 5 methyl C, Y= 5 methyl U
25588	SOD1	AGGZGGAAAYGAA	27	DY547mm0d00000m0mm	ssssssssssso	Z= isobutyl, x = 5 methyl C, Y= 5 methyl U
25589	SOD1	AGGUGGAAAUGAA	28	DY547mm0m00000m0mm	ssssssssssso	Z= isobutyl, x = 5 methyl C, Y= 5 methyl U
25590	SOD1	AGGUGGAAAUGAA	29	DY547mm0m00000m0mm	ssssssssssso	Z= isobutyl, x = 5 methyl C, Y= 5 methyl U

24560	SOD1	AGGUGGAAAUGA A	30	DY547mm0m00000 m0mm	sssssssssssso	
25634 no Fl label	SOD1	GAGAGGCAUGUU A	31	mm0m00m0m0mm m	oooooooooo sso	
25635 no Fl label	SOD1	GAGAGGCAUGUU A	32	mm0m00m0m0mm m	oooooooooo sso	
25636 no Fl label	SOD1	GAGAGGCAUGUU A	33	mm0m00m0m0mm m	oooooooooo sso	
25637 no Fl label	SOD1	GAGAGGCAUGUU A	34	mm0m00m0m0mm m	sssssssssssso	
25638 no Fl label	SOD1	GAGAGGCAUGUU A	35	mm0m00m0m0mm m	sssssssssssso	
25643 no Fl label	SOD1	GAGAGGCAUGUU A	36	mm0m00m0m0mm m	oooooooooo sso	
25644 no Fl label	SOD1	GAGAGGCAUGUU A	37	mm0m00m0m0mm m	sssssssssssso	
25645 no Fl label	SOD1	GAGAGGCAUGUU A	38	mm0m00m0m0mm m	oooooooooo sso	
25652 no Fl label	SOD1	GAGAGGCAUGUU A	39	mm0000m0m0mm m	sssssssssssso	
25568 no Fl label	SOD1	AGGZGGAAAZGAA	40	mm0m00000m0mm	sssssssssssso	Z= octyl, x = 5 methyl C, Y= 5 methyl U
25569 no Fl label	SOD1	AGGYGGAAAZGAA	41	mm0m00000m0mm	sssssssssssso	Z= octyl, x = 5 methyl C, Y= 5 methyl U
25570 no Fl label	SOD1	AGGZGGAAAYGAA	42	mm0m00000m0mm	sssssssssssso	Z= octyl, x = 5 methyl C, Y= 5 methyl U
25571 no Fl label	SOD1	AGGYGGAAAYGAA	43	mm0m00000m0mm	sssssssssssso	Z= octyl, x = 5 methyl C, Y= 5 methyl U
25572 no Fl label	SOD1	AGGUGGAAAUGA A	44	mm0m00000m0mm	sssssssssssso	Z= octyl, x = 5 methyl C, Y= 5 methyl U
25573 no Fl label	SOD1	AGGUGGAAAUGA A	45	mm0m00000m0mm	sssssssssssso	Z= octyl, x = 5 methyl C, Y= 5 methyl U
25574 no Fl label	SOD1	AGGUGGAAAUGA A	46	mm0m00000m0mm	sssssssssssso	Z= octyl, x = 5 methyl C, Y= 5 methyl U
25575 no Fl label	SOD1	AGGUGGAAAUGA A	47	mm0m00000m0mm	sssssssssssso	Z= octyl, x = 5 methyl C, Y= 5 methyl U
25576 no Fl label	SOD1	AGGUGGAAAUGA A	48	mm0m00000m0mm	sssssssssssso	x = 5 methyl C, Y= 5 methyl U
25578 no Fl label	SOD1	AGGYGGAAAZGAA	49	mm0m00000m0mm	sssssssssssso	Z= thiophene, x = 5 methyl C, Y= 5 methyl U
25579 no Fl label	SOD1	AGGZGGAAAYGAA	50	mm0m00000m0mm	sssssssssssso	Z= thiophene, x = 5 methyl C, Y= 5 methyl U

25580 no Fl label	SOD1	AGGYGGAAAYGAA	51	mm0m00000m0mm	ssssssssssso	Z= thiophene, x = 5 methyl C, Y= 5 methyl U
25584 no Fl label	SOD1	AGGUGGAAAUGA A	52	mm0m00000m0mm	ssssssssssso	Z= thiophene, x = 5 methyl C, Y= 5 methyl U
25585 no Fl label	SOD1	AGGUGGAAAUGA A	53	mm0m00000m0mm	ssssssssssso	Z= thiophene, x = 5 methyl C, Y= 5 methyl U
25586 no Fl label	SOD1	AGGZGGAAAZGAA	54	mm0d00000d0mm	ssssssssssso	Z= isobutyl, x = 5 methyl C, Y= 5 methyl U
25587 no Fl label	SOD1	AGGYGGAAAZGAA	55	mm0m00000d0mm	ssssssssssso	Z= isobutyl, x = 5 methyl C, Y= 5 methyl U
25588 no Fl label	SOD1	AGGZGGAAAYGAA	56	mm0d00000m0mm	ssssssssssso	Z= isobutyl, x = 5 methyl C, Y= 5 methyl U
25589 no Fl label	SOD1	AGGUGGAAAUGA A	57	mm0m00000m0mm	ssssssssssso	Z= isobutyl, x = 5 methyl C, Y= 5 methyl U
25590 no Fl label	SOD1	AGGUGGAAAUGA A	58	mm0m00000m0mm	ssssssssssso	Z= isobutyl, x = 5 methyl C, Y= 5 methyl U
24560 no Fl label	SOD1	AGGUGGAAAUGA A	59	mm0m00000m0mm	ssssssssssso	

Table 2. Exemplary antisense oligonucleotides

Oligo Number	Antisense sequence	SEQ ID NO:	AntiSense Chemistry	AntiSense Backbone	Notes
25634	UAACAUGCCUCUC UUCAUCCU	60	Pm00f0f0fffff0fff0fff0	ssssssssssssssssssss o	
25635	UAACAUGCCUCUC UUCAUCCU	61	Pm00f0f0fffffffff0fff0	oooooooooooossss sssso	
25636	UAACAUGCCUCUC UUCAUC	62	Pm00f0f0fffffffff0f0	oooooooooooossss sso	
25637	UAACAUGCCUCUC UUCAUCCU	63	Pm00f0f0fffffffff0fff0	oooooooooooossss sssso	
25600	UAACAUGCCUCUC UUCAUCCU	64	Pm00f0f0fffff0fff0fff0	ssssssssssssssssssss o	
25638	UAACAUGCCUCUC UUCAUCCU	65	Pm00f0f0fffffffff00	oooooooooooossss sssso	
25643	UAACAUGCCUCUC UUCAUC	66	Pm00f0f0fffffffff0f0	ssssssssssssssssso	
25644	UAACAUGCCUCUC UUCAUC	67	Pm00f0f0fffffffff0f0	ssssssssssssssssso	
25645	UAACAUGCCUCUC UUCAUCCU	68	Pm00f0f0fffffffff0fff0	ssssssssssssssssssss o	
25652	UAACAUGCCUCUC UUCAUCCU	69	Pm00f0f0fffff0fff0fff0	ssssssssssssssssssss o	
25568	UUCAUUUCCACCU	70	Pmff0fffff0fmmmm0m	ssssssssssssssssssss	Z= octyl, x = 5

	UUGCCCAA		mf00	o	methyl C, Y= 5 methyl U
25569	UUCAUUUCCACCU UUGCCCAA	71	Pmff0fffff0fmmmm0m mf00	sssssssssssssssssss o	Z= octyl, x = 5 methyl C, Y= 5 methyl U
25570	UUCAUUUCCACCU UUGCCCAA	72	Pmff0fffff0fmmmm0m mf00	sssssssssssssssssss o	Z= octyl, x = 5 methyl C, Y= 5 methyl U
25571	UUCAUUUCCACCU UUGCCCAA	73	Pmff0fffff0fmmmm0m mf00	sssssssssssssssssss o	Z= octyl, x = 5 methyl C, Y= 5 methyl U
25572	YZXAYYZXXAXXZYY GXXXAA	74	Pmff0fffff0fmmmm0m mf00	sssssssssssssssssss o	Z= octyl, x = 5 methyl C, Y= 5 methyl U
25573	YYXAYYZXXAXXZYY GXXXAA	75	Pmff0fffff0fmmmm0m mf00	sssssssssssssssssss o	Z= octyl, x = 5 methyl C, Y= 5 methyl U
25574	YZXAYYYXXAXXZYY GXXXAA	76	Pmff0fffff0fmmmm0m mf00	sssssssssssssssssss o	Z= octyl, x = 5 methyl C, Y= 5 methyl U
25575	YZXAYYZXXAXXYYY GXXXAA	77	Pmff0fffff0fmmmm0m mf00	sssssssssssssssssss o	Z= octyl, x = 5 methyl C, Y= 5 methyl U
25576	YYXAYYYXXAXXYYY GXXXAA	78	Pmff0fffff0fmmmm0m mf00	sssssssssssssssssss o	x = 5 methyl C, Y= 5 methyl U
25578	UUCAUUUCCACCU UUGCCCAA	79	Pmff0fffff0fmmmm0m mf00	sssssssssssssssssss o	Z= thiophene, x = 5 methyl C, Y= 5 methyl U
25579	UUCAUUUCCACCU UUGCCCAA	80	Pmff0fffff0fmmmm0m mf00	sssssssssssssssssss o	Z= thiophene, x = 5 methyl C, Y= 5 methyl U
25580	UUCAUUUCCACCU UUGCCCAA	81	Pmff0fffff0fmmmm0m mf00	sssssssssssssssssss o	Z= thiophene, x = 5 methyl C, Y= 5 methyl U
25584	YZXAYYZXXAXXYYY GXXXAA	82	Pmff0fffff0fmmmm0m mf00	sssssssssssssssssss o	Z= thiophene, x = 5 methyl C, Y= 5 methyl U
25585	YYXAYYYXXAXXYYY GXXXAA	83	Pmff0fffff0fmmmm0m mf00	sssssssssssssssssss o	Z= thiophene, x = 5 methyl C, Y= 5 methyl U
25586	UUCAUUUCCACCU UUGCCCAA	84	Pmff0fffff0fmmmm0m mf00	sssssssssssssssssss o	Z= isobutyl, x = 5 methyl C, Y= 5 methyl U
25587	UUCAUUUCCACCU UUGCCCAA	85	Pmff0fffff0fmmmm0m mf00	sssssssssssssssssss o	Z= isobutyl, x = 5 methyl C, Y= 5 methyl U
25588	UUCAUUUCCACCU UUGCCCAA	86	Pmff0fffff0fmmmm0m mf00	sssssssssssssssssss o	Z= isobutyl, x = 5 methyl C, Y= 5 methyl U
25589	YZXAYYZXXAXXZYY GXXXAA	87	Pmdf0ffdff0fmdmm0m mf00	sssssssssssssssssss o	Z= isobutyl, x = 5 methyl C, Y= 5 methyl U
25590	YYXAYYZXXAXXZYY GXXXAA	88	Pmff0ffdff0fmdmm0m mf00	sssssssssssssssssss o	Z= isobutyl, x = 5 methyl C, Y= 5 methyl U
24560	UUCAUUUCCACCU	89	Pmff0fffff0fmmmm0m	sssssssssssssssssss	

