



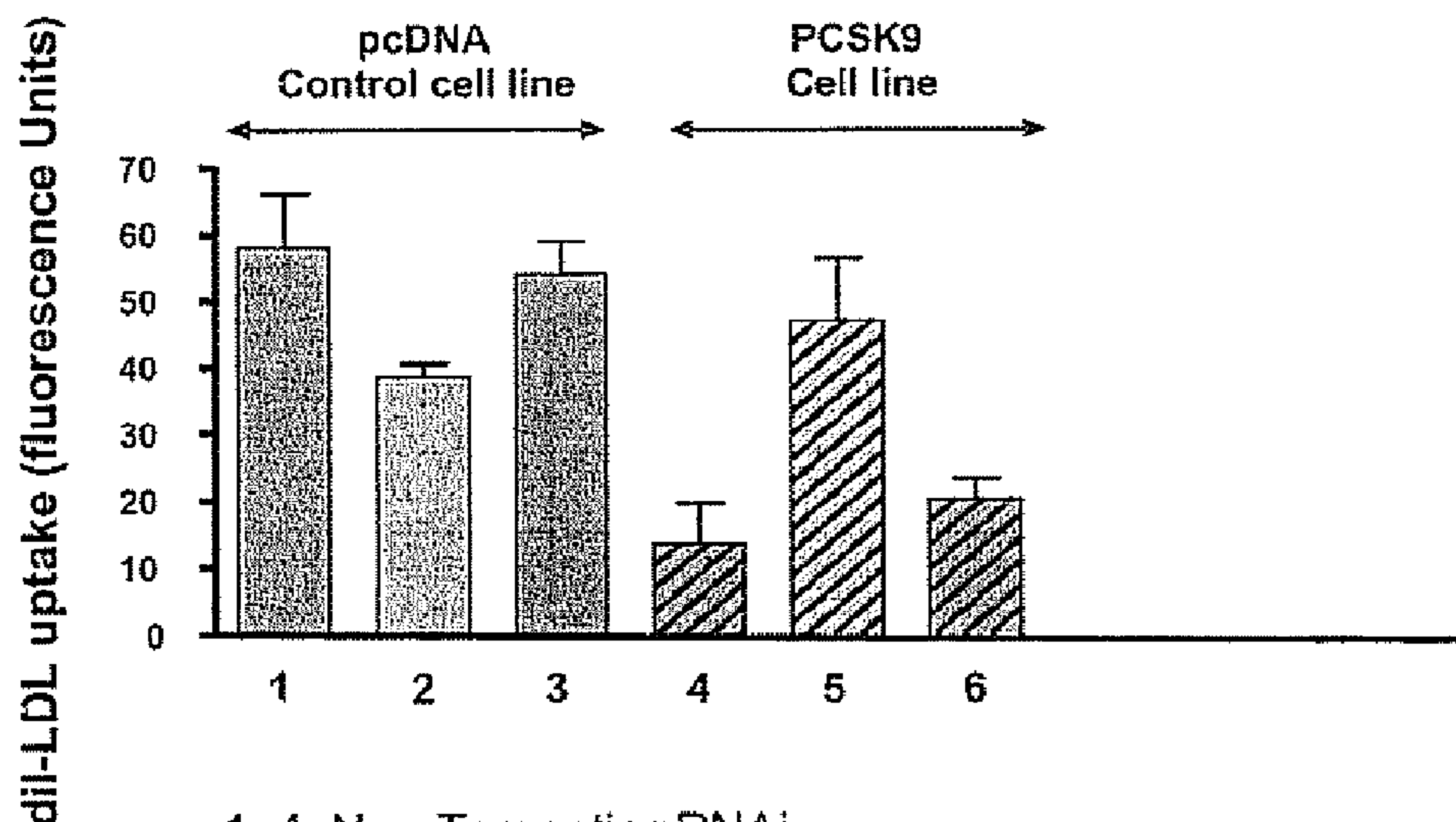
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(71) Demandeur/Applicant:
SIRNA THERAPEUTICS INC., US
(72) Inventeurs/Inventors:
MC SWIGGEN, JAMES, US;
JADHAV, VASANT, US;
VARGESE, CHANDRA, US
(74) Agent: OGILVY RENAULT LLP/S.E.N.C.R.L.,S.R.L.

(54) Titre : INHIBITION MEDIEE PAR UNE INTERFERENCE DE L'ARN DE L'EXPRESSION DU GENE DE LA
PROPROTEINE CONVERTASE SUBTILISINE KEXINE DE TYPE 9 (PCSK9) AU MOYEN DE PETITS ACIDES
NUCLEIQUES INTERFERANTS(ANsi)

(54) Title: RNA INTERFERENCE MEDIATED INHIBITION OF PROPROTEIN CONVERTASE SUBTILISIN KEXIN 9
(PCSK9) GENE EXPRESSION USING SHORT INTERFERING NUCLEIC ACID (siNA)

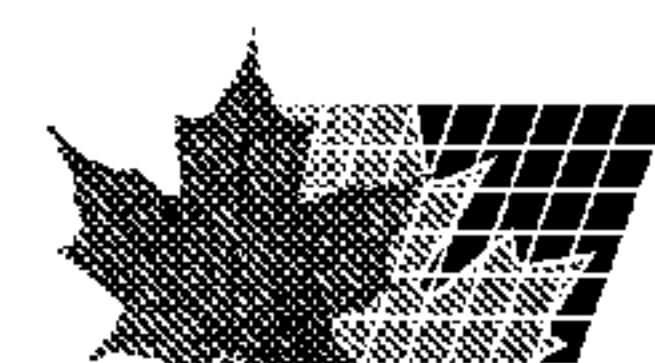
dil - LDL Uptake Assay



1. 4: Non Targeting RNAi
2. 5: PCSK9 Specific RNAi
3. 6: Non Treated cells

(57) Abrégé/Abstract:

The present invention relates to compounds, compositions, and methods for the study, diagnosis, and treatment of traits, diseases and conditions that respond to the modulation of Proprotein Convertase Subtilisin Kexin 9 (PCSK9) gene expression and/or



(57) Abrégé(suite)/Abstract(continued):

activity. The present invention is also directed to compounds, compositions, and methods relating to traits, diseases and conditions that respond to the modulation of expression and/or activity of genes involved in Proprotein Convertase Subtilisin Kexin 9 (PCSK9) gene expression pathways or other cellular processes that mediate the maintenance or development of such traits, diseases and conditions. Specifically, the invention relates to double stranded nucleic acid molecules including small nucleic acid molecules, such as short interfering nucleic acid (siNA), short interfering RNA (siRNA), double-stranded RNA (dsRNA), micro- RNA (miRNA), and short hairpin RNA (shRNA) molecules capable of mediating RNA interference (RNAi) against Proprotein Convertase Subtilisin Kexin 9 (PCSK9) gene expression, including cocktails of such small nucleic acid molecules and lipid nanoparticle (LNP) formulations of such small nucleic acid molecules. The present invention also relates to small nucleic acid molecules, such as siNA, siRNA, and others that can inhibit the function of endogenous RNA molecules, such as endogenous micro-RNA (miRNA) (e.g, miRNA inhibitors) or endogenous short interfering RNA (siRNA), (e.g., siRNA inhibitors) or that can inhibit the function of RISC (e.g., RISC inhibitors), to modulate gene expression by interfering with the regulatory function of such endogenous RNAs or proteins associated with such endogenous RNAs (e.g., RISC), including cocktails of such small nucleic acid molecules and lipid nanoparticle (LNP) formulations of such small nucleic acid molecules. Such small nucleic acid molecules and are useful, for example, in providing compositions to prevent, inhibit, or reduce metabolic diseases, traits and conditions, including but not limited to hyperlipidemia, hypercholesterolemia, cardiovascular disease, atherosclerosis, hypertension, diabetes (e.g., type I and/or type II diabetes), insulin resistance, obesity and/or other disease states, conditions, or traits that are associated with gene expression or activity in a subject or organism.

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(71) Applicant (for all designated States except US): **SIRNA THERAPEUTICS INC.** [US/US]; 1700 Owens St., 4th Floor, San Francisco, CA 94158 (US).

(72) Inventors; and

(75) Inventors/Applicants (for US only): **MCSWIGGEN, James** [US/US]; 4866 Franklin Drive, Boulder, CO 80301 (US). **JADHAV, Vasant** [IN/US]; 4735 Lucca Drive, Longmont, CO 80503 (US). **VAISH, Narendra, K.** [IN/US]; 1313 Williams Street #503, Denver, CO 80218 (US). **GUERCIOLINI, Roberto** [IT/US]; 5525 Racquet Lane, Boulder, CO. **VARGESE, Chandra** [US/US]; 14010 Westhampton Point, Broomfield, CO (US).(74) Agent: **TERPSTRA, Anita, J.**; McDonnell Boehnen Hulbert & Berghoff LLP, 300 South Wacker Drive, Suite 3100, Chicago, IL 60606 (US).

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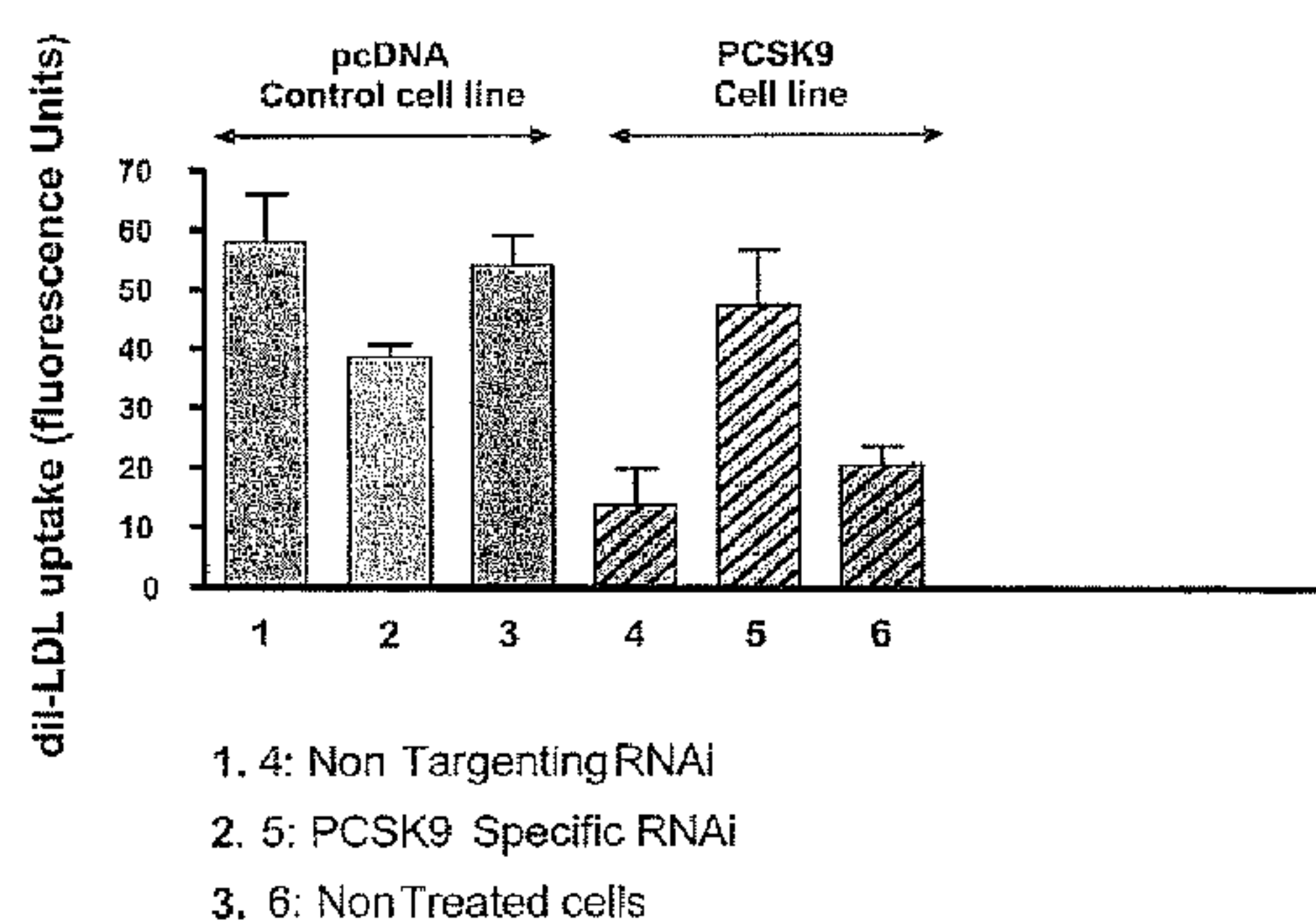
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(54) Title: RNA INTERFERENCE MEDIATED INHIBITION OF PROPROTEIN CONVERTASE SUBTILISIN KEXIN 9 (PCSK9) GENE EXPRESSION USING SHORT INTERFERING NUCLEIC ACID (SINA)

dil - LDL Uptake Assay



(57) Abstract: The present invention relates to compounds, compositions, and methods for the study, diagnosis, and treatment of traits, diseases and conditions that respond to the modulation of Proprotein Convertase Subtilisin Kexin 9 (PCSK9) gene expression and/or activity. The present invention is also directed to compounds, compositions, and methods relating to traits, diseases and conditions that respond to the modulation of expression and/or activity of genes involved in Proprotein Convertase Subtilisin Kexin 9 (PCSK9) gene expression pathways or other cellular processes that mediate the maintenance or development of such traits, diseases and conditions. Specifically, the invention relates to double stranded nucleic acid molecules including small nucleic acid molecules, such as short interfering nucleic acid (siNA), short interfering RNA (siRNA), double-stranded RNA (dsRNA), micro- RNA (miRNA), and short hairpin RNA (shRNA) molecules capable of mediating RNA interference (RNAi) against Proprotein Convertase Subtilisin Kexin 9 (PCSK9) gene expression, including cocktails of such small nucleic acid molecules and lipid nanoparticle (LNP) formulations

of such small nucleic acid molecules. The present invention also relates to small nucleic acid molecules, such as siNA, siRNA, and others that can inhibit the function of endogenous RNA molecules, such as endogenous micro-RNA (miRNA) (e.g., miRNA inhibitors) or endogenous short interfering RNA (siRNA), (e.g., siRNA inhibitors) or that can inhibit the function of RISC (e.g., RISC inhibitors), to modulate gene expression by interfering with the regulatory function of such endogenous RNAs or proteins associated with such endogenous RNAs (e.g., RISC), including cocktails of such small nucleic acid molecules and lipid nanoparticle (LNP) formulations of such small nucleic acid molecules. Such small nucleic acid molecules and are useful, for example, in providing compositions to prevent, inhibit, or reduce metabolic diseases, traits and conditions, including but not limited to hyperlipidemia, hypercholesterolemia, cardiovascular disease, atherosclerosis, hypertension, diabetes (e.g., type I and/or type II diabetes), insulin resistance, obesity and/or other disease states, conditions, or traits that are associated with gene expression or activity in a subject or organism.

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**RNA INTERFERENCE MEDIATED INHIBITION OF PROPROTEIN
CONVERTASE SUBTILISIN KEXIN 9 (PCSK9) GENE EXPRESSION USING
SHORT INTERFERING NUCLEIC ACID (siNA)**

[0001] This application is a continuation-in-part of U.S. Patent Application No. 11/487,788 filed July 17, 2006, which is a continuation-in-part 11/369,108 filed March 6, 2006. This application is also a continuation-in-part of U.S. Patent Application No. 11/299,254, filed December 8, 2005, which is a continuation-in-part of U.S. Patent Application No. 11/234,730, filed September 23, 2005, which is a continuation-in-part of U.S. Patent Application No. 11/205,646, filed August 17, 2005, which is a continuation-in-part of U.S. Patent Application No. 11/098,303, filed April 4, 2005, which is a continuation-in-part of U.S. Patent Application No. 10/923,536, filed August 20, 2004, which is a continuation-in-part of International Patent Application No. PCT/US04/22658, filed July 14, 2004, which claims the benefit of Provisional Application No. 60/487,214, filed July 14, 2003. This application is also a continuation-in-part of International Patent Application No. PCT/US04/16390, filed May 24, 2004, which is a continuation-in-part of U.S. Patent Application No. 10/826,966, filed April 16, 2004, which is continuation-in-part of U.S. Patent Application No. 10/757,803, filed January 14, 2004, which is a continuation-in-part of U.S. Patent Application No. 10/720,448, filed November 24, 2003, which is a continuation-in-part of U.S. Patent Application No. 10/693,059, filed October 23, 2003, which is a continuation-in-part of U.S. Patent Application No. 10/444,853, filed May 23, 2003, which is a continuation-in-part of International Patent Application No. PCT/US03/05346, filed February 20, 2003, and a continuation-in-part of International Patent Application No. PCT/US03/05028, filed February 20, 2003, both of which claim the benefit of U.S. Provisional Application No. 60/358,580 filed February 20, 2002, U.S. Provisional Application No. 60/363,124 filed March 11, 2002, U.S. Provisional Application No. 60/386,782 filed June 6, 2002, U.S. Provisional Application No. 60/406,784 filed August 29, 2002, U.S. Provisional Application No. 60/408,378 filed September 5, 2002, U.S. Provisional Application No. 60/409,293 filed September 9, 2002, and U.S. Provisional Application No. 60/440,129 filed January 15, 2003. This application is also a continuation-in-part of International Patent Application No. PCT/US04/13456, filed April 30, 2004, which is a continuation-in-part of U.S. Patent Application No. 10/780,447, filed February 13, 2004, which is a

continuation-in-part of U.S. Patent Application No. 10/427,160, filed April 30, 2003, which is a continuation-in-part of International Patent Application No. PCT/US02/15876 filed May 17, 2002, which claims the benefit of U.S. Provisional Application No. 60/292,217, filed May 18, 2001, U.S. Provisional Application No. 60/362,016, filed March 6, 2002, U.S. Provisional Application No. 60/306,883, filed July 20, 2001, and U.S. Provisional Application No. 60/311,865, filed August 13, 2001. This application is also a continuation-in-part of U.S. Patent Application No. 10/727,780 filed December 3, 2003. This application is also a continuation-in-part of International Patent Application No. PCT/US05/04270, filed February 9, 2005 which claims the benefit of U.S. Provisional Application No. 60/543,480, filed February 10, 2004. This application is also a continuation-in-part of U.S. Patent Application No. 11/353,630, filed February 14, 2006, which claims the benefit of U.S. Provisional Patent Application No. 60/652,787 filed February 14, 2005, U.S. Provisional Patent Application No. 60/678,531, filed May 6, 2005, U.S. Provisional Patent Application No. 60/703,946, filed July 29, 2005, and U.S. Provisional Patent Application No. 60/737,024, filed November 15, 2005. The instant application claims the benefit of all the listed applications, which are hereby incorporated by reference herein in their entireties, including the drawings.

FIELD OF THE INVENTION

[0002] The present invention relates to compounds, compositions, and methods for the study, diagnosis, and treatment of traits, diseases and conditions that respond to the modulation of Proprotein Convertase Subtilisin Kexin 9 (PCSK9) gene expression and/or activity. The present invention is also directed to compounds, compositions, and methods relating to traits, diseases and conditions that respond to the modulation of expression and/or activity of genes involved in Proprotein Convertase Subtilisin Kexin 9 (PCSK9) gene expression pathways or other cellular processes that mediate the maintenance or development of such traits, diseases and conditions. Specifically, the invention relates to nucleic acid molecules including small nucleic acid molecules, such as short interfering nucleic acid (siNA), short interfering RNA (siRNA), double-stranded RNA (dsRNA), micro-RNA (miRNA), and short hairpin RNA (shRNA) molecules capable of mediating or that mediate RNA interference (RNAi) against Proprotein Convertase Subtilisin Kexin 9 (PCSK9) gene expression, including cocktails of such

small nucleic acid molecules and lipid nanoparticle (LNP) formulations of such small nucleic acid molecules. The present invention also relates to small nucleic acid molecules, such as siNA, siRNA, and others that can inhibit the function of endogenous RNA molecules, such as endogenous micro-RNA (miRNA) (e.g, miRNA inhibitors) or endogenous short interfering RNA (siRNA), (e.g., siRNA inhibitors) or that can inhibit the function of RISC (e.g., RISC inhibitors), to modulate PCSK9 gene expression by interfering with the regulatory function of such endogenous RNAs or proteins associated with such endogenous RNAs (e.g., RISC), including cocktails of such small nucleic acid molecules and lipid nanoparticle (LNP) formulations of such small nucleic acid molecules. Such small nucleic acid molecules are useful, for example, in providing compositions to prevent, inhibit, or reduce metabolic diseases traits and conditions, including but not limited to hyperlipidemia, hypercholesterolemia, cardiovascular disease, atherosclerosis, hypertension, diabetes (e.g., type I and/or type II diabetes), insulin resistance, obesity and/or other disease states, conditions, or traits associated with PCSK9 gene expression or activity in a subject or organism.

BACKGROUND OF THE INVENTION

[0003] The following is a discussion of relevant art pertaining to RNAi. The discussion is provided only for understanding of the invention that follows. The summary is not an admission that any of the work described below is prior art to the claimed invention.

[0004] RNA interference refers to the process of sequence-specific post-transcriptional gene silencing in animals mediated by short interfering RNAs (siRNAs) (Zamore *et al.*, 2000, *Cell*, 101, 25-33; Fire *et al.*, 1998, *Nature*, 391, 806; Hamilton *et al.*, 1999, *Science*, 286, 950-951; Lin *et al.*, 1999, *Nature*, 402, 128-129; Sharp, 1999, *Genes & Dev.*, 13:139-141; and Strauss, 1999, *Science*, 286, 886). The corresponding process in plants (Heifetz *et al.*, International PCT Publication No. WO 99/61631) is commonly referred to as post-transcriptional gene silencing or RNA silencing and is also referred to as quelling in fungi. The process of post-transcriptional gene silencing is thought to be an evolutionarily-conserved cellular defense mechanism used to prevent the expression of foreign genes and is commonly shared by diverse flora and phyla (Fire *et al.*, 1999, *Trends Genet.*, 15, 358). Such protection from foreign gene expression may

have evolved in response to the production of double-stranded RNAs (dsRNAs) derived from viral infection or from the random integration of transposon elements into a host genome via a cellular response that specifically destroys homologous single-stranded RNA or viral genomic RNA. The presence of dsRNA in cells triggers the RNAi response through a mechanism that has yet to be fully characterized. This mechanism appears to be different from other known mechanisms involving double stranded RNA-specific ribonucleases, such as the interferon response that results from dsRNA-mediated activation of protein kinase PKR and 2',5'-oligoadenylate synthetase resulting in non-specific cleavage of mRNA by ribonuclease L (see for example US Patent Nos. 6,107,094; 5,898,031; Clemens *et al.*, 1997, *J. Interferon & Cytokine Res.*, 17, 503-524; Adah *et al.*, 2001, *Curr. Med. Chem.*, 8, 1189).

[0005] The presence of long dsRNAs in cells stimulates the activity of a ribonuclease III enzyme referred to as dicer (Bass, 2000, *Cell*, 101, 235; Zamore *et al.*, 2000, *Cell*, 101, 25-33; Hammond *et al.*, 2000, *Nature*, 404, 293). Dicer is involved in the processing of the dsRNA into short pieces of dsRNA known as short interfering RNAs (siRNAs) (Zamore *et al.*, 2000, *Cell*, 101, 25-33; Bass, 2000, *Cell*, 101, 235; Bernstein *et al.*, 2001, *Nature*, 409, 363). Short interfering RNAs derived from dicer activity are typically about 21 to about 23 nucleotides in length and comprise about 19 base pair duplexes (Zamore *et al.*, 2000, *Cell*, 101, 25-33; Elbashir *et al.*, 2001, *Genes Dev.*, 15, 188). Dicer has also been implicated in the excision of 21- and 22-nucleotide small temporal RNAs (stRNAs) from precursor RNA of conserved structure that are implicated in translational control (Hutvagner *et al.*, 2001, *Science*, 293, 834). The RNAi response also features an endonuclease complex, commonly referred to as an RNA-induced silencing complex (RISC), which mediates cleavage of single-stranded RNA having sequence complementary to the antisense strand of the siRNA duplex. Cleavage of the target RNA takes place in the middle of the region complementary to the antisense strand of the siRNA duplex (Elbashir *et al.*, 2001, *Genes Dev.*, 15, 188).

[0006] RNAi has been studied in a variety of systems. Fire *et al.*, 1998, *Nature*, 391, 806, were the first to observe RNAi in *C. elegans*. Bahramian and Zarbl, 1999, *Molecular and Cellular Biology*, 19, 274-283 and Wianny and Goetz, 1999, *Nature Cell Biol.*, 2, 70, describe RNAi mediated by dsRNA in mammalian systems. Hammond *et*

al., 2000, *Nature*, 404, 293, describe RNAi in *Drosophila* cells transfected with dsRNA. Elbashir *et al.*, 2001, *Nature*, 411, 494 and Tuschl *et al.*, International PCT Publication No. WO 01/75164, describe RNAi induced by introduction of duplexes of synthetic 21-nucleotide RNAs in cultured mammalian cells including human embryonic kidney and HeLa cells. Recent work in *Drosophila* embryonic lysates (Elbashir *et al.*, 2001, *EMBO J.*, 20, 6877 and Tuschl *et al.*, International PCT Publication No. WO 01/75164) has revealed certain requirements for siRNA length, structure, chemical composition, and sequence that are essential to mediate efficient RNAi activity. These studies have shown that 21-nucleotide siRNA duplexes are most active when containing 3'-terminal dinucleotide overhangs. Furthermore, complete substitution of one or both siRNA strands with 2'-deoxy (2'-H) or 2'-O-methyl nucleotides abolishes RNAi activity, whereas substitution of the 3'-terminal siRNA overhang nucleotides with 2'-deoxy nucleotides (2'-H) was shown to be tolerated. Single mismatch sequences in the center of the siRNA duplex were also shown to abolish RNAi activity. In addition, these studies also indicate that the position of the cleavage site in the target RNA is defined by the 5'-end of the siRNA guide sequence rather than the 3'-end of the guide sequence (Elbashir *et al.*, 2001, *EMBO J.*, 20, 6877). Other studies have indicated that a 5'-phosphate on the target-complementary strand of a siRNA duplex is required for siRNA activity and that ATP is utilized to maintain the 5'-phosphate moiety on the siRNA (Nykanen *et al.*, 2001, *Cell*, 107, 309).

[0007] Studies have shown that replacing the 3'-terminal nucleotide overhanging segments of a 21-mer siRNA duplex having two-nucleotide 3'-overhangs with deoxyribonucleotides does not have an adverse effect on RNAi activity. Replacing up to four nucleotides on each end of the siRNA with deoxyribonucleotides has been reported to be well tolerated, whereas complete substitution with deoxyribonucleotides results in no RNAi activity (Elbashir *et al.*, 2001, *EMBO J.*, 20, 6877 and Tuschl *et al.*, International PCT Publication No. WO 01/75164). In addition, Elbashir *et al.*, *supra*, also report that substitution of siRNA with 2'-O-methyl nucleotides completely abolishes RNAi activity. Li *et al.*, International PCT Publication No. WO 00/44914, and Beach *et al.*, International PCT Publication No. WO 01/68836 preliminarily suggest that siRNA may include modifications to either the phosphate-sugar backbone or the nucleoside to include at least one of a nitrogen or sulfur heteroatom, however, neither application

postulates to what extent such modifications would be tolerated in siRNA molecules, nor provides any further guidance or examples of such modified siRNA. Kreutzer *et al.*, Canadian Patent Application No. 2,359,180, also describe certain chemical modifications for use in dsRNA constructs in order to counteract activation of double-stranded RNA-dependent protein kinase PKR, specifically 2'-amino or 2'-O-methyl nucleotides, and nucleotides containing a 2'-O or 4'-C methylene bridge. However, Kreutzer *et al.* similarly fails to provide examples or guidance as to what extent these modifications would be tolerated in dsRNA molecules.

[0008] Parrish *et al.*, 2000, *Molecular Cell*, 6, 1077-1087, tested certain chemical modifications targeting the *unc-22* gene in *C. elegans* using long (>25 nt) siRNA transcripts. The authors describe the introduction of thiophosphate residues into these siRNA transcripts by incorporating thiophosphate nucleotide analogs with T7 and T3 RNA polymerase and observed that RNAs with two phosphorothioate modified bases also had substantial decreases in effectiveness as RNAi. Further, Parrish *et al.* reported that phosphorothioate modification of more than two residues greatly destabilized the RNAs *in vitro* such that interference activities could not be assayed. *Id.* at 1081. The authors also tested certain modifications at the 2'-position of the nucleotide sugar in the long siRNA transcripts and found that substituting deoxynucleotides for ribonucleotides produced a substantial decrease in interference activity, especially in the case of Uridine to Thymidine and/or Cytidine to deoxy-Cytidine substitutions. *Id.* In addition, the authors tested certain base modifications, including substituting, in sense and antisense strands of the siRNA, 4-thiouracil, 5-bromouracil, 5-iodouracil, and 3-(aminoallyl)uracil for uracil, and inosine for guanosine. Whereas 4-thiouracil and 5-bromouracil substitution appeared to be tolerated, Parrish reported that inosine produced a substantial decrease in interference activity when incorporated in either strand. Parrish also reported that incorporation of 5-iodouracil and 3-(aminoallyl)uracil in the antisense strand resulted in a substantial decrease in RNAi activity as well.

[0009] The use of longer dsRNA has been described. For example, Beach *et al.*, International PCT Publication No. WO 01/68836, describes specific methods for attenuating gene expression using endogenously-derived dsRNA. Tuschl *et al.*, International PCT Publication No. WO 01/75164, describe a *Drosophila in vitro* RNAi

system and the use of specific siRNA molecules for certain functional genomic and certain therapeutic applications; although Tuschl, 2001, *Chem. Biochem.*, 2, 239-245, doubts that RNAi can be used to cure genetic diseases or viral infection due to the danger of activating interferon response. Li *et al.*, International PCT Publication No. WO 00/44914, describe the use of specific long (141 bp-488 bp) enzymatically synthesized or vector expressed dsRNAs for attenuating the expression of certain target genes. Zernicka-Goetz *et al.*, International PCT Publication No. WO 01/36646, describe certain methods for inhibiting the expression of particular genes in mammalian cells using certain long (550 bp-714 bp), enzymatically synthesized or vector expressed dsRNA molecules. Fire *et al.*, International PCT Publication No. WO 99/32619, describe particular methods for introducing certain long dsRNA molecules into cells for use in inhibiting gene expression in nematodes. Plaetinck *et al.*, International PCT Publication No. WO 00/01846, describe certain methods for identifying specific genes responsible for conferring a particular phenotype in a cell using specific long dsRNA molecules. Mello *et al.*, International PCT Publication No. WO 01/29058, describe the identification of specific genes involved in dsRNA-mediated RNAi. Pachuck *et al.*, International PCT Publication No. WO 00/63364, describe certain long (at least 200 nucleotide) dsRNA constructs. Deschamps Depaillette *et al.*, International PCT Publication No. WO 99/07409, describe specific compositions consisting of particular dsRNA molecules combined with certain anti-viral agents. Waterhouse *et al.*, International PCT Publication No. 99/53050 and 1998, *PNAS*, 95, 13959-13964, describe certain methods for decreasing the phenotypic expression of a nucleic acid in plant cells using certain dsRNAs. Driscoll *et al.*, International PCT Publication No. WO 01/49844, describe specific DNA expression constructs for use in facilitating gene silencing in targeted organisms.

[0010] Others have reported on various RNAi and gene-silencing systems. For example, Parrish *et al.*, 2000, *Molecular Cell*, 6, 1077-1087, describe specific chemically-modified dsRNA constructs targeting the unc-22 gene of *C. elegans*. Grossniklaus, International PCT Publication No. WO 01/38551, describes certain methods for regulating polycomb gene expression in plants using certain dsRNAs. Churikov *et al.*, International PCT Publication No. WO 01/42443, describe certain methods for modifying genetic characteristics of an organism using certain dsRNAs.

Cogoni *et al.*, International PCT Publication No. WO 01/53475, describe certain methods for isolating a *Neurospora* silencing gene and uses thereof. Reed *et al.*, International PCT Publication No. WO 01/68836, describe certain methods for gene silencing in plants. Honer *et al.*, International PCT Publication No. WO 01/70944, describe certain methods of drug screening using transgenic nematodes as Parkinson's Disease models using certain dsRNAs. Deak *et al.*, International PCT Publication No. WO 01/72774, describe certain *Drosophila*-derived gene products that may be related to RNAi in *Drosophila*. Arndt *et al.*, International PCT Publication No. WO 01/92513 describe certain methods for mediating gene suppression by using factors that enhance RNAi. Tuschl *et al.*, International PCT Publication No. WO 02/44321, describe certain synthetic siRNA constructs. Pachuk *et al.*, International PCT Publication No. WO 00/63364, and Satishchandran *et al.*, International PCT Publication No. WO 01/04313, describe certain methods and compositions for inhibiting the function of certain polynucleotide sequences using certain long (over 250 bp), vector expressed dsRNAs. Echeverri *et al.*, International PCT Publication No. WO 02/38805, describe certain *C. elegans* genes identified via RNAi. Kreutzer *et al.*, International PCT Publications Nos. WO 02/055692, WO 02/055693, and EP 1144623 B1 describes certain methods for inhibiting gene expression using dsRNA. Graham *et al.*, International PCT Publications Nos. WO 99/49029 and WO 01/70949, and AU 4037501 describe certain vector expressed siRNA molecules. Fire *et al.*, US 6,506,559, describe certain methods for inhibiting gene expression in vitro using certain long dsRNA (299 bp-1033 bp) constructs that mediate RNAi. Martinez *et al.*, 2002, *Cell*, 110, 563-574, describe certain single stranded siRNA constructs, including certain 5'-phosphorylated single stranded siRNAs that mediate RNA interference in Hela cells. Harborth *et al.*, 2003, *Antisense & Nucleic Acid Drug Development*, 13, 83-105, describe certain chemically and structurally modified siRNA molecules. Chiu and Rana, 2003, *RNA*, 9, 1034-1048, describe certain chemically and structurally modified siRNA molecules. Woolf *et al.*, International PCT Publication Nos. WO 03/064626 and WO 03/064625 describe certain chemically modified dsRNA constructs. Hornung *et al.*, 2005, *Nature Medicine*, 11, 263 – 270, describe the sequence-specific potent induction of IFN- α by short interfering RNA in plasmacytoid dendritic cells through TLR7. Judge *et al.*, 2005, *Nature Biotechnology*, Published online: 20 March 2005, describe the sequence-dependent stimulation of the

mammalian innate immune response by synthetic siRNA. Yuki *et al.*, International PCT Publication Nos. WO 05/049821 and WO 04/048566, describe certain methods for designing short interfering RNA sequences and certain short interfering RNA sequences with optimized activity. Saigo *et al.*, US Patent Application Publication No. US20040539332, describe certain methods of designing oligo- or polynucleotide sequences, including short interfering RNA sequences, for achieving RNA interference. Tei *et al.*, International PCT Publication No. WO 03/044188, describe certain methods for inhibiting expression of a target gene, which comprises transfecting a cell, tissue, or individual organism with a double-stranded polynucleotide comprising DNA and RNA having a substantially identical nucleotide sequence with at least a partial nucleotide sequence of the target gene.

[0011] Mattick, 2005, *Science*, 309, 1527-1528; Claverie, 2005, *Science*, 309, 1529-1530; Sethupathy *et al.*, 2006, *RNA*, 12, 192-197; and Czech, 2006 *NEJM*, 354, 11: 1194-1195; Hutvagner *et al.*, US 20050227256, and Tuschl *et al.*, US 20050182005, all describe antisense molecules that can inhibit miRNA function via steric blocking and are all incorporated by reference herein in their entirety.

[0012] Lalanne *et al.*, 2005, *Journal of Lipid Research*, 46, 1312-1319, describe certain siRNA molecules targeting PCSK9.