	UUGCCCAA		mf00	o	
25634 no Fl label	UAACAUGCCUCUC UUCAUCCU	90	Pm00f0f0fffff0fff0fff0	sssssssssssssssssss o	
25635 no Fl label	UAACAUGCCUCUC UUCAUCCU	91	Pm00f0f0ffffffff0fff0	oooooooooooossss sssso	
25636 no Fl label	UAACAUGCCUCUC UUCAUC	92	Pm00f0f0ffffffff0f0	oooooooooooossss sso	
25637 no Fl label	UAACAUGCCUCUC UUCAUCCU	93	Pm00f0f0ffffffff0fff0	oooooooooooossss sssso	
25638 no Fl label	UAACAUGCCUCUC UUCAUCCU	94	Pm00f0f0ffffffff00	oooooooooooossss sssso	
25643 no Fl label	UAACAUGCCUCUC UUCAUC	95	Pm00f0f0ffffffff0f0	ssssssssssssssssso	
25644 no Fl label	UAACAUGCCUCUC UUCAUC	96	Pm00f0f0ffffffff0f0	ssssssssssssssssso	
25645 no Fl label	UAACAUGCCUCUC UUCAUCCU	97	Pm00f0f0ffffffff0fff0	sssssssssssssssssss o	
25652 no Fl label	UAACAUGCCUCUC UUCAUCCU	98	Pm00f0f0fffff0fff0fff0	sssssssssssssssssss o	
25568 no Fl label	UUCAUUUCCACCU UUGCCCAA	99	Pmff0ffff0fmmmm0m mf00	sssssssssssssssssss o	Z= octyl, x = 5 methyl C, Y= 5 methyl U
25569 no Fl label	UUCAUUUCCACCU UUGCCCAA	100	Pmff0ffff0fmmmm0m mf00	sssssssssssssssssss o	Z= octyl, x = 5 methyl C, Y= 5 methyl U
25570 no Fl label	UUCAUUUCCACCU UUGCCCAA	101	Pmff0ffff0fmmmm0m mf00	sssssssssssssssssss o	Z= octyl, x = 5 methyl C, Y= 5 methyl U
25571 no Fl label	UUCAUUUCCACCU UUGCCCAA	102	Pmff0ffff0fmmmm0m mf00	sssssssssssssssssss o	Z= octyl, x = 5 methyl C, Y= 5 methyl U
25572 no Fl label	YZXAYYZXXAXXZYY GXXXAA	103	Pmff0ffff0fmmmm0m mf00	sssssssssssssssssss o	Z= octyl, x = 5 methyl C, Y= 5 methyl U
25573 no Fl label	YYXAYYZXXAXXZYY GXXXAA	104	Pmff0ffff0fmmmm0m mf00	sssssssssssssssssss o	Z= octyl, x = 5 methyl C, Y= 5 methyl U
25574 no Fl label	YZXAYYYXXAXXZYY GXXXAA	105	Pmff0ffff0fmmmm0m mf00	sssssssssssssssssss o	Z= octyl, x = 5 methyl C, Y= 5 methyl U
25575 no Fl label	YZXAYYZXXAXXYYY GXXXAA	106	Pmff0ffff0fmmmm0m mf00	sssssssssssssssssss o	Z= octyl, x = 5 methyl C, Y= 5 methyl U
25576 no Fl label	YYXAYYYXXAXXYYY GXXXAA	107	Pmff0ffff0fmmmm0m mf00	sssssssssssssssssss o	x = 5 methyl C, Y= 5 methyl U
25578 no Fl label	UUCAUUUCCACCU UUGCCCAA	108	Pmff0ffff0fmmmm0m mf00	sssssssssssssssssss o	Z= thiophene, x = 5 methyl C, Y= 5 methyl U
25579 no Fl label	UUCAUUUCCACCU UUGCCCAA	109	Pmff0ffff0fmmmm0m mf00	sssssssssssssssssss o	Z= thiophene, x = 5 methyl C, Y= 5 methyl U
25580 no Fl label	UUCAUUUCCACCU UUGCCCAA	110	Pmff0ffff0fmmmm0m mf00	sssssssssssssssssss o	Z= thiophene, x = 5 methyl C, Y= 5 methyl U

25584 no Fl label	YZXAYYZXXAXXYYY GXXXAA	111	Pmff0ffff0fmmmm0m mf00	sssssssssssssssssss o	Z= thiophene, x = 5 methyl C, Y= 5 methyl U
25585 no Fl label	YYXAYYYXXAXXYYY GXXXAA	112	Pmff0ffff0fmmmm0m mf00	sssssssssssssssssss o	Z= thiophene, x = 5 methyl C, Y= 5 methyl U
25586 no Fl label	UUCAUUUCCACCU UUGCCCAA	113	Pmff0ffff0fmmmm0m mf00	sssssssssssssssssss o	Z= isobutyl, x = 5 methyl C, Y= 5 methyl U
25587 no Fl label	UUCAUUUCCACCU UUGCCCAA	114	Pmff0ffff0fmmmm0m mf00	sssssssssssssssssss o	Z= isobutyl, x = 5 methyl C, Y= 5 methyl U
25588 no Fl label	UUCAUUUCCACCU UUGCCCAA	115	Pmff0ffff0fmmmm0m mf00	sssssssssssssssssss o	Z= isobutyl, x = 5 methyl C, Y= 5 methyl U
25589 no Fl label	YZXAYYZXXAXXZYY GXXXAA	116	Pmdf0ffdff0fmdmm0m mf00	sssssssssssssssssss o	Z= isobutyl, x = 5 methyl C, Y= 5 methyl U
25590 no Fl label	YYXAYYZXXAXXZYY GXXXAA	117	Pmff0ffdff0fmdmm0m mf00	sssssssssssssssssss o	Z= isobutyl, x = 5 methyl C, Y= 5 methyl U
24560 no Fl label	UUCAUUUCCACCU UUGCCCAA	118	Pmff0ffff0fmmmm0m mf00	sssssssssssssssssss o	

Key for Tables 1 and 2:	
f	= 2'fluoro
m	= 2'Ome
P	= 5' phosphate
s	= phosphorothioate linkage
o	= phosphodiester linkage
DY547	= DY547 dye
d	= deoxyribose

The human SOD1 sequence is represented by GenBank accession number NM_000454.4 (SEQ ID NO: 119) listed below. Multiple mutations of the SOD1 sequence have been identified (Rosen et al (1993) *Nature*; Deng et al (1993) Science; De Belleroche et al. (1995) *J Med Genet.*; Orrel et al (1997) *J Neurol.*; Cudkowicz (1997) *Ann. Neurol.*; and Anderson et al (1995) *Nature Genet.*) and can also be targeted utilizing the sequences outlined in this application:

GTTTGGGGCCAGAGTGGGCGAGGCGCGGAGGTCTGGCCTATAAAGTAGTCGCGG
AGACGGGGTGCTGGTTTGCGTCGTAGTCTCCTGCAGCGTCTGGGGTTTCCGTTGC
AGTCCTCGGAACCAGGACCTCGGCGTGGCCTAGCGAGTTATGGCGACGAAGGCC
GTGTGCGTGCTGAAGGGCGACGGCCCAGTGCAGGGCATCATCAATTTCGAGCAG
AAGGAAAGTAATGGACCAGTGAAGGTGTGGGGAAGCATTAAAGGACTGACTGA

AGGCCTGCATGGATTCCATGTTCATGAGTTTGGAGATAATACAGCAGGCTGTACC
AGTGCAGGTCCTCACTTTAATCCTCTATCCAGAAAACACGGTGGGCCAAAGGATG
AAGAGAGGCATGTTGGAGACTTGGGCAATGTGACTGCTGACAAAGATGGTGTGG
CCGATGTGTCTATTGAAGATTCTGTGATCTCACTCTCAGGAGACCATTGCATCATT
GGCCGCACACTGGTGGTCCATGAAAAAGCAGATGACTTGGGCAAAGGTGGAAAT
GAAGAAAGTACAAAGACAGGAAACGCTGGAAGTCGTTTGGCTTGTGGTGTAATT
GGGATCGCCCAATAAACATTCCCTTGGATGTAGTCTGAGGCCCCTTAACTCATCT
GTTATCCTGCTAGCTGTAGAAATGTATCCTGATAAACATTAAACACTGTAATCTT
AAAAGTGTAATTGTGTGACTTTTTCAGAGTTGCTTTAAAGTACCTGTAGTGAGAA
ACTGATTTATGATCACTTGGAAGATTTGTATAGTTTTATAAAACTCAGTTAAAAT
GTCTGTTTCAATGACCTGTATTTTGCCAGACTTAAATCACAGATGGGTATTAAAC
TTGTCAGAATTTCTTTGTCATTCAAGCCTGTGAATAAAAACCCTGTATGGCACTTA
TTATGAGGCTATTAAAAGAATCCAAATTCAAACTAAAAAAAAAAAAAAAAAA
(SEQ ID NO: 119)

Table 3. Reducing Phosphorothioate Content Results in Active ps-rxRNA Variants with Reduced Cellular Toxicity *in vitro* (sequences corresponding to FIG. 1)

Oligo ID			SEQ ID NO :	Total # PS
25634	PS	DY547.mG.mA. G.mA. G. G.mC. A.mU. G.mU*mU*mA	120	22
	GS	P.mU* A* A*fC* A*fU* G*fC*fC*fU*fC*fU* C*fU*fU*fC* A*fU*fC*fC* U	121	
25635	PS	DY547.mG.mA. G.mA. G. G.mC. A.mU. G.mU*mU*mA	122	10
	GS	P.mU. A. A.fC. A.fU. G.fC.fC.fU.fC.fU.fC*fU*fU*fC* A*fU*fC*fC* U	123	
25636	PS	DY547.mG.mA. G.mA. G. G.mC. A.mU. G.mU*mU*mA	124	8
	GS	P.mU. A. A.fC. A.fU. G.fC.fC.fU.fC.fU. C*fU*fU*fC* A*fU* C	125	
25637	PS	DY547.mG*mA* G*mA* G* G*mC* A*mU* G*mU*mU*mA	126	20
	GS			
25600 (Parent ps-rxRNA)	PS	mG*mA* G* A* G* G*mC* A*mU* G*mU*mU*mA	127	32
	GS	P.mU* A* A*fC* A*fU* G*fC*fC*fU*fC*fU* C*fU*fU*fC* A*fU*fC*fC* U	128	

Table 4. Reducing Phosphorothioate Content Results in Active ps-rxRNA Variants with Reduced Cellular Toxicity *in vitro* (sequences corresponding to FIG. 2)

Oligo ID			SEQ ID NO:	Total # PS
25638	PS	DY547.mG*mA* G*mA* G* G*mC* A*mU* G*mU*mU*mA	129	18
	GS	P.mU. A. A.fC. A.fU. G.fC.fC.fU.fC.fU.fC*fU*fU*fC* A*fU*fC*fC* U	130	
25643	PS	DY547.mG.mA. G.mA. G. G.mC. A.mU. G.mU*mU*mA	131	20
	GS	P.mU* A* A*fC* A*fU* G*fC*fC*fU*fC*fU*fC*fU*fU*fC* A*fU* C	132	
25644	PS	DY547.mG*mA* G*mA* G* G*mC* A*mU* G*mU*mU*mA	133	30
	GS	P.mU* A* A*fC* A*fU* G*fC*fC*fU*fC*fU*fC*fU*fU*fC* A*fU* C	134	
25645	PS	DY547.mG.mA. G.mA. G. G.mC. A.mU. G.mU*mU*mA	135	22
	GS	P.mU* A* A*fC* A*fU* G*fC*fC*fU*fC*fU*fC*fU*fU*fC* A*fU*fC*fC* U	136	
25600 (Parent ps-rxRNA)	PS	mG*mA* G* A* G* G*mC* A*mU* G*mU*mU*mA	137	32
	GS	P.mU* A* A*fC* A*fU* G*fC*fC*fU*fC*fU* C*fU*fU*fC* A*fU*fC*fC* U	138	

Key for Tables 3 and 4:	
f	= 2'fluoro
m	= 2'Ome
P	= 5' phosphate
*	= phosphorothioate linkage
.	= phosphodiester linkage
DY547	= DY547 dye
d	= deoxyribose

It should be appreciated that while some of the sequences presented in Tables 1-8 are depicted as being attached to the dye DY547, all of the sequences presented in Tables 1-8 are also disclosed herein in the absence of DY547.

Example 2: sd-rxRNA variant uptake in CNS

FIGs. 3-5 demonstrate that sd-rxRNA chemical variants, with varying levels of phosphorothioates, are taken up and delivered to cells in the CNS. To determine the tissue distribution of the sd-rxRNA chemical variants, fluorescently labeled compounds targeting SOD1 were administered to Sprague Dawley rats by intracisternal injection (IC injection), with 15 µL of a 15 mg/mL solution. Twenty four hours post injection tissues were harvested and processed for confocal microscopy. Confocal imaging was used to detect cellular uptake of sd-rxRNA variants. The level of phosphorothioate content correlated with the observed levels of cellular uptake in the CNS (e.g. increased levels of phosphorothioate content resulted in greater uptake).

Example 3: sd-rxRNA variant silencing of SOD1 in CNS

FIG. 6 demonstrates SOD1 silencing *in vivo* (mouse, lumbar spinal cord (LSC)) following 14 day intrathecal administration of a SOD1 targeting sd-rxRNA variant (Oligo ID 25652). A 37% reduction of SOD1 mRNA levels was observed in mice treated with the SOD1-targeting sd-rxRNA variant compared to the non-targeting control (FIG. 6).

Methods: SOD1-targeting sd-rxRNA variant or non-targeting control (NTC) was administered by intrathecal infusion, using an osmotic pump (filled with 100 µL of a 10 mg/mL solution of compound) for 14 days. Terminal biopsy samples of the spinal cord were harvested on Day 14. RNA was isolated and subjected to gene expression analysis by qPCR. Data were normalized to the level of the cyclophilin B (PPIB) housekeeping gene and graphed relative to the non-targeting control set at 1.0. Error bars represent standard deviation between the individual biopsy samples. P value for SOD1-targeting sd-rxRNA variant - treated group vs PBS group was * p <0.001.

Example 4: sd-rxRNA variant silencing of SOD1 in CNS

FIG. 7 demonstrates SOD1 silencing, *in vivo* (mouse, lumbar spinal cord (LSC)) following 14 day intrathecal administration of a SOD1 targeting sd-rxRNA variant (Oligo ID 25645). A statistically significant 24% reduction of SOD1 mRNA levels was observed in mice treated with the SOD1-targeting sd-rxRNA variant compared to the non-targeting control (FIG. 9).

Methods: SOD1-targeting sd-rxRNA variant or non-targeting control (NTC) was administered by intrathecal infusion, using an osmotic pump (filled with 100 µL of a 10 mg/mL solution of compound) for 14 days. Terminal biopsy samples of the spinal cord were harvested on Day 14. RNA was isolated and subjected to gene expression analysis by qPCR. Data were normalized to the level of the cyclophilin B (PPIB) housekeeping gene and graphed relative to the non-targeting control set at 1.0. Error bars represent standard deviation between the individual biopsy samples. P value for SOD1-targeting sd-rxRNA variant - treated group vs non-targeting control group was * p <0.01.

Example 5: Identification of SOD1-Targeting sd-rxRNA variants

sd-rxRNA variants, containing hydrophobic modifications on position 4 or 5 of the base, targeting SOD1 were designed, synthesized and screened *in vitro* to determine the ability of the sd-rRNA variant to reduce target gene mRNA levels. The sd-rxRNA variants were tested for activity in HeLa cells (human cervical carcinoma cell line, 10,000 cells/well, 96 well plate). HeLa cells were treated with varying concentrations of a panel of SOD1-targeting sd-rxRNAs variants or non-targeting control in serum containing media. Concentrations tested were 5, 1 and 0.1 µM. The non-targeting control sd-rxRNA is of similar structure to the SOD1-targeting sd-rxRNA variants and contains similar stabilizing modifications throughout both strands. Forty eight hours post administration, cells were lysed and mRNA levels determined by the Quantigene branched DNA assay according to the manufacture's protocol using gene-specific probes (Affymetrix, Santa Clara, CA). FIGs. 8-11 demonstrate the SOD1-targeting sd-rxRNA variants, containing hydrophobic modifications on position 4 or 5 of the base, significantly reduce target gene mRNA levels *in vitro* in HeLa cells. Sequences corresponding to FIGs. 8-11 can be found in Tables 5-8, respectively. Data were normalized to a house keeping gene (PPIB) and graphed with respect to the non-targeting control. Error bars represent the standard deviation from the mean of biological duplicates.

Table 5. SOD1 sd-rxRNA Variant Octyl Modifications

ID	Z=	Passenger Strand	SEQ ID NO:	Guide Strand	SEQ ID NO:
25568	octyl U	DY547.mA*mG* G*mZ* G* G* A* A* A*mZ* G*mA*mA	139	P.mU*fU*fC* A*fU*fU*fU*fC*fC* A*fC*mC*mU*mU*mU* G*mC*mC*fC* A* A	145
25569	octyl U	DY547.mA*mG* G*mY* G* G* A* A* A*mZ* G*mA*mA	140	P.mU*fU*fC* A*fU*fU*fU*fC*fC* A*fC*mC*mU*mU*mU* G*mC*mC*fC* A* A	146
25570	octyl U	DY547.mA*mG* G*mZ* G* G* A* A* A*mY* G*mA*mA	141	P.mU*fU*fC* A*fU*fU*fU*fC*fC* A*fC*mC*mU*mU*mU* G*mC*mC*fC* A* A	147
25571		DY547.mA*mG* G*mY* G* G* A* A* A*mY* G*mA*mA	142	P.mU*fU*fC* A*fU*fU*fU*fC*fC* A*fC*mC*mU*mU*mU* G*mC*mC*fC* A* A	148
25572	octyl U	DY547.mA*mG* G*mU* G* G* A* A* A*mU* G*mA*mA	143	P.mY*fZ*fX* A*fY*fY*fZ*fX*fX* A*fX*mX*mZ*mY*mY* G*mX*mX*fX* A* A	149
24560		DY547.mA*mG* G*mU* G* G* A* A* A*mU* G*mA*mA	144	P.mU*fU*fC* A*fU*fU*fU*fC*fC* A*fC*mC*mU*mU*mU* G*mC*mC*fC* A* A	150

Table 6. SOD1 sd-rxRNA Variant Octyl Modifications

ID	Z=	Passenger Strand	SEQ ID NO:	Guide Strand	SEQ ID NO:
25573	octyl	DY547.mA*mG* G*mU* G* G* A* A* A*mU* G*mA*mA	151	P.mY*fY*fX* A*fY*fY*fZ*fX*fX* A*fX*mX*mZ*mY*mY* G*mX*mX*fX* A* A	157
25574	octyl	DY547.mA*mG* G*mU* G* G* A* A* A*mU* G*mA*mA	152	P.mY*fZ*fX* A*fY*fY*fY*fX*fX* A*fX*mX*mZ*mY*mY* G*mX*mX*fX* A* A	158
25575	octyl	DY547.mA*mG* G*mU* G* G* A* A* A*mU* G*mA*mA	153	P.mY*fZ*fX* A*fY*fY*fZ*fX*fX* A*fX*mX*mY*mY*mY* G*mX*mX*fX* A* A	159
25576		DY547.mA*mG* G*mU* G* G* A* A* A*mU* G*mA*mA	154	P.mY*fY*fX* A*fY*fY*fY*fX*fX* A*fX*mX*mY*mY*mY* G*mX*mX*fX* A* A	160
25577	thiophene	DY547.mA*mG* G*mZ* G* G* A* A* A*mZ* G*mA*mA	155	P.mU*fU*fC* A*fU*fU*fU*fC*fC* A*fC*mC*mU*mU*mU* G*mC*mC*fC* A* A	161
24560		DY547.mA*mG* G*mU* G* G* A* A* A*mU* G*mA*mA	156	P.mU*fU*fC* A*fU*fU*fU*fC*fC* A*fC*mC*mU*mU*mU* G*mC*mC*fC* A* A	162

Table 7. SOD1 sd-rxRNA Variant Thiophene Modifications

ID	Z=	Passenger Strand	SEQ ID NO:	Guide Strand	SEQ ID NO:
25578	thiophene	DY547.mA*mG* G*mY* G* G* A* A* A*mZ* G*mA*mA	163	P.mU*fU*fC* A*fU*fU*fU*fC*fC* A*fC*mC*mU*mU*mU* G*mC*mC*fC* A* A	169
25579	thiophene	DY547.mA*mG* G*mZ* G* G* A* A* A*mY* G*mA*mA	164	P.mU*fU*fC* A*fU*fU*fU*fC*fC* A*fC*mC*mU*mU*mU* G*mC*mC*fC* A* A	170
25580		DY547.mA*mG* G*mY* G* G* A* A* A*mY* G*mA*mA	165	P.mU*fU*fC* A*fU*fU*fU*fC*fC* A*fC*mC*mU*mU*mU* G*mC*mC*fC* A* A	171
25584	thiophene	DY547.mA*mG* G*mU* G* G* A* A* A*mU* G*mA*mA	166	P.mY*fZ*fX* A*fY*fY*fZ*fX*fX* A*fX*mX*mY*mY*mY* G*mX*mX*fX* A* A	172
25585	thiophene	DY547.mA*mG* G*mU*	167	P.mY*fY*fX* A*fY*fY*fY*fX*fX*	173

		G* G* A* A* A*mU* G*mA*mA		A*fX*mX*mY*mY*mY* G*mX*mX*fX* A* A	
24560		DY547.mA*mG* G*mU* G* G* A* A* A*mU* G*mA*mA	168	P.mU*fU*fC* A*fU*fU*fU*fC*fC* A*fC*mC*mU*mU*mU* G*mC*mC*fC* A* A	174

Table 8. SOD1 sd-rxRNA Variant Isobutyl Modifications

ID	Z=	Passenger Strand	SEQ ID NO:	Guide Strand	SEQ ID NO:
25586	isobutyl	DY547.mA*mG* G*dZ* G* G* A* A* A*dZ* G*mA*mA	175	P.mU*fU*fC* A*fU*fU*fU*fC*fC* A*fC*mC*mU*mU*mU* G*mC*mC*fC* A* A	181
25587	isobutyl	DY547.mA*mG* G*mY* G* G* A* A* A*dZ* G*mA*mA	176	P.mU*fU*fC* A*fU*fU*fU*fC*fC* A*fC*mC*mU*mU*mU* G*mC*mC*fC* A* A	182
25588	isobutyl	DY547.mA*mG* G*dZ* G* G* A* A* A*mY* G*mA*mA	177	P.mU*fU*fC* A*fU*fU*fU*fC*fC* A*fC*mC*mU*mU*mU* G*mC*mC*fC* A* A	183
25589	isobutyl	DY547.mA*mG* G*mU* G* G* A* A* A*mU* G*mA*mA	178	P.mY*dZ*fX* A*fY*fY*dZ*fX*fX* A*fX*mX*dZ*mY*mY* G*mX*mX*fX* A* A	184
25590	isobutyl	DY547.mA*mG* G*mU* G* G* A* A* A*mU* G*mA*mA	179	P.mY*fY*fX* A*fY*fY*dZ*fX*fX* A*fX*mX*dZ*mY*mY* G*mX*mX*fX* A* A	185
24560		DY547.mA*mG* G*mU* G* G* A* A* A*mU* G*mA*mA	180	P.mU*fU*fC* A*fU*fU*fU*fC*fC* A*fC*mC*mU*mU*mU* G*mC*mC*fC* A* A	186

Key for Tables 5 through 8:	
X	= 5 methyl C
Y	= 5 methyl U
f	= 2'fluoro
m	= 2'Ome
P	= 5' phosphate
*	= phosphorothioate linkage
.	= phosphodiester linkage
DY547	= DY547 dye
d	= deoxyribose

EQUIVALENTS

Those skilled in the art will recognize, or be able to ascertain using no more than routine experimentation, many equivalents to the specific embodiments of the invention described herein. Such equivalents are intended to be encompassed by the following claims.