SUMMARY OF THE INVENTION

[0013] This invention relates to compounds, compositions, and methods useful for modulating the expression of Proprotein Convertase Subtilisin Kexin 9 (PCSK9) genes, such as those PCSK9 genes associated with the development or maintenance of metabolic diseases traits and conditions, including but not limited to hyperlipidemia, hypercholesterolemia, cardiovascular disease, atherosclerosis, hypertension, diabetes (e.g., type I and/or type II diabetes), insulin resistance, obesity and/or any other diseases, traits, and conditions that are related to PCSK9 gene expression or activity, by RNA interference (RNAi) using short interfering nucleic acid (siNA) molecules. This invention also relates to compounds, compositions, and methods useful for modulating the expression and activity of other genes involved in pathways of PCSK9 gene expression and/or activity by RNA interference (RNAi) using small nucleic acid

molecules. In particular, the instant invention features small nucleic acid molecules, such as short interfering nucleic acid (siNA), short interfering RNA (siRNA), double-stranded RNA (dsRNA), micro-RNA (miRNA), and short hairpin RNA (shRNA) molecules and methods used to modulate the expression of PCSK9 genes and/or other genes involved in pathways of PCSK9 gene expression and/or activity.

[0014] The instant invention also relates to small nucleic acid molecules, such as siNA, siRNA, and others that can inhibit the function of endogenous RNA molecules, such as endogenous micro-RNA (miRNA) (e.g, miRNA inhibitors) or endogenous short interfering RNA (siRNA), (e.g., siRNA inhibitors) or that can inhibit the function of RISC (e.g., RISC inhibitors), to modulate PCSK9 gene expression by interfering with the regulatory function of such endogenous RNAs or proteins associated with such endogenous RNAs (e.g., RISC). Such molecules are collectively referred to herein as RNAi inhibitors.

[0015] A siNA or RNAi inhibitor of the invention can be unmodified or chemically-modified. A siNA or RNAi inhibitor of the instant invention can be chemically synthesized, expressed from a vector or enzymatically synthesized. The instant invention also features various chemically-modified synthetic short interfering nucleic acid (siNA) molecules capable of modulating target gene expression or activity in cells by RNA interference (RNAi). The instant invention also features various chemically-modified synthetic short nucleic acid (siNA) molecules capable of modulating RNAi activity in cells by interacting with miRNA, siRNA, or RISC, and hence down regulating or inhibiting RNA interference (RNAi), translational inhibition, or transcriptional silencing in a cell or organism. The use of chemically-modified siNA and/or RNAi inhibitors improves various properties of native siNA molecules and/or RNAi inhibitors through increased resistance to nuclease degradation in vivo and/or through improved cellular uptake. Further, contrary to earlier published studies, siNA molecules of the invention having multiple chemical modifications, including fully modified siNA, retains its RNAi activity. Therefore, Applicant teaches herein chemically modified siRNA that retains or improves upon the activity of native siRNA. The siNA molecules of the instant invention provide useful reagents and methods for a variety of therapeutic,

prophylactic, veterinary, diagnostic, target validation, genomic discovery, genetic engineering, and pharmacogenomic applications.

[0016] In one embodiment, the invention features one or more siNA molecules and/or RNAi inhibitors and methods that independently or in combination modulate the expression of gene(s) encoding Proprotein Convertase Subtilisin Kexin 9 (PCSK9) and/or PCSK9 pathway genes, such as genes encoding sequences comprising those sequences referred to by GenBank Accession Nos. shown in Table I, and USSN 10/923,536 and PCT/US03/05028, both incorporated by reference herein referred to herein generally as “PCSK9”. The description below of the various aspects and embodiments of the invention is provided with reference to exemplary Proprotein Convertase Subtilisin Kexin 9 (PCSK9) gene referred to herein generally as PCSK9, but also known as neural apoptosis-regulated convertase 1 or NARC1. The present invention is also directed to compounds, compositions, and methods relating to traits, diseases and conditions that respond to the modulation of expression and/or activity of genes involved in Proprotein Convertase Subtilisin Kexin 9 (PCSK9) gene expression pathways or other cellular processes that mediate the maintenance or development of such traits, diseases and conditions. However, such reference is meant to be exemplary only and the various aspects and embodiments of the invention are also directed to other genes that express alternate PCSK9 genes, such as mutant PCSK9 genes, splice variants of PCSK9 genes, PCSK9 variants from species to species or subject to subject, and other PCSK9 pathway genes including certain genes described in PCT/US03/05028 and USSN 10/923,536, both incorporated by reference herein. Such additional genes can be analyzed for target sites using the methods described herein for exemplary PCSK9 genes and sequences herein. Thus, the modulation of other genes and the effects of such modulation of the other genes can be performed, determined, and measured as described herein. In other words, the term “PCSK9” as it is defined herein below and recited in the described embodiments, is meant to encompass genes associated with the development and/or maintenance of diseases, traits and conditions herein, such as genes which encode PCSK9 polypeptides, PCSK9 regulatory polynucleotides (e.g., PCSK9 miRNAs and siRNAs), mutant PCSK9 genes, and splice variants of PCSK9 genes, as well as other genes involved in PCSK9 pathways of gene expression and/or activity. Thus, each of the embodiments described herein with reference to the term “PCSK9” are

applicable to all of the protein, peptide, polypeptide, and/or polynucleotide molecules covered by the term “PCSK9”, as that term is defined herein. Comprehensively, such gene targets are also referred to herein generally as “target” sequences.

[0017] In one embodiment, the invention features a composition comprising two or more different siNA molecules and/or RNAi inhibitors of the invention (e.g., siNA, duplex forming siNA, or multifunctional siNA or any combination thereof) targeting different polynucleotide targets, such as different regions of PCSK9 RNA or DNA (e.g., two different target sites such as provided herein or any combination of PCSK9 targets or PCSK9 pathway targets) or both coding and non-coding targets. Such pools of siNA molecules can provide increased therapeutic effect.

[0018] In one embodiment, the invention features a pool of two or more different siNA molecules of the invention (e.g., siNA, duplex forming siNA, or multifunctional siNA or any combination thereof) that have specificity for different polynucleotide targets, such as different regions of PCSK9 RNA or DNA (e.g., two different target sites herein or any combination of PCSK9 targets or PCSK9 pathway targets) or both coding and non-coding targets, wherein the pool comprises siNA molecules targeting about 2, 3, 4, 5, 6, 7, 8, 9, 10 or more different PCSK9 targets.

[0019] Due to the potential for sequence variability of the PCSK9 genome across different organisms or different subjects, selection of siNA molecules for broad therapeutic applications likely involve the conserved regions of the PCSK9 gene. In one embodiment, the present invention relates to siNA molecules and/or RNAi inhibitors that target conserved regions of the PCSK9 genome or regions that are conserved across different PCSK9 targets. siNA molecules and/or RNAi inhibitors designed to target conserved regions of various PCSK9 targets enable efficient inhibition of PCSK9 target gene expression in diverse patient populations.

[0020] In one embodiment, the invention features a double stranded nucleic acid molecule, such as an siNA molecule, where one of the strands comprises nucleotide sequence having complementarity to a predetermined nucleotide sequence in a target nucleic acid molecule, or a portion thereof. In one embodiment, the predetermined nucleotide sequence is a nucleotide target sequence described herein. In another

embodiment, the predetermined nucleotide sequence is a target sequence as is known in the art.

[0021] In one embodiment, the invention features a double-stranded short interfering nucleic acid (siNA) molecule that down-regulates expression of a target gene or that directs cleavage of a target RNA, wherein said siNA molecule comprises about 15 to about 28 base pairs. For example, in one embodiment, the invention features a double-stranded short interfering nucleic acid (siNA) molecule that down-regulates expression of a target gene or that directs cleavage of a target RNA, wherein said siNA molecule comprises about 18 to about 27 base pairs.

[0022] In one embodiment, the invention features a double-stranded short interfering nucleic acid or polynucleotide (siNA) molecule that directs cleavage of a target RNA, wherein said siNA molecule comprises about 15 to about 28 base pairs.

[0023] In one embodiment, the invention features a double stranded short interfering nucleic acid or polynucleotide (siNA) molecule that directs cleavage of a target RNA via RNA interference (RNAi), wherein the double stranded siNA molecule comprises a first strand and a second strand, each strand of the siNA molecule is about 18 to about 28 (e.g., about 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, or 28) nucleotides in length, the first strand of the siNA molecule comprises nucleotide sequence having sufficient complementarity to the target RNA for the siNA molecule to direct cleavage of the target RNA via RNA interference, and the second strand of said siNA molecule comprises nucleotide sequence that is complementary to the first strand. In one specific embodiment, for example, each strand of the siNA molecule is about 18 to about 27 nucleotides in length.

[0024] In one embodiment, the invention features a double stranded short interfering nucleic acid or polynucleotide (siNA) molecule that directs cleavage of a target RNA via RNA interference (RNAi), wherein the double stranded siNA molecule comprises a first strand and a second strand, each strand of the siNA molecule is about 18 to about 23 (e.g., about 18, 19, 20, 21, 22, or 23) nucleotides in length, the first strand of the siNA molecule comprises nucleotide sequence having sufficient complementarity to the target RNA for the siNA molecule to direct cleavage of the target RNA via RNA interference,

and the second strand of said siNA molecule comprises nucleotide sequence that is complementary to the first strand.

[0025] In one embodiment, the invention features a chemically synthesized double stranded short interfering nucleic acid or polynucleotide (siNA) molecule that directs cleavage of a target RNA via RNA interference (RNAi), wherein each strand of the siNA molecule is about 18 to about 28 nucleotides in length; and one strand of the siNA molecule comprises nucleotide sequence having sufficient complementarity to the target RNA for the siNA molecule to direct cleavage of the target RNA via RNA interference.

[0026] In one embodiment, the invention features a chemically synthesized double stranded short interfering nucleic acid or polynucleotide (siNA) molecule that directs cleavage of a target RNA via RNA interference (RNAi), wherein each strand of the siNA molecule is about 18 to about 23 nucleotides in length; and one strand of the siNA molecule comprises nucleotide sequence having sufficient complementarity to the target RNA for the siNA molecule to direct cleavage of the target RNA via RNA interference.

[0027] In one embodiment, the invention features a siNA molecule that down-regulates expression of a target gene or that directs cleavage of a target RNA, for example, wherein the target gene or RNA comprises protein encoding sequence. In one embodiment, the invention features a siNA molecule that down-regulates expression of a target gene or that directs cleavage of a target RNA, for example, wherein the target gene or RNA comprises non-coding sequence or regulatory elements involved in target gene expression (*e.g.*, non-coding RNA, miRNA, stRNA, shRNA etc.).

[0028] In another embodiment of the invention, the strand or strands of dsRNA are 100 bases (or base pairs) or less. In other embodiments the strand or strands of the dsRNA are less than 70 bases in length. With respect to those embodiments, each of the dsRNA strand or strands may be from 5-70, 10-65, 20-60, 30-55, 40-50 bases or base pairs in length. In certain aspects, each of the strands are from 15-30 base pairs in length, while in other aspects, the each of the strands is from 19-23 base pairs in length. A dsRNA that has a complementarity region equal to or less than 30 base pairs, *e.g.*, one in which each of the strands are 30 bases or fewer in length or a single stranded hairpin RNA in which the stem or complementary portion is less than or equal to 30 base pairs

are especially preferred since such strands are able to evade a mammalian's cell antiviral response. Thus, a hairpin dsRNA (one strand) may be 70 or fewer bases in length with a complementary region of 30 base pairs or fewer.

[0029] Hairpin interfering RNA: A hairpin interfering RNA is a single molecule (e.g., a single oligonucleotide chain) that comprises both the sense and antisense strands of an interfering RNA in a stem-loop or hairpin structure (e.g., a shRNA). For example, shRNAs can be expressed from DNA vectors in which the DNA oligonucleotides encoding a sense interfering RNA strand are linked to the DNA oligonucleotides encoding the reverse complementary antisense interfering RNA strand by a short spacer. If needed for the chosen expression vector, 3' terminal T's and nucleotides forming restriction sites may be added. The resulting RNA transcript folds back onto itself to form a stem-loop structure.

[0030] In one embodiment, RNAi constructs of the invention are used to down regulate or inhibit the expression of PCSK9 proteins arising from PCSK9 haplotype polymorphisms that are associated with a disease or condition, (e.g., hypercholesterolemia, hyperlipidemia, and cardiovascular disease). Analysis of PCSK9 genes, or PCSK9 protein or RNA levels can be used to identify subjects with such polymorphisms or those subjects who are at risk of developing traits, conditions, or diseases described herein. These subjects are amenable to treatment, for example, treatment with RNAi constructs of the invention and any other composition useful in treating diseases related to PCSK9 gene expression. As such, analysis of PCSK9 protein or RNA levels can be used to determine treatment type and the course of therapy in treating a subject. Monitoring of PCSK9 protein or RNA levels can be used to predict treatment outcome and to determine the efficacy of compounds and compositions that modulate the level and/or activity of certain PCSK9 proteins associated with a trait, condition, or disease.

[0031] Further, the antisense strand of the RNAi construct of the invention detailed above and elsewhere in the application, hybridizes under physiological conditions to a portion of a PCSK9 mRNA. The administration of such a composition attenuates the expression of the cholesterol modulating target mRNA of the subject.

[0032] In one embodiment, a siNA of the invention is used to inhibit the expression of target genes or a target gene family (e.g., different PCSK9 genes), wherein the genes or gene family sequences share sequence homology. Such homologous sequences can be identified as is known in the art, for example using sequence alignments. siNA molecules can be designed to target such homologous sequences, for example using perfectly complementary sequences or by incorporating non-canonical base pairs, for example mismatches and/or wobble base pairs, that can provide additional target sequences. In instances where mismatches are identified, non-canonical base pairs (for example, mismatches and/or wobble bases) can be used to generate siNA molecules that target more than one gene sequence. In a non-limiting example, non-canonical base pairs such as UU and CC base pairs are used to generate siNA molecules that are capable of targeting sequences for differing polynucleotide targets that share sequence homology. As such, one advantage of using siNAs of the invention is that a single siNA can be designed to include nucleic acid or polynucleotide sequence that is complementary to the nucleotide sequence that is conserved between the homologous genes. In this approach, a single siNA can be used to inhibit expression of more than one gene instead of using more than one siNA molecule to target the different genes.

[0033] In one embodiment, the invention features a siNA molecule having RNAi activity against target RNA (e.g., coding or non-coding RNA), wherein the siNA molecule comprises a sequence complementary to any RNA sequence, such as those sequences having GenBank Accession Nos. shown in Table I, PCT/US03/05028, U.S. Provisional Patent Application No. 60/363,124, or USSN 10/923536, all of which are incorporated by reference herein. In another embodiment, the invention features a siNA molecule having RNAi activity against target RNA, wherein the siNA molecule comprises a sequence complementary to an RNA having variant encoding sequence, for example other mutant genes known in the art to be associated with the maintenance and/or development of diseases, traits, disorders, and/or conditions described herein or otherwise known in the art. Chemical modifications as shown in Tables III and IV or otherwise described herein can be applied to any siNA construct of the invention. In another embodiment, a siNA molecule of the invention includes a nucleotide sequence that can interact with nucleotide sequence of a PCSK9 gene and thereby mediate silencing of PCSK9 gene expression, for example, wherein the siNA mediates regulation

of PCSK9 gene expression by cellular processes that modulate the chromatin structure or methylation patterns of the PCSK9 gene and prevent transcription of the PCSK9 gene.

[0034] In one embodiment, siNA molecules of the invention are used to down regulate or inhibit the expression of proteins arising from haplotype polymorphisms that are associated with a trait, disease or condition in a subject or organism. Analysis of genes, or protein or RNA levels can be used to identify subjects with such polymorphisms or those subjects who are at risk of developing traits, conditions, or diseases described herein. These subjects are amenable to treatment, for example, treatment with siNA molecules of the invention and any other composition useful in treating diseases related to target gene expression. As such, analysis of target protein or RNA levels can be used to determine treatment type and the course of therapy in treating a subject. Monitoring of target protein or RNA levels can be used to predict treatment outcome and to determine the efficacy of compounds and compositions that modulate the level and/or activity of certain target proteins associated with a trait, disorder, condition, or disease.

[0035] In one embodiment of the invention a siNA molecule comprises an antisense strand comprising a nucleotide sequence that is complementary to a nucleotide sequence or a portion thereof encoding a target protein. The siNA further comprises a sense strand, wherein said sense strand comprises a nucleotide sequence of a target gene or a portion thereof.

[0036] In another embodiment, a siNA molecule comprises an antisense region comprising a nucleotide sequence that is complementary to a nucleotide sequence encoding a target protein or a portion thereof. The siNA molecule further comprises a sense region, wherein said sense region comprises a nucleotide sequence of a target gene or a portion thereof.

[0037] In another embodiment, the invention features a siNA molecule comprising nucleotide sequence, for example, nucleotide sequence in the antisense region of the siNA molecule that is complementary to a nucleotide sequence or portion of sequence of a target gene. In another embodiment, the invention features a siNA molecule

comprising a region, for example, the antisense region of the siNA construct, complementary to a sequence comprising a target gene sequence or a portion thereof.

[0038] In one embodiment, the sense region or sense strand of a siNA molecule of the invention is complementary to that portion of the antisense region or antisense strand of the siNA molecule that is complementary to a target polynucleotide sequence.

[0039] In yet another embodiment, the invention features a siNA molecule comprising a sequence, for example, the antisense sequence of the siNA construct, complementary to a sequence or portion of sequence comprising sequence represented by GenBank Accession Nos. shown in PCT/US03/05028, U.S. Provisional Patent Application No. 60/363,124, and/or in USSN 10/923,536, all of which are incorporated by reference herein. Chemical modifications in **Tables III and IV** and otherwise described herein can be applied to any siNA construct of the invention. LNP formulations described in **Table VI** can be applied to any siNA molecule or combination of siNA molecules herein.

[0040] In one embodiment of the invention a siNA molecule comprises an antisense strand having about 15 to about 30 (e.g., about 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, or 30) nucleotides, wherein the antisense strand is complementary to a PCSK9 target RNA sequence or a portion thereof, and wherein said siNA further comprises a sense strand having about 15 to about 30 (e.g., about 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, or 30) nucleotides, and wherein said sense strand and said antisense strand are distinct nucleotide sequences where at least about 15 nucleotides in each strand are complementary to the other strand.

[0041] In one embodiment, a siNA molecule of the invention (e.g., a double stranded nucleic acid or polynucleotide molecule) comprises an antisense (guide) strand having about 15 to about 30 (e.g., about 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, or 30) nucleotides that are complementary to a target RNA sequence or a portion thereof. In one embodiment, at least 15 nucleotides (e.g., 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, or 30 nucleotides) of a target RNA sequence are complementary to the antisense (guide) strand of a siNA molecule of the invention. In one embodiment, a siNA molecule of the invention (e.g., a double stranded nucleic acid or polynucleotide

molecule) comprises a sense (passenger) strand having about 15 to about 30 (e.g., about 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, or 30) nucleotides that comprise sequence of a target RNA or a portion thereof. In one embodiment, at least 15 nucleotides (e.g., about 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, or 30) nucleotides of a target RNA sequence comprise the sense (passenger) strand of a siNA molecule of the invention.

[0042] In another embodiment of the invention a siNA molecule of the invention comprises an antisense region having about 15 to about 30 (e.g., about 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, or 30) nucleotides, wherein the antisense region is complementary to a target DNA sequence, and wherein said siNA further comprises a sense region having about 15 to about 30 (e.g., about 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, or 30) nucleotides, wherein said sense region and said antisense region are comprised in a linear molecule where the sense region comprises at least about 15 nucleotides that are complementary to the antisense region.

[0043] In one embodiment, a siNA molecule of the invention has RNAi activity that modulates expression of RNA encoded by a PCSK9 gene. Because PCSK9 genes can share some degree of sequence homology with each other, siNA molecules can be designed to target a class of PCSK9 genes by selecting sequences that are either shared amongst different PCSK9 targets or alternatively that are unique for a specific PCSK9 target. Therefore, in one embodiment, the siNA molecule can be designed to target conserved regions of PCSK9 RNA sequences having homology among several gene variants so as to target a class of PCSK9 genes with one siNA molecule. Accordingly, in one embodiment, the siNA molecule of the invention modulates the expression of one or more PCSK9 isoforms or variants in a subject or organism. In another embodiment, the siNA molecule can be designed to target a sequence that is unique to a specific PCSK9 polynucleotide sequence (e.g., a single PCSK9 isoform or PCSK9 single nucleotide polymorphism (SNP)) due to the high degree of specificity that the siNA molecule requires to mediate RNAi activity.

[0044] In one embodiment, nucleic acid or polynucleotide molecules of the invention that act as mediators of the RNA interference gene silencing response are double-stranded nucleic acid or polynucleotide molecules. In another embodiment, the siNA

molecules of the invention consist of duplex nucleic acid or polynucleotide molecules containing about 15 to about 30 base pairs between oligonucleotides comprising about 15 to about 30 (e.g., about 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, or 30) nucleotides. In yet another embodiment, siNA molecules of the invention comprise duplex nucleic acid or polynucleotide molecules with overhanging ends of about 1 to about 3 (e.g., about 1, 2, or 3) nucleotides, for example, about 21-nucleotide duplexes with about 19 base pairs and 3'-terminal mononucleotide, dinucleotide, or trinucleotide overhangs. In yet another embodiment, siNA molecules of the invention comprise duplex nucleic acid or polynucleotide molecules with blunt ends, where both ends are blunt, or alternatively, where one of the ends is blunt.

[0045] In one embodiment, a double stranded nucleic acid or polynucleotide (e.g., siNA) molecule comprises nucleotide or non-nucleotide overhangs. By “overhang” is meant a terminal portion of the nucleotide sequence that is not base paired between the two strands of a double stranded molecule (see for example **Figure 6**). In one embodiment, a double stranded nucleic acid or polynucleotide molecule of the invention can comprise nucleotide or non-nucleotide overhangs at the 3'-end of one or both strands of the double stranded nucleic acid or polynucleotide molecule. For example, a double stranded nucleic acid or polynucleotide molecule of the invention can comprise a nucleotide or non-nucleotide overhang at the 3'-end of the guide strand or antisense strand/region, the 3'-end of the passenger strand or sense strand/region, or both the guide strand or antisense strand/region and the passenger strand or sense strand/region of the double stranded molecule. In another embodiment, the nucleotide overhang portion of a double stranded nucleic acid or polynucleotide (siNA) molecule of the invention comprises 2'-O-methyl, 2'-deoxy, 2'-deoxy-2'-fluoro, 2'-deoxy-2'-fluoroarabino (FANA), 4'-thio, 2'-O-trifluoromethyl, 2'-O-ethyl-trifluoromethoxy, 2'-O-difluoromethoxy-ethoxy, universal base, acyclic, or 5-C-methyl nucleotides. In another embodiment, the non-nucleotide overhang portion of a double stranded nucleic acid or polynucleotide (siNA) molecule of the invention comprises glyceryl, abasic, or inverted deoxy abasic non-nucleotides.

[0046] In one embodiment, the nucleotides comprising the overhang portions of a double stranded nucleic acid or polynucleotide (e.g., siNA) molecule of the invention

correspond to the nucleotides comprising the target polynucleotide sequence of the siNA molecule. Accordingly, in such embodiments, the nucleotides comprising the overhang portion of a siNA molecule of the invention comprise sequence based on the target polynucleotide sequence in which nucleotides comprising the overhang portion of the guide strand or antisense strand/region of a siNA molecule of the invention can be complementary to nucleotides in the target polynucleotide sequence and nucleotides comprising the overhang portion of the passenger strand or sense strand/region of a siNA molecule of the invention can comprise the nucleotides in the target polynucleotide sequence. Such nucleotide overhangs comprise sequence that would result from Dicer processing of a native dsRNA into siRNA.

[0047] In one embodiment, the nucleotides comprising the overhang portion of a double stranded nucleic acid or polynucleotide (*e.g.*, siNA) molecule of the invention are complementary to the target polynucleotide sequence and are optionally chemically modified as described herein. As such, in one embodiment, the nucleotides comprising the overhang portion of the guide strand or antisense strand/region of a siNA molecule of the invention can be complementary to nucleotides in the target polynucleotide sequence, *i.e.* those nucleotide positions in the target polynucleotide sequence that are complementary to the nucleotide positions of the overhang nucleotides in the guide strand or antisense strand/region of a siNA molecule. In another embodiment, the nucleotides comprising the overhang portion of the passenger strand or sense strand/region of a siNA molecule of the invention can comprise the nucleotides in the target polynucleotide sequence, *i.e.* those nucleotide positions in the target polynucleotide sequence that correspond to same the nucleotide positions of the overhang nucleotides in the passenger strand or sense strand/region of a siNA molecule. In one embodiment, the overhang comprises a two nucleotide (*e.g.*, 3'-GA; 3'-GU; 3'-GG; 3'-GC; 3'-CA; 3'-CU; 3'-CG; 3'-CC; 3'-UA; 3'-UU; 3'-UG; 3'-UC; 3'-AA; 3'-AU; 3'-AG; 3'-AC; 3'-TA; 3'-TU; 3'-TG; 3'-TC; 3'-AT; 3'-UT; 3'-GT; 3'-CT) overhang that is complementary to a portion of the target polynucleotide sequence. In one embodiment, the overhang comprises a two nucleotide (*e.g.*, 3'-GA; 3'-GU; 3'-GG; 3'-GC; 3'-CA; 3'-CU; 3'-CG; 3'-CC; 3'-UA; 3'-UU; 3'-UG; 3'-UC; 3'-AA; 3'-AU; 3'-AG; 3'-AC; 3'-TA; 3'-TU; 3'-TG; 3'-TC; 3'-AT; 3'-UT; 3'-GT; 3'-CT) overhang that is not complementary to a portion of the target polynucleotide sequence. In another

embodiment, the overhang nucleotides of a siNA molecule of the invention are 2'-O-methyl nucleotides, 2'-deoxy-2'-fluoroarabino, and/or 2'-deoxy-2'-fluoro nucleotides. In another embodiment, the overhang nucleotides of a siNA molecule of the invention are 2'-O-methyl nucleotides in the event the overhang nucleotides are purine nucleotides and/or 2'-deoxy-2'-fluoro nucleotides or 2'-deoxy-2'-fluoroarabino nucleotides in the event the overhang nucleotides are pyrimidines nucleotides. In another embodiment, the purine nucleotide (when present) in an overhang of siNA molecule of the invention is 2'-O-methyl nucleotides. In another embodiment, the pyrimidine nucleotides (when present) in an overhang of siNA molecule of the invention are 2'-deoxy-2'-fluoro or 2'-deoxy-2'-fluoroarabino nucleotides.

[0048] In one embodiment, the nucleotides comprising the overhang portion of a double stranded nucleic acid or polynucleotide (*e.g.*, siNA) molecule of the invention are not complementary to the target polynucleotide sequence and are optionally chemically modified as described herein. In one embodiment, the overhang comprises a 3'-UU overhang that is not complementary to a portion of the target polynucleotide sequence. In another embodiment, the nucleotides comprising the overhanging portion of a siNA molecule of the invention are 2'-O-methyl nucleotides, 2'-deoxy-2'-fluoroarabino and/or 2'-deoxy-2'-fluoro nucleotides.

[0049] In one embodiment, the double stranded nucleic molecule (*e.g.* siNA) of the invention comprises a two or three nucleotide overhang, wherein the nucleotides in the overhang are the same or different. In one embodiment, the double stranded nucleic molecule (*e.g.* siNA) of the invention comprises a two or three nucleotide overhang, wherein the nucleotides in the overhang are the same or different and wherein one or more nucleotides in the overhang are chemically modified at the base, sugar and/or phosphate backbone.

[0050] In one embodiment, the invention features one or more chemically-modified siNA constructs having specificity for PCSK9 target nucleic acid molecules, such as RNA encoding a protein or non-coding RNA associated with the expression of PCSK9 target genes. In one embodiment, the invention features a RNA based siNA molecule (*e.g.*, a siNA comprising 2'-OH nucleotides) having specificity for nucleic acid molecules that includes one or more chemical modifications described herein. Non-

limiting examples of such chemical modifications include without limitation phosphorothioate internucleotide linkages, 2'-deoxyribonucleotides, 2'-O-methyl ribonucleotides, 2'-deoxy-2'-fluoro ribonucleotides, 4'-thio ribonucleotides, 2'-O-trifluoromethyl nucleotides, 2'-O-ethyl-trifluoromethoxy nucleotides, 2'-O-difluoromethoxy-ethoxy nucleotides (see for example USSN 10/981,966 filed November 5, 2004, incorporated by reference herein), "universal base" nucleotides, "acyclic" nucleotides, 5-C-methyl nucleotides, 2'-deoxy-2'-fluoroarabino (FANA, see for example Dowler et al., 2006, Nucleic Acids Research, 34, 1669-1675) and terminal glyceryl and/or inverted deoxy abasic residue incorporation. These chemical modifications, when used in various siNA constructs, (e.g., RNA based siNA constructs), are shown to preserve RNAi activity in cells while at the same time, dramatically increasing the serum stability of these compounds.

[0051] In one embodiment, a siNA molecule of the invention comprises chemical modifications described herein (e.g., 2'-O-methyl ribonucleotides, 2'-deoxy-2'-fluoro ribonucleotides, 4'-thio ribonucleotides, 2'-O-trifluoromethyl nucleotides, 2'-O-ethyl-trifluoromethoxy nucleotides, 2'-O-difluoromethoxy-ethoxy nucleotides, LNA) at the internal positions of the siNA molecule. By "internal position" is meant the base paired positions of a siNA duplex. In one embodiment, a siNA molecule of the invention comprises modified nucleotides while maintaining the ability to mediate RNAi. The modified nucleotides can be used to improve *in vitro* or *in vivo* characteristics such as stability, activity, toxicity, immune response, and/or bioavailability. For example, a siNA molecule of the invention can comprise modified nucleotides as a percentage of the total number of nucleotides present in the siNA molecule. As such, a siNA molecule of the invention can generally comprise about 5% to about 100% modified nucleotides (e.g., about 5%, 10%, 15%, 20%, 25%, 30%, 35%, 40%, 45%, 50%, 55%, 60%, 65%, 70%, 75%, 80%, 85%, 90%, 95% or 100% modified nucleotides). For example, in one embodiment, between about 5% to about 100% (e.g., about 5%, 10%, 15%, 20%, 25%, 30%, 35%, 40%, 45%, 50%, 55%, 60%, 65%, 70%, 75%, 80%, 85%, 90%, 95% or 100% modified nucleotides) of the nucleotide positions in a siNA molecule of the invention comprise a nucleic acid sugar modification, such as a 2'-sugar modification, e.g., 2'-O-methyl nucleotides, 2'-deoxy-2'-fluoro nucleotides, 2'-deoxy-2'-fluoroarabino, 2'-O-methoxyethyl nucleotides, 2'-O-trifluoromethyl nucleotides, 2'-O-

ethyl-trifluoromethoxy nucleotides, 2'-O-difluoromethoxy-ethoxy nucleotides, or 2'-deoxy nucleotides. In another embodiment, between about 5% to about 100% (e.g., about 5%, 10%, 15%, 20%, 25%, 30%, 35%, 40%, 45%, 50%, 55%, 60%, 65%, 70%, 75%, 80%, 85%, 90%, 95% or 100% modified nucleotides) of the nucleotide positions in a siNA molecule of the invention comprise a nucleic acid base modification, such as inosine, purine, pyridin-4-one, pyridin-2-one, phenyl, pseudouracil, 2, 4, 6-trimethoxy benzene, 3-methyl uracil, dihydrouridine, naphthyl, aminophenyl, 5-alkylcytidines (e.g., 5-methylcytidine), 5-alkyluridines (e.g., ribothymidine), 5-halouridine (e.g., 5-bromouridine) or 6-azapyrimidines or 6-alkylpyrimidines (e.g. 6-methyluridine), or propyne modifications. In another embodiment, between about 5% to about 100% (e.g., about 5%, 10%, 15%, 20%, 25%, 30%, 35%, 40%, 45%, 50%, 55%, 60%, 65%, 70%, 75%, 80%, 85%, 90%, 95% or 100% modified nucleotides) of the nucleotide positions in a siNA molecule of the invention comprise a nucleic acid backbone modification, such as a backbone modification having Formula I herein. In another embodiment, between about 5% to about 100% (e.g., about 5%, 10%, 15%, 20%, 25%, 30%, 35%, 40%, 45%, 50%, 55%, 60%, 65%, 70%, 75%, 80%, 85%, 90%, 95% or 100% modified nucleotides) of the nucleotide positions in a siNA molecule of the invention comprise a nucleic acid sugar, base, or backbone modification or any combination thereof (e.g., any combination of nucleic acid sugar, base, backbone or non-nucleotide modifications herein). In one embodiment, a siNA molecule of the invention comprises at least about 20%, 25%, 30%, 35%, 40%, 45%, 50%, 55%, 60%, 65%, 70%, 75%, 80%, 85%, 90%, 95% or 100% modified nucleotides. The actual percentage of modified nucleotides present in a given siNA molecule will depend on the total number of nucleotides present in the siNA. If the siNA molecule is single stranded, the percent modification can be based upon the total number of nucleotides present in the single stranded siNA molecules. Likewise, if the siNA molecule is double stranded, the percent modification can be based upon the total number of nucleotides present in the sense strand, antisense strand, or both the sense and antisense strands.

[0052] A nucleic acid or polynucleotide (e.g., siNA) molecule of the invention can comprise modified nucleotides at various locations within the nucleic acid or polynucleotide molecule. In one embodiment, a double stranded nucleic acid or polynucleotide (e.g., siNA) molecule of the invention comprises modified nucleotides at

internal base paired positions within the siNA duplex. For example, internal positions can comprise positions from about 3 to about 19 nucleotides from the 5'-end of either sense or antisense strand or region of a 21 nucleotide siNA duplex having 19 base pairs and two nucleotide 3'-overhangs. In another embodiment, a double stranded nucleic acid or polynucleotide molecule of the invention comprises modified nucleotides at non-base paired or overhang regions of the siNA molecule. By "non-base paired" is meant, the nucleotides are not base paired between the sense strand or sense region and the antisense strand or antisense region of the nucleic acid or polynucleotide molecule. The overhang nucleotides can be complementary or base paired to a corresponding target polynucleotide sequence (see for example Figure 6C). For example, overhang positions can comprise positions from about 20 to about 21 nucleotides from the 5'-end of either sense or antisense strand or region of a 21 nucleotide siNA duplex having 19 base pairs and two nucleotide 3'-overhangs. In another embodiment, a double stranded nucleic acid or polynucleotide molecule of the invention comprises modified nucleotides at terminal positions of the nucleic acid or polynucleotide molecule. For example, such terminal regions include the 3'-position, 5'-position, for both 3' and 5'-positions of the sense and/or antisense strand or region of the nucleic acid or polynucleotide molecule. In another embodiment, a double stranded nucleic acid or polynucleotide molecule of the invention comprises modified nucleotides at base-paired or internal positions, non-base paired or overhang regions, and/or terminal regions, or any combination thereof.

[0053] One aspect of the invention features a double-stranded short interfering nucleic acid or polynucleotide (*e.g.*, siNA) molecule that down-regulates expression of a target gene or that directs cleavage of a target RNA. In one embodiment, the double stranded nucleic acid or polynucleotide molecule comprises one or more chemical modifications and each strand of the double-stranded nucleic acid or polynucleotide is about 21 nucleotides long. In one embodiment, the double-stranded nucleic acid or polynucleotide molecule does not contain any ribonucleotides. In another embodiment, the double-stranded nucleic acid or polynucleotide molecule comprises one or more ribonucleotides. In one embodiment, each strand of the double-stranded nucleic acid or polynucleotide molecule independently comprises about 15 to about 30 (*e.g.*, about 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, or 30) nucleotides, wherein each strand comprises about 15 to about 30 (*e.g.*, about 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29,

or 30) nucleotides that are complementary to the nucleotides of the other strand. In one embodiment, one of the strands of the double-stranded nucleic acid or polynucleotide molecule comprises a nucleotide sequence that is complementary to a nucleotide sequence or a portion thereof of the target gene, and the second strand of the double-stranded nucleic acid or polynucleotide (*e.g.*, molecule comprises a nucleotide sequence substantially similar to the nucleotide sequence of the target gene or a portion thereof.