All references, including patent documents, disclosed herein are incorporated by reference in their entirety. This application incorporates by reference the entire contents, including all the drawings and all parts of the specification (including sequence listing or amino acid / polynucleotide sequences) of PCT Publication No. WO2010/033247 (Application No. PCT/US2009/005247), filed on September 22, 2009, and entitled "REDUCED SIZE SELF-DELIVERING RNAI COMPOUNDS," US Patent No. 8,796,443, issued on August 5, 2014, published as US 2012/0040459 on February 16, 2012, entitled "REDUCED SIZE SELF-DELIVERING RNAI COMPOUNDS," US Patent No. 9,175,289, issued on November 3, 2015, entitled "REDUCED SIZE SELF-DELIVERING RNAI COMPOUNDS," PCT Publication No. WO2009/102427 (Application No. PCT/US2009/000852), filed on February 11, 2009, and entitled, "MODIFIED RNAI POLYNUCLEOTIDES AND USES THEREOF," and US Patent Publication No. 2011/0039914, published on February 17, 2011 and entitled "MODIFIED RNAI POLYNUCLEOTIDES AND USES THEREOF," PCT Publication No. WO 2011/119852, filed on March 24, 2011 and entitled "REDUCED SIZE SELF-DELIVERING RNAI COMPOUNDS," and US Patent No. 9,080,171, issued on July 14, 2015, and entitled "REDUCED SIZE SELF-DELIVERING RNAI COMPOUNDS."

CLAIMS

1. An isolated double stranded nucleic acid molecule directed against superoxide dismutase 1 (SOD1) comprising a guide strand and a passenger strand, wherein the isolated double stranded nucleic acid molecule includes a double stranded region and a single stranded region, wherein the region of the molecule that is double stranded is from 8-15 nucleotides long, wherein the guide strand contains a single stranded region that is 2-14 nucleotides long, wherein the guide strand contains 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21 or 22 phosphorothioate modifications, wherein the passenger strand is 8 to 15 nucleotides long, wherein the passenger strand contains 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13 or 14 phosphorothioate modifications, wherein at least 40% of the nucleotides of the isolated double stranded nucleic acid molecule are modified, and wherein the isolated double stranded nucleic acid molecule comprises at least 12 contiguous nucleotides of a sequence selected from the sequences within Tables 1-8, including the modification pattern provided in Tables 1-8.

2. The isolated double stranded nucleic acid molecule of claim 1, wherein at least 60% of the nucleotides are modified.

3. The isolated double stranded nucleic acid molecule of claim 1 or claim 2, wherein at least one of the nucleotides of the isolated double stranded nucleic acid molecule that is modified comprises a 2'O-methyl or a 2'-fluoro modification.

4. The isolated double stranded nucleic acid molecule of any one of claims 1 to 3, wherein at least one strand of the isolated double stranded nucleic acid molecule is completely phosphorothioated, or is completely phosphorothioated with the exception of one residue.

5. The isolated double stranded nucleic acid molecule of any one of claims 1 to 4, wherein a plurality of the U's and/or C's include a hydrophobic modification, selected from the group consisting of methyl, isobutyl, octyl, imidazole or thiophene and wherein the modifications are located on positions 4 or 5 of U's and/or C's.

6. An isolated double stranded nucleic acid molecule that comprises at least 12 contiguous nucleotides of a sequence selected from the sequences within Tables 1-8, wherein if the isolated double stranded nucleic acid molecule comprises at least 12

contiguous nucleotides of a sequence selected from SEQ ID NOs: 70, 71, 72, 73, 79, 80, 81, or 84 in Table 2, then the guide strand contains more than 6 phosphorothioate modifications.

7. The isolated double stranded nucleic acid molecule of any one of claims 1 to 6, wherein the isolated double stranded nucleic acid molecule further comprises a hydrophobic conjugate that is attached to the isolated double stranded nucleic acid molecule.

8. The isolated double stranded nucleic acid molecule of any one of claims 1 to 7, wherein the sense strand comprises SEQ ID NO: 2, SEQ ID NO: 32, or SEQ ID NO: 122, and the guide strand comprises SEQ ID NO: 61, SEQ ID NO: 91, or SEQ ID NO: 123.

9. The isolated double stranded nucleic acid molecule of any one of claims 1 to 7, wherein the sense strand comprises SEQ ID NO: 4, SEQ ID NO: 34, or SEQ ID NO: 126, and the guide strand comprises SEQ ID NO: 63 or SEQ ID NO: 93.

10. The isolated double stranded nucleic acid molecule of any one of claims 1 to 7, wherein the sense strand comprises SEQ ID NO: 9, SEQ ID NO: 38, or SEQ ID NO:135, and the guide strand comprises SEQ ID NO: 68, SEQ ID NO: 97, or SEQ ID NO: 136.

11. The isolated double stranded nucleic acid molecule of any one of claims 1 to 7, wherein the sense strand comprises SEQ ID NO: 10 or SEQ ID NO: 39, and the guide strand comprises SEQ ID NO: 69 or SEQ ID NO: 98.

12. The isolated double stranded nucleic acid molecule of any one of claims 1 to 7, wherein the sense strand comprises SEQ ID NO: 5, SEQ ID NO: 127 or SEQ ID NO: 137, and the guide strand comprises SEQ ID NO: 64, SEQ ID NO: 128 or SEQ ID NO: 138.

13. A composition comprising the isolated double stranded nucleic acid molecule of any one of claims 1 to 12.

14. The composition of claim 13, further comprising a pharmaceutically acceptable carrier.

15. The composition of claim 13 or claim 14 further comprising a second therapeutic agent.

16. A method for treating ALS comprising administering to a subject in need thereof a therapeutically effective amount of an isolated double stranded nucleic acid molecule of any one of claims 1 to 12, or a composition of any one of claims 13 to 15.

17. A method for treating ALS comprising administering to a subject in need thereof a therapeutically effective amount of an isolated double stranded nucleic acid molecule directed against superoxide dismutase 1 (SOD1) comprising a guide strand and a passenger strand, wherein the isolated double stranded nucleic acid molecule includes a double stranded region and a single stranded region, wherein the region of the molecule that is double stranded is from 8-15 nucleotides long, wherein the guide strand contains a single stranded region that is 2-14 nucleotides long, wherein the guide strand contains 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21 or 22 phosphorothioate modifications, wherein the passenger strand is 8 to 15 nucleotides long, wherein the passenger strand contains 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13 or 14 phosphorothioate modifications, wherein at least 40% of the nucleotides of the isolated double stranded nucleic acid molecule are modified, and wherein the isolated double stranded nucleic acid molecule comprises at least 12 contiguous nucleotides of a sequence selected from the sequences within Tables 1-8, including the modification pattern provided in Tables 1-8.

18. The method of claim 17, wherein the isolated double stranded nucleic acid molecule further comprises a hydrophobic conjugate that is attached to the isolated double stranded nucleic acid molecule.

19. The method of claim 17 or claim 18, wherein at least one strand of the isolated double stranded nucleic acid molecule is completely phosphorothioated, or is completely phosphorothioated with the exception of one residue.

20. The method of any one of claims 17 to 19, wherein the isolated double stranded nucleic acid molecule is formulated for delivery to the central nervous system.

21. The method of any one of claims 17 to 20, wherein the sense strand of the isolated double stranded nucleic acid molecule comprises SEQ ID NO: 2, SEQ ID

NO: 32, or SEQ ID NO: 122, and the guide strand of the isolated double stranded nucleic acid molecule comprises SEQ ID NO: 61, SEQ ID NO: 91, or SEQ ID NO: 123.

22. The method of any one of claims 17 to 20, wherein the sense strand of the isolated double stranded nucleic acid molecule comprises SEQ ID NO: 4, SEQ ID NO: 34, or SEQ ID NO: 126, and the guide strand of the isolated double stranded nucleic acid molecule comprises SEQ ID NO: 63 or SEQ ID NO: 93.

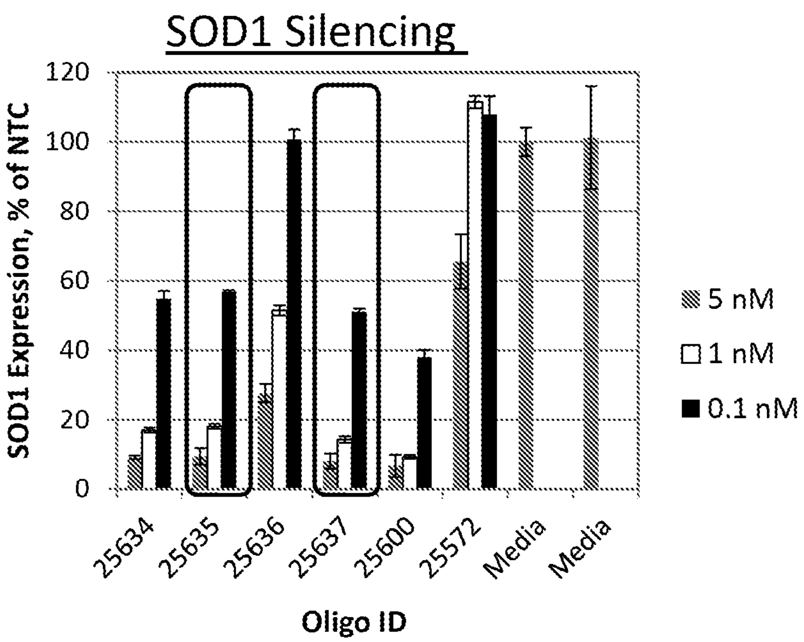
23. The method of any one of claims 17 to 20, wherein the sense strand of the isolated double stranded nucleic acid molecule comprises SEQ ID NO: 9, SEQ ID NO: 38, or SEQ ID NO:135, and the guide strand of the isolated double stranded nucleic acid molecule comprises SEQ ID NO: 68, SEQ ID NO: 97, or SEQ ID NO: 136.

24. The method of any one of claims 17 to 20, wherein the sense strand of the isolated double stranded nucleic acid molecule comprises SEQ ID NO: 10 or SEQ ID NO: 39, and the guide strand of the isolated double stranded nucleic acid molecule comprises SEQ ID NO: 69 or SEQ ID NO: 98.

25. The method of any one of claims 17 to 20, wherein the sense strand of the isolated double stranded nucleic acid comprises SEQ ID NO: 5, SEQ ID NO: 127 or SEQ ID NO: 137, and the guide strand of the isolated double stranded nucleic acid comprises SEQ ID NO: 64, SEQ ID NO: 128 or SEQ ID NO: 138.

26. The method of any one of claims 17 to 25, wherein the isolated double stranded nucleic acid molecule is administered via intrathecal infusion and/or injection.