[0054] In another embodiment, the invention features a double-stranded short interfering nucleic acid or polynucleotide (*e.g.*, siNA) molecule that down-regulates expression of a target gene or that directs cleavage of a target RNA, comprising an antisense region, wherein the antisense region comprises a nucleotide sequence that is complementary to a nucleotide sequence of the target gene or a portion thereof, and a sense region, wherein the sense region comprises a nucleotide sequence substantially similar to the nucleotide sequence of the target gene or a portion thereof. In one embodiment, the antisense region and the sense region independently comprise about 15 to about 30 (*e.g.* about 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, or 30) nucleotides, wherein the antisense region comprises about 15 to about 30 (*e.g.* about 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, or 30) nucleotides that are complementary to nucleotides of the sense region.

[0055] In another embodiment, the invention features a double-stranded short interfering nucleic acid or polynucleotide (*e.g.*, siNA) molecule that down-regulates expression of a target gene or that directs cleavage of a target RNA, comprising a sense region and an antisense region, wherein the antisense region comprises a nucleotide sequence that is complementary to a nucleotide sequence of RNA encoded by the target gene or a portion thereof and the sense region comprises a nucleotide sequence that is complementary to the antisense region.

[0056] In one embodiment, a nucleic acid or polynucleotide (*e.g.*, siNA) molecule of the invention comprises blunt ends, *i.e.*, ends that do not include any overhanging nucleotides. For example, a siNA molecule—comprising modifications described herein (*e.g.*, comprising nucleotides having Formulae I-VII or siNA constructs comprising “Stab 00”-“Stab 36” or “Stab 3F”-“Stab 36F” (Table IV) or any combination thereof)

and/or any length described herein can comprise blunt ends or ends with no overhanging nucleotides.

[0057] In one embodiment, any nucleic acid or polynucleotide (*e.g.*, siNA) molecule of the invention can comprise one or more blunt ends, i.e. where a blunt end does not have any overhanging nucleotides. In one embodiment, the blunt ended siNA molecule has a number of base pairs equal to the number of nucleotides present in each strand of the siNA molecule. In another embodiment, the siNA molecule comprises one blunt end, for example wherein the 5'-end of the antisense strand and the 3'-end of the sense strand do not have any overhanging nucleotides. In another example, the siNA molecule comprises one blunt end, for example wherein the 3'-end of the antisense strand and the 5'-end of the sense strand do not have any overhanging nucleotides. In another example, a siNA molecule comprises two blunt ends, for example wherein the 3'-end of the antisense strand and the 5'-end of the sense strand as well as the 5'-end of the antisense strand and 3'-end of the sense strand do not have any overhanging nucleotides. A blunt ended siNA molecule can comprise, for example, from about 15 to about 30 nucleotides (*e.g.*, about 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, or 30 nucleotides). Other nucleotides present in a blunt ended siNA molecule can comprise, for example, mismatches, bulges, loops, or wobble base pairs to modulate the activity of the siNA molecule to mediate RNA interference.

[0058] By “blunt ends” is meant symmetric termini or termini of a double stranded nucleic acid or polynucleotide (*e.g.*, siNA) molecule having no overhanging nucleotides. The two strands of a double stranded siNA molecule align with each other without overhanging nucleotides at the termini. For example, a blunt ended siNA construct comprises terminal nucleotides that are complementary between the sense and antisense regions of the siNA molecule.

[0059] In one embodiment, the invention features a double-stranded short interfering nucleic acid or polynucleotide (*e.g.*, siNA) molecule that down-regulates expression of a target gene or that directs cleavage of a target RNA, wherein the siNA molecule is assembled from two separate oligonucleotide fragments wherein one fragment comprises the sense region and the second fragment comprises the antisense region of the siNA

molecule. The sense region can be connected to the antisense region via a linker molecule, such as a polynucleotide linker or a non-nucleotide linker.

[0060] In one embodiment, a double stranded nucleic acid or polynucleotide (*e.g.*, siNA) molecule of the invention comprises ribonucleotides at positions that maintain or enhance RNAi activity. In one embodiment, ribonucleotides are present in the sense strand or sense region of the siNA molecule, which can provide for RNAi activity by allowing cleavage of the sense strand or sense region by an enzyme within the RISC (*e.g.*, ribonucleotides present at the position of passenger strand, sense strand, or sense region cleavage, such as position 9 of the passenger strand of a 19 base-pair duplex, which is cleaved in the RISC by AGO2 enzyme, see for example Matranga *et al.*, 2005, *Cell*, 123:1-114 and Rand *et al.*, 2005, *Cell*, 123:621-629). In another embodiment, one or more (for example 1, 2, 3, 4 or 5) nucleotides at the 5'-end of the guide strand or guide region (also known as antisense strand or antisense region) of the siNA molecule are ribonucleotides.

[0061] In one embodiment, a double stranded nucleic acid or polynucleotide (*e.g.*, siNA)) molecule of the invention comprises one or more ribonucleotides at positions within the passenger strand or passenger region (also known as the sense strand or sense region) that allows cleavage of the passenger strand or passenger region by an enzyme in the RISC complex, (*e.g.*, ribonucleotides present at the position of passenger strand such as position 9 of the passenger strand of a 19 base-pair duplex is cleaved in the RISC by AGO2 enzyme, see for example Matranga *et al.*, 2005, *Cell*, 123:1-114 and Rand *et al.*, 2005, *Cell*, 123:621-629).

[0062] In one embodiment, a nucleic acid or polynucleotide (*e.g.*, siNA) molecule of the invention contains at least 2, 3, 4, 5, or more chemical modifications that can be the same or different. In another embodiment, a siNA molecule of the invention contains at least 2, 3, 4, 5, or more different chemical modifications.

[0063] In one embodiment, a nucleic acid or polynucleotide (*e.g.*, siNA) molecule of the invention is a double-stranded short interfering nucleic acid or polynucleotide (*e.g.*, siNA), wherein the double stranded nucleic acid molecule comprises about 15 to about 30 (*e.g.* about 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, or 30) base pairs,

and wherein one or more (e.g., at least 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, or 30) of the nucleotide positions in each strand of the siNA molecule comprises a chemical modification. In another embodiment, the siNA contains at least 2, 3, 4, 5, or more different chemical modifications.

[0064] In one embodiment, the invention features double-stranded short interfering nucleic acid or polynucleotide (e.g., siNA) molecule that down-regulates expression of a target gene or that directs cleavage of a target RNA, wherein the siNA molecule comprises about 15 to about 30 (e.g. about 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, or 30) base pairs, and wherein each strand of the siNA molecule comprises one or more chemical modifications. In one embodiment, each strand of the double stranded siNA molecule comprises at least two (e.g., 2, 3, 4, 5, or more) different chemical modifications, e.g., different nucleotide sugar, base, or backbone modifications. In another embodiment, one of the strands of the double-stranded siNA molecule comprises a nucleotide sequence that is complementary to a nucleotide sequence of a target gene or a portion thereof, and the second strand of the double-stranded siNA molecule comprises a nucleotide sequence substantially similar to the nucleotide sequence or a portion thereof of the target gene. In another embodiment, one of the strands of the double-stranded siNA molecule comprises a nucleotide sequence that is complementary to a nucleotide sequence of a target gene or portion thereof, and the second strand of the double-stranded siNA molecule comprises a nucleotide sequence substantially similar to the nucleotide sequence or portion thereof of the target gene. In another embodiment, each strand of the siNA molecule comprises about 15 to about 30 (e.g. about 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, or 30) nucleotides, and each strand comprises at least about 15 to about 30 (e.g. about 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, or 30) nucleotides that are complementary to the nucleotides of the other strand. The target gene can comprise, for example, sequences referred to herein or incorporated herein by reference. The target gene can comprise, for example, sequences referred to by GenBank Accession number herein.

[0065] In one embodiment, each strand of a double stranded nucleic acid or polynucleotide (e.g., siNA) molecule of the invention comprises a different pattern of

chemical modifications, such as any “Stab 00”-“Stab 36” or “Stab 3F”-“Stab 36F” (Table IV) modification patterns herein or any combination thereof (see **Table IV**). Non-limiting examples of sense and antisense strands of such siNA molecules having various modification patterns are shown in Table III and Figures 4 and 5.

[0066] In one embodiment, a nucleic acid or polynucleotide (*e.g.*, siNA) molecule of the invention comprises no ribonucleotides. In another embodiment, a siNA molecule of the invention comprises one or more ribonucleotides (*e.g.*, 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, or more ribonucleotides).

[0067] In one embodiment, a nucleic acid or polynucleotide (*e.g.*, siNA) molecule of the invention comprises an antisense region comprising a nucleotide sequence that is complementary to a nucleotide sequence of a PCSK9 target gene or a portion thereof, and the siNA further comprises a sense region comprising a nucleotide sequence substantially similar to the nucleotide sequence of the PCSK9 target gene or a portion thereof. In another embodiment, the antisense region and the sense region each comprise about 15 to about 30 (*e.g.* about 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, or 30) nucleotides and the antisense region comprises at least about 15 to about 30 (*e.g.* about 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, or 30) nucleotides that are complementary to nucleotides of the sense region. In one embodiment, each strand of the double stranded siNA molecule comprises at least two (*e.g.*, 2, 3, 4, 5, or more) different chemical modifications, *e.g.*, different nucleotide sugar, base, or backbone modifications. The PCSK9 target gene can comprise, for example, sequences referred to herein or incorporated by reference herein. In another embodiment, the siNA is a double stranded nucleic acid molecule, where each of the two strands of the siNA molecule independently comprise about 15 to about 40 (*e.g.* about 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, or 40) nucleotides, and where one of the strands of the siNA molecule comprises at least about 15 (*e.g.* about 15, 16, 17, 18, 19, 20, 21, 22, 23, 24 or 25 or more) nucleotides that are complementary to the nucleic acid sequence of the PCSK9 target gene or a portion thereof.

[0068] In one embodiment, a nucleic acid or polynucleotide (*e.g.*, siNA) molecule of the invention comprises a sense region and an antisense region, wherein the antisense

region comprises a nucleotide sequence that is complementary to a nucleotide sequence of RNA encoded by a PCSK9 target gene, or a portion thereof, and the sense region comprises a nucleotide sequence that is complementary to the antisense region. In one embodiment, the siNA molecule is assembled from two separate oligonucleotide fragments, wherein one fragment comprises the sense region and the second fragment comprises the antisense region of the siNA molecule. In another embodiment, the sense region is connected to the antisense region via a linker molecule. In another embodiment, the sense region is connected to the antisense region via a linker molecule, such as a nucleotide or non-nucleotide linker. In one embodiment, each strand of the double stranded siNA molecule comprises at least two (e.g., 2, 3, 4, 5, or more) different chemical modifications, e.g., different nucleotide sugar, base, or backbone modifications. The PCSK9 target gene can comprise, for example, sequences referred herein or incorporated by reference herein.

[0069] In one embodiment, a nucleic acid or polynucleotide (e.g., siNA) molecule of the invention comprises one or more (e.g., 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20 or more) 2'-deoxy-2'-fluoro pyrimidine modifications (e.g., where one or more or all pyrimidine (e.g., U or C) positions of the siNA are modified with 2'-deoxy-2'-fluoro nucleotides). In one embodiment, the 2'-deoxy-2'-fluoro pyrimidine modifications are present in the sense strand. In one embodiment, the 2'-deoxy-2'-fluoro pyrimidine modifications are present in the antisense strand. In one embodiment, the 2'-deoxy-2'-fluoro pyrimidine modifications are present in both the sense strand and the antisense strand of the siNA molecule.

[0070] In one embodiment, a nucleic acid or polynucleotide (e.g., siNA) molecule of the invention comprises one or more (e.g., 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20 or more) 2'-O-methyl purine modifications (e.g., where one or more or all purine (e.g., A or G) positions of the siNA are modified with 2'-O-methyl nucleotides). In one embodiment, the 2'-O-methyl purine modifications are present in the sense strand. In one embodiment, the 2'-O-methyl purine modifications are present in the antisense strand. In one embodiment, the 2'-O-methyl purine modifications are present in both the sense strand and the antisense strand of the siNA molecule.

[0071] In one embodiment, a nucleic acid or polynucleotide (*e.g.*, siNA) molecule of the invention comprises one or more (*e.g.*, 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20 or more) 2'-deoxy purine modifications (*e.g.*, where one or more or all purine (*e.g.*, A or G) positions of the siNA are modified with 2'-deoxy nucleotides). In one embodiment, the 2'-deoxy purine modifications are present in the sense strand. In one embodiment, the 2'-deoxy purine modifications are present in the antisense strand. In one embodiment, the 2'-deoxy purine modifications are present in both the sense strand and the antisense strand of the siNA molecule.

[0072] In one embodiment, the invention features a double-stranded short interfering nucleic acid or polynucleotide (*e.g.*, siNA) molecule that down-regulates expression of a target gene or that directs cleavage of a PCSK9 target RNA, comprising a sense region and an antisense region, wherein the antisense region comprises a nucleotide sequence that is complementary to a nucleotide sequence of RNA encoded by the target gene or a portion thereof and the sense region comprises a nucleotide sequence that is complementary to the antisense region, and wherein the siNA molecule has one or more modified pyrimidine and/or purine nucleotides. In one embodiment, each strand of the double stranded siNA molecule comprises at least two (*e.g.*, 2, 3, 4, 5, or more) different chemical modifications, *e.g.*, different nucleotide sugar, base, or backbone modifications. In one embodiment, the pyrimidine nucleotides in the sense region are 2'-O-methyl pyrimidine nucleotides or 2'-deoxy-2'-fluoro pyrimidine nucleotides and the purine nucleotides present in the sense region are 2'-deoxy purine nucleotides. In another embodiment, the pyrimidine nucleotides in the sense region are 2'-deoxy-2'-fluoro pyrimidine nucleotides and the purine nucleotides present in the sense region are 2'-O-methyl purine nucleotides. In another embodiment, the pyrimidine nucleotides in the sense region are 2'-deoxy-2'-fluoro pyrimidine nucleotides and the purine nucleotides present in the sense region are 2'-deoxy purine nucleotides. In one embodiment, the pyrimidine nucleotides in the antisense region are 2'-deoxy-2'-fluoro pyrimidine nucleotides and the purine nucleotides present in the antisense region are 2'-O-methyl or 2'-deoxy purine nucleotides. In another embodiment of any of the above-described siNA molecules, any nucleotides present in a non-complementary region of the sense strand (*e.g.* overhang region) are 2'-deoxy nucleotides.

[0073] In one embodiment, the invention features a double-stranded short interfering nucleic acid or polynucleotide (*e.g.*, siNA) molecule that down-regulates expression of a target gene or that directs cleavage of a target RNA, wherein the siNA molecule is assembled from two separate oligonucleotide fragments wherein one fragment comprises the sense region and the second fragment comprises the antisense region of the siNA molecule, and wherein the fragment comprising the sense region includes a terminal cap moiety at the 5'-end, the 3'-end, or both of the 5' and 3' ends of the fragment. In one embodiment, the terminal cap moiety is an inverted deoxy abasic moiety or glyceryl moiety. In one embodiment, each of the two fragments of the siNA molecule independently comprise about 15 to about 30 (*e.g.* about 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, or 30) nucleotides. In another embodiment, each of the two fragments of the siNA molecule independently comprise about 15 to about 40 (*e.g.* about 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, or 40) nucleotides. In a non-limiting example, each of the two fragments of the siNA molecule comprise about 21 nucleotides.

[0074] In one embodiment, the invention features a nucleic acid or polynucleotide (*e.g.*, siNA) molecule comprising at least one modified nucleotide, wherein the modified nucleotide is a 2'-deoxy-2'-fluoro nucleotide, 2'-deoxy-2'-fluoroarabino, 2'-O-trifluoromethyl nucleotide, 2'-O-ethyl-trifluoromethoxy nucleotide, or 2'-O-difluoromethoxy-ethoxy nucleotide or any other modified nucleoside/nucleotide described herein, and in USSN 10/981,966 filed November 5, 2004, incorporated by reference herein. In one embodiment, the invention features a siNA molecule comprising at least two (*e.g.*, 2, 3, 4, 5, 6, 7, 8, 9, 10, or more) modified nucleotides, wherein the modified nucleotide is selected from the group consisting of 2'-deoxy-2'-fluoro nucleotide, 2'-deoxy-2'-fluoroarabino, 2'-O-trifluoromethyl nucleotide, 2'-O-ethyl-trifluoromethoxy nucleotide, or 2'-O-difluoromethoxy-ethoxy nucleotide or any other modified nucleoside/nucleotide described herein and in USSN 10/981,966, filed November 5, 2004, incorporated by reference herein. The modified nucleotide/nucleoside can be the same or different. The siNA can be, for example, about 15 to about 40 nucleotides in length. In one embodiment, all pyrimidine nucleotides present in the siNA are 2'-deoxy-2'-fluoro, 2'-deoxy-2'-fluoroarabino, 2'-O-trifluoromethyl, 2'-O-ethyl-trifluoromethoxy, or 2'-O-difluoromethoxy-ethoxy, 4'-thio

pyrimidine nucleotides. In one embodiment, the modified nucleotides in the siNA include at least one 2'-deoxy-2'-fluoro cytidine or 2'-deoxy-2'-fluoro uridine nucleotide. In another embodiment, the modified nucleotides in the siNA include at least one 2'-deoxy-2'-fluoro cytidine and at least one 2'-deoxy-2'-fluoro uridine nucleotides. In one embodiment, all uridine nucleotides present in the siNA are 2'-deoxy-2'-fluoro uridine nucleotides. In one embodiment, all cytidine nucleotides present in the siNA are 2'-deoxy-2'-fluoro cytidine nucleotides. In one embodiment, all adenosine nucleotides present in the siNA are 2'-deoxy-2'-fluoro adenosine nucleotides. In one embodiment, all guanosine nucleotides present in the siNA are 2'-deoxy-2'-fluoro guanosine nucleotides. The siNA can further comprise at least one modified internucleotidic linkage, such as phosphorothioate linkage. In one embodiment, the 2'-deoxy-2'-fluoronucleotides are present at specifically selected locations in the siNA that are sensitive to cleavage by ribonucleases, such as locations having pyrimidine nucleotides.

[0075] In one embodiment, the invention features a method of increasing the stability of a nucleic acid or polynucleotide (*e.g.*, siNA) molecule against cleavage by ribonucleases comprising introducing at least one modified nucleotide into the siNA molecule, wherein the modified nucleotide is a 2'-deoxy-2'-fluoro nucleotide. In one embodiment, all pyrimidine nucleotides present in the siNA are 2'-deoxy-2'-fluoro pyrimidine nucleotides. In one embodiment, the modified nucleotides in the siNA include at least one 2'-deoxy-2'-fluoro cytidine or 2'-deoxy-2'-fluoro uridine nucleotide. In another embodiment, the modified nucleotides in the siNA include at least one 2'-fluoro cytidine and at least one 2'-deoxy-2'-fluoro uridine nucleotides. In one embodiment, all uridine nucleotides present in the siNA are 2'-deoxy-2'-fluoro uridine nucleotides. In one embodiment, all cytidine nucleotides present in the siNA are 2'-deoxy-2'-fluoro cytidine nucleotides. In one embodiment, all adenosine nucleotides present in the siNA are 2'-deoxy-2'-fluoro adenosine nucleotides. In one embodiment, all guanosine nucleotides present in the siNA are 2'-deoxy-2'-fluoro guanosine nucleotides. The siNA can further comprise at least one modified internucleotidic linkage, such as a phosphorothioate linkage. In one embodiment, the 2'-deoxy-2'-fluoronucleotides are present at specifically selected locations in the siNA that are sensitive to cleavage by ribonucleases, such as locations having pyrimidine nucleotides.

[0076] In one embodiment, the invention features a method of increasing the stability of a nucleic acid or polynucleotide (*e.g.*, siNA) molecule against cleavage by ribonucleases comprising introducing at least one modified nucleotide into the siNA molecule, wherein the modified nucleotide is a 2'-deoxy-2'-fluoroarabino nucleotide. In one embodiment, all pyrimidine nucleotides present in the siNA are 2'-deoxy-2'-fluoroarabino pyrimidine nucleotides. In one embodiment, the modified nucleotides in the siNA include at least one 2'-deoxy-2'-fluoroarabino cytidine or 2'-deoxy-2'-fluoroarabino uridine nucleotide. In another embodiment, the modified nucleotides in the siNA include at least one 2'-fluoro cytidine and at least one 2'-deoxy-2'-fluoroarabino uridine nucleotides. In one embodiment, all uridine nucleotides present in the siNA are 2'-deoxy-2'-fluoroarabino uridine nucleotides. In one embodiment, all cytidine nucleotides present in the siNA are 2'-deoxy-2'-fluoroarabino cytidine nucleotides. In one embodiment, all adenosine nucleotides present in the siNA are 2'-deoxy-2'-fluoroarabino adenosine nucleotides. In one embodiment, all guanosine nucleotides present in the siNA are 2'-deoxy-2'-fluoroarabino guanosine nucleotides. The siNA can further comprise at least one modified internucleotidic linkage, such as a phosphorothioate linkage. In one embodiment, the 2'-deoxy-2'-fluoroarabinonucleotides are present at specifically selected locations in the siNA that are sensitive to cleavage by ribonucleases, such as locations having pyrimidine nucleotides.

[0077] In one embodiment, the invention features a double-stranded short interfering nucleic acid or polynucleotide (*e.g.*, siNA) molecule that down-regulates expression of a target gene or that directs cleavage of a target RNA, comprising a sense region and an antisense region, wherein the antisense region comprises a nucleotide sequence that is complementary to a nucleotide sequence of RNA encoded by the target gene or a portion thereof and the sense region comprises a nucleotide sequence that is complementary to the antisense region, and wherein the purine nucleotides present in the antisense region comprise 2'-deoxy- purine nucleotides. In an alternative embodiment, the purine nucleotides present in the antisense region comprise 2'-O-methyl purine nucleotides. In either of the above embodiments, the antisense region can comprise a phosphorothioate internucleotide linkage at the 3' end of the antisense region. Alternatively, in either of the above embodiments, the antisense region can comprise a glyceryl modification at the 3' end of the antisense region. In another embodiment of any of the above-described

siNA molecules, any nucleotides present in a non-complementary region of the antisense strand (e.g. overhang region) are 2'-deoxy nucleotides.

[0078] In one embodiment, the antisense region of a nucleic acid or polynucleotide (*e.g.*, siNA) molecule of the invention comprises sequence complementary to a portion of an endogenous transcript having sequence unique to a particular disease or trait related allele in a subject or organism, such as sequence comprising a single nucleotide polymorphism (SNP) associated with the disease or trait specific allele. As such, the antisense region of a siNA molecule of the invention can comprise sequence complementary to sequences that are unique to a particular allele to provide specificity in mediating selective RNAi against the disease, condition, or trait related allele.

[0079] In one embodiment, the invention features a double-stranded short interfering nucleic acid or polynucleotide (*e.g.*, siNA) molecule that down-regulates expression of a target gene or that directs cleavage of a target RNA, wherein the siNA molecule is assembled from two separate oligonucleotide fragments wherein one fragment comprises the sense region and the second fragment comprises the antisense region of the siNA molecule. In one embodiment, each strand of the double stranded siNA molecule is about 21 nucleotides long where about 19 nucleotides of each fragment of the siNA molecule are base-paired to the complementary nucleotides of the other fragment of the siNA molecule, wherein at least two 3' terminal nucleotides of each fragment of the siNA molecule are not base-paired to the nucleotides of the other fragment of the siNA molecule. In another embodiment, the siNA molecule is a double stranded nucleic acid molecule, where each strand is about 19 nucleotides long and where the nucleotides of each fragment of the siNA molecule are base-paired to the complementary nucleotides of the other fragment of the siNA molecule to form at least about 15 (*e.g.*, 15, 16, 17, 18, or 19) base pairs, wherein one or both ends of the siNA molecule are blunt ends. In one embodiment, each of the two 3' terminal nucleotides of each fragment of the siNA molecule is a 2'-deoxy-pyrimidine nucleotide, such as a 2'-deoxy-thymidine. In one embodiment, each of the two 3' terminal nucleotides of each fragment of the siNA molecule is a 2'-O-methyl pyrimidine nucleotide, such as a 2'-O-methyl uridine, cytidine, or thymidine. In another embodiment, all nucleotides of each fragment of the siNA molecule are base-paired to the complementary nucleotides of the other fragment

of the siNA molecule. In another embodiment, the siNA molecule is a double stranded nucleic acid molecule of about 19 to about 25 base pairs having a sense region and an antisense region, where about 19 nucleotides of the antisense region are base-paired to the nucleotide sequence or a portion thereof of the RNA encoded by the target gene. In another embodiment, about 21 nucleotides of the antisense region are base-paired to the nucleotide sequence or a portion thereof of the RNA encoded by the target gene. In any of the above embodiments, the 5'-end of the fragment comprising said antisense region can optionally include a phosphate group.

[0080] In one embodiment, the invention features a double-stranded short interfering nucleic acid or polynucleotide (*e.g.*, siNA) molecule that inhibits the expression of a target RNA sequence, wherein the siNA molecule does not contain any ribonucleotides and wherein each strand of the double-stranded siNA molecule is about 15 to about 30 nucleotides. In one embodiment, the siNA molecule is 21 nucleotides in length. Examples of non-ribonucleotide containing siNA constructs are combinations of stabilization chemistries shown in Table IV in any combination of Sense/Antisense chemistries, such as Stab 7/8, Stab 7/11, Stab 8/8, Stab 18/8, Stab 18/11, Stab 12/13, Stab 7/13, Stab 18/13, Stab 7/19, Stab 8/19, Stab 18/19, Stab 7/20, Stab 8/20, Stab 18/20, Stab 7/32, Stab 8/32, or Stab 18/32 (*e.g.*, any siNA having Stab 7, 8, 11, 12, 13, 14, 15, 17, 18, 19, 20, or 32 sense or antisense strands or any combination thereof). Herein, numeric Stab chemistries can include both 2'-fluoro and 2'-OCF₃ versions of the chemistries shown in Table IV. For example, "Stab 7/8" refers to both Stab 7/8 and Stab 7F/8F etc. In one embodiment, the invention features a chemically synthesized double stranded RNA molecule that directs cleavage of a target RNA via RNA interference, wherein each strand of said RNA molecule is about 15 to about 30 nucleotides in length; one strand of the RNA molecule comprises nucleotide sequence having sufficient complementarity to the target RNA for the RNA molecule to direct cleavage of the target RNA via RNA interference; and wherein at least one strand of the RNA molecule optionally comprises one or more chemically modified nucleotides described herein, such as without limitation deoxynucleotides, 2'-O-methyl nucleotides, 2'-deoxy-2'-fluoro nucleotides, 2'-deoxy-2'-fluoroarabino, 2'-O-methoxyethyl nucleotides, 4'-thio nucleotides, 2'-O-trifluoromethyl nucleotides, 2'-O-ethyl-

trifluoromethoxy nucleotides, 2'-O-difluoromethoxy-ethoxy nucleotides, etc. or any combination thereof.

[0081] In one embodiment, a target RNA of the invention comprises sequence encoding a protein.

[0082] In one embodiment, target RNA of the invention comprises non-coding RNA sequence (e.g., miRNA, snRNA, siRNA etc.), see for example Mattick, 2005, Science, 309, 1527-1528; Claverie, 2005, Science, 309, 1529-1530; Sethupathy et al., 2006, RNA, 12, 192-197; and Czech, 2006 NEJM, 354, 11: 1194-1195.

[0083] In one embodiment, the invention features a medicament comprising a siNA molecule of the invention.

[0084] In one embodiment, the invention features an active ingredient comprising a siNA molecule of the invention.

[0085] In one embodiment, the invention features the use of a double-stranded short interfering nucleic acid or polynucleotide (e.g., siNA) molecule to inhibit, down-regulate, or reduce expression of a target gene, wherein the siNA molecule comprises one or more chemical modifications and each strand of the double-stranded siNA is independently about 15 to about 30 or more (e.g., about 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29 or 30 or more) nucleotides long. In one embodiment, the siNA molecule of the invention is a double stranded nucleic acid molecule comprising one or more chemical modifications, where each of the two fragments of the siNA molecule independently comprise about 15 to about 40 (e.g. about 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, or 40) nucleotides and where one of the strands comprises at least 15 nucleotides that are complementary to nucleotide sequence of target encoding RNA or a portion thereof. In a non-limiting example, each of the two fragments of the siNA molecule comprise about 21 nucleotides. In another embodiment, the siNA molecule is a double stranded nucleic acid molecule comprising one or more chemical modifications, where each strand is about 21 nucleotide long and where about 19 nucleotides of each fragment of the siNA molecule are base-paired to the complementary nucleotides of the other fragment of the siNA

molecule, wherein at least two 3' terminal nucleotides of each fragment of the siNA molecule are not base-paired to the nucleotides of the other fragment of the siNA molecule. In another embodiment, the siNA molecule is a double stranded nucleic acid molecule comprising one or more chemical modifications, where each strand is about 19 nucleotide long and where the nucleotides of each fragment of the siNA molecule are base-paired to the complementary nucleotides of the other fragment of the siNA molecule to form at least about 15 (e.g., 15, 16, 17, 18, or 19) base pairs, wherein one or both ends of the siNA molecule are blunt ends. In one embodiment, each of the two 3' terminal nucleotides of each fragment of the siNA molecule is a 2'-deoxy-pyrimidine nucleotide, such as a 2'-deoxy-thymidine. In one embodiment, each of the two 3' terminal nucleotides of each fragment of the siNA molecule is a 2'-O-methyl pyrimidine nucleotide, such as a 2'-O-methyl uridine, cytidine, or thymidine. In another embodiment, all nucleotides of each fragment of the siNA molecule are base-paired to the complementary nucleotides of the other fragment of the siNA molecule. In another embodiment, the siNA molecule is a double stranded nucleic acid molecule of about 19 to about 25 base pairs having a sense region and an antisense region and comprising one or more chemical modifications, where about 19 nucleotides of the antisense region are base-paired to the nucleotide sequence or a portion thereof of the RNA encoded by the target. In another embodiment, about 21 nucleotides of the antisense region are base-paired to the nucleotide sequence or a portion thereof of the RNA encoded by the target. In any of the above embodiments, the 5'-end of the fragment comprising said antisense region can optionally include a phosphate group.

[0086] In one embodiment, the invention features the use of a double-stranded short interfering nucleic acid or polynucleotide (*e.g.*, siNA) molecule that inhibits, down-regulates, or reduces expression of a target gene, wherein one of the strands of the double-stranded siNA molecule is an antisense strand which comprises nucleotide sequence that is complementary to nucleotide sequence of target RNA or a portion thereof, the other strand is a sense strand which comprises nucleotide sequence that is complementary to a nucleotide sequence of the antisense strand. In one embodiment, each strand has at least two (*e.g.*, 2, 3, 4, 5, or more) chemical modifications, which can be the same or different, such as nucleotide, sugar, base, or backbone modifications. In one embodiment, a majority of the pyrimidine nucleotides present in the double-stranded

siNA molecule comprises a sugar modification. In one embodiment, a majority of the purine nucleotides present in the double-stranded siNA molecule comprises a sugar modification.

[0087] In one embodiment, the invention features a double-stranded short interfering nucleic acid or polynucleotide (*e.g.*, siNA) molecule that inhibits, down-regulates, or reduces expression of a target gene, wherein one of the strands of the double-stranded siNA molecule is an antisense strand which comprises nucleotide sequence that is complementary to nucleotide sequence of target RNA or a portion thereof, wherein the other strand is a sense strand which comprises nucleotide sequence that is complementary to a nucleotide sequence of the antisense strand. In one embodiment, each strand has at least two (*e.g.*, 2, 3, 4, 5, or more) chemical modifications, which can be the same or different, such as nucleotide, sugar, base, or backbone modifications. In one embodiment, a majority of the pyrimidine nucleotides present in the double-stranded siNA molecule comprises a sugar modification. In one embodiment, a majority of the purine nucleotides present in the double-stranded siNA molecule comprises a sugar modification.

[0088] In one embodiment, the invention features a double-stranded short interfering nucleic acid or polynucleotide (*e.g.*, siNA) molecule that inhibits, down-regulates, or reduces expression of a target gene, wherein one of the strands of the double-stranded siNA molecule is an antisense strand which comprises nucleotide sequence that is complementary to nucleotide sequence of target RNA that encodes a protein or portion thereof, the other strand is a sense strand which comprises nucleotide sequence that is complementary to a nucleotide sequence of the antisense strand and wherein a majority of the pyrimidine nucleotides present in the double-stranded siNA molecule comprises a sugar modification. In one embodiment, each strand of the siNA molecule comprises about 15 to about 30 or more (*e.g.*, about 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, or 30 or more) nucleotides, wherein each strand comprises at least about 15 nucleotides that are complementary to the nucleotides of the other strand. In one embodiment, the siNA molecule is assembled from two oligonucleotide fragments, wherein one fragment comprises the nucleotide sequence of the antisense strand of the siNA molecule and a second fragment comprises nucleotide sequence of the sense region

of the siNA molecule. In one embodiment, the sense strand is connected to the antisense strand via a linker molecule, such as a polynucleotide linker or a non-nucleotide linker. In a further embodiment, the pyrimidine nucleotides present in the sense strand are 2'-deoxy-2'-fluoro pyrimidine nucleotides and the purine nucleotides present in the sense region are 2'-deoxy purine nucleotides. In another embodiment, the pyrimidine nucleotides present in the sense strand are 2'-deoxy-2'-fluoro pyrimidine nucleotides and the purine nucleotides present in the sense region are 2'-O-methyl purine nucleotides. In still another embodiment, the pyrimidine nucleotides present in the antisense strand are 2'-deoxy-2'-fluoro pyrimidine nucleotides and any purine nucleotides present in the antisense strand are 2'-deoxy purine nucleotides. In another embodiment, the antisense strand comprises one or more 2'-deoxy-2'-fluoro pyrimidine nucleotides and one or more 2'-O-methyl purine nucleotides. In another embodiment, the pyrimidine nucleotides present in the antisense strand are 2'-deoxy-2'-fluoro pyrimidine nucleotides and any purine nucleotides present in the antisense strand are 2'-O-methyl purine nucleotides. In a further embodiment the sense strand comprises a 3'-end and a 5'-end, wherein a terminal cap moiety (e.g., an inverted deoxy abasic moiety or inverted deoxy nucleotide moiety such as inverted thymidine) is present at the 5'-end, the 3'-end, or both of the 5' and 3' ends of the sense strand. In another embodiment, the antisense strand comprises a phosphorothioate internucleotide linkage at the 3' end of the antisense strand. In another embodiment, the antisense strand comprises a glyceryl modification at the 3' end. In another embodiment, the 5'-end of the antisense strand optionally includes a phosphate group.

[0089] In any of the above-described embodiments of a double-stranded short interfering nucleic acid or polynucleotide (e.g., siNA) molecule that inhibits, down-regulates, or reduces expression of a target gene, wherein a majority of the pyrimidine nucleotides present in the double-stranded siNA molecule comprises a sugar modification, each of the two strands of the siNA molecule can comprise about 15 to about 30 or more (e.g., about 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, or 30 or more) nucleotides. In one embodiment, about 15 to about 30 or more (e.g., about 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, or 30 or more) nucleotides of each strand of the siNA molecule are base-paired to the complementary nucleotides of the other strand of the siNA molecule. In another embodiment, about 15 to about 30 or

more (e.g., about 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, or 30 or more) nucleotides of each strand of the siNA molecule are base-paired to the complementary nucleotides of the other strand of the siNA molecule, wherein at least two 3' terminal nucleotides of each strand of the siNA molecule are not base-paired to the nucleotides of the other strand of the siNA molecule. In another embodiment, each of the two 3' terminal nucleotides of each fragment of the siNA molecule is a 2'-deoxy-pyrimidine, such as 2'-deoxy-thymidine. In one embodiment, each strand of the siNA molecule is base-paired to the complementary nucleotides of the other strand of the siNA molecule. In one embodiment, about 15 to about 30 (e.g., about 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, or 30) nucleotides of the antisense strand are base-paired to the nucleotide sequence of the target RNA or a portion thereof. In one embodiment, about 18 to about 25 (e.g., about 18, 19, 20, 21, 22, 23, 24, or 25) nucleotides of the antisense strand are base-paired to the nucleotide sequence of the target RNA or a portion thereof.

[0090] In one embodiment, the invention features a double-stranded short interfering nucleic acid or polynucleotide (e.g., siNA) molecule that inhibits expression of a target gene, wherein one of the strands of the double-stranded siNA molecule is an antisense strand which comprises nucleotide sequence that is complementary to nucleotide sequence of target RNA or a portion thereof, the other strand is a sense strand which comprises nucleotide sequence that is complementary to a nucleotide sequence of the antisense strand. In one embodiment, each strand has at least two (e.g., 2, 3, 4, 5, or more) different chemical modifications, such as nucleotide sugar, base, or backbone modifications. In one embodiment, a majority of the pyrimidine nucleotides present in the double-stranded siNA molecule comprises a sugar modification. In one embodiment, a majority of the purine nucleotides present in the double-stranded siNA molecule comprises a sugar modification. In one embodiment, the 5'-end of the antisense strand optionally includes a phosphate group.

[0091] In one embodiment, the invention features a double-stranded short interfering nucleic acid or polynucleotide (e.g., siNA) molecule that inhibits expression of a target gene, wherein one of the strands of the double-stranded siNA molecule is an antisense strand which comprises nucleotide sequence that is complementary to nucleotide sequence of target RNA or a portion thereof, the other strand is a sense strand which

comprises nucleotide sequence that is complementary to a nucleotide sequence of the antisense strand and wherein a majority of the pyrimidine nucleotides present in the double-stranded siNA molecule comprises a sugar modification, and wherein the nucleotide sequence or a portion thereof of the antisense strand is complementary to a nucleotide sequence of the untranslated region or a portion thereof of the target RNA.

[0092] In one embodiment, the invention features a double-stranded short interfering nucleic acid or polynucleotide (*e.g.*, siNA) molecule that inhibits expression of a target gene, wherein one of the strands of the double-stranded siNA molecule is an antisense strand which comprises nucleotide sequence that is complementary to nucleotide sequence of target RNA or a portion thereof, wherein the other strand is a sense strand which comprises nucleotide sequence that is complementary to a nucleotide sequence of the antisense strand, wherein a majority of the pyrimidine nucleotides present in the double-stranded siNA molecule comprises a sugar modification, and wherein the nucleotide sequence of the antisense strand is complementary to a nucleotide sequence of the target RNA or a portion thereof that is present in the target RNA.

[0093] In one embodiment, the invention features a composition comprising a nucleic acid or polynucleotide (*e.g.*, siNA) molecule of the invention in a pharmaceutically acceptable carrier or diluent. In another embodiment, the invention features two or more differing siNA molecules of the invention (*e.g.* siNA molecules that target different regions of target RNA or siNA molecules that target RNA and cellular targets) in a pharmaceutically acceptable carrier or diluent.

[0094] In a non-limiting example, the introduction of chemically-modified nucleotides into nucleic acid or polynucleotide (*e.g.*, siNA) molecules provides a powerful tool in overcoming potential limitations of *in vivo* stability and bioavailability inherent to native RNA molecules that are delivered exogenously. For example, the use of chemically-modified nucleic acid molecules can enable a lower dose of a particular nucleic acid molecule for a given therapeutic effect since chemically-modified nucleic acid molecules tend to have a longer half-life in serum. Furthermore, certain chemical modifications can improve the bioavailability of nucleic acid molecules by targeting particular cells or tissues and/or improving cellular uptake of the nucleic acid molecule.

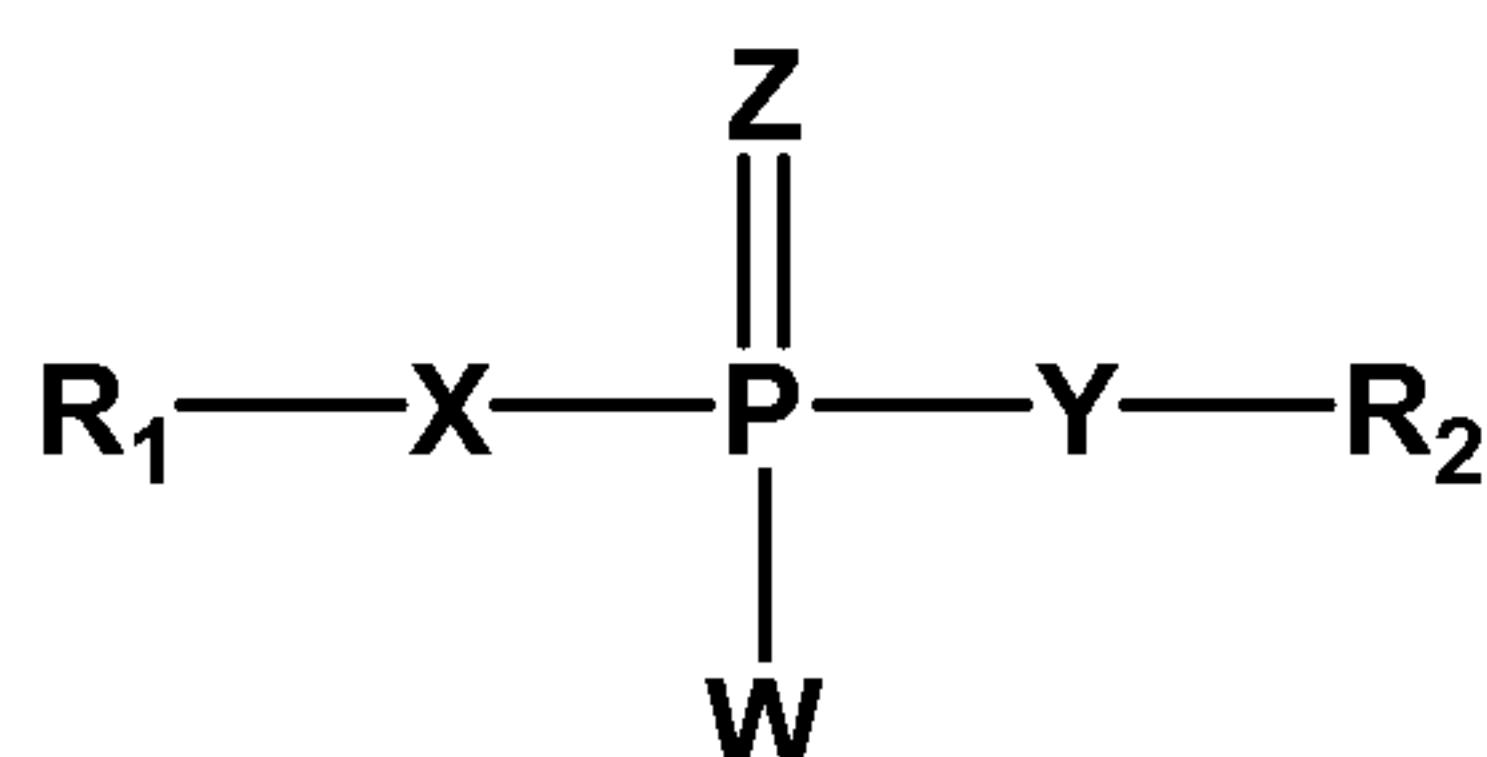
Therefore, even if the activity of a chemically-modified nucleic acid molecule is reduced as compared to a native nucleic acid molecule, for example, when compared to an all-RNA nucleic acid molecule, the overall activity of the modified nucleic acid molecule can be greater than that of the native molecule due to improved stability and/or delivery of the molecule. Unlike native unmodified siNA, chemically-modified siNA can also minimize the possibility of activating interferon activity or immunostimulation in humans. These properties therefore improve upon native siRNA or minimally modified siRNA's ability to mediate RNAi in various in vitro and in vivo settings, including use in both research and therapeutic applications. Applicant describes herein chemically modified siNA molecules with improved RNAi activity compared to corresponding unmodified or minimally modified siRNA molecules. The chemically modified siNA motifs disclosed herein provide the capacity to maintain RNAi activity that is substantially similar to unmodified or minimally modified active siRNA (see for example Elbashir et al., 2001, EMBO J., 20:6877-6888) while at the same time providing nuclease resistance and pharmacokinetic properties suitable for use in therapeutic applications.

[0095] In any of the embodiments of nucleic acid or polynucleotide (*e.g.*, siNA) molecules described herein, the antisense region of a siNA molecule of the invention can comprise a phosphorothioate internucleotide linkage at the 3'-end of said antisense region. In any of the embodiments of siNA molecules described herein, the antisense region can comprise about one to about five phosphorothioate internucleotide linkages at the 5'-end of said antisense region. In any of the embodiments of siNA molecules described herein, the 3'-terminal nucleotide overhangs of a siNA molecule of the invention can comprise ribonucleotides or deoxyribonucleotides that are chemically-modified at a nucleic acid sugar, base, or backbone. In any of the embodiments of siNA molecules described herein, the 3'-terminal nucleotide overhangs can comprise one or more universal base ribonucleotides. In any of the embodiments of siNA molecules described herein, the 3'-terminal nucleotide overhangs can comprise one or more acyclic nucleotides.

[0096] One embodiment of the invention provides an expression vector comprising a nucleic acid sequence encoding at least one polynucleotide (*e.g.*, siNA) molecule of the

invention in a manner that allows expression of the nucleic acid molecule. Another embodiment of the invention provides a mammalian cell comprising such an expression vector. The mammalian cell can be a human cell. The siNA molecule of the expression vector can comprise a sense region and an antisense region. The antisense region can comprise sequence complementary to a RNA or DNA sequence encoding target and the sense region can comprise sequence complementary to the antisense region. The siNA molecule can comprise two distinct strands having complementary sense and antisense regions. The siNA molecule can comprise a single strand having complementary sense and antisense regions.

[0097] In one embodiment, the invention features a chemically-modified short interfering nucleic acid or polynucleotide (*e.g.*, siNA) molecule capable of mediating RNA interference (RNAi) inside a cell or reconstituted in vitro system, wherein the chemical modification comprises one or more (*e.g.*, about 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, or more) nucleotides comprising a backbone modified internucleotide linkage having Formula I:

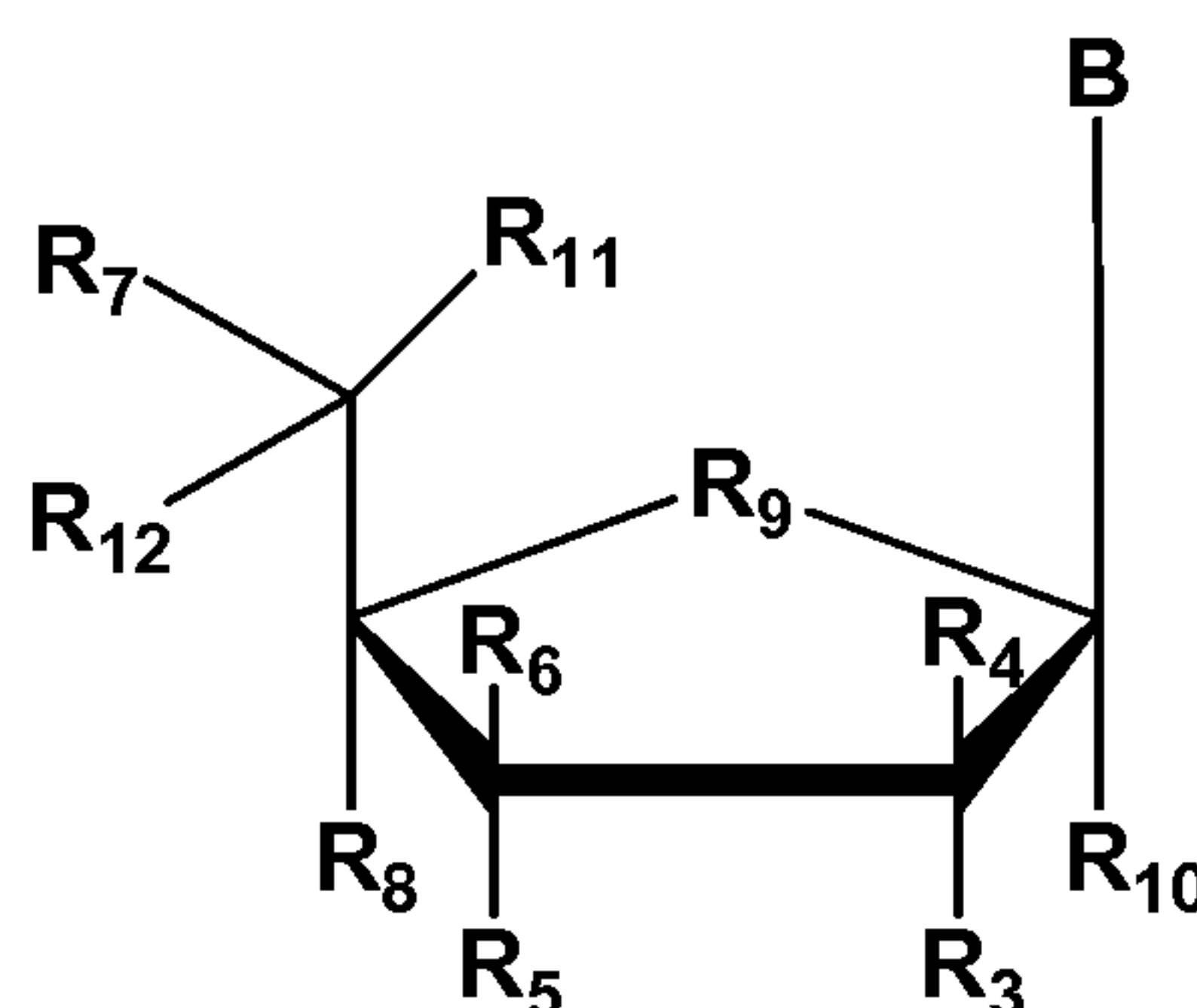


[0098] wherein each R1 and R2 is independently any nucleotide, non-nucleotide, or polynucleotide which can be naturally-occurring or chemically-modified and which can be included in the structure of the siNA molecule or serve as a point of attachment to the siNA molecule, each X and Y is independently O, S, N, alkyl, or substituted alkyl, each Z and W is independently O, S, N, alkyl, substituted alkyl, O-alkyl, S-alkyl, alkaryl, aralkyl, or acetyl and wherein W, X, Y, and Z are optionally not all O. In another embodiment, a backbone modification of the invention comprises a phosphonoacetate and/or thiophosphonoacetate internucleotide linkage (see for example Sheehan et al., 2003, *Nucleic Acids Research*, 31, 4109-4118).

[0099] The chemically-modified internucleotide linkages having Formula I, for example, wherein any Z, W, X, and/or Y independently comprises a sulphur atom, can

be present in one or both oligonucleotide strands of the nucleic acid or polynucleotide (*e.g.*, siNA) duplex, for example, in the sense strand, the antisense strand, or both strands. The siNA molecules of the invention can comprise one or more (*e.g.*, about 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, or more) chemically-modified internucleotide linkages having Formula I at the 3'-end, the 5'-end, or both of the 3' and 5'-ends of the sense strand, the antisense strand, or both strands. For example, an exemplary siNA molecule of the invention can comprise about 1 to about 5 or more (*e.g.*, about 1, 2, 3, 4, 5, or more) chemically-modified internucleotide linkages having Formula I at the 5'-end of the sense strand, the antisense strand, or both strands. In another non-limiting example, an exemplary siNA molecule of the invention can comprise one or more (*e.g.*, about 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, or more) pyrimidine nucleotides with chemically-modified internucleotide linkages having Formula I in the sense strand, the antisense strand, or both strands. In yet another non-limiting example, an exemplary siNA molecule of the invention can comprise one or more (*e.g.*, about 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, or more) purine nucleotides with chemically-modified internucleotide linkages having Formula I in the sense strand, the antisense strand, or both strands. In another embodiment, a siNA molecule of the invention having internucleotide linkage(s) of Formula I also comprises a chemically-modified nucleotide or non-nucleotide having any of Formulae I-VII.

[00100] In one embodiment, the invention features a chemically-modified short interfering nucleic acid or polynucleotide (*e.g.*, siNA) molecule capable of mediating RNA interference (RNAi) inside a cell or reconstituted in vitro system, wherein the chemical modification comprises one or more (*e.g.*, about 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, or more) nucleotides or non-nucleotides having Formula II:

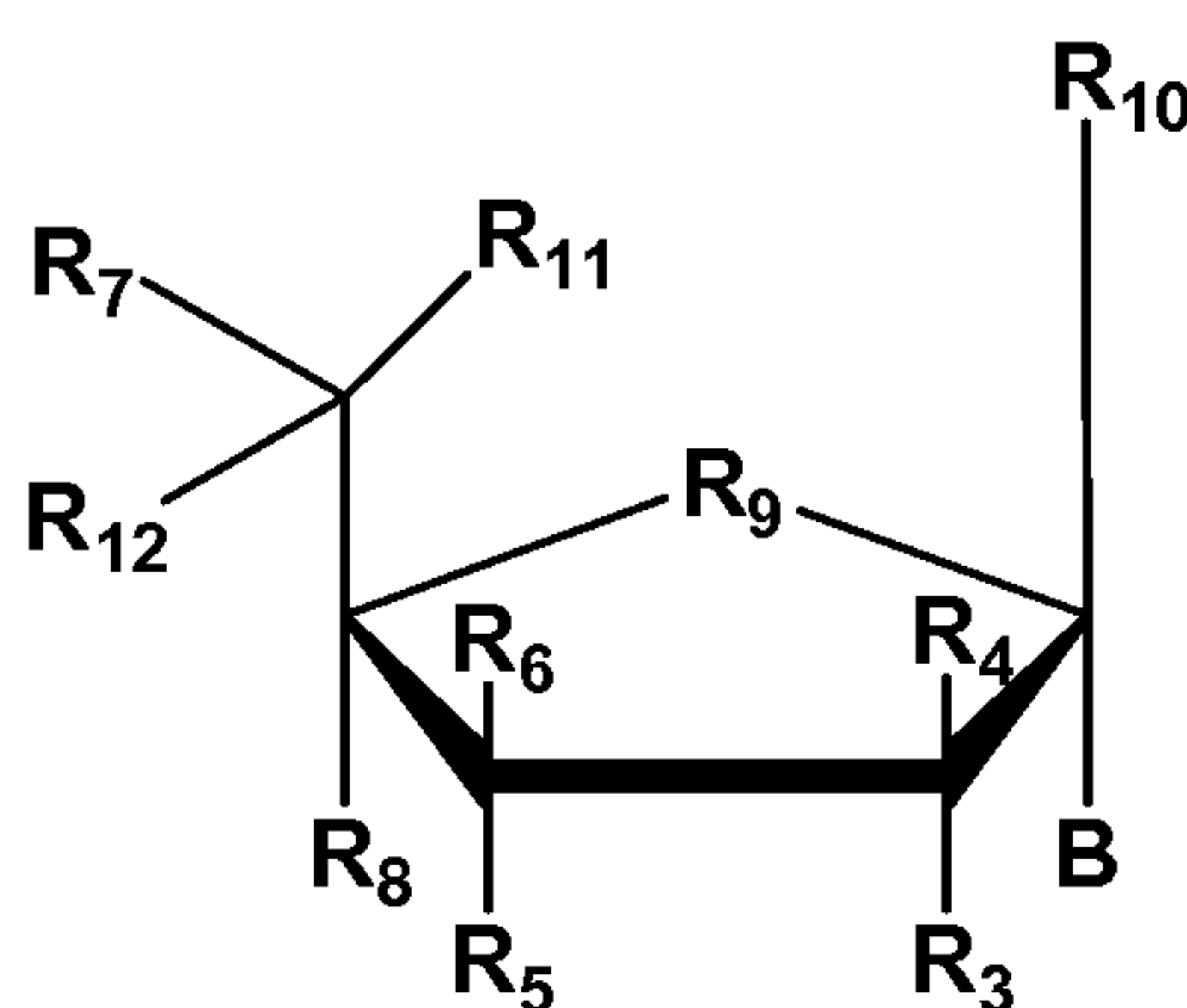


wherein each R3, R4, R5, R6, R7, R8, R10, R11 and R12 is independently H, OH, alkyl, substituted alkyl, alkaryl or aralkyl, F, Cl, Br, CN, CF3, OCF3, OCH3, OCN, O-alkyl, S-alkyl, N-alkyl, O-alkenyl, S-alkenyl, N-alkenyl, SO-alkyl, alkyl-OSH, alkyl-OH, O-alkyl-OH, O-alkyl-SH, S-alkyl-OH, S-alkyl-SH, alkyl-S-alkyl, alkyl-O-alkyl, ONO2, NO2, N3, NH2, aminoalkyl, aminoacid, aminoacyl, ONH2, O-aminoalkyl, O-aminoacid, O-aminoacyl, heterocycloalkyl, heterocycloalkaryl, aminoalkylamino, polyalkylamino, substituted silyl, or a group having any of Formula I, II, III, IV, V, VI and/or VII, any of which can be included in the structure of the siNA molecule or serve as a point of attachment to the siNA molecule; R9 is O, S, CH2, S=O, CHF, or CF2, and B is a nucleosidic base such as adenine, guanine, uracil, cytosine, thymine, 2-aminoadenosine, 5-methylcytosine, 2,6-diaminopurine, or any other non-naturally occurring base that can be complementary or non-complementary to target RNA or a non-nucleosidic base such as phenyl, naphthyl, 3-nitropyrrole, 5-nitroindole, nebularine, pyridone, pyridinone, or any other non-naturally occurring universal base that can be complementary or non-complementary to target RNA. In one embodiment, R3 and/or R7 comprises a conjugate moiety and a linker (*e.g.*, a nucleotide or non-nucleotide linker as described herein or otherwise known in the art). Non-limiting examples of conjugate moieties include ligands for cellular receptors, such as peptides derived from naturally occurring protein ligands; protein localization sequences, including cellular ZIP code sequences; antibodies; nucleic acid aptamers; vitamins and other co-factors, such as folate and N-acetylgalactosamine; polymers, such as polyethyleneglycol (PEG); phospholipids; cholesterol; steroids, and polyamines, such as PEI, spermine or spermidine. In one embodiment, a nucleotide of the invention having Formula II is a 2'-deoxy-2'-fluoro nucleotide. In one embodiment, a nucleotide of the invention having Formula II is a 2'-O-methyl nucleotide. In one embodiment, a nucleotide of the invention having Formula II is a 2'-deoxy nucleotide.

[00101] The chemically-modified nucleotide or non-nucleotide of Formula II can be present in one or both oligonucleotide strands of the siNA duplex, for example in the sense strand, the antisense strand, or both strands. The nucleic acid or polynucleotide (*e.g.*, siNA) molecules of the invention can comprise one or more chemically-modified nucleotides or non-nucleotides of Formula II at the 3'-end, the 5'-end, or both of the 3' and 5'-ends of the sense strand, the antisense strand, or both strands. For example, an

exemplary siNA molecule of the invention can comprise about 1 to about 5 or more (e.g., about 1, 2, 3, 4, 5, or more) chemically-modified nucleotides or non-nucleotides of Formula II at the 5'-end of the sense strand, the antisense strand, or both strands. In another non-limiting example, an exemplary siNA molecule of the invention can comprise about 1 to about 5 or more (e.g., about 1, 2, 3, 4, 5, or more) chemically-modified nucleotides or non-nucleotides of Formula II at the 3'-end of the sense strand, the antisense strand, or both strands.

[00102] In one embodiment, the invention features a chemically-modified short interfering nucleic acid or polynucleotide (e.g., siNA) molecule capable of mediating RNA interference (RNAi) inside a cell or reconstituted in vitro system, wherein the chemical modification comprises one or more (e.g., about 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, or more) nucleotides or non-nucleotides having Formula III:



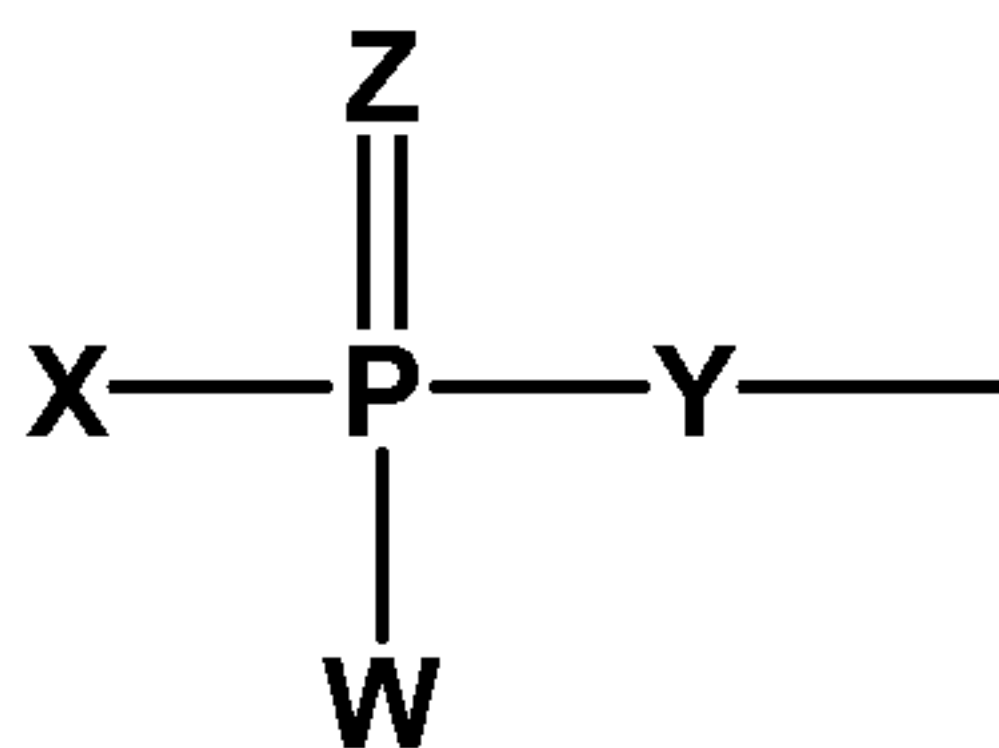
[00103] wherein each R3, R4, R5, R6, R7, R8, R10, R11 and R12 is independently H, OH, alkyl, substituted alkyl, alkaryl or aralkyl, F, Cl, Br, CN, CF3, OCF3, OCH3, OCN, O-alkyl, S-alkyl, N-alkyl, O-alkenyl, S-alkenyl, N-alkenyl, SO-alkyl, alkyl-OSH, alkyl-OH, O-alkyl-OH, O-alkyl-SH, S-alkyl-OH, S-alkyl-SH, alkyl-S-alkyl, alkyl-O-alkyl, ONO2, NO2, N3, NH2, aminoalkyl, aminoacid, aminoacyl, ONH2, O-aminoalkyl, O-aminoacid, O-aminoacyl, heterocycloalkyl, heterocycloalkaryl, aminoalkylamino, polyalkylamino, substituted silyl, or a group having any of Formula I, II, III, IV, V, VI and/or VII, any of which can be included in the structure of the siNA molecule or serve as a point of attachment to the siNA molecule; R9 is O, S, CH2, S=O, CHF, or CF2, and B is a nucleosidic base such as adenine, guanine, uracil, cytosine, thymine, 2-aminoadenosine, 5-methylcytosine, 2,6-diaminopurine, or any other non-naturally occurring base that can be employed to be complementary or non-complementary to

target RNA or a non-nucleosidic base such as phenyl, naphthyl, 3-nitropyrrole, 5-nitroindole, nebularine, pyridone, pyridinone, or any other non-naturally occurring universal base that can be complementary or non-complementary to target RNA. In one embodiment, R3 and/or R7 comprises a conjugate moiety and a linker (e.g., a nucleotide or non-nucleotide linker as described herein or otherwise known in the art). Non-limiting examples of conjugate moieties include ligands for cellular receptors, such as peptides derived from naturally occurring protein ligands; protein localization sequences, including cellular ZIP code sequences; antibodies; nucleic acid aptamers; vitamins and other co-factors, such as folate and N-acetylgalactosamine; polymers, such as polyethyleneglycol (PEG); phospholipids; cholesterol; steroids, and polyamines, such as PEI, spermine or spermidine.

[00104] The chemically-modified nucleotide or non-nucleotide of Formula III can be present in one or both oligonucleotide strands of the siNA duplex, for example, in the sense strand, the antisense strand, or both strands. The nucleic acid or polynucleotide (e.g., siNA) molecules of the invention can comprise one or more chemically-modified nucleotides or non-nucleotides of Formula III at the 3'-end, the 5'-end, or both of the 3' and 5'-ends of the sense strand, the antisense strand, or both strands. For example, an exemplary siNA molecule of the invention can comprise about 1 to about 5 or more (e.g., about 1, 2, 3, 4, 5, or more) chemically-modified nucleotide(s) or non-nucleotide(s) of Formula III at the 5'-end of the sense strand, the antisense strand, or both strands. In another non-limiting example, an exemplary siNA molecule of the invention can comprise about 1 to about 5 or more (e.g., about 1, 2, 3, 4, 5, or more) chemically-modified nucleotide or non-nucleotide of Formula III at the 3'-end of the sense strand, the antisense strand, or both strands.

[00105] In another embodiment, a nucleic acid or polynucleotide (e.g., siNA) molecule of the invention comprises a nucleotide having Formula II or III, wherein the nucleotide having Formula II or III is in an inverted configuration. For example, the nucleotide having Formula II or III is connected to the siNA construct in a 3'-3', 3'-2', 2'-3', or 5'-5' configuration, such as at the 3'-end, the 5'-end, or both of the 3' and 5'-ends of one or both siNA strands.

[00106] In one embodiment, the invention features a chemically-modified short interfering nucleic acid or polynucleotide (*e.g.*, siNA) molecule capable of mediating RNA interference (RNAi) inside a cell or reconstituted in vitro system, wherein the chemical modification comprises a 5'-terminal phosphate group having Formula IV:



wherein each X and Y is independently O, S, N, alkyl, substituted alkyl, or alkylhalo; wherein each Z and W is independently O, S, N, alkyl, substituted alkyl, O-alkyl, S-alkyl, alkaryl, aralkyl, alkylhalo, or acetyl; and wherein W, X, Y and Z are optionally not all O and Y serves as a point of attachment to the siNA molecule.

[00107] In one embodiment, the invention features a nucleic acid or polynucleotide (*e.g.*, siNA) molecule having a 5'-terminal phosphate group having Formula IV on the target-complementary strand, for example, a strand complementary to a target RNA, wherein the siNA molecule comprises an all RNA siNA molecule. In another embodiment, the invention features a siNA molecule having a 5'-terminal phosphate group having Formula IV on the target-complementary strand wherein the siNA molecule also comprises about 1 to about 3 (*e.g.*, about 1, 2, or 3) nucleotide 3'-terminal nucleotide overhangs having about 1 to about 4 (*e.g.*, about 1, 2, 3, or 4) deoxyribonucleotides on the 3'-end of one or both strands. In another embodiment, a 5'-terminal phosphate group having Formula IV is present on the target-complementary strand of a siNA molecule of the invention, for example a siNA molecule having chemical modifications having any of Formulae I-VII.

[00108] In one embodiment, the invention features a chemically-modified short interfering nucleic acid or polynucleotide (*e.g.*, siNA) molecule capable of mediating RNA interference (RNAi) inside a cell or reconstituted in vitro system, wherein the chemical modification comprises one or more phosphorothioate internucleotide linkages. For example, in a non-limiting example, the invention features a chemically-modified short interfering nucleic acid or polynucleotide (*e.g.*, siNA) having about 1, 2, 3, 4, 5, 6,

7, 8 or more phosphorothioate internucleotide linkages in one siNA strand. In yet another embodiment, the invention features a chemically-modified short interfering nucleic acid or polynucleotide (*e.g.*, siNA) individually having about 1, 2, 3, 4, 5, 6, 7, 8 or more phosphorothioate internucleotide linkages in both siNA strands. The phosphorothioate internucleotide linkages can be present in one or both oligonucleotide strands of the siNA duplex, for example in the sense strand, the antisense strand, or both strands. The siNA molecules of the invention can comprise one or more phosphorothioate internucleotide linkages at the 3'-end, the 5'-end, or both of the 3'- and 5'-ends of the sense strand, the antisense strand, or both strands. For example, an exemplary siNA molecule of the invention can comprise about 1 to about 5 or more (*e.g.*, about 1, 2, 3, 4, 5, or more) consecutive phosphorothioate internucleotide linkages at the 5'-end of the sense strand, the antisense strand, or both strands. In another non-limiting example, an exemplary siNA molecule of the invention can comprise one or more (*e.g.*, about 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, or more) pyrimidine phosphorothioate internucleotide linkages in the sense strand, the antisense strand, or both strands. In yet another non-limiting example, an exemplary siNA molecule of the invention can comprise one or more (*e.g.*, about 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, or more) purine phosphorothioate internucleotide linkages in the sense strand, the antisense strand, or both strands.

[00109] Each strand of the double stranded nucleic acid or polynucleotide (*e.g.*, siNA) molecule can have one or more chemical modifications such that each strand comprises a different pattern of chemical modifications. Several non-limiting examples of modification schemes that could give rise to different patterns of modifications are provided herein.

[00110] In one embodiment, the invention features a nucleic acid or polynucleotide (*e.g.*, siNA) molecule, wherein the sense strand comprises one or more, for example, about 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, or more phosphorothioate internucleotide linkages, and/or one or more (*e.g.*, about 1, 2, 3, 4, 5, 6, 7, 8, 9, 10 or more) 2'-deoxy, 2'-O-methyl, 2'-deoxy-2'-fluoro, 2'-O-trifluoromethyl, 2'-O-ethyl-trifluoromethoxy, 2'-O-difluoromethoxy-ethoxy and/or about one or more (*e.g.*, about 1, 2, 3, 4, 5, 6, 7, 8, 9, 10 or more) universal base modified nucleotides, and optionally a terminal cap molecule at the 3'-end, the 5'-end, or both of the 3'- and 5'-ends of the sense strand; and wherein the

antisense strand comprises about 1 to about 10 or more, specifically about 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, or more phosphorothioate internucleotide linkages, and/or one or more (e.g., about 1, 2, 3, 4, 5, 6, 7, 8, 9, 10 or more) 2'-deoxy, 2'-O-methyl, 2'-deoxy-2'-fluoro, 2'-O-trifluoromethyl, 2'-O-ethyl-trifluoromethoxy, 2'-O-difluoromethoxy-ethoxy, 4'-thio and/or one or more (e.g., about 1, 2, 3, 4, 5, 6, 7, 8, 9, 10 or more) universal base modified nucleotides, and optionally a terminal cap molecule at the 3'-end, the 5'-end, or both of the 3'- and 5'-ends of the antisense strand. In another embodiment, one or more, for example about 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, or more, pyrimidine nucleotides of the sense and/or antisense siNA strand are chemically-modified with 2'-deoxy, 2'-O-methyl, 2'-O-trifluoromethyl, 2'-O-ethyl-trifluoromethoxy, 2'-O-difluoromethoxy-ethoxy, 4'-thio and/or 2'-deoxy-2'-fluoro nucleotides, with or without one or more, for example about 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, or more, phosphorothioate internucleotide linkages and/or a terminal cap molecule at the 3'-end, the 5'-end, or both of the 3'- and 5'-ends, being present in the same or different strand.

[00111] In another embodiment, the invention features a nucleic acid or polynucleotide (e.g., siNA) molecule, wherein the sense strand comprises about 1 to about 5, specifically about 1, 2, 3, 4, or 5 phosphorothioate internucleotide linkages, and/or one or more (e.g., about 1, 2, 3, 4, 5, or more) 2'-deoxy, 2'-O-methyl, 2'-deoxy-2'-fluoro, 2'-O-trifluoromethyl, 2'-O-ethyl-trifluoromethoxy, 2'-O-difluoromethoxy-ethoxy, 4'-thio and/or one or more (e.g., about 1, 2, 3, 4, 5, or more) universal base modified nucleotides, and optionally a terminal cap molecule at the 3'-end, the 5'-end, or both of the 3'- and 5'-ends of the sense strand; and wherein the antisense strand comprises about 1 to about 5 or more, specifically about 1, 2, 3, 4, 5, or more phosphorothioate internucleotide linkages, and/or one or more (e.g., about 1, 2, 3, 4, 5, 6, 7, 8, 9, 10 or more) 2'-deoxy, 2'-O-methyl, 2'-deoxy-2'-fluoro, 2'-O-trifluoromethyl, 2'-O-ethyl-trifluoromethoxy, 2'-O-difluoromethoxy-ethoxy, 4'-thio and/or one or more (e.g., about 1, 2, 3, 4, 5, 6, 7, 8, 9, 10 or more) universal base modified nucleotides, and optionally a terminal cap molecule at the 3'-end, the 5'-end, or both of the 3'- and 5'-ends of the antisense strand. In another embodiment, one or more, for example about 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, or more, pyrimidine nucleotides of the sense and/or antisense siNA strand are chemically-modified with 2'-deoxy, 2'-O-methyl, 2'-O-trifluoromethyl, 2'-O-ethyl-trifluoromethoxy, 2'-O-difluoromethoxy-ethoxy, 4'-thio and/or 2'-deoxy-2'-fluoro

nucleotides, with or without about 1 to about 5 or more, for example about 1, 2, 3, 4, 5, or more phosphorothioate internucleotide linkages and/or a terminal cap molecule at the 3'-end, the 5'-end, or both of the 3'- and 5'-ends, being present in the same or different strand.