Reducing Phosphorothioate Content Results in Active ps-rxRNA Variants with Reduced Cellular Toxicity *in vitro*



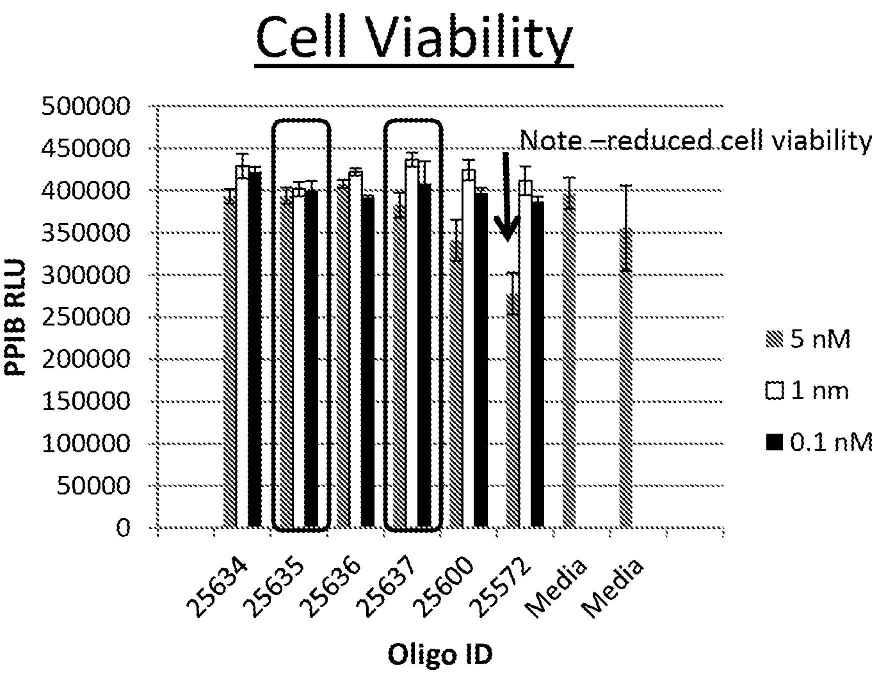


FIG. 1

Reducing Phosphorothioate Content Results in Active ps-rxRNA Variants with Reduced Cellular Toxicity *in vitro*

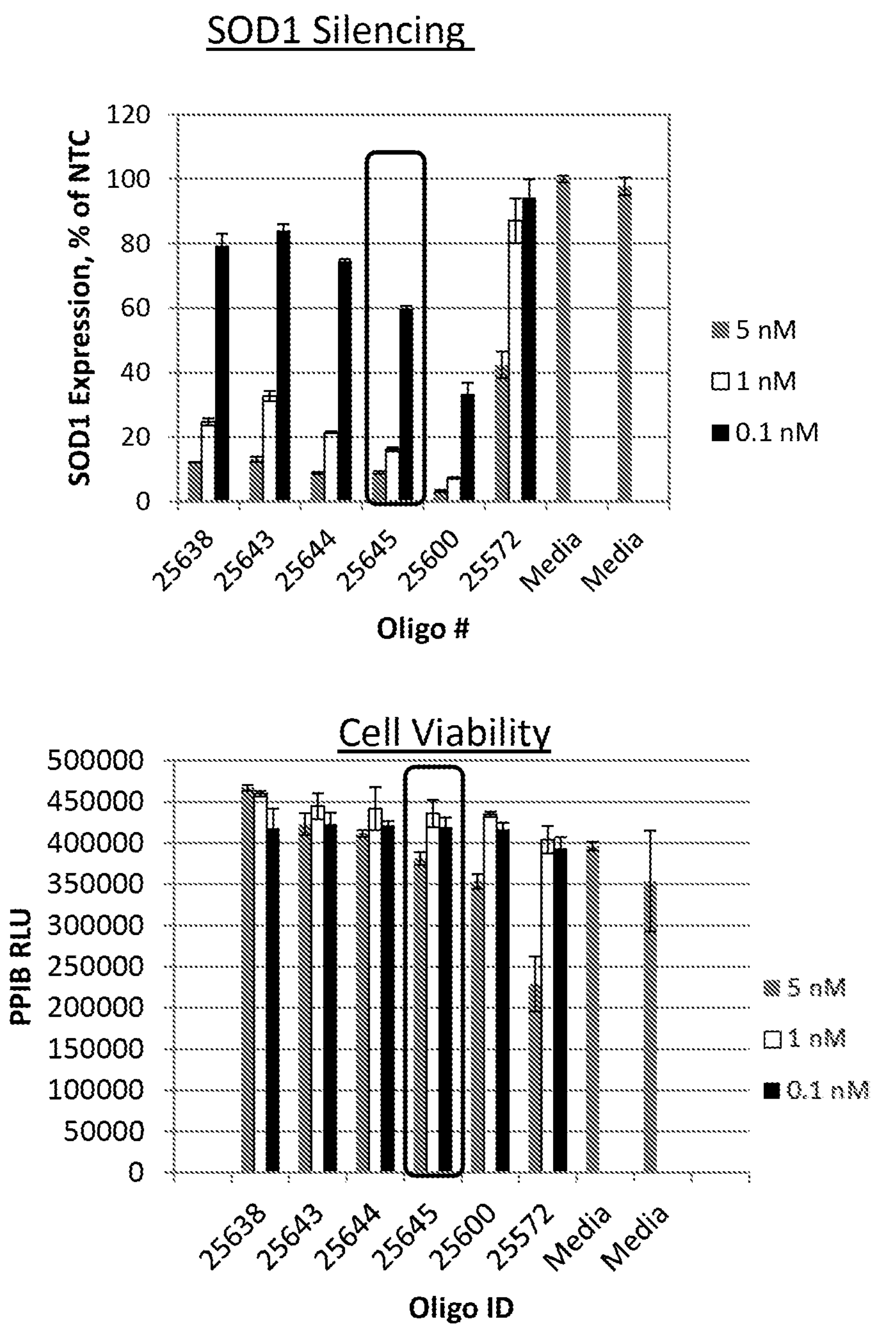


FIG. 2

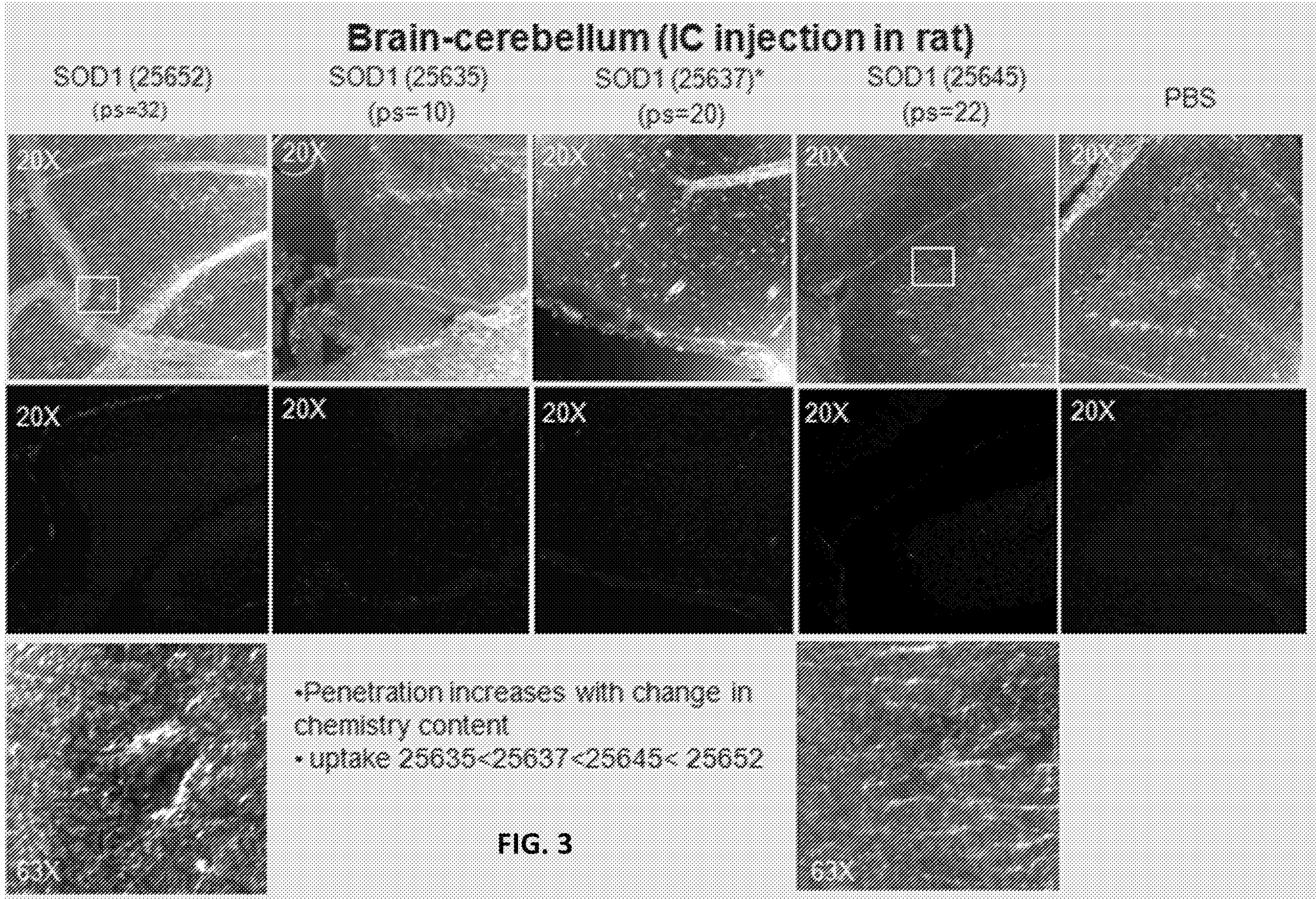

Brain-cerebellum (IC injection in rat)
SOD1 (25652)
(ps=32)
SOD1 (25635)
(ps=10)
SOD1 (25637)*
(ps=20)
SOD1 (25645)
(ps=22)
PBS
20X
63X
•Penetration increases with change in chemistry content
• uptake 25635<25637<25645< 25652

FIG. 3

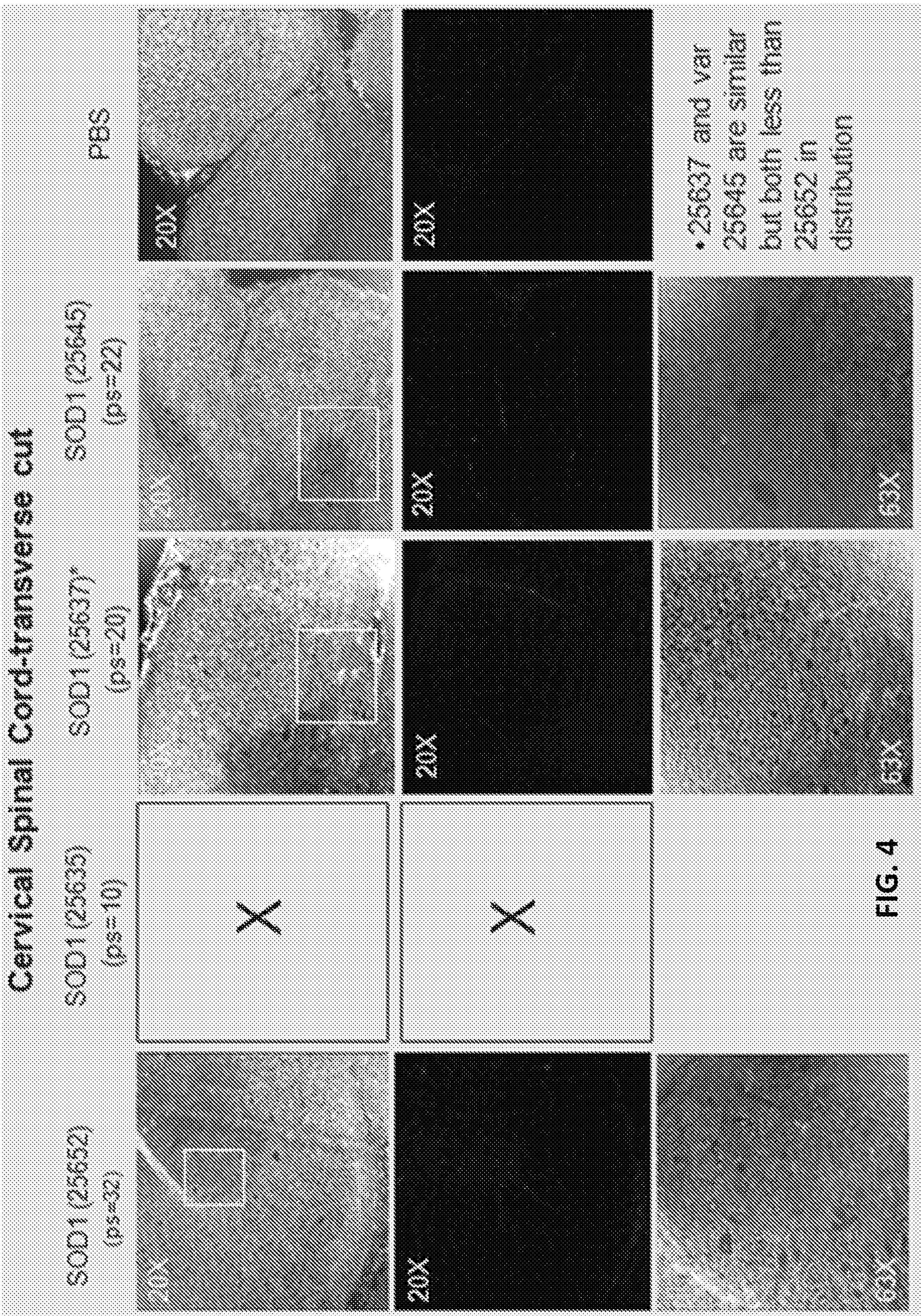

Cervical Spinal Cord-transverse cut
SOD1 (25652) (ps=32)
SOD1 (25635) (ps=10)
SOD1 (25637)* (ps=20)
SOD1 (25645) (ps=22)
PBS
20X
63X
• 25637 and var 25645 are similar but both less than 25652 in distribution