[00112] In one embodiment, the invention features a nucleic acid or polynucleotide (*e.g.*, siNA) molecule, wherein the antisense strand comprises one or more, for example, about 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, or more phosphorothioate internucleotide linkages, and/or about one or more (*e.g.*, about 1, 2, 3, 4, 5, 6, 7, 8, 9, 10 or more) 2'-deoxy, 2'-O-methyl, 2'-deoxy-2'-fluoro, 2'-O-trifluoromethyl, 2'-O-ethyl-trifluoromethoxy, 2'-O-difluoromethoxy-ethoxy, 4'-thio and/or one or more (*e.g.*, about 1, 2, 3, 4, 5, 6, 7, 8, 9, 10 or more) universal base modified nucleotides, and optionally a terminal cap molecule at the 3'-end, the 5'-end, or both of the 3'- and 5'-ends of the sense strand; and wherein the antisense strand comprises about 1 to about 10 or more, specifically about 1, 2, 3, 4, 5, 6, 7, 8, 9, 10 or more phosphorothioate internucleotide linkages, and/or one or more (*e.g.*, about 1, 2, 3, 4, 5, 6, 7, 8, 9, 10 or more) 2'-deoxy, 2'-O-methyl, 2'-deoxy-2'-fluoro, 2'-O-trifluoromethyl, 2'-O-ethyl-trifluoromethoxy, 2'-O-difluoromethoxy-ethoxy, 4'-thio and/or one or more (*e.g.*, about 1, 2, 3, 4, 5, 6, 7, 8, 9, 10 or more) universal base modified nucleotides, and optionally a terminal cap molecule at the 3'-end, the 5'-end, or both of the 3'- and 5'-ends of the antisense strand. In another embodiment, one or more, for example about 1, 2, 3, 4, 5, 6, 7, 8, 9, 10 or more pyrimidine nucleotides of the sense and/or antisense siNA strand are chemically-modified with 2'-deoxy, 2'-O-methyl, 2'-O-trifluoromethyl, 2'-O-ethyl-trifluoromethoxy, 2'-O-difluoromethoxy-ethoxy, 4'-thio and/or 2'-deoxy-2'-fluoro nucleotides, with or without one or more, for example, about 1, 2, 3, 4, 5, 6, 7, 8, 9, 10 or more phosphorothioate internucleotide linkages and/or a terminal cap molecule at the 3'-end, the 5'-end, or both of the 3' and 5'-ends, being present in the same or different strand.

[00113] In another embodiment, the invention features a nucleic acid or polynucleotide (*e.g.*, siNA) molecule, wherein the antisense strand comprises about 1 to about 5 or more, specifically about 1, 2, 3, 4, 5 or more phosphorothioate internucleotide linkages, and/or one or more (*e.g.*, about 1, 2, 3, 4, 5, 6, 7, 8, 9, 10 or more) 2'-deoxy, 2'-O-methyl, 2'-deoxy-2'-fluoro, 2'-O-trifluoromethyl, 2'-O-ethyl-trifluoromethoxy, 2'-O-

difluoromethoxy-ethoxy, 4'-thio and/or one or more (e.g., about 1, 2, 3, 4, 5, 6, 7, 8, 9, 10 or more) universal base modified nucleotides, and optionally a terminal cap molecule at the 3'-end, the 5'-end, or both of the 3'- and 5'-ends of the sense strand; and wherein the antisense strand comprises about 1 to about 5 or more, specifically about 1, 2, 3, 4, 5 or more phosphorothioate internucleotide linkages, and/or one or more (e.g., about 1, 2, 3, 4, 5, 6, 7, 8, 9, 10 or more) 2'-deoxy, 2'-O-methyl, 2'-deoxy-2'-fluoro, 2'-O-trifluoromethyl, 2'-O-ethyl-trifluoromethoxy, 2'-O-difluoromethoxy-ethoxy, 4'-thio and/or one or more (e.g., about 1, 2, 3, 4, 5, 6, 7, 8, 9, 10 or more) universal base modified nucleotides, and optionally a terminal cap molecule at the 3'-end, the 5'-end, or both of the 3'- and 5'-ends of the antisense strand. In another embodiment, one or more, for example about 1, 2, 3, 4, 5, 6, 7, 8, 9, 10 or more pyrimidine nucleotides of the sense and/or antisense siNA strand are chemically-modified with 2'-deoxy, 2'-O-methyl, 2'-O-trifluoromethyl, 2'-O-ethyl-trifluoromethoxy, 2'-O-difluoromethoxy-ethoxy, 4'-thio and/or 2'-deoxy-2'-fluoro nucleotides, with or without about 1 to about 5, for example about 1, 2, 3, 4, 5 or more phosphorothioate internucleotide linkages and/or a terminal cap molecule at the 3'-end, the 5'-end, or both of the 3'- and 5'-ends, being present in the same or different strand.

[00114] In one embodiment, the invention features a chemically-modified short interfering nucleic acid or polynucleotide (*e.g.*, siNA) molecule having about 1 to about 5 or more (specifically about 1, 2, 3, 4, 5 or more) phosphorothioate internucleotide linkages in each strand of the siNA molecule.

[00115] In another embodiment, the invention features a nucleic acid or polynucleotide (*e.g.*, siNA) molecule comprising 2'-5' internucleotide linkages. The 2'-5' internucleotide linkage(s) can be at the 3'-end, the 5'-end, or both of the 3'- and 5'-ends of one or both siNA sequence strands. In addition, the 2'-5' internucleotide linkage(s) can be present at various other positions within one or both siNA sequence strands, for example, about 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, or more including every internucleotide linkage of a pyrimidine nucleotide in one or both strands of the siNA molecule can comprise a 2'-5' internucleotide linkage, or about 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, or more including every internucleotide linkage of a purine nucleotide in one or both strands of the siNA molecule can comprise a 2'-5' internucleotide linkage.

[00116] In another embodiment, a chemically-modified nucleic acid or polynucleotide (*e.g.*, siNA) molecule of the invention comprises a duplex having two strands, one or both of which can be chemically-modified, wherein each strand is independently about 15 to about 30 (*e.g.*, about 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, or 30) nucleotides in length, wherein the duplex has about 15 to about 30 (*e.g.*, about 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, or 30) base pairs, and wherein the chemical modification comprises a structure having any of Formulae I-VII. For example, an exemplary chemically-modified siNA molecule of the invention comprises a duplex having two strands, one or both of which can be chemically-modified with a chemical modification having any of Formulae I-VII or any combination thereof, wherein each strand consists of about 21 nucleotides, each having a 2-nucleotide 3'-terminal nucleotide overhang, and wherein the duplex has about 19 base pairs. In another embodiment, a siNA molecule of the invention comprises a single stranded hairpin structure, wherein the siNA is about 36 to about 70 (*e.g.*, about 36, 40, 45, 50, 55, 60, 65, or 70) nucleotides in length having about 15 to about 30 (*e.g.*, about 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, or 30) base pairs, and wherein the siNA can include a chemical modification comprising a structure having any of Formulae I-VII or any combination thereof. For example, an exemplary chemically-modified siNA molecule of the invention comprises a linear oligonucleotide having about 42 to about 50 (*e.g.*, about 42, 43, 44, 45, 46, 47, 48, 49, or 50) nucleotides that is chemically-modified with a chemical modification having any of Formulae I-VII or any combination thereof, wherein the linear oligonucleotide forms a hairpin structure having about 19 to about 21 (*e.g.*, 19, 20, or 21) base pairs and a 2-nucleotide 3'-terminal nucleotide overhang. In another embodiment, a linear hairpin siNA molecule of the invention contains a stem loop motif, wherein the loop portion of the siNA molecule is biodegradable. For example, a linear hairpin siNA molecule of the invention is designed such that degradation of the loop portion of the siNA molecule *in vivo* can generate a double-stranded siNA molecule with 3'-terminal overhangs, such as 3'-terminal nucleotide overhangs comprising about 2 nucleotides.

[00117] In another embodiment, a nucleic acid or polynucleotide (*e.g.*, siNA) molecule of the invention comprises a hairpin structure, wherein the siNA is about 25 to about 50 (*e.g.*, about 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, or 50) nucleotides in length having about 3 to about 25 (e.g., about 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, or 25) base pairs, and wherein the siNA can include one or more chemical modifications comprising a structure having any of Formulae I-VII or any combination thereof. For example, an exemplary chemically-modified siNA molecule of the invention comprises a linear oligonucleotide having about 25 to about 35 (e.g., about 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, or 35) nucleotides that is chemically-modified with one or more chemical modifications having any of Formulae I-VII or any combination thereof, wherein the linear oligonucleotide forms a hairpin structure having about 3 to about 25 (e.g., about 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, or 25) base pairs and a 5'-terminal phosphate group that can be chemically modified as described herein (for example a 5'-terminal phosphate group having Formula IV). In another embodiment, a linear hairpin siNA molecule of the invention contains a stem loop motif, wherein the loop portion of the siNA molecule is biodegradable. In one embodiment, a linear hairpin siNA molecule of the invention comprises a loop portion comprising a non-nucleotide linker.

[00118] In another embodiment, a nucleic acid or polynucleotide (e.g., siNA) molecule of the invention comprises an asymmetric hairpin structure, wherein the siNA is about 25 to about 50 (e.g., about 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, or 50) nucleotides in length having about 3 to about 25 (e.g., about 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, or 25) base pairs, and wherein the siNA can include one or more chemical modifications comprising a structure having any of Formulae I-VII or any combination thereof. For example, an exemplary chemically-modified siNA molecule of the invention comprises a linear oligonucleotide having about 25 to about 35 (e.g., about 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, or 35) nucleotides that is chemically-modified with one or more chemical modifications having any of Formulae I-VII or any combination thereof, wherein the linear oligonucleotide forms an asymmetric hairpin structure having about 3 to about 25 (e.g., about 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, or 25) base pairs and a 5'-terminal phosphate group that can be chemically modified as described herein (for example a 5'-terminal phosphate group having Formula IV). In one embodiment, an asymmetric hairpin siNA molecule of the

invention contains a stem loop motif, wherein the loop portion of the siNA molecule is biodegradable. In another embodiment, an asymmetric hairpin siNA molecule of the invention comprises a loop portion comprising a non-nucleotide linker.

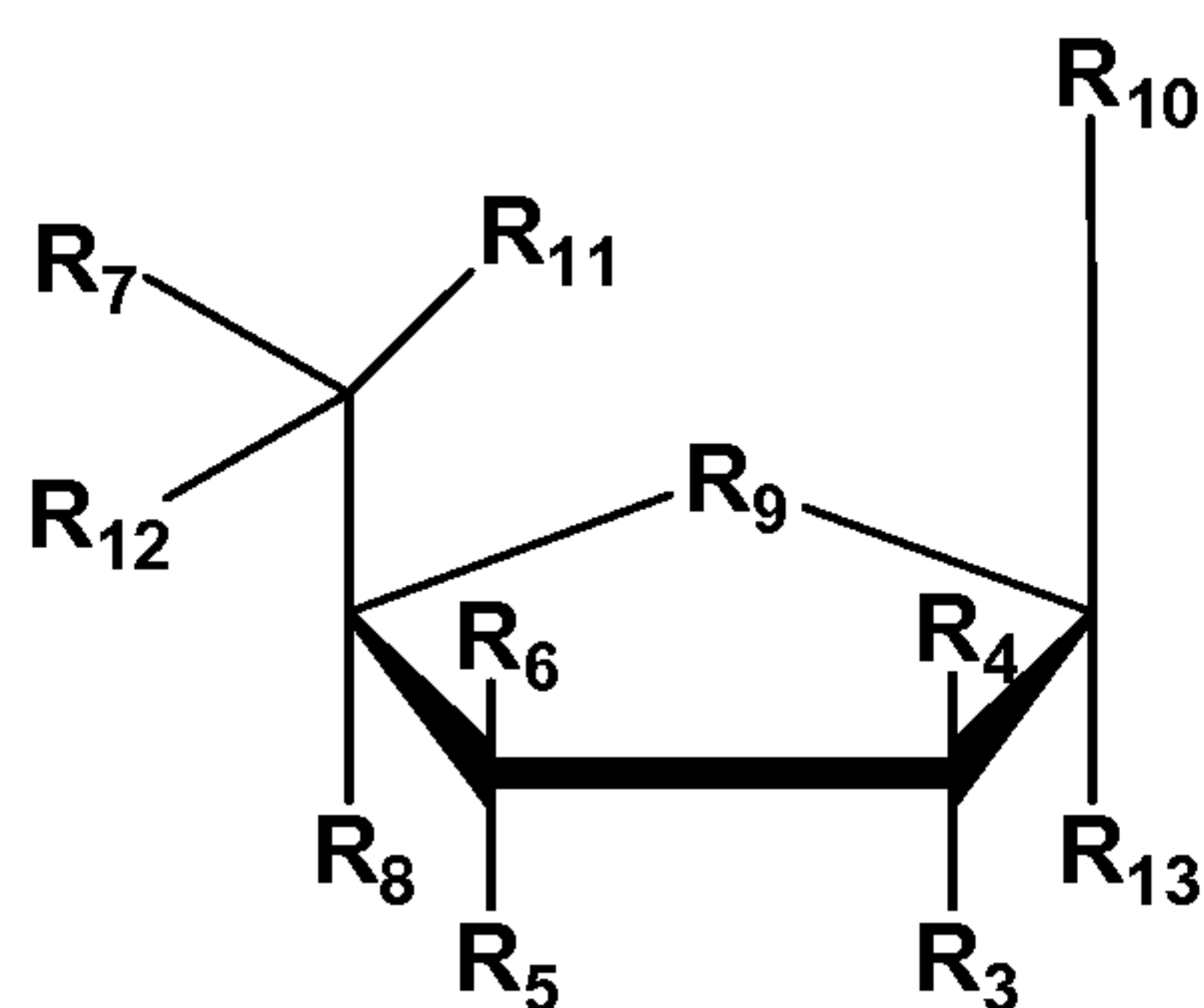
[00119] In another embodiment, a nucleic acid or polynucleotide (*e.g.*, siNA) molecule of the invention comprises an asymmetric double stranded structure having separate polynucleotide strands comprising sense and antisense regions, wherein the antisense region is about 15 to about 30 (*e.g.*, about 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, or 30) nucleotides in length, wherein the sense region is about 3 to about 25 (*e.g.*, about 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, or 25) nucleotides in length, wherein the sense region and the antisense region have at least 3 complementary nucleotides, and wherein the siNA can include one or more chemical modifications comprising a structure having any of Formulae I-VII or any combination thereof. For example, an exemplary chemically-modified siNA molecule of the invention comprises an asymmetric double stranded structure having separate polynucleotide strands comprising sense and antisense regions, wherein the antisense region is about 18 to about 23 (*e.g.*, about 18, 19, 20, 21, 22, or 23) nucleotides in length and wherein the sense region is about 3 to about 15 (*e.g.*, about 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, or 15) nucleotides in length, wherein the sense region the antisense region have at least 3 complementary nucleotides, and wherein the siNA can include one or more chemical modifications comprising a structure having any of Formulae I-VII or any combination thereof. In another embodiment, the asymmetric double stranded siNA molecule can also have a 5'-terminal phosphate group that can be chemically modified as described herein (for example a 5'-terminal phosphate group having Formula IV).

[00120] In another embodiment, a nucleic acid or polynucleotide (*e.g.*, siNA) molecule of the invention comprises a circular nucleic acid molecule, wherein the siNA is about 38 to about 70 (*e.g.*, about 38, 40, 45, 50, 55, 60, 65, or 70) nucleotides in length having about 15 to about 30 (*e.g.*, about 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, or 30) base pairs, and wherein the siNA can include a chemical modification, which comprises a structure having any of Formulae I-VII or any combination thereof. For example, an exemplary chemically-modified siNA molecule of the invention comprises a circular oligonucleotide having about 42 to about 50 (*e.g.*, about 42, 43, 44, 45, 46, 47,

48, 49, or 50) nucleotides that is chemically-modified with a chemical modification having any of Formulae I-VII or any combination thereof, wherein the circular oligonucleotide forms a dumbbell shaped structure having about 19 base pairs and 2 loops.

[00121] In another embodiment, a circular nucleic acid or polynucleotide (*e.g.*, siNA) molecule of the invention contains two loop motifs, wherein one or both loop portions of the siNA molecule is biodegradable. For example, a circular siNA molecule of the invention is designed such that degradation of the loop portions of the siNA molecule in vivo can generate a double-stranded siNA molecule with 3'-terminal overhangs, such as 3'-terminal nucleotide overhangs comprising about 2 nucleotides.

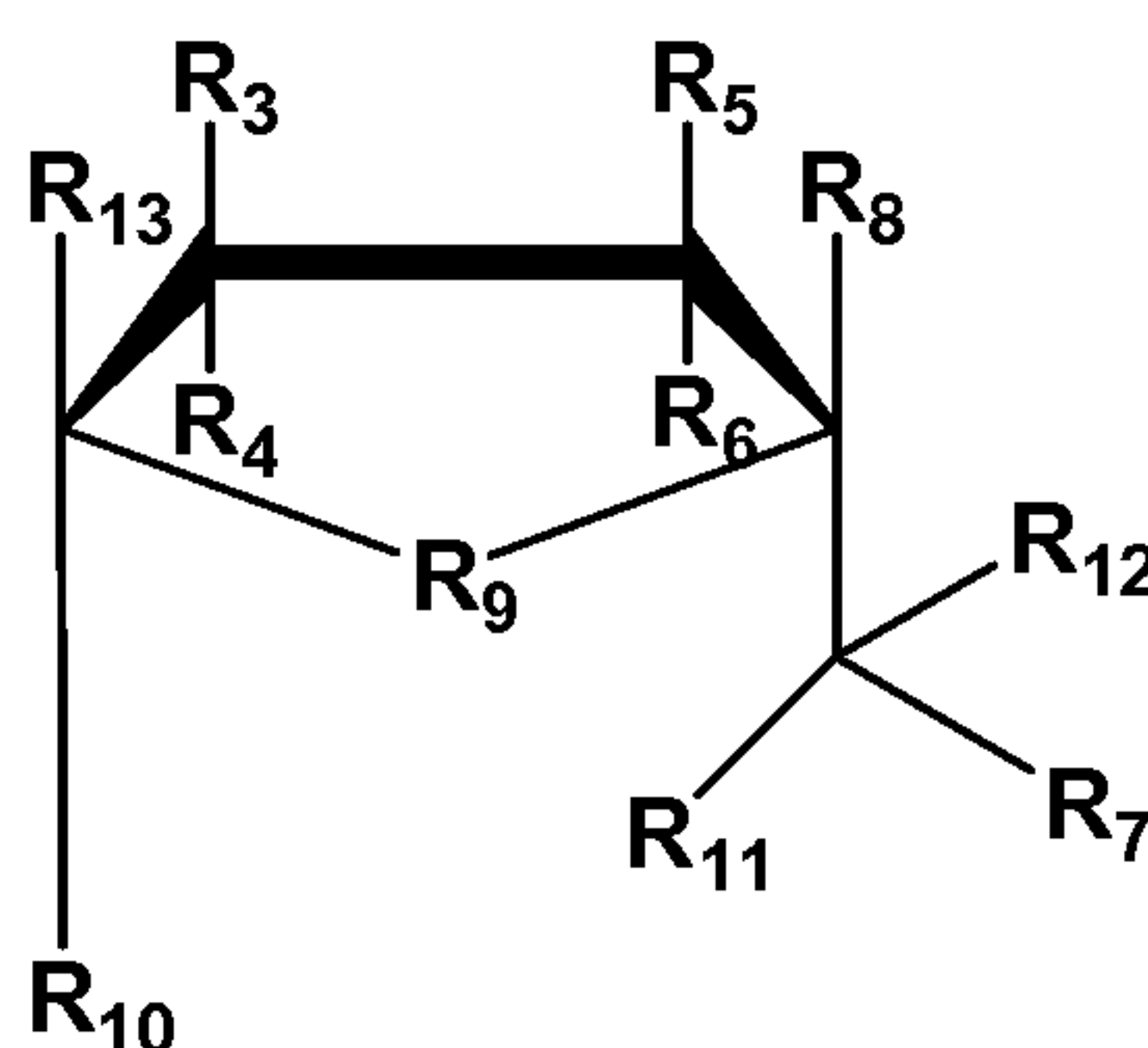
[00122] In one embodiment, a nucleic acid or polynucleotide (*e.g.*, siNA) molecule of the invention comprises at least one (*e.g.*, about 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, or more) abasic moiety, for example a compound having Formula V:



wherein each R3, R4, R5, R6, R7, R8, R10, R11, R12, and R13 is independently H, OH, alkyl, substituted alkyl, alkaryl or aralkyl, F, Cl, Br, CN, CF3, OCF3, OCH3, OCN, O-alkyl, S-alkyl, N-alkyl, O-alkenyl, S-alkenyl, N-alkenyl, SO-alkyl, alkyl-OSH, alkyl-OH, O-alkyl-OH, O-alkyl-SH, S-alkyl-OH, S-alkyl-SH, alkyl-S-alkyl, alkyl-O-alkyl, ONO2, NO2, N3, NH2, aminoalkyl, aminoacid, aminoacyl, ONH2, O-aminoalkyl, O-aminoacid, O-aminoacyl, heterocycloalkyl, heterocycloalkaryl, aminoalkylamino, polyalkylamino, substituted silyl, or a group having any of Formula I, II, III, IV, V, VI and/or VII, any of which can be included in the structure of the siNA molecule or serve as a point of attachment to the siNA molecule; R9 is O, S, CH2, S=O, CHF, or CF2. In one embodiment, R3 and/or R7 comprises a conjugate moiety and a linker (*e.g.*, a nucleotide

or non-nucleotide linker as described herein or otherwise known in the art). Non-limiting examples of conjugate moieties include ligands for cellular receptors, such as peptides derived from naturally occurring protein ligands; protein localization sequences, including cellular ZIP code sequences; antibodies; nucleic acid aptamers; vitamins and other co-factors, such as folate and N-acetylgalactosamine; polymers, such as polyethyleneglycol (PEG); phospholipids; cholesterol; steroids, and polyamines, such as PEI, spermine or spermidine.

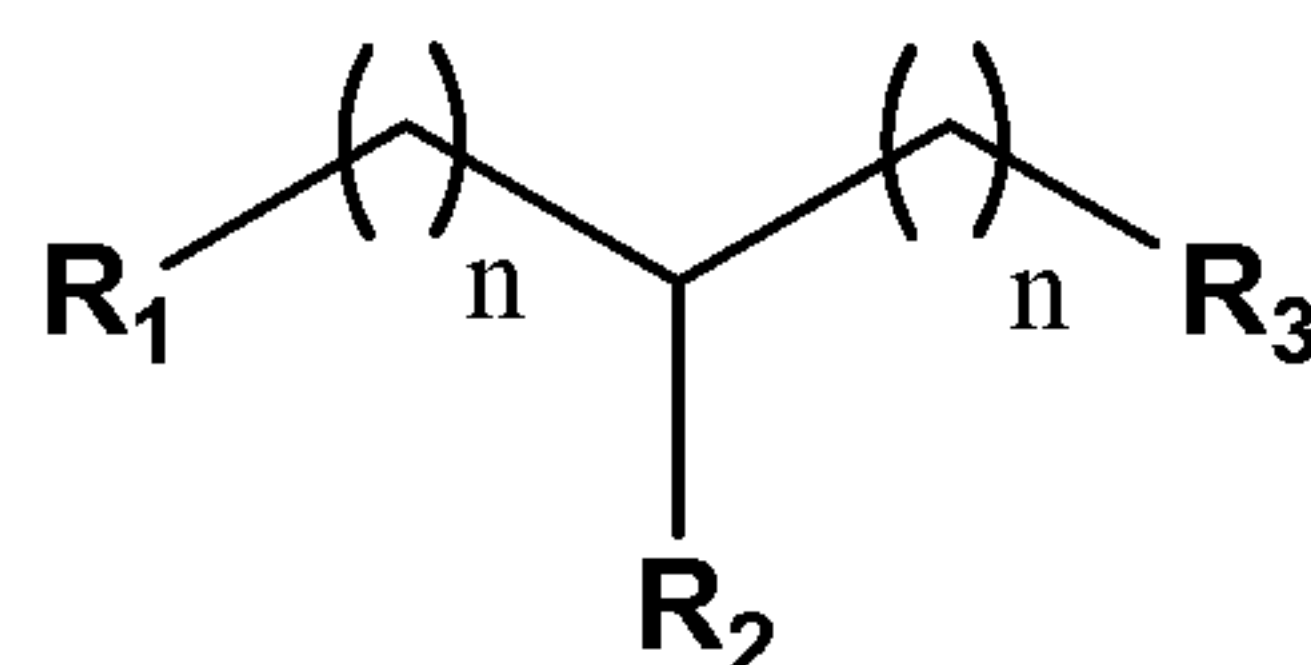
[00123] In one embodiment, a nucleic acid or polynucleotide (*e.g.*, siNA) molecule of the invention comprises at least one (*e.g.*, about 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, or more) inverted abasic moiety, for example a compound having Formula VI:



wherein each R3, R4, R5, R6, R7, R8, R10, R11, R12, and R13 is independently H, OH, alkyl, substituted alkyl, alkaryl or aralkyl, F, Cl, Br, CN, CF3, OCF3, OCH3, OCN, O-alkyl, S-alkyl, N-alkyl, O-alkenyl, S-alkenyl, N-alkenyl, SO-alkyl, alkyl-OSH, alkyl-OH, O-alkyl-OH, O-alkyl-SH, S-alkyl-OH, S-alkyl-SH, alkyl-S-alkyl, alkyl-O-alkyl, ONO2, NO2, N3, NH2, aminoalkyl, aminoacid, aminoacyl, ONH2, O-aminoalkyl, O-aminoacid, O-aminoacyl, heterocycloalkyl, heterocycloalkaryl, aminoalkylamino, polyalkylamino, substituted silyl, or a group having any of Formula I, II, III, IV, V, VI and/or VII, any of which can be included in the structure of the siNA molecule or serve as a point of attachment to the siNA molecule; R9 is O, S, CH2, S=O, CHF, or CF2, and either R2, R3, R8 or R13 serve as points of attachment to the siNA molecule of the invention. In one embodiment, R3 and/or R7 comprises a conjugate moiety and a linker (*e.g.*, a nucleotide or non-nucleotide linker as described herein or otherwise known in the art). Non-limiting examples of conjugate moieties include ligands for cellular receptors, such as peptides derived from naturally occurring protein ligands; protein localization

sequences, including cellular ZIP code sequences; antibodies; nucleic acid aptamers; vitamins and other co-factors, such as folate and N-acetylgalactosamine; polymers, such as polyethyleneglycol (PEG); phospholipids; cholesterol; steroids, and polyamines, such as PEI, spermine or spermidine.

[00124] In another embodiment, a nucleic acid or polynucleotide (*e.g.*, siNA) molecule of the invention comprises at least one (*e.g.*, about 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, or more) substituted polyalkyl moieties, for example a compound having Formula VII:



wherein each n is independently an integer from 1 to 12, each R1, R2 and R3 is independently H, OH, alkyl, substituted alkyl, alkaryl or aralkyl, F, Cl, Br, CN, CF3, OCF3, OCH3, OCN, O-alkyl, S-alkyl, N-alkyl, O-alkenyl, S-alkenyl, N-alkenyl, SO-alkyl, alkyl-OSH, alkyl-OH, O-alkyl-OH, O-alkyl-SH, S-alkyl-OH, S-alkyl-SH, alkyl-S-alkyl, alkyl-O-alkyl, ONO2, NO2, N3, NH2, aminoalkyl, aminoacid, aminoacyl, ONH2, O-aminoalkyl, O-aminoacid, O-aminoacyl, heterocycloalkyl, heterocycloalkaryl, aminoalkylamino, polyalkylamino, substituted silyl, or a group having any of Formula I, II, III, IV, V, VI and/or VII, any of which can be included in the structure of the siNA molecule or serve as a point of attachment to the siNA molecule. In one embodiment, R3 and/or R1 comprises a conjugate moiety and a linker (*e.g.*, a nucleotide or non-nucleotide linker as described herein or otherwise known in the art). Non-limiting examples of conjugate moieties include ligands for cellular receptors, such as peptides derived from naturally occurring protein ligands; protein localization sequences, including cellular ZIP code sequences; antibodies; nucleic acid aptamers; vitamins and other co-factors, such as folate and N-acetylgalactosamine; polymers, such as polyethyleneglycol (PEG); phospholipids; cholesterol; steroids, and polyamines, such as PEI, spermine or spermidine.

[00125] By “ZIP code” sequences is meant, any peptide or protein sequence that is involved in cellular topogenic signaling mediated transport (see for example Ray *et al.*, 2004, *Science*, 306(1501): 1505)

[00126] Each nucleotide within the double stranded nucleic acid or polynucleotide (*e.g.*, siNA) molecule can independently have a chemical modification comprising the structure of any of Formulae I-VII. Thus, in one embodiment, one or more nucleotide positions of a siNA molecule of the invention comprises a chemical modification having structure of any of Formulae I-VII or any other modification herein. In one embodiment, each nucleotide position of a siNA molecule of the invention comprises a chemical modification having structure of any of Formulae I-VII or any other modification herein.

[00127] In one embodiment, one or more nucleotide positions of one or both strands of a double stranded nucleic acid or polynucleotide (*e.g.*, siNA) molecule of the invention comprises a chemical modification having structure of any of Formulae I-VII or any other modification herein. In one embodiment, each nucleotide position of one or both strands of a double stranded siNA molecule of the invention comprises a chemical modification having structure of any of Formulae I-VII or any other modification herein.

[00128] In another embodiment, the invention features a compound having Formula VII, wherein R1 and R2 are hydroxyl (OH) groups, $n = 1$, and R3 comprises O and is the point of attachment to the 3'-end, the 5'-end, or both of the 3' and 5'-ends of one or both strands of a double-stranded nucleic acid or polynucleotide (*e.g.*, siNA) molecule of the invention or to a single-stranded siNA molecule of the invention. This modification is referred to herein as "glyceryl" (for example modification 6 in Figure 10).

[00129] In another embodiment, a chemically modified nucleoside or non-nucleoside (*e.g.* a moiety having any of Formula V, VI or VII) of the invention is at the 3'-end, the 5'-end, or both of the 3' and 5'-ends of a siNA molecule of the invention. For example, chemically modified nucleoside or non-nucleoside (*e.g.*, a moiety having Formula V, VI or VII) can be present at the 3'-end, the 5'-end, or both of the 3' and 5'-ends of the antisense strand, the sense strand, or both antisense and sense strands of the nucleic acid or polynucleotide (*e.g.*, siNA) molecule. In one embodiment, the chemically modified nucleoside or non-nucleoside (*e.g.*, a moiety having Formula V, VI or VII) is present at the 5'-end and 3'-end of the sense strand and the 3'-end of the antisense strand of a double stranded siNA molecule of the invention. In one embodiment, the chemically modified nucleoside or non-nucleoside (*e.g.*, a moiety having Formula V, VI or VII) is

present at the terminal position of the 5'-end and 3'-end of the sense strand and the 3'-end of the antisense strand of a double stranded siNA molecule of the invention. In one embodiment, the chemically modified nucleoside or non-nucleoside (e.g., a moiety having Formula V, VI or VII) is present at the two terminal positions of the 5'-end and 3'-end of the sense strand and the 3'-end of the antisense strand of a double stranded siNA molecule of the invention. In one embodiment, the chemically modified nucleoside or non-nucleoside (e.g., a moiety having Formula V, VI or VII) is present at the penultimate position of the 5'-end and 3'-end of the sense strand and the 3'-end of the antisense strand of a double stranded siNA molecule of the invention. In addition, a moiety having Formula VII can be present at the 3'-end or the 5'-end of a hairpin siNA molecule as described herein.

[00130] In another embodiment, a nucleic acid or polynucleotide (*e.g.*, siNA) molecule of the invention comprises an abasic residue having Formula V or VI, wherein the abasic residue having Formula VI or VI is connected to the siNA construct in a 3'-3', 3'-2', 2'-3', or 5'-5' configuration, such as at the 3'-end, the 5'-end, or both of the 3' and 5'-ends of one or both siNA strands.

[00131] In one embodiment, a nucleic acid or polynucleotide (*e.g.*, siNA) molecule of the invention comprises one or more (e.g., about 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, or more) locked nucleic acid (LNA) nucleotides, for example, at the 5'-end, the 3'-end, both of the 5' and 3'-ends, or any combination thereof, of the siNA molecule.

[00132] In one embodiment, a nucleic acid or polynucleotide (*e.g.*, siNA) molecule of the invention comprises one or more (e.g., about 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, or more) 4'-thio nucleotides, for example, at the 5'-end, the 3'-end, both of the 5' and 3'-ends, or any combination thereof, of the siNA molecule.

[00133] In another embodiment, a nucleic acid or polynucleotide (*e.g.*, siNA) molecule of the invention comprises one or more (e.g., about 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, or more) acyclic nucleotides, for example, at the 5'-end, the 3'-end, both of the 5' and 3'-ends, or any combination thereof, of the siNA molecule.

[00134] In one embodiment, a chemically-modified short interfering nucleic acid or polynucleotide (*e.g.*, siNA) molecule of the invention comprises a sense strand or sense region having one or more (*e.g.*, 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 or more) 2'-O-alkyl (*e.g.* 2'-O-methyl), 2'-deoxy-2'-fluoro, 2'-deoxy, FANA, or abasic chemical modifications or any combination thereof.

[00135] In one embodiment, a chemically-modified short interfering nucleic acid or polynucleotide (*e.g.*, siNA) molecule of the invention comprises an antisense strand or antisense region having one or more (*e.g.*, 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 or more) 2'-O-alkyl (*e.g.* 2'-O-methyl), 2'-deoxy-2'-fluoro, 2'-deoxy, FANA, or abasic chemical modifications or any combination thereof.

[00136] In one embodiment, a chemically-modified short interfering nucleic acid or polynucleotide (*e.g.*, siNA) molecule of the invention comprises a sense strand or sense region and an antisense strand or antisense region, each having one or more (*e.g.*, 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 or more) 2'-O-alkyl (*e.g.* 2'-O-methyl), 2'-deoxy-2'-fluoro, 2'-deoxy, FANA, or abasic chemical modifications or any combination thereof.

[00137] In one embodiment, the invention features a chemically-modified short interfering nucleic acid or polynucleotide (*e.g.*, siNA) molecule of the invention comprising a sense region, wherein any (*e.g.*, one or more or all) pyrimidine nucleotides present in the sense region are 2'-deoxy-2'-fluoro pyrimidine nucleotides (*e.g.*, wherein all pyrimidine nucleotides are 2'-deoxy-2'-fluoro pyrimidine nucleotides or alternately a plurality (*ie.* more than one) of pyrimidine nucleotides are 2'-deoxy-2'-fluoro pyrimidine nucleotides).

[00138] In one embodiment, the invention features a chemically-modified short interfering nucleic acid or polynucleotide (*e.g.*, siNA) molecule of the invention comprising a sense region, wherein any (*e.g.*, one or more or all) pyrimidine nucleotides present in the sense region are FANA pyrimidine nucleotides (*e.g.*, wherein all

pyrimidine nucleotides are FANA pyrimidine nucleotides or alternately a plurality (ie. more than one) of pyrimidine nucleotides are FANA pyrimidine nucleotides).

[00139] In one embodiment, the invention features a chemically-modified short interfering nucleic acid or polynucleotide (*e.g.*, siNA) molecule of the invention comprising an antisense region, wherein any (*e.g.*, one or more or all) pyrimidine nucleotides present in the antisense region are 2'-deoxy-2'-fluoro pyrimidine nucleotides (*e.g.*, wherein all pyrimidine nucleotides are 2'-deoxy-2'-fluoro pyrimidine nucleotides or alternately a plurality (ie. more than one) of pyrimidine nucleotides are 2'-deoxy-2'-fluoro pyrimidine nucleotides).

[00140] In one embodiment, the invention features a chemically-modified short interfering nucleic acid or polynucleotide (*e.g.*, siNA) molecule of the invention comprising a sense region and an antisense region, wherein any (*e.g.*, one or more or all) pyrimidine nucleotides present in the sense region and the antisense region are 2'-deoxy-2'-fluoro pyrimidine nucleotides (*e.g.*, wherein all pyrimidine nucleotides are 2'-deoxy-2'-fluoro pyrimidine nucleotides or alternately a plurality (ie. more than one) of pyrimidine nucleotides are 2'-deoxy-2'-fluoro pyrimidine nucleotides).

[00141] In one embodiment, the invention features a chemically-modified short interfering nucleic acid or polynucleotide (*e.g.*, siNA) molecule of the invention comprising a sense region, wherein any (*e.g.*, one or more or all) purine nucleotides present in the sense region are 2'-deoxy purine nucleotides (*e.g.*, wherein all purine nucleotides are 2'-deoxy purine nucleotides or alternately a plurality (ie. more than one) of purine nucleotides are 2'-deoxy purine nucleotides).

[00142] In one embodiment, the invention features a chemically-modified short interfering nucleic acid or polynucleotide (*e.g.*, siNA) molecule of the invention comprising an antisense region, wherein any (*e.g.*, one or more or all) purine nucleotides present in the antisense region are 2'-O-methyl purine nucleotides (*e.g.*, wherein all purine nucleotides are 2'-O-methyl purine nucleotides or alternately a plurality (ie. more than one) of pyrimidine nucleotides are 2'-O-methyl purine nucleotides).

[00143] In one embodiment, the invention features a chemically-modified short interfering nucleic acid or polynucleotide (*e.g.*, siNA) molecule of the invention comprising a sense region, wherein any (*e.g.*, one or more or all) pyrimidine nucleotides present in the sense region are 2'-deoxy-2'-fluoro pyrimidine nucleotides (*e.g.*, wherein all pyrimidine nucleotides are 2'-deoxy-2'-fluoro pyrimidine nucleotides or alternately a plurality (*ie.* more than one) of pyrimidine nucleotides are 2'-deoxy-2'-fluoro pyrimidine nucleotides), and wherein any (*e.g.*, one or more or all) purine nucleotides present in the sense region are 2'-deoxy purine nucleotides (*e.g.*, wherein all purine nucleotides are 2'-deoxy purine nucleotides or alternately a plurality (*ie.* more than one) of purine nucleotides are 2'-deoxy purine nucleotides).

[00144] In one embodiment, the invention features a chemically-modified short interfering nucleic acid or polynucleotide (*e.g.*, siNA) molecule of the invention comprising a sense region, wherein any (*e.g.*, one or more or all) pyrimidine nucleotides present in the sense region are 2'-deoxy-2'-fluoro, 4'-thio, 2'-O-trifluoromethyl, 2'-O-ethyl-trifluoromethoxy, or 2'-O-difluoromethoxy-ethoxy pyrimidine nucleotides (*e.g.*, wherein all pyrimidine nucleotides are 2'-deoxy-2'-fluoro, 4'-thio, 2'-O-trifluoromethyl, 2'-O-ethyl-trifluoromethoxy, or 2'-O-difluoromethoxy-ethoxy pyrimidine nucleotides or alternately a plurality (*ie.* more than one) of pyrimidine nucleotides are 2'-deoxy-2'-fluoro, 4'-thio, 2'-O-trifluoromethyl, 2'-O-ethyl-trifluoromethoxy, or 2'-O-difluoromethoxy-ethoxy pyrimidine nucleotides), and wherein any (*e.g.*, one or more or all) purine nucleotides present in the sense region are 2'-deoxy purine nucleotides (*e.g.*, wherein all purine nucleotides are 2'-deoxy purine nucleotides or alternately a plurality (*ie.* more than one) of purine nucleotides are 2'-deoxy purine nucleotides), wherein any nucleotides comprising a 3'-terminal nucleotide overhang that are present in said sense region are 2'-deoxy nucleotides.

[00145] In one embodiment, the invention features a chemically-modified short interfering nucleic acid or polynucleotide (*e.g.*, siNA) molecule of the invention comprising a sense region, wherein any (*e.g.*, one or more or all) pyrimidine nucleotides present in the sense region are 2'-deoxy-2'-fluoro, 4'-thio, 2'-O-trifluoromethyl, 2'-O-ethyl-trifluoromethoxy, or 2'-O-difluoromethoxy-ethoxy pyrimidine nucleotides (*e.g.*, wherein all pyrimidine nucleotides are 2'-deoxy-2'-fluoro, 4'-thio, 2'-O-trifluoromethyl,

2'-O-ethyl-trifluoromethoxy, or 2'-O-difluoromethoxy-ethoxy pyrimidine nucleotides or alternately a plurality (ie. more than one) of pyrimidine nucleotides are 2'-deoxy-2'-fluoro, 4'-thio, 2'-O-trifluoromethyl, 2'-O-ethyl-trifluoromethoxy, or 2'-O-difluoromethoxy-ethoxy pyrimidine nucleotides), and wherein any (e.g., one or more or all) purine nucleotides present in the sense region are 2'-O-methyl purine nucleotides (e.g., wherein all purine nucleotides are 2'-O-methyl, 4'-thio, 2'-O-trifluoromethyl, 2'-O-ethyl-trifluoromethoxy, or 2'-O-difluoromethoxy-ethoxy purine nucleotides or alternately a plurality (ie. more than one) of purine nucleotides are 2'-O-methyl, 4'-thio, 2'-O-trifluoromethyl, 2'-O-ethyl-trifluoromethoxy, or 2'-O-difluoromethoxy-ethoxy purine nucleotides).

[00146] In one embodiment, the invention features a chemically-modified short interfering nucleic acid or polynucleotide (e.g., siNA) molecule of the invention comprising a sense region, wherein any (e.g., one or more or all) pyrimidine nucleotides present in the sense region are 2'-deoxy-2'-fluoro, 4'-thio, 2'-O-trifluoromethyl, 2'-O-ethyl-trifluoromethoxy, or 2'-O-difluoromethoxy-ethoxy pyrimidine nucleotides (e.g., wherein all pyrimidine nucleotides are 2'-deoxy-2'-fluoro, 4'-thio, 2'-O-trifluoromethyl, 2'-O-ethyl-trifluoromethoxy, or 2'-O-difluoromethoxy-ethoxy pyrimidine nucleotides or alternately a plurality (ie. more than one) of pyrimidine nucleotides are 2'-deoxy-2'-fluoro, 4'-thio, 2'-O-trifluoromethyl, 2'-O-ethyl-trifluoromethoxy, or 2'-O-difluoromethoxy-ethoxy pyrimidine nucleotides), wherein any (e.g., one or more or all) purine nucleotides present in the sense region are 2'-O-methyl, 4'-thio, 2'-O-trifluoromethyl, 2'-O-ethyl-trifluoromethoxy, or 2'-O-difluoromethoxy-ethoxy purine nucleotides (e.g., wherein all purine nucleotides are 2'-O-methyl, 4'-thio, 2'-O-trifluoromethyl, 2'-O-ethyl-trifluoromethoxy, or 2'-O-difluoromethoxy-ethoxy purine nucleotides or alternately a plurality (ie. more than one) of purine nucleotides are 2'-O-methyl, 4'-thio, 2'-O-trifluoromethyl, 2'-O-ethyl-trifluoromethoxy, or 2'-O-difluoromethoxy-ethoxy purine nucleotides), and wherein any nucleotides comprising a 3'-terminal nucleotide overhang that are present in said sense region are 2'-deoxy nucleotides.

[00147] In one embodiment, the invention features a chemically-modified short interfering nucleic acid or polynucleotide (e.g., siNA) molecule of the invention

comprising an antisense region, wherein any (e.g., one or more or all) pyrimidine nucleotides present in the antisense region are 2'-deoxy-2'-fluoro, 4'-thio, 2'-O-trifluoromethyl, 2'-O-ethyl-trifluoromethoxy, or 2'-O-difluoromethoxy-ethoxy pyrimidine nucleotides (e.g., wherein all pyrimidine nucleotides are 2'-deoxy-2'-fluoro, 4'-thio, 2'-O-trifluoromethyl, 2'-O-ethyl-trifluoromethoxy, or 2'-O-difluoromethoxy-ethoxy pyrimidine nucleotides or alternately a plurality (ie. more than one) of pyrimidine nucleotides are 2'-deoxy-2'-fluoro, 4'-thio, 2'-O-trifluoromethyl, 2'-O-ethyl-trifluoromethoxy, or 2'-O-difluoromethoxy-ethoxy pyrimidine nucleotides), and wherein any (e.g., one or more or all) purine nucleotides present in the antisense region are 2'-O-methyl, 4'-thio, 2'-O-trifluoromethyl, 2'-O-ethyl-trifluoromethoxy, or 2'-O-difluoromethoxy-ethoxy purine nucleotides (e.g., wherein all purine nucleotides are 2'-O-methyl, 4'-thio, 2'-O-trifluoromethyl, 2'-O-ethyl-trifluoromethoxy, or 2'-O-difluoromethoxy-ethoxy purine nucleotides or alternately a plurality (ie. more than one) of purine nucleotides are 2'-O-methyl, 4'-thio, 2'-O-trifluoromethyl, 2'-O-ethyl-trifluoromethoxy, or 2'-O-difluoromethoxy-ethoxy purine nucleotides).

[00148] In one embodiment, the invention features a chemically-modified short interfering nucleic acid or polynucleotide (e.g., siNA) molecule of the invention comprising an antisense region, wherein any (e.g., one or more or all) pyrimidine nucleotides present in the antisense region are 2'-deoxy-2'-fluoro, 4'-thio, 2'-O-trifluoromethyl, 2'-O-ethyl-trifluoromethoxy, or 2'-O-difluoromethoxy-ethoxy pyrimidine nucleotides (e.g., wherein all pyrimidine nucleotides are 2'-deoxy-2'-fluoro, 4'-thio, 2'-O-trifluoromethyl, 2'-O-ethyl-trifluoromethoxy, or 2'-O-difluoromethoxy-ethoxy pyrimidine nucleotides or alternately a plurality (ie. more than one) of pyrimidine nucleotides are 2'-deoxy-2'-fluoro, 4'-thio, 2'-O-trifluoromethyl, 2'-O-ethyl-trifluoromethoxy, or 2'-O-difluoromethoxy-ethoxy pyrimidine nucleotides), wherein any (e.g., one or more or all) purine nucleotides present in the antisense region are 2'-O-methyl, 4'-thio, 2'-O-trifluoromethyl, 2'-O-ethyl-trifluoromethoxy, or 2'-O-difluoromethoxy-ethoxy purine nucleotides (e.g., wherein all purine nucleotides are 2'-O-methyl, 4'-thio, 2'-O-trifluoromethyl, 2'-O-ethyl-trifluoromethoxy, or 2'-O-difluoromethoxy-ethoxy purine nucleotides or alternately a plurality (ie. more than one) of purine nucleotides are 2'-O-methyl, 4'-thio, 2'-O-trifluoromethyl, 2'-O-ethyl-trifluoromethoxy, or 2'-O-difluoromethoxy-ethoxy purine nucleotides), and wherein any

nucleotides comprising a 3'-terminal nucleotide overhang that are present in said antisense region are 2'-deoxy nucleotides.

[00149] In one embodiment, the invention features a chemically-modified short interfering nucleic acid or polynucleotide (*e.g.*, siNA) molecule of the invention comprising an antisense region, wherein any (*e.g.*, one or more or all) pyrimidine nucleotides present in the antisense region are 2'-deoxy-2'-fluoro, 4'-thio, 2'-O-trifluoromethyl, 2'-O-ethyl-trifluoromethoxy, or 2'-O-difluoromethoxy-ethoxy pyrimidine nucleotides (*e.g.*, wherein all pyrimidine nucleotides are 2'-deoxy-2'-fluoro, 4'-thio, 2'-O-trifluoromethyl, 2'-O-ethyl-trifluoromethoxy, or 2'-O-difluoromethoxy-ethoxy pyrimidine nucleotides or alternately a plurality (*ie.* more than one) of pyrimidine nucleotides are 2'-deoxy-2'-fluoro, 4'-thio, 2'-O-trifluoromethyl, 2'-O-ethyl-trifluoromethoxy, or 2'-O-difluoromethoxy-ethoxy pyrimidine nucleotides), and wherein any (*e.g.*, one or more or all) purine nucleotides present in the antisense region are 2'-deoxy purine nucleotides (*e.g.*, wherein all purine nucleotides are 2'-deoxy purine nucleotides or alternately a plurality (*ie.* more than one) of purine nucleotides are 2'-deoxy purine nucleotides).

[00150] In one embodiment, the invention features a chemically-modified short interfering nucleic acid or polynucleotide (*e.g.*, siNA) molecule of the invention comprising an antisense region, wherein any (*e.g.*, one or more or all) pyrimidine nucleotides present in the antisense region are 2'-deoxy-2'-fluoro, 4'-thio, 2'-O-trifluoromethyl, 2'-O-ethyl-trifluoromethoxy, or 2'-O-difluoromethoxy-ethoxy pyrimidine nucleotides (*e.g.*, wherein all pyrimidine nucleotides are 2'-deoxy-2'-fluoro, 4'-thio, 2'-O-trifluoromethyl, 2'-O-ethyl-trifluoromethoxy, or 2'-O-difluoromethoxy-ethoxy pyrimidine nucleotides or alternately a plurality (*ie.* more than one) of pyrimidine nucleotides are 2'-deoxy-2'-fluoro, 4'-thio, 2'-O-trifluoromethyl, 2'-O-ethyl-trifluoromethoxy, or 2'-O-difluoromethoxy-ethoxy pyrimidine nucleotides), and wherein any (*e.g.*, one or more or all) purine nucleotides present in the antisense region are 2'-O-methyl, 4'-thio, 2'-O-trifluoromethyl, 2'-O-ethyl-trifluoromethoxy, or 2'-O-difluoromethoxy-ethoxy purine nucleotides (*e.g.*, wherein all purine nucleotides are 2'-O-methyl, 4'-thio, 2'-O-trifluoromethyl, 2'-O-ethyl-trifluoromethoxy, or 2'-O-difluoromethoxy-ethoxy purine nucleotides or alternately a plurality (*ie.* more than one)

of purine nucleotides are 2'-O-methyl, 4'-thio, 2'-O-trifluoromethyl, 2'-O-ethyl-trifluoromethoxy, or 2'-O-difluoromethoxy-ethoxy purine nucleotides).

[00151] In one embodiment, the invention features a chemically-modified short interfering nucleic acid or polynucleotide (*e.g.*, siNA) molecule of the invention capable of mediating RNA interference (RNAi) inside a cell or reconstituted in vitro system comprising a sense region, wherein one or more pyrimidine nucleotides present in the sense region are 2'-deoxy-2'-fluoro, 4'-thio, 2'-O-trifluoromethyl, 2'-O-ethyl-trifluoromethoxy, or 2'-O-difluoromethoxy-ethoxy pyrimidine nucleotides (*e.g.*, wherein all pyrimidine nucleotides are 2'-deoxy-2'-fluoro, 4'-thio, 2'-O-trifluoromethyl, 2'-O-ethyl-trifluoromethoxy, or 2'-O-difluoromethoxy-ethoxy pyrimidine nucleotides or alternately a plurality (*ie.* more than one) of pyrimidine nucleotides are 2'-deoxy-2'-fluoro, 4'-thio, 2'-O-trifluoromethyl, 2'-O-ethyl-trifluoromethoxy, or 2'-O-difluoromethoxy-ethoxy pyrimidine nucleotides), and one or more purine nucleotides present in the sense region are 2'-deoxy purine nucleotides (*e.g.*, wherein all purine nucleotides are 2'-deoxy purine nucleotides or alternately a plurality (*ie.* more than one) of purine nucleotides are 2'-deoxy purine nucleotides), and an antisense region, wherein one or more pyrimidine nucleotides present in the antisense region are 2'-deoxy-2'-fluoro, 4'-thio, 2'-O-trifluoromethyl, 2'-O-ethyl-trifluoromethoxy, or 2'-O-difluoromethoxy-ethoxy pyrimidine nucleotides (*e.g.*, wherein all pyrimidine nucleotides are 2'-deoxy-2'-fluoro, 4'-thio, 2'-O-trifluoromethyl, 2'-O-ethyl-trifluoromethoxy, or 2'-O-difluoromethoxy-ethoxy pyrimidine nucleotides or alternately a plurality (*ie.* more than one) of pyrimidine nucleotides are 2'-deoxy-2'-fluoro, 4'-thio, 2'-O-trifluoromethyl, 2'-O-ethyl-trifluoromethoxy, or 2'-O-difluoromethoxy-ethoxy pyrimidine nucleotides), and one or more purine nucleotides present in the antisense region are 2'-O-methyl, 4'-thio, 2'-O-trifluoromethyl, 2'-O-ethyl-trifluoromethoxy, or 2'-O-difluoromethoxy-ethoxy purine nucleotides (*e.g.*, wherein all purine nucleotides are 2'-O-methyl, 4'-thio, 2'-O-trifluoromethyl, 2'-O-ethyl-trifluoromethoxy, or 2'-O-difluoromethoxy-ethoxy purine nucleotides or alternately a plurality (*ie.* more than one) of purine nucleotides are 2'-O-methyl, 4'-thio, 2'-O-trifluoromethyl, 2'-O-ethyl-trifluoromethoxy, or 2'-O-difluoromethoxy-ethoxy purine nucleotides). The sense region and/or the antisense region can have a terminal cap modification, such as any modification described herein or shown in Figure 10, that is optionally present at the 3'-

end, the 5'-end, or both of the 3' and 5'-ends of the sense and/or antisense sequence. The sense and/or antisense region can optionally further comprise a 3'-terminal nucleotide overhang having about 1 to about 4 (e.g., about 1, 2, 3, or 4) 2'-deoxynucleotides. The overhang nucleotides can further comprise one or more (e.g., about 1, 2, 3, 4 or more) phosphorothioate, phosphonoacetate, and/or thiophosphonoacetate internucleotide linkages. Non-limiting examples of these chemically-modified siNAs are shown in Figures 4 and 5 and Table III herein. In any of these described embodiments, the purine nucleotides present in the sense region are alternatively 2'-O-methyl, 4'-thio, 2'-O-trifluoromethyl, 2'-O-ethyl-trifluoromethoxy, or 2'-O-difluoromethoxy-ethoxy purine nucleotides (e.g., wherein all purine nucleotides are 2'-O-methyl, 4'-thio, 2'-O-trifluoromethyl, 2'-O-ethyl-trifluoromethoxy, or 2'-O-difluoromethoxy-ethoxy purine nucleotides or alternately a plurality of purine nucleotides are 2'-O-methyl, 4'-thio, 2'-O-trifluoromethyl, 2'-O-ethyl-trifluoromethoxy, or 2'-O-difluoromethoxy-ethoxy purine nucleotides) and one or more purine nucleotides present in the antisense region are 2'-O-methyl, 4'-thio, 2'-O-trifluoromethyl, 2'-O-ethyl-trifluoromethoxy, or 2'-O-difluoromethoxy-ethoxy purine nucleotides (e.g., wherein all purine nucleotides are 2'-O-methyl, 4'-thio, 2'-O-trifluoromethyl, 2'-O-ethyl-trifluoromethoxy, or 2'-O-difluoromethoxy-ethoxy purine nucleotides or alternately a plurality (ie. more than one) of purine nucleotides are 2'-O-methyl, 4'-thio, 2'-O-trifluoromethyl, 2'-O-ethyl-trifluoromethoxy, or 2'-O-difluoromethoxy-ethoxy purine nucleotides). Also, in any of these embodiments, one or more purine nucleotides present in the sense region are alternatively purine ribonucleotides (e.g., wherein all purine nucleotides are purine ribonucleotides or alternately a plurality (ie. more than one) of purine nucleotides are purine ribonucleotides) and any purine nucleotides present in the antisense region are 2'-O-methyl, 4'-thio, 2'-O-trifluoromethyl, 2'-O-ethyl-trifluoromethoxy, or 2'-O-difluoromethoxy-ethoxy purine nucleotides (e.g., wherein all purine nucleotides are 2'-O-methyl, 4'-thio, 2'-O-trifluoromethyl, 2'-O-ethyl-trifluoromethoxy, or 2'-O-difluoromethoxy-ethoxy purine nucleotides or alternately a plurality (ie. more than one) of purine nucleotides are 2'-O-methyl, 4'-thio, 2'-O-trifluoromethyl, 2'-O-ethyl-trifluoromethoxy, or 2'-O-difluoromethoxy-ethoxy purine nucleotides). Additionally, in any of these embodiments, one or more purine nucleotides present in the sense region and/or present in the antisense region are alternatively selected from the group consisting

of 2'-deoxy nucleotides, locked nucleic acid (LNA) nucleotides, 2'-methoxyethyl nucleotides, 4'-thionucleotides, 2'-O-trifluoromethyl nucleotides, 2'-O-ethyl-trifluoromethoxy nucleotides, 2'-O-difluoromethoxy-ethoxy nucleotides and 2'-O-methyl nucleotides (e.g., wherein all purine nucleotides are selected from the group consisting of 2'-deoxy nucleotides, locked nucleic acid (LNA) nucleotides, 2'-methoxyethyl nucleotides, 4'-thionucleotides, 2'-O-trifluoromethyl nucleotides, 2'-O-ethyl-trifluoromethoxy nucleotides, 2'-O-difluoromethoxy-ethoxy nucleotides and 2'-O-methyl nucleotides or alternately a plurality (ie. more than one) of purine nucleotides are selected from the group consisting of 2'-deoxy nucleotides, locked nucleic acid (LNA) nucleotides, 2'-methoxyethyl nucleotides, 4'-thionucleotides, 2'-O-trifluoromethyl nucleotides, 2'-O-ethyl-trifluoromethoxy nucleotides, 2'-O-difluoromethoxy-ethoxy nucleotides and 2'-O-methyl nucleotides).

[00152] In another embodiment, any modified nucleotides present in the nucleic acid or polynucleotide (e.g., siNA) molecules of the invention, preferably in the antisense strand of the siNA molecules of the invention, but also optionally in the sense and/or both antisense and sense strands, comprise modified nucleotides having properties or characteristics similar to naturally occurring ribonucleotides. For example, the invention features siNA molecules including modified nucleotides having a Northern conformation (e.g., Northern pseudorotation cycle, see for example Saenger, Principles of Nucleic Acid Structure, Springer-Verlag ed., 1984) otherwise known as a "ribo-like" or "A-form helix" configuration. As such, chemically modified nucleotides present in the siNA molecules of the invention, preferably in the antisense strand of the siNA molecules of the invention, but also optionally in the sense and/or both antisense and sense strands, are resistant to nuclease degradation while at the same time maintaining the capacity to mediate RNAi. Non-limiting examples of nucleotides having a northern configuration include locked nucleic acid (LNA) nucleotides (e.g., 2'-O, 4'-C-methylene-(D-ribofuranosyl) nucleotides); 2'-methoxyethoxy (MOE) nucleotides; 2'-methyl-thio-ethyl, 2'-deoxy-2'-fluoro nucleotides, 2'-deoxy-2'-chloro nucleotides, 2'-azido nucleotides, 2'-O-trifluoromethyl nucleotides, 2'-O-ethyl-trifluoromethoxy nucleotides, 2'-O-difluoromethoxy-ethoxy nucleotides, 4'-thio nucleotides and 2'-O-methyl nucleotides.

[00153] In one embodiment, the sense strand of a double stranded nucleic acid or polynucleotide (*e.g.*, siNA) molecule of the invention comprises a terminal cap moiety, (see for example Figure 10) such as an inverted deoxyabaisc moiety, at the 3'-end, 5'-end, or both 3' and 5'-ends of the sense strand.

[00154] In one embodiment, the invention features a chemically-modified short interfering nucleic acid or polynucleotide molecule (*e.g.*, siNA) capable of mediating RNA interference (RNAi) inside a cell or reconstituted in vitro system, wherein the chemical modification comprises a conjugate covalently attached to the chemically-modified siNA molecule. Non-limiting examples of conjugates contemplated by the invention include conjugates and ligands described in Vargeese et al., USSN 10/427,160, filed April 30, 2003, incorporated by reference herein in its entirety, including the drawings. In another embodiment, the conjugate is covalently attached to the chemically-modified siNA molecule via a biodegradable linker. In one embodiment, the conjugate molecule is attached at the 3'-end of either the sense strand, the antisense strand, or both strands of the chemically-modified siNA molecule. In another embodiment, the conjugate molecule is attached at the 5'-end of either the sense strand, the antisense strand, or both strands of the chemically-modified siNA molecule. In yet another embodiment, the conjugate molecule is attached both the 3'-end and 5'-end of either the sense strand, the antisense strand, or both strands of the chemically-modified siNA molecule, or any combination thereof. In one embodiment, a conjugate molecule of the invention comprises a molecule that facilitates delivery of a chemically-modified siNA molecule into a biological system, such as a cell. In another embodiment, the conjugate molecule attached to the chemically-modified siNA molecule is a cholesterol, polyethylene glycol, human serum albumin, or a ligand for a cellular receptor, such as peptides derived from naturally occurring protein ligands; protein localization sequences, including cellular ZIP code sequences; antibodies; nucleic acid aptamers; vitamins and other co-factors, such as folate and N-acetylgalactosamine; polymers, such as polyethyleneglycol (PEG); phospholipids; cholesterol; steroids, and polyamines, such as PEI, spermine or spermidine. Examples of specific conjugate molecules contemplated by the instant invention that can be attached to chemically-modified siNA molecules are described in Vargeese et al., U.S. Serial No. 10/201,394, filed July 22, 2002 incorporated by reference herein. The type of conjugates used and the extent of conjugation of siNA

molecules of the invention can be evaluated for improved pharmacokinetic profiles, bioavailability, and/or stability of siNA constructs while at the same time maintaining the ability of the siNA to mediate RNAi activity. As such, one skilled in the art can screen siNA constructs that are modified with various conjugates to determine whether the siNA conjugate complex possesses improved properties while maintaining the ability to mediate RNAi, for example in animal models as are generally known in the art.

[00155] In one embodiment, the invention features a short interfering nucleic acid or polynucleotide (*e.g.*, siNA) molecule of the invention, wherein the siNA further comprises a nucleotide, non-nucleotide, or mixed nucleotide/non-nucleotide linker that joins the sense region of the siNA to the antisense region of the siNA. In one embodiment, a nucleotide, non-nucleotide, or mixed nucleotide/non-nucleotide linker is used, for example, to attach a conjugate moiety to the siNA. In one embodiment, a nucleotide linker of the invention can be a linker of ≥ 2 nucleotides in length, for example about 3, 4, 5, 6, 7, 8, 9, or 10 nucleotides in length. In another embodiment, the nucleotide linker can be a nucleic acid aptamer. By “aptamer” or “nucleic acid aptamer” as used herein is meant a nucleic acid molecule that binds specifically to a target molecule wherein the nucleic acid molecule has sequence that comprises a sequence recognized by the target molecule in its natural setting. Alternately, an aptamer can be a nucleic acid molecule that binds to a target molecule where the target molecule does not naturally bind to a nucleic acid. The target molecule can be any molecule of interest. For example, the aptamer can be used to bind to a ligand-binding domain of a protein, thereby preventing interaction of the naturally occurring ligand with the protein. This is a non-limiting example and those in the art will recognize that other embodiments can be readily generated using techniques generally known in the art. (See, for example, Gold et al., 1995, *Annu. Rev. Biochem.*, 64, 763; Brody and Gold, 2000, *J. Biotechnol.*, 74, 5; Sun, 2000, *Curr. Opin. Mol. Ther.*, 2, 100; Kusser, 2000, *J. Biotechnol.*, 74, 27; Hermann and Patel, 2000, *Science*, 287, 820; and Jayasena, 1999, *Clinical Chemistry*, 45, 1628.)

[00156] In yet another embodiment, a non-nucleotide linker of the invention comprises abasic nucleotide, polyether, polyamine, polyamide, peptide, carbohydrate, lipid, polyhydrocarbon, or other polymeric compounds (*e.g.* polyethylene glycols such as those having between 2 and 100 ethylene glycol units). Specific examples include those

described by Seela and Kaiser, Nucleic Acids Res. 1990, 18:6353 and Nucleic Acids Res. 1987, 15:3113; Cload and Schepartz, J. Am. Chem. Soc. 1991, 113:6324; Richardson and Schepartz, J. Am. Chem. Soc. 1991, 113:5109; Ma et al., Nucleic Acids Res. 1993, 21:2585 and Biochemistry 1993, 32:1751; Durand et al., Nucleic Acids Res. 1990, 18:6353; McCurdy et al., Nucleosides & Nucleotides 1991, 10:287; Jsche et al., Tetrahedron Lett. 1993, 34:301; Ono et al., Biochemistry 1991, 30:9914; Arnold et al., International Publication No. WO 89/02439; Usman et al., International Publication No. WO 95/06731; Dudycz et al., International Publication No. WO 95/11910 and Ferentz and Verdine, J. Am. Chem. Soc. 1991, 113:4000, all hereby incorporated by reference herein. A "non-nucleotide" further means any group or compound that can be incorporated into a nucleic acid chain in the place of one or more nucleotide units, including either sugar and/or phosphate substitutions, and allows the siNA molecule to retain RNAi activity or RNAi inhibitory to retain its inhibition activity. The group or compound can be abasic in that it does not contain a commonly recognized nucleotide base, such as adenosine, guanine, cytosine, uracil or thymine, for example at the C1 position of the sugar.

Targeting Moieties

[00157] In one embodiment of the invention, the properties of a nucleic acid or polynucleotide (e.g., siNA) agent, including its pharmacological properties, can be influenced and tailored, for example, by the introduction of ligands, e.g. tethered ligands. A wide variety of entities, e.g., ligands, can be tethered to a siNA agent, e.g., to the carrier of a ligand-conjugated monomer subunit. Examples are described below in the context of a ligand-conjugated monomer subunit but that is only preferred, entities can be coupled at other points to a siNA agent.

[00158] In a preferred embodiment moieties which are ligands, are coupled, preferably covalently, either directly or indirectly via an intervening tether, to the carrier. In certain embodiments, the ligand is attached to the carrier via an intervening tether. The ligand or tethered ligand may be present on the ligand-conjugated monomer when the ligand-conjugated monomer is incorporated into the growing strand. In some embodiments, the ligand may be incorporated into a "precursor" ligand-conjugated monomer subunit after a "precursor" ligand-conjugated monomer subunit has been incorporated into the

growing strand. For example, a monomer having, e.g., an amino-terminated tether, e.g., TAP-(CH₂)_nNH₂ may be incorporated into a growing sense or antisense strand. In a subsequent operation, i.e., after incorporation of the precursor monomer subunit into the strand, a ligand having an electrophilic group, e.g., a pentafluorophenyl ester or aldehyde group, can subsequently be attached to the precursor ligand-conjugated monomer by coupling the electrophilic group of the ligand with the terminal nucleophilic group of the precursor ligand-conjugated monomer subunit tether.

[00159] In another embodiment, a ligand alters the distribution, targeting or lifetime of a nucleic acid or polynucleotide (*e.g.*, siNA) agent into which it is incorporated. In certain embodiments a ligand provides an enhanced affinity for a selected target, *e.g.*, molecule, cell or cell type, compartment, *e.g.*, a cellular or organ compartment, tissue, organ or region of the body, as, *e.g.*, compared to a species absent such a ligand.

[00160] In a preferred embodiment ligands can improve transport, hybridization, and specificity properties and may also improve nuclease resistance of the resultant natural or modified oligoribonucleotide, or a polymeric molecule comprising any combination of monomers described herein and/or natural or modified ribonucleotides.

[00161] Ligands in general can include therapeutic modifiers, *e.g.*, for enhancing uptake; diagnostic compounds or reporter groups *e.g.*, for monitoring distribution; cross-linking agents; nuclease-resistance conferring moieties; and natural or unusual nucleobases. General examples include lipophiles, lipids, steroids (*e.g.*, uvaol, hecigenin, diosgenin), terpenes (*e.g.*, triterpenes, *e.g.*, sarsasapogenin, Friedelin, epifriedelanol derivatized lithocholic acid), vitamins (*e.g.*, folic acid, vitamin A, biotin, pyridoxal), carbohydrates, proteins, protein binding agents, integrin targeting molecules, polycationics, peptides, polyamines, and peptide mimics.

[00162] In another embodiment ligands can include a naturally occurring substance, (*e.g.*, human serum albumin (HSA), low-density lipoprotein (LDL), or globulin); carbohydrate (*e.g.*, a dextran, pullulan, chitin, chitosan, inulin, cyclodextrin or hyaluronic acid); amino acid, or a lipid. The ligand may also be a recombinant or synthetic molecule, such as a synthetic polymer, *e.g.*, a synthetic polyamino acid. Examples of polyamino acids include polyamino acid is a polylysine (PLL), poly L-aspartic acid, poly

L-glutamic acid, styrene-maleic acid anhydride copolymer, poly(L-lactide-co-glycolide) copolymer, divinyl ether-maleic anhydride copolymer, N-(2-hydroxypropyl)methacrylamide copolymer (HMPA), polyethylene glycol (PEG), polyvinyl alcohol (PVA), polyurethane, poly(2-ethylacrylic acid), N-isopropylacrylamide polymers, or polyphosphazene. Example of polyamines include: polyethylenimine, polylysine (PLL), spermine, spermidine, polyamine, pseudopeptide-polyamine, peptidomimetic polyamine, dendrimer polyamine, arginine, amidine, protamine, cationic moieties, e.g., cationic lipid, cationic porphyrin, quaternary salt of a polyamine, or an alpha helical peptide.

[00163] In yet another embodiment ligands can include targeting groups, e.g., a cell or tissue targeting agent, e.g., a lectin, glycoprotein, lipid or protein, e.g., an antibody, that binds to a specified cell type such as a liver cell or a cell of the jejunum. A targeting group can be a thyrotropin, melanotropin, lectin, glycoprotein, surfactant protein A, Mucin carbohydrate, multivalent lactose, multivalent galactose, N-acetyl-galactosamine, N-acetyl-gulucosamine multivalent mannose, multivalent fucose, glycosylated polyaminoacids, multivalent galactose, transferrin, bisphosphonate, polyglutamate, polyaspartate, a lipid, cholesterol, a steroid, bile acid, folate, vitamin B12, biotin, or an RGD peptide or RGD peptide mimetic. The antibody need not be a whole antibody but may also include fragments thereof. See 2005/0250722, FcγRIIIA-specific nucleic acid interference, filed November 15, 2004; US 20050267059, filed November 15, 2004, which teaches a methodology involving the use of RNAi constructs that are targeted to a Syk mRNA sequence. See also Song E, Zhu P, Lee S-K, Chowdhury D, Kussman S, Dykxhoorn DM, Feng Y, Palliser D, Weiner DB, Shankar P, Marasco WA, Lieberman J. Antibody-mediated delivery of small interfering RNAs via cell surface receptors. *Nature Biotech* 2005; 9:347-351; Ted C. Chu, Karen Y. Twu, Andrew D. Ellington and Matthew Levy, Aptamer mediated siRNA delivery, *Nucleic Acid Research*, 34: e73, published online June 1, 2006.