FIG. 4

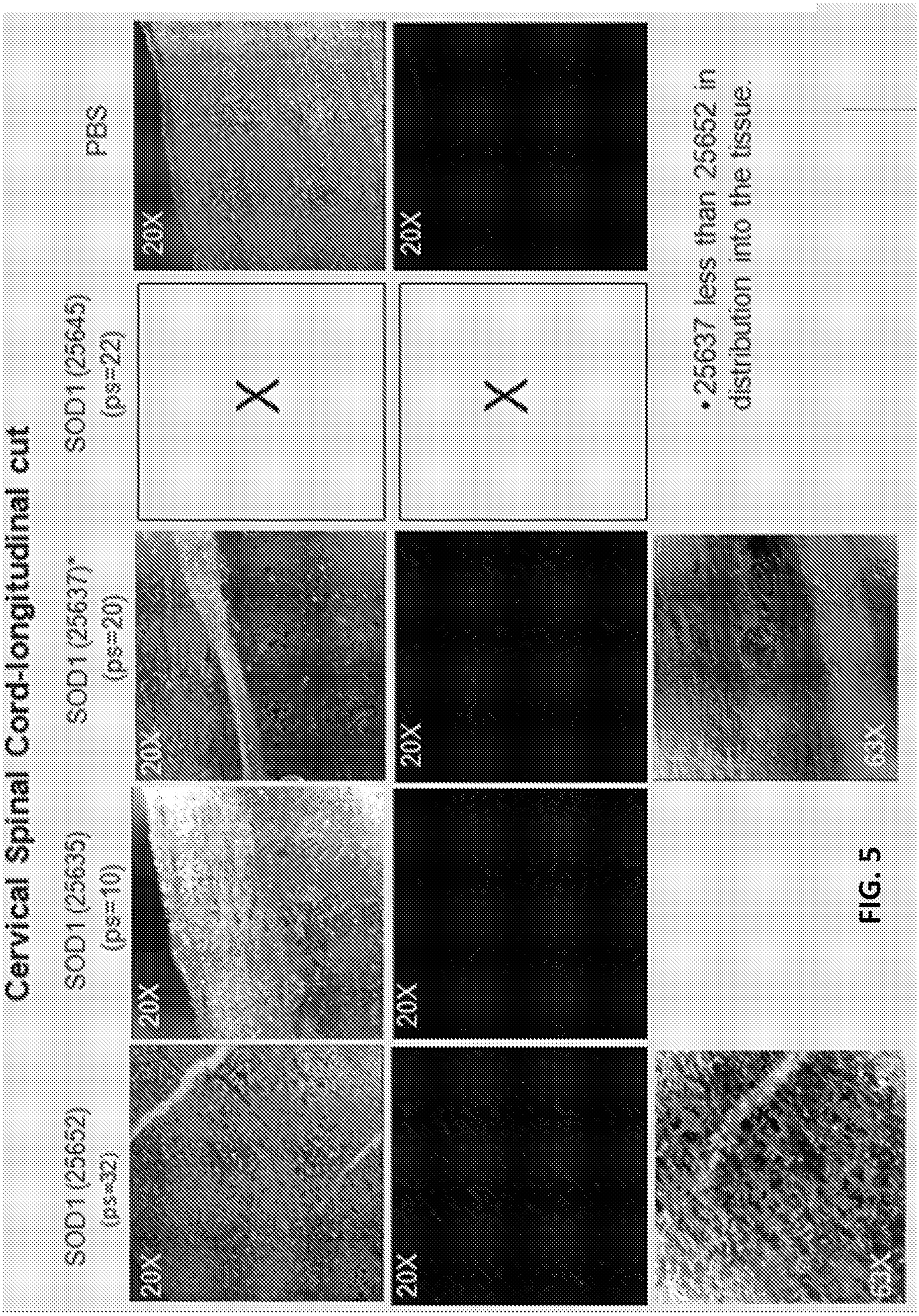

Cervical Spinal Cord-longitudinal cut
SOD1 (25652) (ps=32)
SOD1 (25635) (ps=10)
SOD1 (25637)* (ps=20)
SOD1 (25645) (ps=22)
PBS
20X
63X
* 25637 less than 25652 in distribution into the tissue.

FIG. 5

Reduction of SOD1 mRNA following a 14-day Intrathecal Infusion of SOD1 Targeting sd-rxRNA in Normal Mice

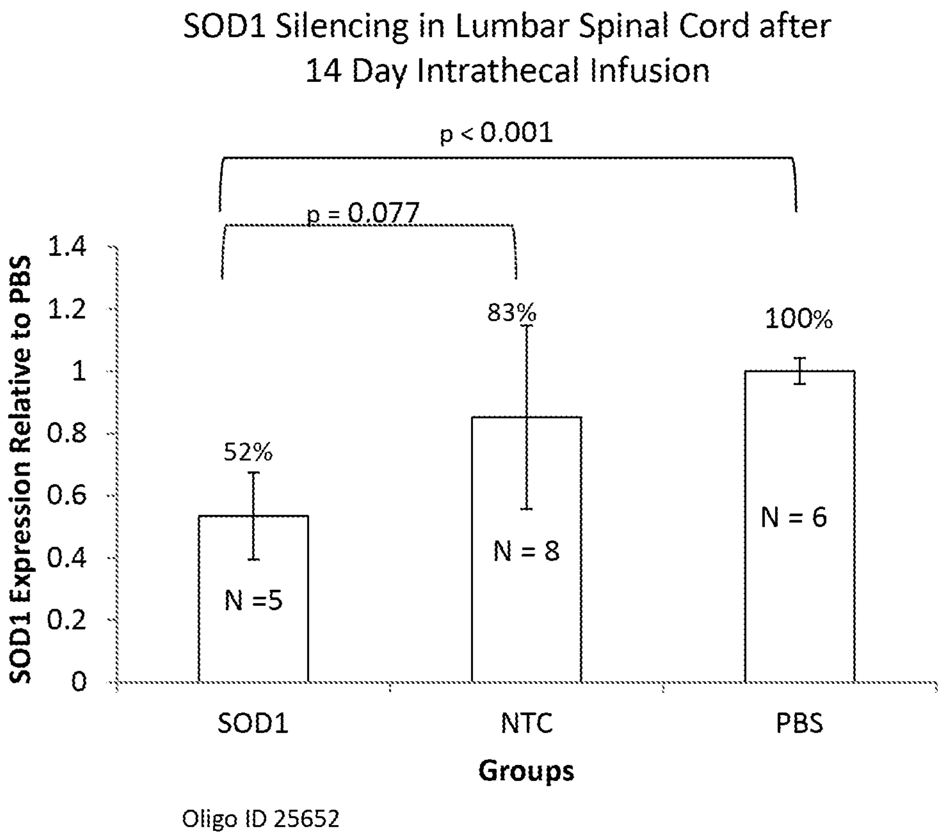
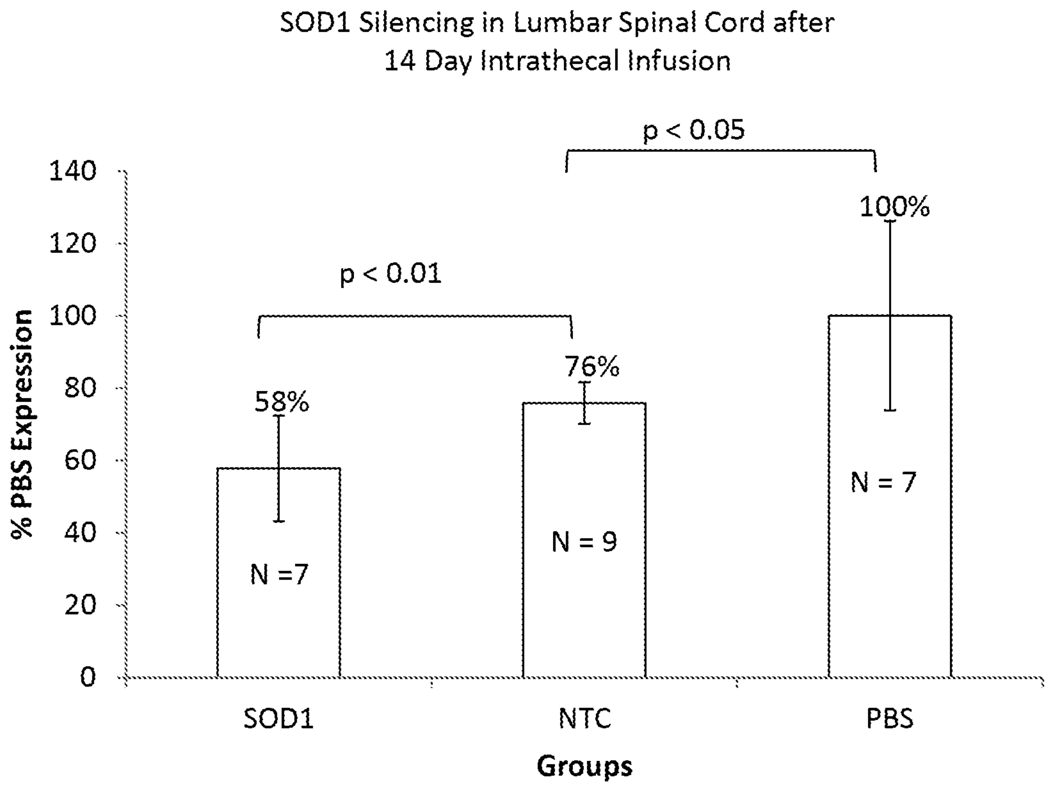


FIG. 6

Reduction of SOD1 mRNA following a 14-day Intrathecal Infusion of SOD1 Targeting sd-rxRNA Varient 3 in Normal Mice