[00164] In one embodiment examples of ligands include dyes, intercalating agents (e.g. acridines), cross-linkers (e.g. psoralene, mitomycin C), porphyrins (TPPC4, texaphyrin, Sapphyrin), polycyclic aromatic hydrocarbons (e.g., phenazine, dihydrophenazine), artificial endonucleases (e.g. EDTA), lipophilic molecules, e.g, cholesterol, cholic acid,

adamantane acetic acid, 1-pyrene butyric acid, dihydrotestosterone, glycerol (e.g., esters and ethers thereof, e.g., C₁₀, C₁₁, C₁₂, C₁₃, C₁₄, C₁₅, C₁₆, C₁₇, C₁₈, C₁₉, or C₂₀ alkyl; e.g., 1,3-bis-O(hexadecyl)glycerol, 1,3-bis-O(octaadecyl)glycerol), geranyloxyhexyl group, hexadecylglycerol, borneol, menthol, 1,3-propanediol, heptadecyl group, palmitic acid, myristic acid, O3-(oleoyl)lithocholic acid, O3-(oleoyl)cholenic acid, dimethoxytrityl, or phenoxazine) and peptide conjugates (e.g., antennapedia peptide, Tat peptide), alkylating agents, phosphate, amino, mercapto, PEG (e.g., PEG-40K), MPEG, [MPEG]₂, polyamino, alkyl, substituted alkyl, radiolabeled markers, enzymes, haptens (e.g. biotin), transport/absorption facilitators (e.g., aspirin, vitamin E, folic acid), synthetic ribonucleases (e.g., imidazole, bisimidazole, histamine, imidazole clusters, acridine-imidazole conjugates, Eu³⁺ complexes of tetraazamacrocycles), dinitrophenyl, HRP, or AP.

[00165] In another embodiment, ligands can be proteins, e.g., glycoproteins, or peptides, e.g., molecules having a specific affinity for a co-ligand, or antibodies e.g., an antibody, that binds to a specified cell type such as a cancer cell, endothelial cell, or bone cell. Ligands may also include hormones and hormone receptors. They can also include non-peptidic species, such as lipids, lectins, carbohydrates, vitamins, cofactors, multivalent lactose, multivalent galactose, N-acetyl-galactosamine, N-acetyl-gulucosamine multivalent mannose, or multivalent fucose. The ligand can be, for example, a lipopolysaccharide, an activator of p38 MAP kinase, or an activator of NF- κ B.

[00166] In other embodiment of the invention, the ligand can be a substance, e.g. a drug, which can increase the uptake of the nucleic acid or polynucleotide (e.g., siNA) agent into the cell, for example, by disrupting the cell's cytoskeleton, e.g., by disrupting the cell's microtubules, microfilaments, and/or intermediate filaments. The drug can be, for example, taxon, vincristine, vinblastine, cytochalasin, nocodazole, japlakinolide, latrunculin A, phalloidin, swinholide A, indanocine, or myoservin. Ligands that are capable of increasing the uptake of the siRNA agent into the cell are also within the scope of the invention.

[00167] Lipid or lipid-based molecules are also considered ligands. Such a lipid or lipid-based molecule may bind a serum protein, e.g., human serum albumin (HSA). For

example, an HSA binding ligand allows for distribution of the conjugate to a target tissue, e.g., liver tissue, including parenchymal cells of the liver. Other molecules that can bind HSA can also be used as ligands. For example, neproxin or aspirin can be used. A lipid or lipid-based ligand can (a) increase resistance to degradation of the conjugate, (b) increase targeting or transport into a target cell or cell membrane, and/or (c) can be used to adjust binding to a serum protein, e.g., HSA. See for example, US 2005/0064595 and references cited therein, filed July 16, 2004 which provides for lipid based molecules for delivering RNAi agents. The content of this publication are incorporated by reference herein in its entirety.

[00168] A lipid based ligand can be used to modulate, e.g., control the binding of the conjugate to a target tissue. For example, a lipid or lipid-based ligand that binds to HSA more strongly will be less likely to be targeted to the kidney and therefore less likely to be cleared from the body. A lipid or lipid-based ligand that binds to HSA less strongly can be used to target the conjugate to the kidney.

[00169] In another embodiment of the invention, the ligand is a moiety, e.g., a vitamin, which is taken up by a target cell, e.g., a proliferating cell. These are particularly useful for treating disorders characterized by unwanted cell proliferation, e.g., of the malignant or non-malignant type, e.g., cancer cells. Exemplary vitamins include vitamin A, E, and K. Other exemplary vitamins include are B vitamin, e.g., folic acid, B12, riboflavin, biotin, pyridoxal or other vitamins or nutrients taken up by cancer cells. Also included are HSA and low density lipoprotein (LDL).

[00170] In another embodiment of the invention, the ligand is a cell-permeation agent, preferably a helical cell-permeation agent. In one embodiment, the agent is amphipathic. An exemplary agent is a peptide such as tat or antennopodia. If the agent is a peptide, it can be modified, including a peptidylmimetic, invertomers, non-peptide or pseudo-peptide linkages, and use of D-amino acids. The helical agent is an alpha-helical agent, which preferably has a lipophilic and a lipophobic phase.

[00171] Peptides that target markers enriched in proliferating cells can also be used. E.g., RGD containing peptides and petomimetics can target cancer cells, in particular cells that exhibit an $\alpha_v\beta_3$ integrin. Thus, one could use RGD peptides, cyclic peptides

containing RGD, RGD peptides that include D-amino acids, as well as synthetic RGD mimics. In addition to RGD, one can use other moieties that target the $\alpha_v\beta_3$ integrin ligand. Generally, such ligands can be used to control proliferating cells and angiogenesis. Preferred conjugates of this type include a nucleic acid or polynucleotide (e.g., siNA) agent that targets PECAM-1, PCSK9, or other cancer gene, e.g., a cancer gene described herein.

[00172] A targeting agent that incorporates a sugar, e.g., galactose and/or analogues thereof, is particularly useful. These agents target, in particular, the parenchymal cells of the liver. For example, a targeting moiety can include more than one or preferably two or three galactose moieties, spaced about 15 angstroms from each other. The targeting moiety can alternatively be lactose (e.g., three lactose moieties), which is glucose coupled to a galactose. The targeting moiety can also be N-Acetyl-Galactosamine, N-Ac-Glucosamine. A mannose or mannose-6-phosphate targeting moiety can be used for macrophage targeting.

[00173] The ligand can be a peptide or peptidomimetic. A peptidomimetic (also referred to herein as an oligopeptidomimetic) is a molecule capable of folding into a defined three-dimensional structure similar to a natural peptide. The attachment of peptide and peptidomimetics to siRNA agents can affect pharmacokinetic distribution of the siRNA, such as by enhancing cellular recognition and absorption. The peptide or peptidomimetic moiety can be about 5-50 amino acids long, e.g., about 5, 10, 15, 20, 25, 30, 35, 40, 45, or 50 amino acids long.

5'-Phosphate Modifications

[00174] In certain embodiments of the invention, the siRNA agent is 5' phosphorylated or include a phosphoryl analog at the 5' prime terminus. 5'-phosphate modifications of the antisense strand include those which are compatible with RISC mediated gene silencing. Suitable modifications include: 5'-monophosphate ((HO)₂(O)P--O-5'); 5'-diphosphate ((HO)₂(O)P--O--P(HO)(O)--O-5'); 5'-triphosphate ((HO)₂(O)P--O--(HO)(O)P--O--P(HO)(O)--O-5'); 5'-guanosine cap (7-methylated or non-methylated) (7m-G-O-5'-(HO)(O)P--O--(HO)(O)P--O--P(HO)(O)--O-5'); 5'-adenosine cap (Appp), and any modified or unmodified nucleotide cap structure (N--O-5'-(HO)(O)P--O--

(HO)(O)P--O--P(HO)(O)--O-5'); 5'-monothiophosphate (phosphorothioate; (HO)₂(S)P--O-5'); 5'-monodithiophosphate (phosphorodithioate; (HO)(HS)(S)P--O-5'), 5'-phosphorothiolate ((HO)₂(O)P--S-5'); any additional combination of oxygen/sulfur replaced monophosphate, diphosphate and triphosphates (e.g. 5'-alpha-thiotriphosphate, 5'-gamma-thiotriphosphate, etc.), 5'-phosphoramidates ((HO)₂(O)P--NH-5', (HO)(NH₂)(O)P--O-5'), 5'-alkylphosphonates (R=alkyl=methyl, ethyl, isopropyl, propyl, etc., e.g. RP(OH)(O)--O-5', (OH)₂(O)P-5'-CH₂-), 5'-alkyletherphosphonates (R=alkylether=methoxymethyl (MeOCH₂-), ethoxymethyl, etc., e.g. RP(OH)(O)--O-5'-).

[00175] In one embodiment of the invention the sense strand can also be modified in order to inactivate the sense strand and prevent formation of an active RISC, thereby potentially reducing off-target effects. This can be accomplished by a modification which prevents 5'-phosphorylation of the sense strand, e.g., by modification with a 5'-O-methyl ribonucleotide (see Nykanen et al., (2001) ATP requirements and small interfering RNA structure in the RNA interference pathway. Cell 107, 309-321.) Other modifications which prevent phosphorylation can also be used, e.g., simply substituting the 5'-OH by H rather than O-Me. Alternatively, a large bulky group may be added to the 5'-phosphate turning it into a phosphodiester linkage.

[00176] In one embodiment, the invention features a short interfering nucleic acid or polynucleotide (e.g., siNA) molecule capable of mediating RNA interference (RNAi) inside a cell or reconstituted in vitro system, wherein one or both strands of the siNA molecule that are assembled from two separate oligonucleotides do not comprise any ribonucleotides (e.g., one or both strands of the siNA molecule are 100% chemically modified). For example, a siNA molecule can be assembled from a single oligonucleotide where the sense and antisense regions of the siNA comprise separate oligonucleotides that do not have any ribonucleotides (e.g., nucleotides having a 2'-OH group) present in the oligonucleotides. In another example, a siNA molecule can be assembled from a single oligonucleotide where the sense and antisense regions of the siNA are linked or circularized by a nucleotide or non-nucleotide linker as described herein, wherein the oligonucleotide does not have any ribonucleotides (e.g., nucleotides having a 2'-OH group) present in the oligonucleotide. Applicant has surprisingly found that the presense of ribonucleotides (e.g., nucleotides having a 2'-hydroxyl group) within

the siNA molecule is not required or essential to support RNAi activity. As such, in one embodiment, all positions within the siNA can include chemically modified nucleotides and/or non-nucleotides such as nucleotides and or non-nucleotides having Formula I, II, III, IV, V, VI, or VII or any combination thereof to the extent that the ability of the siNA molecule to support RNAi activity in a cell is maintained.

[00177] In one embodiment, a siNA molecule of the invention is a single stranded nucleic acid or polynucleotide (*e.g.*, siNA) molecule that mediates RNAi activity in a cell or reconstituted in vitro system comprising a single stranded polynucleotide having complementarity to a target nucleic acid sequence. In another embodiment, the single stranded siNA molecule of the invention comprises a 5'-terminal phosphate group. In another embodiment, the single stranded siNA molecule of the invention comprises a 5'-terminal phosphate group and a 3'-terminal phosphate group (*e.g.*, a 2',3'-cyclic phosphate). In another embodiment, the single stranded siNA molecule of the invention comprises about 15 to about 30 (*e.g.*, about 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, or 30) nucleotides. In yet another embodiment, the single stranded siNA molecule of the invention comprises one or more chemically modified nucleotides or non-nucleotides described herein. For example, all the positions within the siNA molecule can include chemically-modified nucleotides such as nucleotides having any of Formulae I-VII, or any combination thereof to the extent that the ability of the siNA molecule to support RNAi activity in a cell is maintained.

[00178] In one embodiment, a nucleic acid or polynucleotide (*e.g.*, siNA) molecule of the invention is a single stranded siNA molecule that mediates RNAi activity or that alternately modulates RNAi activity in a cell or reconstituted in vitro system comprising a single stranded polynucleotide having complementarity to a target nucleic acid sequence, wherein one or more pyrimidine nucleotides present in the siNA are 2'-deoxy-2'-fluoro, 4'-thio, 2'-O-trifluoromethyl, 2'-O-ethyl-trifluoromethoxy, or 2'-O-difluoromethoxy-ethoxy pyrimidine nucleotides (*e.g.*, wherein all pyrimidine nucleotides are 2'-deoxy-2'-fluoro, 4'-thio, 2'-O-trifluoromethyl, 2'-O-ethyl-trifluoromethoxy, or 2'-O-difluoromethoxy-ethoxy pyrimidine nucleotides or alternately a plurality of pyrimidine nucleotides are 2'-deoxy-2'-fluoro, 4'-thio, 2'-O-trifluoromethyl, 2'-O-ethyl-trifluoromethoxy, or 2'-O-difluoromethoxy-ethoxy pyrimidine nucleotides), and wherein

any purine nucleotides present in the antisense region are 2'-O-methyl, 4'-thio, 2'-O-trifluoromethyl, 2'-O-ethyl-trifluoromethoxy, or 2'-O-difluoromethoxy-ethoxy purine nucleotides (e.g., wherein all purine nucleotides are 2'-O-methyl, 4'-thio, 2'-O-trifluoromethyl, 2'-O-ethyl-trifluoromethoxy, or 2'-O-difluoromethoxy-ethoxy purine nucleotides or alternately a plurality of purine nucleotides are 2'-O-methyl, 4'-thio, 2'-O-trifluoromethyl, 2'-O-ethyl-trifluoromethoxy, or 2'-O-difluoromethoxy-ethoxy purine nucleotides), and a terminal cap modification, such as any modification described herein or shown in Figure 10, that is optionally present at the 3'-end, the 5'-end, or both of the 3' and 5'-ends of the antisense sequence. The siNA optionally further comprises about 1 to about 4 or more (e.g., about 1, 2, 3, 4 or more) terminal 2'-deoxynucleotides at the 3'-end of the siNA molecule, wherein the terminal nucleotides can further comprise one or more (e.g., 1, 2, 3, 4 or more) phosphorothioate, phosphonoacetate, and/or thiophosphonoacetate internucleotide linkages, and wherein the siNA optionally further comprises a terminal phosphate group, such as a 5'-terminal phosphate group. In any of these embodiments, any purine nucleotides present in the antisense region are alternatively 2'-deoxy purine nucleotides (e.g., wherein all purine nucleotides are 2'-deoxy purine nucleotides or alternately a plurality of purine nucleotides are 2'-deoxy purine nucleotides). Also, in any of these embodiments, any purine nucleotides present in the siNA (i.e., purine nucleotides present in the sense and/or antisense region) can alternatively be locked nucleic acid (LNA) nucleotides (e.g., wherein all purine nucleotides are LNA nucleotides or alternately a plurality of purine nucleotides are LNA nucleotides). Also, in any of these embodiments, any purine nucleotides present in the siNA are alternatively 2'-methoxyethyl purine nucleotides (e.g., wherein all purine nucleotides are 2'-methoxyethyl purine nucleotides or alternately a plurality of purine nucleotides are 2'-methoxyethyl purine nucleotides). In another embodiment, any modified nucleotides present in the single stranded siNA molecules of the invention comprise modified nucleotides having properties or characteristics similar to naturally occurring ribonucleotides. For example, the invention features siNA molecules including modified nucleotides having a Northern conformation (e.g., Northern pseudorotation cycle, see for example Saenger, Principles of Nucleic Acid Structure, Springer-Verlag ed., 1984). As such, chemically modified nucleotides present in the single stranded siNA

molecules of the invention are preferably resistant to nuclease degradation while at the same time maintaining the capacity to mediate RNAi.

[00179] In one embodiment, a chemically-modified short interfering nucleic acid or polynucleotide (*e.g.*, siNA) molecule of the invention comprises a sense strand or sense region having two or more (*e.g.*, 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 or more) 2'-O-alkyl (*e.g.* 2'-O-methyl) modifications or any combination thereof. In another embodiment, the 2'-O-alkyl modification is at alternating position in the sense strand or sense region of the siNA, such as position 1, 3, 5, 7, 9, 11, 13, 15, 17, 19, 21 etc. or position 2, 4, 6, 8, 10, 12, 14, 16, 18, 20 etc.

[00180] In one embodiment, a chemically-modified short interfering nucleic acid or polynucleotide (*e.g.*, siNA) molecule of the invention comprises an antisense strand or antisense region having two or more (*e.g.*, 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 or more) 2'-O-alkyl (*e.g.* 2'-O-methyl) modifications or any combination thereof. In another embodiment, the 2'-O-alkyl modification is at alternating position in the antisense strand or antisense region of the siNA, such as position 1, 3, 5, 7, 9, 11, 13, 15, 17, 19, 21 etc. or position 2, 4, 6, 8, 10, 12, 14, 16, 18, 20 etc.

[00181] In one embodiment, a chemically-modified short interfering nucleic acid or polynucleotide (*e.g.*, siNA) molecule of the invention comprises a sense strand or sense region and an antisense strand or antisense region, each having two or more (*e.g.*, 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 or more) 2'-O-alkyl (*e.g.* 2'-O-methyl), 2'-deoxy-2'-fluoro, 2'-deoxy, or abasic chemical modifications or any combination thereof. In another embodiment, the 2'-O-alkyl modification is at alternating position in the sense strand or sense region of the siNA, such as position 1, 3, 5, 7, 9, 11, 13, 15, 17, 19, 21 etc. or position 2, 4, 6, 8, 10, 12, 14, 16, 18, 20 etc. In another embodiment, the 2'-O-alkyl modification is at alternating position in the antisense strand or antisense region of the siNA, such as position 1, 3, 5, 7, 9, 11, 13, 15, 17, 19, 21 etc. or position 2, 4, 6, 8, 10, 12, 14, 16, 18, 20 etc.

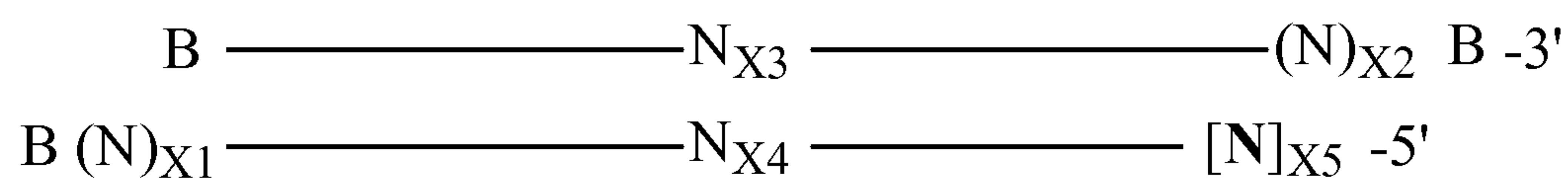
[00182] In one embodiment, a nucleic acid or polynucleotide (*e.g.*, siNA) molecule of the invention comprises chemically modified nucleotides or non-nucleotides (*e.g.*, having any of Formulae I-VII, such as 2'-deoxy, 2'-deoxy-2'-fluoro, 4'-thio, 2'-O-trifluoromethyl, 2'-O-ethyl-trifluoromethoxy, 2'-O-difluoromethoxy-ethoxy or 2'-O-methyl nucleotides) at alternating positions within one or more strands or regions of the siNA molecule. For example, such chemical modifications can be introduced at every other position of a RNA based siNA molecule, starting at either the first or second nucleotide from the 3'-end or 5'-end of the siNA. In a non-limiting example, a double stranded siNA molecule of the invention in which each strand of the siNA is 21 nucleotides in length is featured wherein positions 1, 3, 5, 7, 9, 11, 13, 15, 17, 19 and 21 of each strand are chemically modified (*e.g.*, with compounds having any of Formulae I-VII, such as such as 2'-deoxy, 2'-deoxy-2'-fluoro, 4'-thio, 2'-O-trifluoromethyl, 2'-O-ethyl-trifluoromethoxy, 2'-O-difluoromethoxy-ethoxy or 2'-O-methyl nucleotides). In another non-limiting example, a double stranded siNA molecule of the invention in which each strand of the siNA is 21 nucleotides in length is featured wherein positions 2, 4, 6, 8, 10, 12, 14, 16, 18, and 20 of each strand are chemically modified (*e.g.*, with compounds having any of Formulae I-VII, such as such as 2'-deoxy, 2'-deoxy-2'-fluoro, 4'-thio, 2'-O-trifluoromethyl, 2'-O-ethyl-trifluoromethoxy, 2'-O-difluoromethoxy-ethoxy or 2'-O-methyl nucleotides). In one embodiment, one strand of the double stranded siNA molecule comprises chemical modifications at positions 2, 4, 6, 8, 10, 12, 14, 16, 18, and 20 and chemical modifications at positions 1, 3, 5, 7, 9, 11, 13, 15, 17, 19 and 21. Such siNA molecules can further comprise terminal cap moieties and/or backbone modifications as described herein.

[00183] In one embodiment, a nucleic acid or polynucleotide (*e.g.*, siNA) molecule of the invention comprises the following features: if purine nucleotides are present at the 5'-end (*e.g.*, at any of terminal nucleotide positions 1, 2, 3, 4, 5, or 6 from the 5'-end) of the antisense strand or antisense region (otherwise referred to as the guide sequence or guide strand) of the siNA molecule then such purine nucleosides are ribonucleotides. In another embodiment, the purine ribonucleotides, when present, are base paired to nucleotides of the sense strand or sense region (otherwise referred to as the passenger strand) of the siNA molecule. Such purine ribonucleotides can be present in a siNA stabilization motif that otherwise comprises modified nucleotides.

[00184] In one embodiment, a nucleic acid or polynucleotide (*e.g.*, siNA) molecule of the invention comprises the following features: if pyrimidine nucleotides are present at the 5'-end (*e.g.*, at any of terminal nucleotide positions 1, 2, 3, 4, 5, or 6 from the 5'-end) of the antisense strand or antisense region (otherwise referred to as the guide sequence or guide strand) of the siNA molecule then such pyrimidine nucleosides are ribonucleotides. In another embodiment, the pyrimidine ribonucleotides, when present, are base paired to nucleotides of the sense strand or sense region (otherwise referred to as the passenger strand) of the siNA molecule. Such pyrimidine ribonucleotides can be present in a siNA stabilization motif that otherwise comprises modified nucleotides.

[00185] In one embodiment, a nucleic acid or polynucleotide (*e.g.*, siNA) molecule of the invention comprises the following features: if pyrimidine nucleotides are present at the 5'-end (*e.g.*, at any of terminal nucleotide positions 1, 2, 3, 4, 5, or 6 from the 5'-end) of the antisense strand or antisense region (otherwise referred to as the guide sequence or guide strand) of the siNA molecule then such pyrimidine nucleosides are modified nucleotides. In another embodiment, the modified pyrimidine nucleotides, when present, are base paired to nucleotides of the sense strand or sense region (otherwise referred to as the passenger strand) of the siNA molecule. Non-limiting examples of modified pyrimidine nucleotides include those having any of Formulae I-VII, such as such as 2'-deoxy, 2'-deoxy-2'-fluoro, 4'-thio, 2'-O-trifluoromethyl, 2'-O-ethyl-trifluoromethoxy, 2'-O-difluoromethoxy-ethoxy or 2'-O-methyl nucleotides.

[00186] In one embodiment, the invention features a double stranded nucleic acid or polynucleotide (*e.g.*, siNA) molecule having structure SI:



SI

[00187] wherein each N is independently a nucleotide which can be unmodified or chemically modified; each B is a terminal cap moiety that can be present or absent; (N) represents non-base paired or overhanging nucleotides which can be unmodified or chemically modified; [N] represents nucleotide positions wherein any purine nucleotides

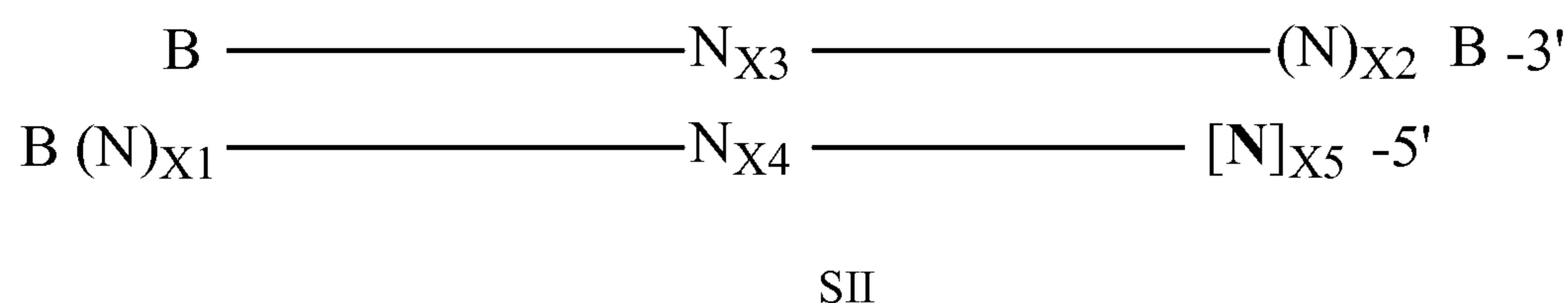
when present are ribonucleotides; X1 and X2 are independently integers from about 0 to about 4; X3 is an integer from about 9 to about 30; X4 is an integer from about 11 to about 30, provided that the sum of X4 and X5 is between 17-36; X5 is an integer from about 1 to about 6; NX3 is complementary to NX4 and NX5, and

[00188] (a) any pyrimidine nucleotides present in the antisense strand (lower strand) are 2'-deoxy-2'-fluoro nucleotides; any purine nucleotides present in the antisense strand (lower strand) other than the purines nucleotides in the [N] nucleotide positions, are independently 2'-O-methyl nucleotides, 2'-deoxyribonucleotides or a combination of 2'-deoxyribonucleotides and 2'-O-methyl nucleotides;

[00189] (b) any pyrimidine nucleotides present in the sense strand (upper strand) are 2'-deoxy-2'-fluoro nucleotides; any purine nucleotides present in the sense strand (upper strand) are independently 2'-deoxyribonucleotides, 2'-O-methyl nucleotides or a combination of 2'-deoxyribonucleotides and 2'-O-methyl nucleotides; and

[00190] (c) any (N) nucleotides are optionally 2'-O-methyl, 2'-deoxy-2'-fluoro, or deoxyribonucleotides.

[00191] In one embodiment, the invention features a double stranded nucleic acid or polynucleotide (*e.g.*, siNA) molecule having structure SII:



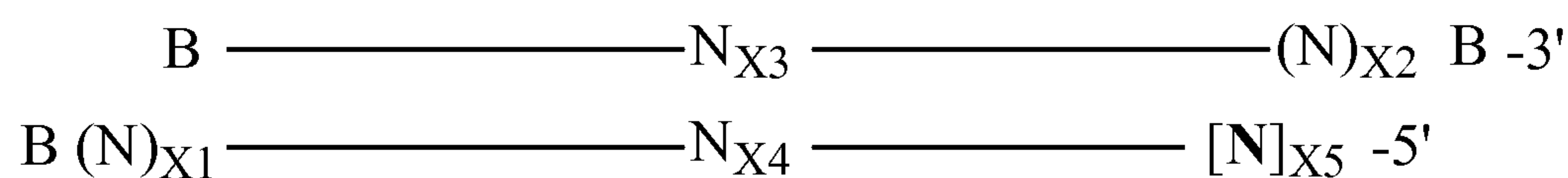
[00192] wherein each N is independently a nucleotide which can be unmodified or chemically modified; each B is a terminal cap moiety that can be present or absent; (N) represents non-base paired or overhanging nucleotides which can be unmodified or chemically modified; [N] represents nucleotide positions wherein any purine nucleotides when present are ribonucleotides; X1 and X2 are independently integers from about 0 to about 4; X3 is an integer from about 9 to about 30; X4 is an integer from about 11 to about 30, provided that the sum of X4 and X5 is between 17-36; X5 is an integer from about 1 to about 6; NX3 is complementary to NX4 and NX5, and

[00193] (a) any pyrimidine nucleotides present in the antisense strand (lower strand) are 2'-deoxy-2'-fluoro nucleotides; any purine nucleotides present in the antisense strand (lower strand) other than the purines nucleotides in the [N] nucleotide positions, are 2'-O-methyl nucleotides;

[00194] (b) any pyrimidine nucleotides present in the sense strand (upper strand) are ribonucleotides; any purine nucleotides present in the sense strand (upper strand) are ribonucleotides; and

[00195] (c) any (N) nucleotides are optionally 2'-O-methyl, 2'-deoxy-2'-fluoro, or deoxyribonucleotides.

[00196] In one embodiment, the invention features a double stranded nucleic acid or polynucleotide (*e.g.*, siNA) molecule having structure SIII:



SIII

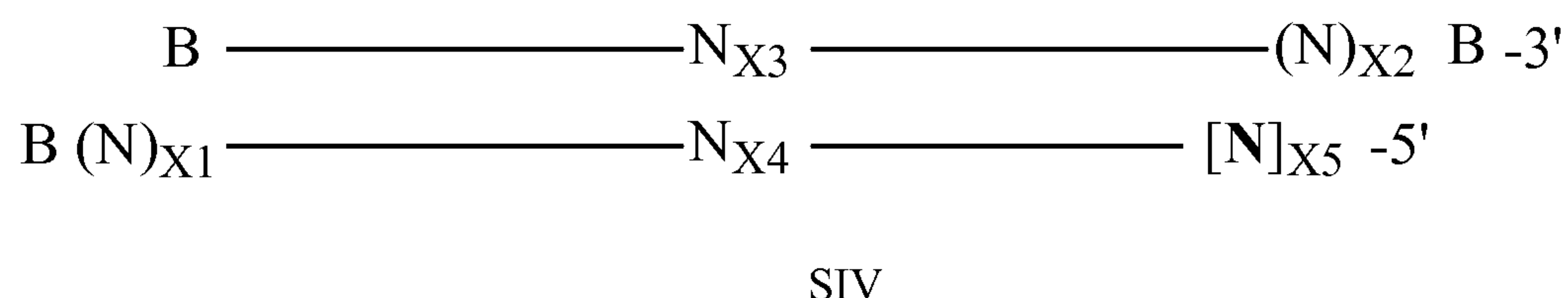
[00197] wherein each N is independently a nucleotide which can be unmodified or chemically modified; each B is a terminal cap moiety that can be present or absent; (N) represents non-base paired or overhanging nucleotides which can be unmodified or chemically modified; [N] represents nucleotide positions wherein any purine nucleotides when present are ribonucleotides; X1 and X2 are independently integers from about 0 to about 4; X3 is an integer from about 9 to about 30; X4 is an integer from about 11 to about 30, provided that the sum of X4 and X5 is between 17-36; X5 is an integer from about 1 to about 6; NX3 is complementary to NX4 and NX5, and

[00198] (a) any pyrimidine nucleotides present in the antisense strand (lower strand) are 2'-deoxy-2'-fluoro nucleotides; any purine nucleotides present in the antisense strand (lower strand) other than the purines nucleotides in the [N] nucleotide positions, are 2'-O-methyl nucleotides;

[00199] (b) any pyrimidine nucleotides present in the sense strand (upper strand) are 2'-deoxy-2'-fluoro nucleotides; any purine nucleotides present in the sense strand (upper strand) are ribonucleotides; and

[00200] (c) any (N) nucleotides are optionally 2'-O-methyl, 2'-deoxy-2'-fluoro, or deoxyribonucleotides.

[00201] In one embodiment, the invention features a double stranded nucleic acid or polynucleotide (*e.g.*, siNA) molecule having structure SIV:



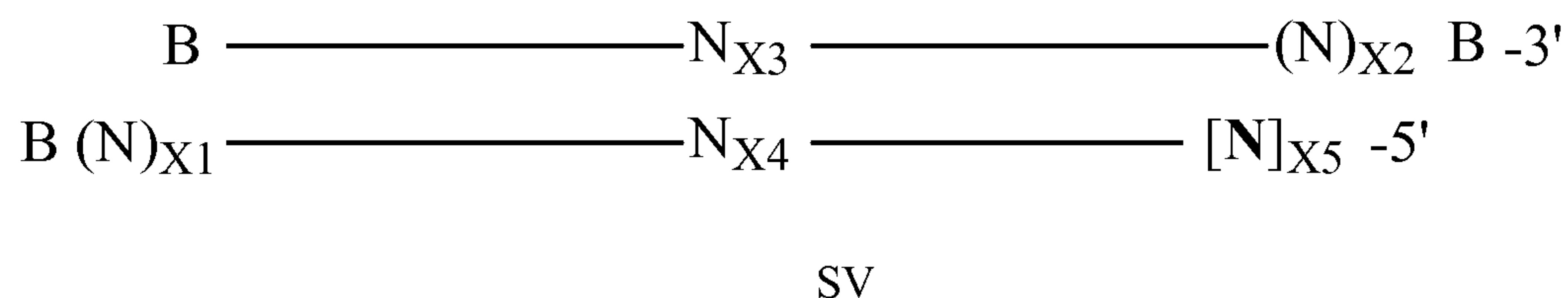
[00202] wherein each N is independently a nucleotide which can be unmodified or chemically modified; each B is a terminal cap moiety that can be present or absent; (N) represents non-base paired or overhanging nucleotides which can be unmodified or chemically modified; [N] represents nucleotide positions wherein any purine nucleotides when present are ribonucleotides; X1 and X2 are independently integers from about 0 to about 4; X3 is an integer from about 9 to about 30; X4 is an integer from about 11 to about 30, provided that the sum of X4 and X5 is between 17-36; X5 is an integer from about 1 to about 6; NX3 is complementary to NX4 and NX5, and

[00203] (a) any pyrimidine nucleotides present in the antisense strand (lower strand) are 2'-deoxy-2'-fluoro nucleotides; any purine nucleotides present in the antisense strand (lower strand) other than the purines nucleotides in the [N] nucleotide positions, are 2'-O-methyl nucleotides;

[00204] (b) any pyrimidine nucleotides present in the sense strand (upper strand) are 2'-deoxy-2'-fluoro nucleotides; any purine nucleotides present in the sense strand (upper strand) are deoxyribonucleotides; and

[00205] (c) any (N) nucleotides are optionally 2'-O-methyl, 2'-deoxy-2'-fluoro, or deoxyribonucleotides.

[00206] In one embodiment, the invention features a double stranded nucleic acid or polynucleotide (*e.g.*, siNA) molecule having structure SV:



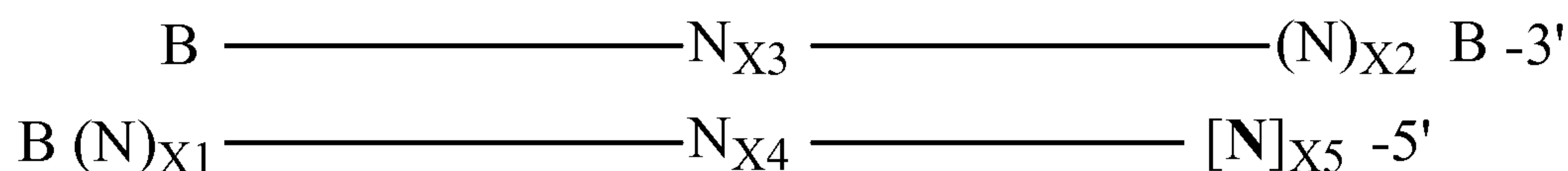
[00207] wherein each N is independently a nucleotide which can be unmodified or chemically modified; each B is a terminal cap moiety that can be present or absent; (N) represents non-base paired or overhanging nucleotides which can be unmodified or chemically modified; [N] represents nucleotide positions wherein any purine nucleotides when present are ribonucleotides; X1 and X2 are independently integers from about 0 to about 4; X3 is an integer from about 9 to about 30; X4 is an integer from about 11 to about 30, provided that the sum of X4 and X5 is between 17-36; X5 is an integer from about 1 to about 6; NX3 is complementary to NX4 and NX5, and

[00208] (a) any pyrimidine nucleotides present in the antisense strand (lower strand) are nucleotides having a ribo-like configuration (*e.g.*, Northern or A-form helix configuration); any purine nucleotides present in the antisense strand (lower strand) other than the purines nucleotides in the [N] nucleotide positions, are 2'-O-methyl nucleotides;

[00209] (b) any pyrimidine nucleotides present in the sense strand (upper strand) are nucleotides having a ribo-like configuration (*e.g.*, Northern or A-form helix configuration); any purine nucleotides present in the sense strand (upper strand) are 2'-O-methyl nucleotides; and

[00210] (c) any (N) nucleotides are optionally 2'-O-methyl, 2'-deoxy-2'-fluoro, or deoxyribonucleotides.