Oligo ID25645

FIG. 7

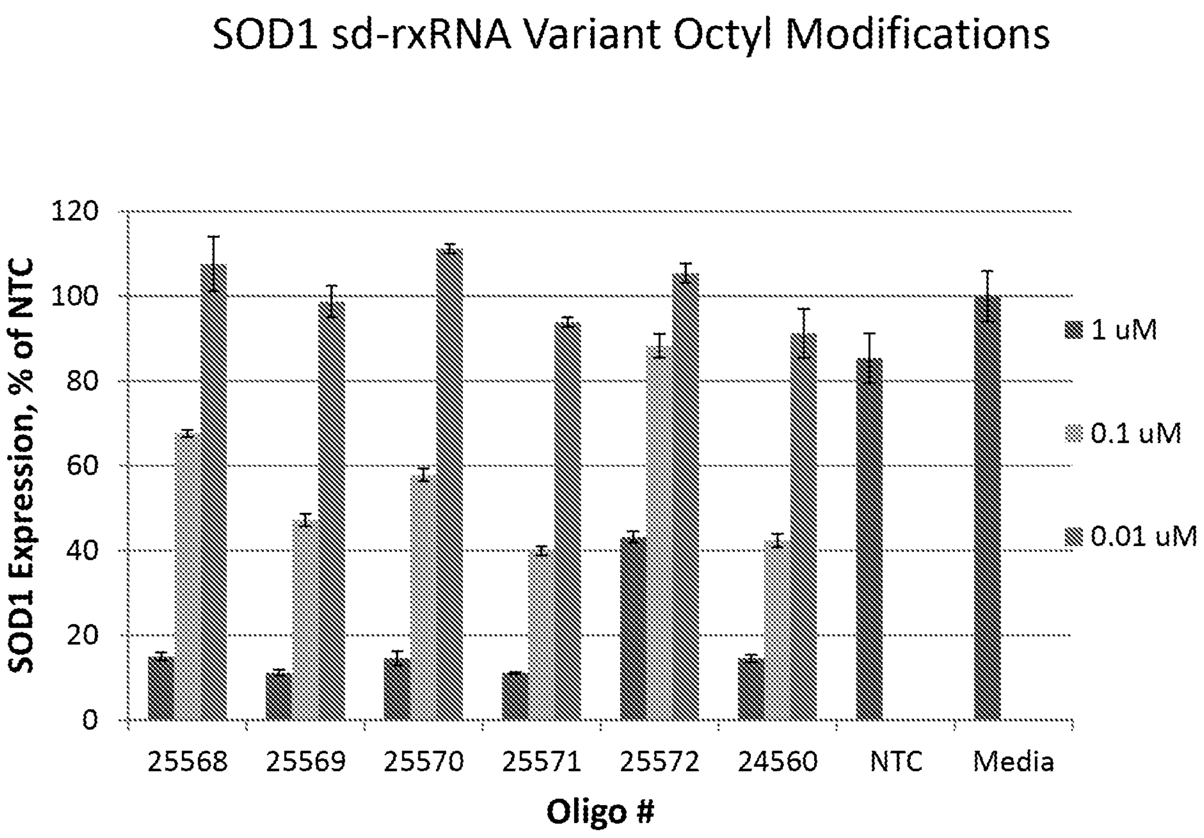

SOD1 sd-rxRNA Variant Octyl Modifications
SOD1 Expression, % of NTC
120
100
80
60
40
20
0
1 uM
0.1 uM
0.01 uM
25568
25569
25570
25571
25572
24560
NTC
Media
Oligo #

FIG. 8

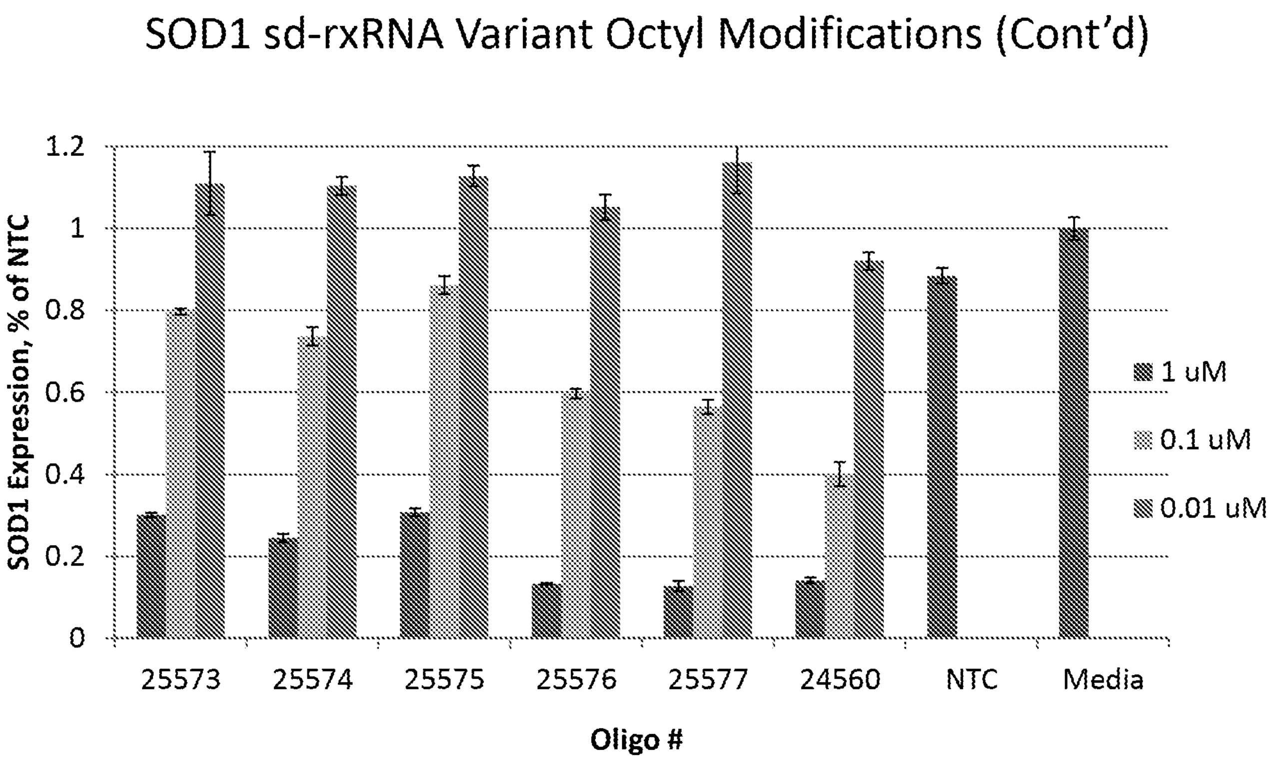

SOD1 sd-rxRNA Variant Octyl Modifications (Cont'd)
SOD1 Expression, % of NTC
1.2
1
0.8
0.6
0.4
0.2
0
25573
25574
25575
25576
25577
24560
NTC
Media
Oligo #
1 uM
0.1 uM
0.01 uM

FIG. 9

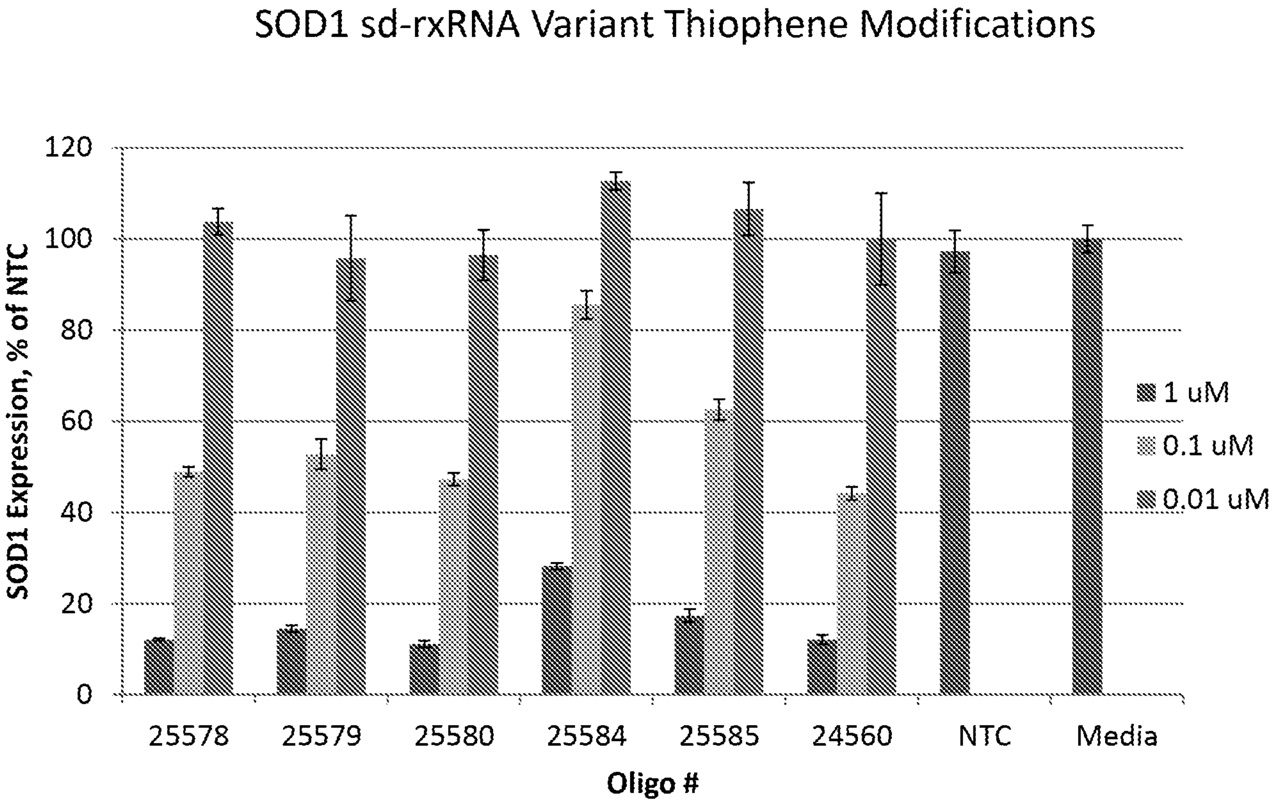

SOD1 sd-rxRNA Variant Thiophene Modifications
SOD1 Expression, % of NTC
120
100
80
60
40
20
0
25578
25579
25580
25584
25585
24560
NTC
Media
Oligo #
1 uM
0.1 uM
0.01 uM

FIG. 10

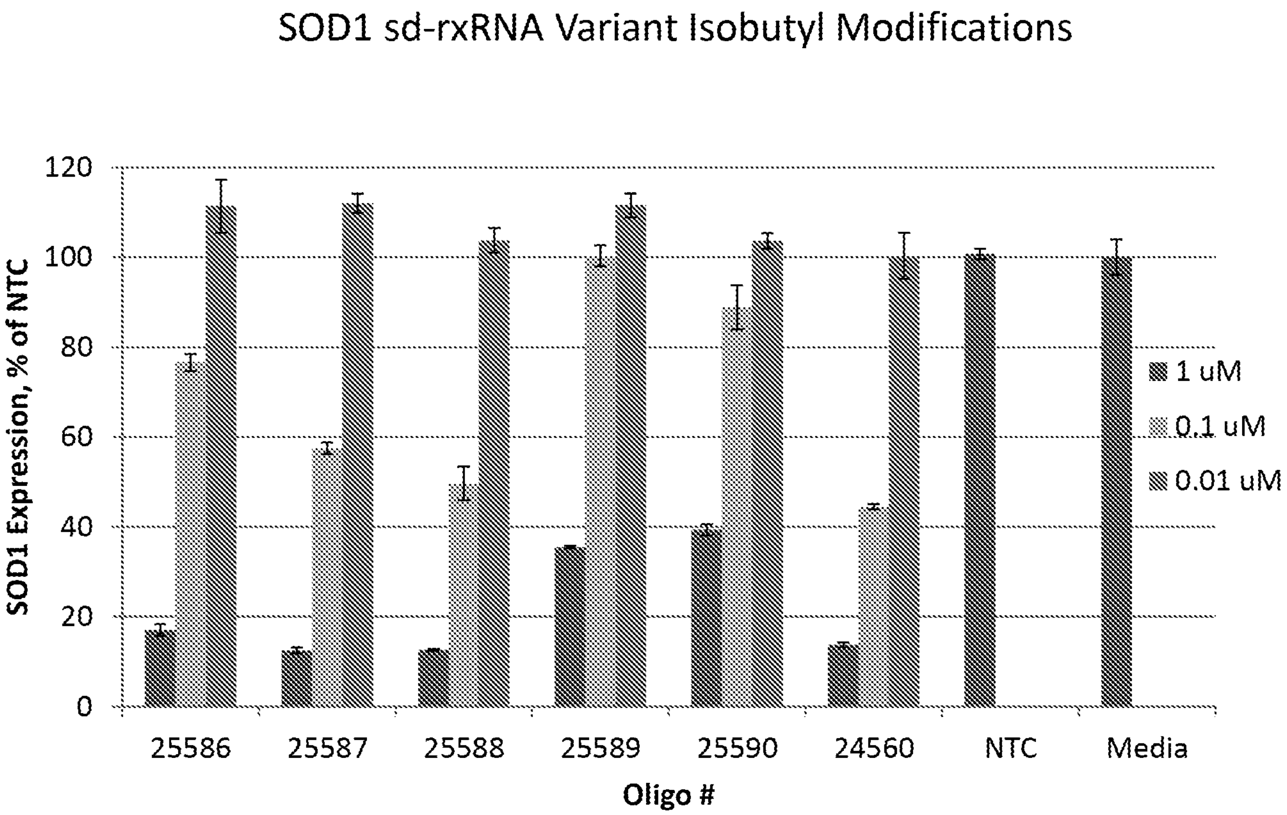

SOD1 sd-rxRNA Variant Isobutyl Modifications
SOD1 Expression, % of NTC
120
100
80
60
40
20
0
1 uM
0.1 uM
0.01 uM
25586
25587
25588
25589
25590
24560
NTC
Media
Oligo #

FIG. 11

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US2016/041095

A. CLASSIFICATION OF SUBJECT MATTER
IPC(8) - A61K 31/713; A61P 25/28; C07H 21/02; C12N 15/113 (2016.01)
CPC - C12N 15/111; C12N 15/113; C12N 15/1137; C12N 2310/14; C12N 2310/315; C12N 2320/5 (2016.11)
According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC - A61K 31/713; A61P 25/28; C07H 21/02; C12N 15/113
CPC - C12N 15/111; C12N 15/113; C12N 15/1137; C12N 2310/14; C12N 2310/315; C12N 2320/50; C12Y 115/01001

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched
USPC - 435/375; 514/44A; 536/24.5; 536/24.1 (keyword delimited)

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)
PatBase, Google Patents, Google Scholar, PubMed

Search terms used: amyotrophic lateral sclerosis ALS superoxide dismutase 1 SOD-1 double strand duplex guide passenger

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	US 2004/0192629 A1 (XU et al) 30 September 2004 (30.09.2004) entire document	1-3, 17-19
A	US 2013/0131141 A1 (KHVOROVA et al) 23 May 2013 (23.05.2013) entire document	1-3, 17-19
A	US 2006/0229268 A1 (BENJAMIN et al) 12 October 2006 (12.10.2006) entire document	1-3, 17-19
A	US 2011/0039914 A1 (PAVCO et al) 17 February 2011 (17.02.2011) entire document	1-3, 17-19
A	US 2014/0364482 A1 (RXI PHARMACEUTICALS CORPORATION) 11 December 2014 (11.12.2014) entire document	1-3, 17-19
A	US 2008/0125386 A1 (RANA et al) 29 May 2008 (29.05.2008) entire document	1-3, 17-19

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

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

Date of the actual completion of the international search	Date of mailing of the international search report
17 November 2016	08 DEC 2016

Name and mailing address of the ISA/US	Authorized officer
Mail Stop PCT, Attn: ISA/US, Commissioner for Patents P.O. Box 1450, Alexandria, VA 22313-1450 Facsimile No. 571-273-8300	Blaine R. Copenheaver PCT Helpdesk: 571-272-4300 PCT OSP: 571-272-7774

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US2016/041095

Box No. I Nucleotide and/or amino acid sequence(s) (Continuation of item 1.c of the first sheet)

1. With regard to any nucleotide and/or amino acid sequence disclosed in the international application, the international search was carried out on the basis of a sequence listing:

   a. [X] forming part of the international application as filed:

      [X] in the form of an Annex C/ST.25 text file.

      [ ] on paper or in the form of an image file.

   b. [ ] furnished together with the international application under PCT Rule 13*ter*.1(a) for the purposes of international search only in the form of an Annex C/ST.25 text file.

   c. [ ] furnished subsequent to the international filing date for the purposes of international search only:

      [ ] in the form of an Annex C/ST.25 text file (Rule 13*ter*.1(a)).

      [ ] on paper or in the form of an image file (Rule 13*ter*.1(b) and Administrative Instructions, Section 713).

2. [ ] In addition, in the case that more than one version or copy of a sequence listing has been filed or furnished, the required statements that the information in the subsequent or additional copies is identical to that forming part of the application as filed or does not go beyond the application as filed, as appropriate, were furnished.

3. Additional comments:

SEQ ID NOs:2 and 61 were searched.

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US2016/041095

Box No. II Observations where certain claims were found unsearchable (Continuation of item 2 of first sheet)

This international search report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐ Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:

2. ☐ Claims Nos.:
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:

3. ☒ Claims Nos.: 4, 5, 7-16, 20-26
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box No. III Observations where unity of invention is lacking (Continuation of item 3 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:
see Extra Sheet(s).

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.

2. ☐ As all searchable claims could be searched without effort justifying additional fees, this Authority did not invite payment of additional fees.

3. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:

4. ☒ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:
1-3 and17-19 restricted to "oligo number 25635" and SEQ ID NO:2 and SEQ ID NO:61.

Remark on Protest

☐ The additional search fees were accompanied by the applicant's protest and, where applicable, the payment of a protest fee.

☐ The additional search fees were accompanied by the applicant's protest but the applicable protest fee was not paid within the time limit specified in the invitation.

☐ No protest accompanied the payment of additional search fees.

Continued from Box No. III Observations where unity of invention is lacking

This application contains the following inventions or groups of inventions which are not so linked as to form a single general inventive concept under PCT Rule 13.1. In order for all inventions to be examined, the appropriate additional examination fees need to be paid.

Group I+: claims 1-3, 6, and 17-19 are drawn to isolated double stranded nucleic acid molecules directed against superoxide dismutase 1 (SOD1), and methods comprising the same.

The first invention of Group I+ is restricted to an isolated double stranded nucleic acid molecule directed against superoxide dismutase 1 (SOD1), and methods comprising the same, wherein the isolated double stranded nucleic acid molecule is selected to be "oligo number 25635", wherein the isolated double stranded nucleic acid molecule comprises a guide strand and a passenger strand, wherein the guide (antisense) strand is selected to be SEQ ID NO:61 with antisense chemistry and antisense backbone as defined in Table 2 of the instant application; wherein the passenger (sense) strand is selected to be SEQ ID NO:2 with sense chemistry and sense backbone as defined in Table 1 of the instant application. It is believed that claims 1-3 and 17-19 read on this first named invention and thus these claims will be searched without fee to the extent that they read on "oligo number 25635" and SEQ ID NO:2 and SEQ ID NO:61.

Applicant is invited to elect additional isolated double stranded nucleic acid molecules with specified SEQ ID NO, chemistry, and backbone to be searched in a specific combination by paying additional fee for each set of election. An exemplary election would be an isolated double stranded nucleic acid molecule directed against superoxide dismutase 1 (SOD1), and methods comprising the same, wherein the isolated double stranded nucleic acid molecule is selected to be "oligo number 25568", wherein the isolated double stranded nucleic acid molecule comprises a guide strand and a passenger strand, wherein the guide (antisense) strand is selected to be SEQ ID NO:70 with antisense chemistry and antisense backbone as defined in Table 2 of the instant application; wherein the passenger (sense) strand is selected to be SEQ ID NO:11 with sense chemistry and sense backbone as defined in Table 1 of the instant application. Additional isolated double stranded nucleic acid molecules with specified SEQ ID NO, chemistry, and backbone will be searched upon the payment of additional fees. Applicants must specify the claims that read on any additional elected inventions. Applicants must further indicate, if applicable, the claims which read on the first named invention if different than what was indicated above for this group. Failure to clearly identify how any paid additional invention fees are to be applied to the "+" group(s) will result in only the first claimed invention to be searched/examined.

The inventions listed in Groups I+ do not relate to a single general inventive concept under PCT Rule 13.1, because under PCT Rule 13.2 they lack the same or corresponding special technical features for the following reasons:

The Groups I+ formulas do not share a significant structural element responsible for treating ALS, requiring the selection of alternatives for the guide and passenger strands of the additional isolated double stranded nucleic acid molecule, where "the isolated double stranded nucleic acid molecule includes a double stranded region and a single 5 stranded region, wherein the region of the molecule that is double stranded is from 8-15 nucleotides long, wherein the guide strand contains a single stranded region that is 2-14 nucleotides long, wherein the guide strand contains 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21 or 22 phosphorothioate modifications, wherein the passenger strand is 8 to 15 nucleotides long, wherein the passenger strand contains 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13 10 or 14 phosphorothioate modifications, wherein at least 40% of the nucleotides of the isolated double stranded nucleic acid molecule are modified, and wherein the isolated double stranded nucleic acid molecule comprises at least 12 contiguous nucleotides of a sequence selected from the sequences within Tables 1-8, including the modification pattern provided in Tables 1-8".

The Groups I+ share the technical features of an isolated double stranded nucleic acid molecule directed against superoxide dismutase 1 (SOD1) comprising a guide strand and a passenger strand, wherein the isolated double stranded nucleic acid molecule includes a double stranded region and a single stranded region; an isolated double stranded nucleic acid molecule that comprises at least 12 contiguous nucleotides of a sequence; a method for treating ALS comprising administering to a subject in need thereof a therapeutically effective amount of an isolated double stranded nucleic acid molecule directed against superoxide dismutase I (SOD1) comprising a guide strand and a passenger strand, wherein the isolated double stranded nucleic acid molecule includes a double stranded region and a single stranded region. However, these shared technical features do not represent a contribution over the prior art.

Specifically, US 2004/0192629 A1 to Xu et al. discloses an isolated double stranded nucleic acid molecule directed against superoxide dismutase 1 (SOD1) ("isolated RNA" (e.g., "isolated siRNA" or "isolated siRNA precursor") refers to RNA molecules which are substantially free of other cellular material, Para. [0048]; introducing double-stranded RNA corresponding to the target gene, Para. [0031]; siRNA duplexes used: mutant siRNA P11 (SEQ ID NO: 5, sense; SEQ ID NO: 6, anti-sense or guide), Para. [0019]; (RNAi) against selected point mutations occurring in a single allele in the mutant gene e.g., the point mutation in the copper zinc superoxide dismutase (SOD1) gene associated with amyotrophic lateral sclerosis (ALS), Para. [0030]) comprising a guide strand and a passenger strand (mutant siRNA P11 (SEQ ID NO: 5, sense; SEQ ID NO: 6, anti-sense or guide), Para. [0019]), wherein the isolated double stranded nucleic acid molecule includes a double stranded region and a single stranded region (double-stranded RNA (siRNA) which complements a region containing a point mutation within the mutant SOD1 mRNA. After introduction of siRNA into neurons, the siRNA partially unwinds, binds to the region containing the point mutation within the SOD1 mRNA in a site-specific manner, Para. [0060]; an overhang of 1, 2 or 3 residues occurs at one or both ends of the duplex when strands are annealed, Para. [0066]); an isolated double stranded nucleic acid molecule that comprises at least 12 contiguous nucleotides of a sequence (siRNA duplexes used: mutant siRNA P11 (SEQ ID NO: 5, sense; SEQ ID NO: 6, anti-sense or guide), Para. [0019]); a method for treating ALS (These methods are applicable to the treatment of diseases that are caused by dominant, gain-of-function type of gene mutations, including, but not limited to, ALS, Para. [0029]) comprising administering to a subject in need thereof a therapeutically effective amount of an isolated double stranded nucleic acid molecule directed against superoxide dismutase I (SOD1) comprising a guide strand and a passenger strand (the method comprising administering to the subject a therapeutically effective amount of an siRNA specific for the mutant allele, Para. [0009];]; siRNA duplexes used: mutant siRNA P11 (SEQ ID NO: 5, sense; SEQ ID NO: 6, anti-sense or guide), Para. [0019]; (RNAi) against selected point mutations occurring in a single allele in the mutant gene e.g., the point mutation in the copper zinc superoxide dismutase (SOD1) gene associated with amyotrophic lateral sclerosis (ALS), Para. [0030]), wherein the isolated double stranded nucleic acid molecule includes a double stranded region and a single stranded region (double-stranded RNA (siRNA) which complements a region containing a point mutation within the mutant SOD1 mRNA. After introduction of siRNA into neurons, the siRNA partially unwinds, binds to the region containing the point mutation within the SOD1 mRNA in a site-specific manner, Para. [0060]; an overhang of 1, 2, or 3 residues occurs at one or both ends of the duplex when strands are annealed, Para. [0066]).

The inventions listed in Groups I+ therefore lack unity under Rule 13 because they do not share a same or corresponding special technical features.