[00211] In one embodiment, the invention features a double stranded nucleic acid or polynucleotide (*e.g.*, siNA) molecule having structure SVI:



SVI

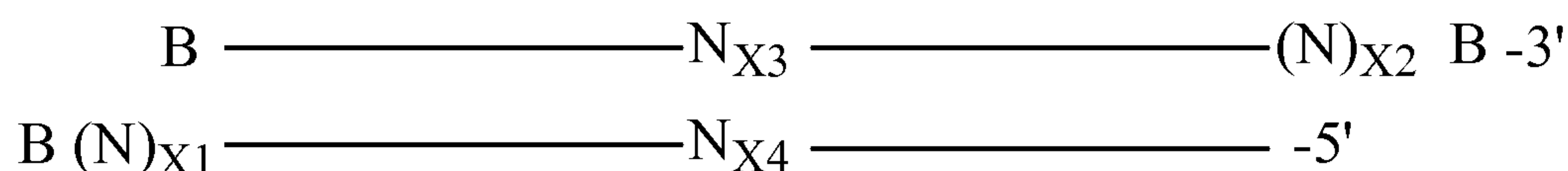
[00212] wherein each N is independently a nucleotide which can be unmodified or chemically modified; each B is a terminal cap moiety that can be present or absent; (N) represents non-base paired or overhanging nucleotides which can be unmodified or chemically modified; [N] represents nucleotide positions comprising sequence that renders the 5'-end of the antisense strand (lower strand) less thermally stable than the 5'-end of the sense strand (upper strand); X1 and X2 are independently integers from about 0 to about 4; X3 is an integer from about 9 to about 30; X4 is an integer from about 11 to about 30, provided that the sum of X4 and X5 is between 17-36; X5 is an integer from about 1 to about 6; NX3 is complementary to NX4 and NX5, and

[00213] (a) any pyrimidine nucleotides present in the antisense strand (lower strand) are 2'-deoxy-2'-fluoro nucleotides; any purine nucleotides present in the antisense strand (lower strand) other than the purines nucleotides in the [N] nucleotide positions, are independently 2'-O-methyl nucleotides, 2'-deoxyribonucleotides or a combination of 2'-deoxyribonucleotides and 2'-O-methyl nucleotides;

[00214] (b) any pyrimidine nucleotides present in the sense strand (upper strand) are 2'-deoxy-2'-fluoro nucleotides; any purine nucleotides present in the sense strand (upper strand) are independently 2'-deoxyribonucleotides, 2'-O-methyl nucleotides or a combination of 2'-deoxyribonucleotides and 2'-O-methyl nucleotides; and

[00215] (c) any (N) nucleotides are optionally 2'-O-methyl, 2'-deoxy-2'-fluoro, or deoxyribonucleotides.

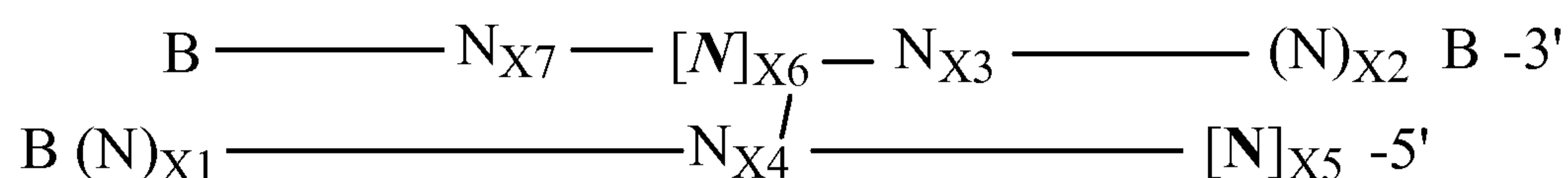
[00216] In one embodiment, the invention features a double stranded nucleic acid or polynucleotide (*e.g.*, siNA) molecule having structure SVII:



SVII

[00217] wherein each N is independently a nucleotide which can be unmodified or chemically modified; each B is a terminal cap moiety that can be present or absent; (N) represents non-base paired or overhanging nucleotides; X1 and X2 are independently integers from about 0 to about 4; X3 is an integer from about 9 to about 30; X4 is an integer from about 11 to about 30; NX3 is complementary to NX4, and any (N) nucleotides are 2'-O-methyl and/or 2'-deoxy-2'-fluoro nucleotides.

[00218] In one embodiment, the invention features a double stranded nucleic acid molecule having structure SVIII:



SVIII

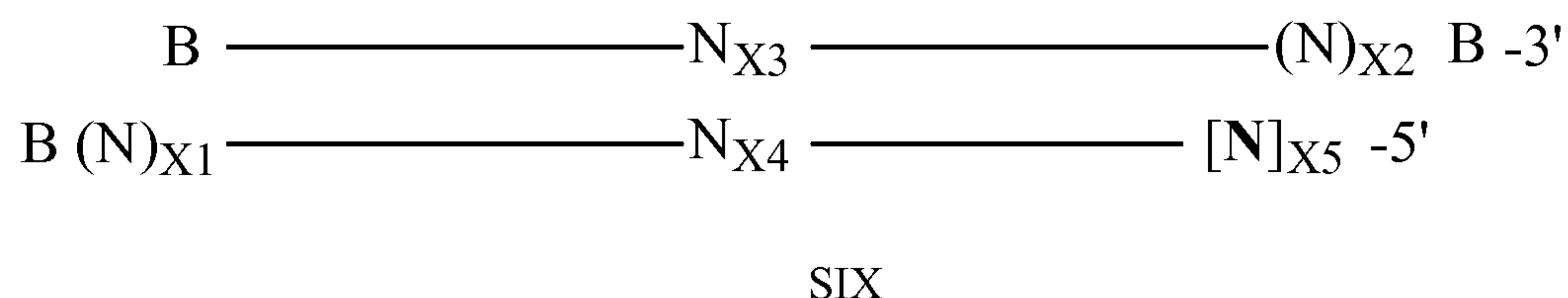
[00219] wherein each N is independently a nucleotide which can be unmodified or chemically modified; each B is a terminal cap moiety that can be present or absent; (N) represents non-base paired or overhanging nucleotides which can be unmodified or chemically modified; [N] represents nucleotide positions comprising sequence that renders the 5'-end of the antisense strand (lower strand) less thermally stable than the 5'-end of the sense strand (upper strand); [N] represents nucleotide positions that are ribonucleotides; X1 and X2 are independently integers from about 0 to about 4; X3 is an integer from about 9 to about 15; X4 is an integer from about 11 to about 30, provided that the sum of X4 and X5 is between 17-36; X5 is an integer from about 1 to about 6; X6 is an integer from about 1 to about 4; X7 is an integer from about 9 to about 15; NX7, NX6, and NX3 are complementary to NX4 and NX5, and

[00220] (a) any pyrimidine nucleotides present in the antisense strand (lower strand) are 2'-deoxy-2'-fluoro nucleotides; any purine nucleotides present in the antisense strand (lower strand) other than the purines nucleotides in the [N] nucleotide positions, are independently 2'-O-methyl nucleotides, 2'-deoxyribonucleotides or a combination of 2'-deoxyribonucleotides and 2'-O-methyl nucleotides;

[00221] (b) any pyrimidine nucleotides present in the sense strand (upper strand) are 2'-deoxy-2'-fluoro nucleotides other than [N] nucleotides; any purine nucleotides present in the sense strand (upper strand) are independently 2'-deoxyribonucleotides, 2'-O-methyl nucleotides or a combination of 2'-deoxyribonucleotides and 2'-O-methyl nucleotides other than [N] nucleotides; and

[00222] (c) any (N) nucleotides are optionally 2'-O-methyl, 2'-deoxy-2'-fluoro, or deoxyribonucleotides.

[00223] In one embodiment, the invention features a double stranded nucleic acid or polynucleotide (*e.g.*, siNA) molecule having structure SIX:



[00224] wherein each N is independently a nucleotide which can be unmodified or chemically modified; each B is a terminal cap moiety that can be present or absent; (N) represents non-base paired or overhanging nucleotides which can be unmodified or chemically modified; [N] represents nucleotide positions that are ribonucleotides; X1 and X2 are independently integers from about 0 to about 4; X3 is an integer from about 9 to about 30; X4 is an integer from about 11 to about 30, provided that the sum of X4 and X5 is between 17-36; X5 is an integer from about 1 to about 6; NX3 is complementary to NX4 and NX5, and

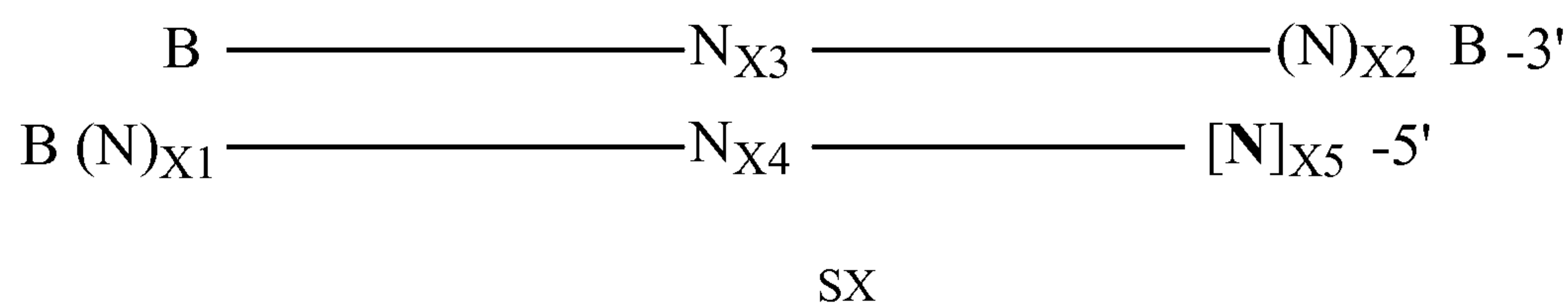
[00225] (a) any pyrimidine nucleotides present in the antisense strand (lower strand) are 2'-deoxy-2'-fluoro nucleotides; any purine nucleotides present in the antisense strand (lower strand) other than the purines nucleotides in the [N] nucleotide positions, are independently 2'-O-methyl nucleotides, 2'-deoxyribonucleotides or a combination of 2'-deoxyribonucleotides and 2'-O-methyl nucleotides;

[00226] (b) any pyrimidine nucleotides present in the sense strand (upper strand) are 2'-deoxy-2'-fluoro nucleotides; any purine nucleotides present in the sense strand (upper

strand) are independently 2'-deoxyribonucleotides, 2'-O-methyl nucleotides or a combination of 2'-deoxyribonucleotides and 2'-O-methyl nucleotides; and

[00227] (c) any (N) nucleotides are optionally 2'-O-methyl, 2'-deoxy-2'-fluoro, or deoxyribonucleotides.

[00228] In one embodiment, the invention features a double stranded nucleic acid or polynucleotide (*e.g.*, siNA) molecule having structure SX:



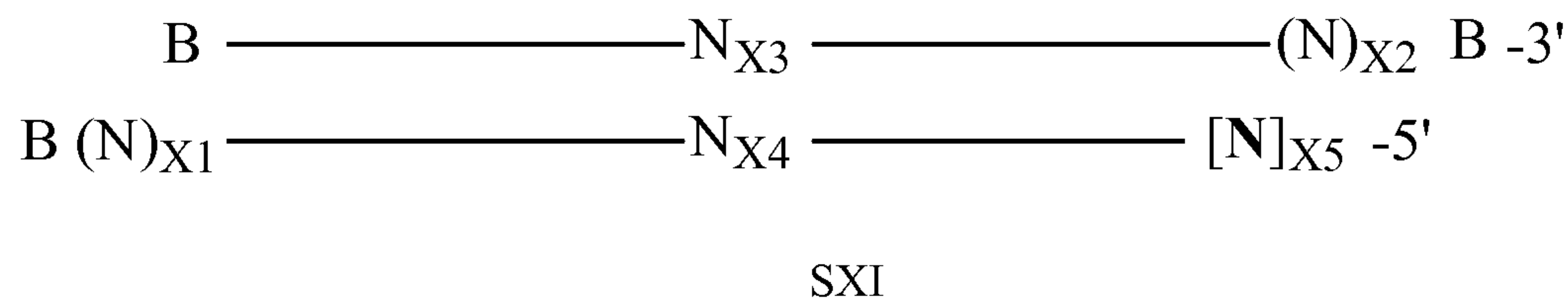
[00229] wherein each N is independently a nucleotide which can be unmodified or chemically modified; each B is a terminal cap moiety that can be present or absent; (N) represents non-base paired or overhanging nucleotides which can be unmodified or chemically modified; [N] represents nucleotide positions that are ribonucleotides; X1 and X2 are independently integers from about 0 to about 4; X3 is an integer from about 9 to about 30; X4 is an integer from about 11 to about 30, provided that the sum of X4 and X5 is between 17-36; X5 is an integer from about 1 to about 6; NX3 is complementary to NX4 and NX5, and

[00230] (a) any pyrimidine nucleotides present in the antisense strand (lower strand) are 2'-deoxy-2'-fluoro nucleotides; any purine nucleotides present in the antisense strand (lower strand) other than the purines nucleotides in the [N] nucleotide positions, are 2'-O-methyl nucleotides;

[00231] (b) any pyrimidine nucleotides present in the sense strand (upper strand) are ribonucleotides; any purine nucleotides present in the sense strand (upper strand) are ribonucleotides; and

[00232] (c) any (N) nucleotides are optionally 2'-O-methyl, 2'-deoxy-2'-fluoro, or deoxyribonucleotides.

[00233] In one embodiment, the invention features a double stranded nucleic acid or polynucleotide (*e.g.*, siNA) molecule having structure SXI:



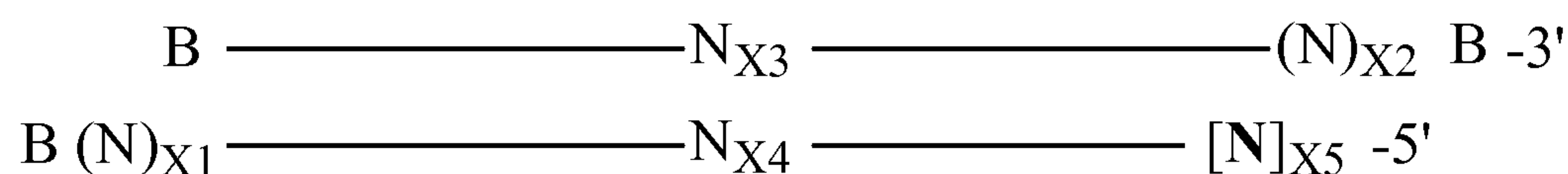
[00234] wherein each N is independently a nucleotide which can be unmodified or chemically modified; each B is a terminal cap moiety that can be present or absent; (N) represents non-base paired or overhanging nucleotides which can be unmodified or chemically modified; [N] represents nucleotide positions that are ribonucleotides; X1 and X2 are independently integers from about 0 to about 4; X3 is an integer from about 9 to about 30; X4 is an integer from about 11 to about 30, provided that the sum of X4 and X5 is between 17-36; X5 is an integer from about 1 to about 6; NX3 is complementary to NX4 and NX5, and

[00235] (a) any pyrimidine nucleotides present in the antisense strand (lower strand) are 2'-deoxy-2'-fluoro nucleotides; any purine nucleotides present in the antisense strand (lower strand) other than the purines nucleotides in the [N] nucleotide positions, are 2'-O-methyl nucleotides;

[00236] (b) any pyrimidine nucleotides present in the sense strand (upper strand) are 2'-deoxy-2'-fluoro nucleotides; any purine nucleotides present in the sense strand (upper strand) are ribonucleotides; and

[00237] (c) any (N) nucleotides are optionally 2'-O-methyl, 2'-deoxy-2'-fluoro, or deoxyribonucleotides.

[00238] In one embodiment, the invention features a double stranded nucleic acid or polynucleotide (*e.g.*, siNA) molecule having structure SXII:



SXII

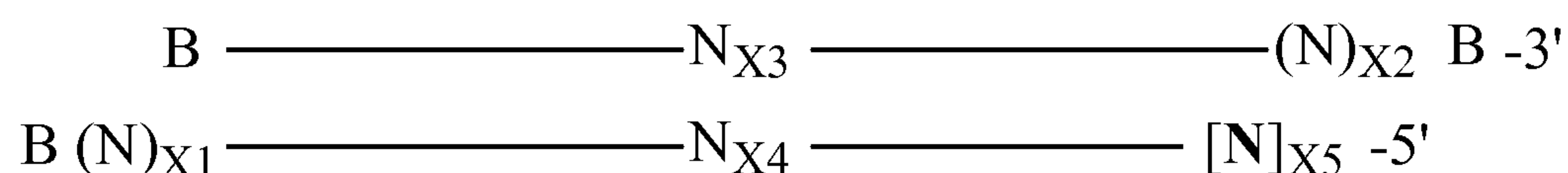
[00239] wherein each N is independently a nucleotide which can be unmodified or chemically modified; each B is a terminal cap moiety that can be present or absent; (N) represents non-base paired or overhanging nucleotides which can be unmodified or chemically modified; [N] represents nucleotide positions that are ribonucleotides; X1 and X2 are independently integers from about 0 to about 4; X3 is an integer from about 9 to about 30; X4 is an integer from about 11 to about 30, provided that the sum of X4 and X5 is between 17-36; X5 is an integer from about 1 to about 6; NX3 is complementary to NX4 and NX5, and

[00240] (a) any pyrimidine nucleotides present in the antisense strand (lower strand) are 2'-deoxy-2'-fluoro nucleotides; any purine nucleotides present in the antisense strand (lower strand) other than the purines nucleotides in the [N] nucleotide positions, are 2'-O-methyl nucleotides;

[00241] (b) any pyrimidine nucleotides present in the sense strand (upper strand) are 2'-deoxy-2'-fluoro nucleotides; any purine nucleotides present in the sense strand (upper strand) are deoxyribonucleotides; and

[00242] (c) any (N) nucleotides are optionally 2'-O-methyl, 2'-deoxy-2'-fluoro, or deoxyribonucleotides.

[00243] In one embodiment, the invention features a double stranded nucleic acid or polynucleotide (*e.g.*, siNA) molecule having structure SXIII:



SXIII

[00244] wherein each N is independently a nucleotide which can be unmodified or chemically modified; each B is a terminal cap moiety that can be present or absent; (N) represents non-base paired or overhanging nucleotides which can be unmodified or chemically modified; [N] represents nucleotide positions that are ribonucleotides; X1

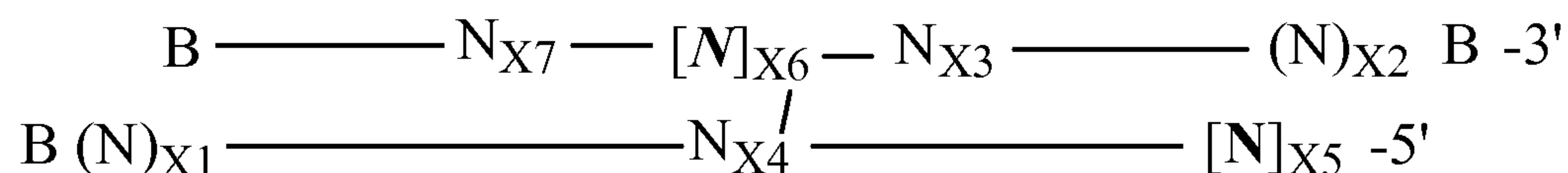
and X2 are independently integers from about 0 to about 4; X3 is an integer from about 9 to about 30; X4 is an integer from about 11 to about 30, provided that the sum of X4 and X5 is between 17-36; X5 is an integer from about 1 to about 6; NX3 is complementary to NX4 and NX5, and

[00245] (a) any pyrimidine nucleotides present in the antisense strand (lower strand) are nucleotides having a ribo-like configuration (e.g., Northern or A-form helix configuration); any purine nucleotides present in the antisense strand (lower strand) other than the purines nucleotides in the [N] nucleotide positions, are 2'-O-methyl nucleotides;

[00246] (b) any pyrimidine nucleotides present in the sense strand (upper strand) are nucleotides having a ribo-like configuration (e.g., Northern or A-form helix configuration); any purine nucleotides present in the sense strand (upper strand) are 2'-O-methyl nucleotides; and

[00247] (c) any (N) nucleotides are optionally 2'-O-methyl, 2'-deoxy-2'-fluoro, or deoxyribonucleotides.

[00248] In one embodiment, the invention features a double stranded nucleic acid or polynucleotide (e.g., siNA) molecule having structure SXIV:



SXIV

[00249] wherein each N is independently a nucleotide which can be unmodified or chemically modified; each B is a terminal cap moiety that can be present or absent; (N) represents non-base paired or overhanging nucleotides which can be unmodified or chemically modified; [N] represents nucleotide positions that are ribonucleotides; [N] represents nucleotide positions that are ribonucleotides; X1 and X2 are independently integers from about 0 to about 4; X3 is an integer from about 9 to about 15; X4 is an integer from about 11 to about 30, provided that the sum of X4 and X5 is between 17-36; X5 is an integer from about 1 to about 6; X6 is an integer from about 1 to about 4;

X7 is an integer from about 9 to about 15; NX7, NX6, and NX3 are complementary to NX4 and NX5, and

[00250] (a) any pyrimidine nucleotides present in the antisense strand (lower strand) are 2'-deoxy-2'-fluoro nucleotides; any purine nucleotides present in the antisense strand (lower strand) other than the purines nucleotides in the [N] nucleotide positions, are independently 2'-O-methyl nucleotides, 2'-deoxyribonucleotides or a combination of 2'-deoxyribonucleotides and 2'-O-methyl nucleotides;

[00251] (b) any pyrimidine nucleotides present in the sense strand (upper strand) are 2'-deoxy-2'-fluoro nucleotides other than [N] nucleotides; any purine nucleotides present in the sense strand (upper strand) are independently 2'-deoxyribonucleotides, 2'-O-methyl nucleotides or a combination of 2'-deoxyribonucleotides and 2'-O-methyl nucleotides other than [N] nucleotides; and

[00252] (c) any (N) nucleotides are optionally 2'-O-methyl, 2'-deoxy-2'-fluoro, or deoxyribonucleotides.

[00253] In one embodiment, a double stranded nucleic acid or polynucleotide (*e.g.*, siNA) molecule having any of structure SI, SII, SIII, SIV, SV, SVI, SVII, SVIII, SIX, SX, SXI, SXII, SXIII, or SXIV comprises a terminal phosphate group at the 5'-end of the antisense strand or antisense region of the nucleic acid molecule.

[00254] In one embodiment, a double stranded nucleic acid or polynucleotide (*e.g.*, siNA) molecule having any of structure SI, SII, SIII, SIV, SV, SVI, SVII, SVIII, SIX, SX, SXI, SXII, SXIII, or SXIV comprises X5 = 1, 2, or 3; each X1 and X2 = 1 or 2; X3 = 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, or 30, and X4 = 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, or 30.

[00255] In one embodiment, a double stranded nucleic acid or polynucleotide (*e.g.*, siNA) molecule having any of structure SI, SII, SIII, SIV, SV, SVI, SVII, SVIII, SIX, SX, SXI, SXII, SXIII, or SXIV comprises X5 = 1; each X1 and X2 = 2; X3 = 19, and X4 = 18.

[00256] In one embodiment, a double stranded nucleic acid or polynucleotide (*e.g.*, siNA) molecule having any of structure SI, SII, SIII, SIV, SV, SVI, SVII, SVIII, SIX, SX, SXI, SXII, SXIII, or SXIV comprises $X5 = 2$; each $X1$ and $X2 = 2$; $X3 = 19$, and $X4 = 17$

[00257] In one embodiment, a double stranded nucleic acid or polynucleotide (*e.g.*, siNA) molecule having any of structure SI, SII, SIII, SIV, SV, SVI, SVII, SVIII, SIX, SX, SXI, SXII, SXIII, or SXIV comprises $X5 = 3$; each $X1$ and $X2 = 2$; $X3 = 19$, and $X4 = 16$.

[00258] In one embodiment, a double stranded nucleic acid or polynucleotide (*e.g.*, siNA) molecule having any of structure SI, SII, SIII, SIV, SV, SVI, SVII, SVIII, SIX, SX, SXI, SXII, SXIII, or SXIV comprises B at the 3' and 5' ends of the sense strand or sense region.

[00259] In one embodiment, a double stranded nucleic acid or polynucleotide (*e.g.*, siNA) molecule having any of structure SI, SII, SIII, SIV, SV, SVI, SVII, SVIII, SIX, SX, SXI, SXII, SXIII, or SXIV comprises B at the 3'-end of the antisense strand or antisense region.

[00260] In one embodiment, a double stranded nucleic acid or polynucleotide (*e.g.*, siNA) molecule having any of structure SI, SII, SIII, SIV, SV, SVI, SVII, SVIII, SIX, SX, SXI, SXII, SXIII, or SXIV comprises B at the 3' and 5' ends of the sense strand or sense region and B at the 3'-end of the antisense strand or antisense region.

[00261] In one embodiment, a double stranded nucleic acid or polynucleotide (*e.g.*, siNA) molecule having any of structure SI, SII, SIII, SIV, SV, SVI, SVII, SVIII, SIX, SX, SXI, SXII, SXIII, or SXIV further comprises one or more phosphorothioate internucleotide linkages at the first terminal (N) on the 3'end of the sense strand, antisense strand, or both sense strand and antisense strands of the nucleic acid molecule. For example, a double stranded nucleic acid molecule can comprise $X1$ and/or $X2 = 2$ having overhanging nucleotide positions with a phosphorothioate internucleotide linkage, *e.g.*, (NsN) where "s" indicates phosphorothioate.

[00262] In one embodiment, a double stranded nucleic acid or polynucleotide (*e.g.*, siNA) molecule having any of structure SI, SII, SIII, SIV, SV, SVI, SVII, SVIII, SIX, SX, SXI, SXII, SXIII, or SXIV comprises (N) nucleotides that are 2'-O-methyl nucleotides.

[00263] In one embodiment, a double stranded nucleic acid or polynucleotide (*e.g.*, siNA) molecule having any of structure SI, SII, SIII, SIV, SV, SVI, SVII, SVIII, SIX, SX, SXI, SXII, SXIII, or SXIV comprises (N) nucleotides that are 2'-deoxy nucleotides.

[00264] In one embodiment, a double stranded nucleic acid or polynucleotide (*e.g.*, siNA) molecule having any of structure SI, SII, SIII, SIV, SV, SVI, SVII, SVIII, SIX, SX, SXI, SXII, SXIII, or SXIV comprises (N) nucleotides in the antisense strand (lower strand) that are complementary to nucleotides in a target polynucleotide sequence (*e.g.*, PCSK9 target and/or PCSK9 pathway target sequence) having complementary to the N and [N] nucleotides of the antisense (lower) strand.

[00265] In one embodiment, a double stranded nucleic acid or polynucleotide (*e.g.*, siNA) molecule having any of structure SI, SII, SIII, SIV, SV, SVI, SVII, SVIII, SIX, SX, SXI, SXII, SXIII, or SXIV comprises (N) nucleotides in the sense strand (upper strand) that comprise a contiguous nucleotide sequence of about 15 to about 30 nucleotides of a target polynucleotide sequence (*e.g.*, PCSK9 target and/or PCSK9 pathway target sequence).

[00266] In one embodiment, a double stranded nucleic acid or polynucleotide (*e.g.*, siNA) molecule having any of structure SI, SII, SIII, SIV, SV, SVI, SVII, SVIII, SIX, SX, SXI, SXII, SXIII, or SXIV comprises (N) nucleotides in the sense strand (upper strand) that comprise nucleotide sequence corresponding a target polynucleotide sequence (*e.g.*, PCSK9 target and/or PCSK9 pathway target sequence) having complementary to the antisense (lower) strand such that the contiguous (N) and N nucleotide sequence of the sense strand comprises nucleotide sequence of the target nucleic acid sequence (*e.g.*, PCSK9 target and/or PCSK9 pathway target sequence).

[00267] In one embodiment, a double stranded nucleic acid or polynucleotide (*e.g.*, siNA) molecule having any of structure SVIII or SXIV comprises B only at the 5'-end of the sense (upper) strand of the double stranded nucleic acid molecule.

[00268] In one embodiment, a double stranded nucleic acid or polynucleotide (*e.g.*, siNA) molecule having any of structure SI, SII, SIII, SIV, SV, SVI, SVII, SVIII, SIX, SX, SXI, SXII, SXIII, or SXIV further comprises an unpaired terminal nucleotide at the 5'-end of the antisense (lower) strand. The unpaired nucleotide is not complementary to the sense (upper) strand. In one embodiment, the unpaired terminal nucleotide is complementary to a target polynucleotide sequence having complementary to the N and [N] nucleotides of the antisense (lower) strand. In another embodiment, the unpaired terminal nucleotide is not complementary to a target polynucleotide sequence having complementary to the N and [N] nucleotides of the antisense (lower) strand.

[00269] In one embodiment, a double stranded nucleic acid or polynucleotide (*e.g.*, siNA) molecule having any of structure SVIII or SXIV comprises $X6 = 1$ and $X3 = 10$.

[00270] In one embodiment, a double stranded nucleic acid molecule having any of structure SVIII or SXIV comprises $X6 = 2$ and $X3 = 9$.

[00271] In one embodiment, the invention features a composition comprising a double stranded nucleic acid or polynucleotide (*e.g.*, siNA) molecule or RNAi inhibitor formulated as any of formulation shown in Table VI, for example LNP-051; LNP-053; LNP-054; LNP-069; LNP-073; LNP-077; LNP-080; LNP-082; LNP-083; LNP-060; LNP-061; LNP-086; LNP-097; LNP-098; LNP-099; LNP-100; LNP-101; LNP-102; LNP-103; or LNP-104 (see Table VI).

[00272] In one embodiment, the invention features a composition comprising a first double stranded nucleic acid and a second double stranded nucleic acid molecule each having a first strand and a second strand that are complementary to each other, wherein the second strand of the first double stranded nucleic acid molecule comprises sequence complementary to a first target sequence and the second strand of the second double stranded nucleic acid molecule comprises sequence complementary to a second target or pathway target sequence(*e.g.*, PCSK9). In one embodiment, the composition further

comprises a cationic lipid, a neutral lipid, and a polyethyleneglycol-conjugate. In one embodiment, the composition further comprises a cationic lipid, a neutral lipid, a polyethyleneglycol-conjugate, and a cholesterol. In one embodiment, the composition further comprises a polyethyleneglycol-conjugate, a cholesterol, and a surfactant. In one embodiment, the cationic lipid is selected from the group consisting of CLinDMA, pCLinDMA, eCLinDMA, DMOBA, and DMLBA. In one embodiment, the neutral lipid is selected from the group consisting of DSPC, DOBA, and cholesterol. In one embodiment, the polyethyleneglycol-conjugate is selected from the group consisting of a PEG-dimyristoyl glycerol and PEG-cholesterol. In one embodiment, the PEG is 2KPEG. In one embodiment, the surfactant is selected from the group consisting of palmityl alcohol, stearyl alcohol, oleyl alcohol and linoleyl alcohol. In one embodiment, the cationic lipid is CLinDMA, the neutral lipid is DSPC, the polyethylene glycol conjugate is 2KPEG-DMG, the cholesterol is cholesterol, and the surfactant is linoleyl alcohol. In one embodiment, the CLinDMA, the DSPC, the 2KPEG-DMG, the cholesterol, and the linoleyl alcohol are present in molar ratio of 43:38:10:2:7 respectively.

[00273] In any of the embodiments herein, the nucleic acid or polynucleotide (*e.g.*, siNA) molecule of the invention modulates expression of one or more targets via RNA interference or the inhibition of RNA interference. In one embodiment, the RNA interference is RISC mediated cleavage of the target (*e.g.*, siRNA mediated RNA interference). In one embodiment, the RNA interference is translational inhibition of the target (*e.g.*, miRNA mediated RNA interference). In one embodiment, the RNA interference is transcriptional inhibition of the target (*e.g.*, siRNA mediated transcriptional silencing). In one embodiment, the RNA interference takes place in the cytoplasm. In one embodiment, the RNA interference takes place in the nucleus.

[00274] In any of the embodiments herein, the nucleic acid or polynucleotide (*e.g.*, siNA) molecule of the invention modulates expression of one or more targets via inhibition of an endogenous target RNA, such as an endogenous mRNA, siRNA, miRNA, or alternately through inhibition of RISC.

[00275] In one embodiment, the invention features one or more RNAi inhibitors that modulate the expression of one or more gene targets by miRNA inhibition, siRNA inhibition, or RISC inhibition.

[00276] In one embodiment, a RNAi inhibitor of the invention is a nucleic acid or polynucleotide (*e.g.*, siNA) molecule as described herein that has one or more strands that are complementary to one or more target miRNA or siRNA molecules.

[00277] In one embodiment, the RNAi inhibitor of the invention is an antisense molecule that is complementary to a target miRNA or siRNA molecule or a portion thereof. An antisense RNAi inhibitor of the invention can be of length of about 10 to about 40 nucleotides in length (*e.g.*, 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, or 40 nucleotides in length). An antisense RNAi inhibitor of the invention can comprise one or more modified nucleotides or non-nucleotides as described herein (see for example molecules having any of Formulae I-VII herein or any combination thereof). In one embodiment, an antisense RNAi inhibitor of the invention can comprise one or more or all 2'-O-methyl nucleotides. In one embodiment, an antisense RNAi inhibitor of the invention can comprise one or more or all 2'-deoxy-2'-fluoro nucleotides. In one embodiment, an antisense RNAi inhibitor of the invention can comprise one or more or all 2'-O-methoxy-ethyl (also known as 2'-methoxyethoxy or MOE) nucleotides. In one embodiment, an antisense RNAi inhibitor of the invention can comprise one or more or all phosphorothioate internucleotide linkages. In one embodiment, an antisense RNA inhibitor of the invention can comprise a terminal cap moiety at the 3'-end, the 5'-end, or both the 5' and 3' ends of the the antisense RNA inhibitor.

[00278] In one embodiment, a RNAi inhibitor of the invention is a nucleic acid aptamer having binding affinity for RISC, such as a regulatable aptamer (see for example An et al., 2006, RNA, 12:710-716). An aptamer RNAi inhibitor of the invention can be of length of about 10 to about 50 nucleotides in length (*e.g.*, 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, or 50 nucleotides in length). An aptamer RNAi inhibitor of the invention can comprise one or more modified nucleotides or non-nucleotides as described herein (see for example molecules having any of

Formulae I-VII herein or any combination thereof). In one embodiment, an aptamer RNAi inhibitor of the invention can comprise one or more or all 2'-O-methyl nucleotides. In one embodiment, an aptamer RNAi inhibitor of the invention can comprise one or more or all 2'-deoxy-2'-fluoro nucleotides. In one embodiment, an aptamer RNAi inhibitor of the invention can comprise one or more or all 2'-O-methoxyethyl (also known as 2'-methoxyethoxy or MOE) nucleotides. In one embodiment, an aptamer RNAi inhibitor of the invention can comprise one or more or all phosphorothioate internucleotide linkages. In one embodiment, an aptamer RNA inhibitor of the invention can comprise a terminal cap moiety at the 3'-end, the 5'-end, or both the 5' and 3' ends of the the aptamer RNA inhibitor.

[00279] In one embodiment, the invention features a method for modulating the expression of a target gene within a cell comprising: (a) synthesizing a siNA molecule of the invention, which can be chemically-modified or unmodified, wherein one of the siNA strands comprises a sequence complementary to RNA of the target; and (b) introducing the siNA molecule into a cell under conditions suitable to modulate (e.g., inhibit) the expression of the target gene in the cell.

[00280] In one embodiment, the invention features a method for modulating the expression of a target gene within a cell comprising: (a) synthesizing a siNA molecule of the invention, which can be chemically-modified or unmodified, wherein one of the siNA strands comprises a sequence complementary to RNA of the target gene and wherein the sense strand sequence of the siNA comprises a sequence identical or substantially similar to the sequence of the target RNA; and (b) introducing the siNA molecule into a cell under conditions suitable to modulate (e.g., inhibit) the expression of the target gene in the cell.

[00281] In another embodiment, the invention features a method for modulating the expression of more than one target gene within a cell comprising: (a) synthesizing siNA molecules of the invention, which can be chemically-modified or unmodified, wherein one of the siNA strands comprises a sequence complementary to RNA of the target genes; and (b) introducing the siNA molecules into a cell under conditions suitable to modulate (e.g., inhibit) the expression of the target genes in the cell.

[00282] In another embodiment, the invention features a method for modulating the expression of two or more target genes within a cell comprising: (a) synthesizing one or more siNA molecules of the invention, which can be chemically-modified or unmodified, wherein the siNA strands comprise sequences complementary to RNA of the target genes and wherein the sense strand sequences of the siNAs comprise sequences identical or substantially similar to the sequences of the target RNAs; and (b) introducing the siNA molecules into a cell under conditions suitable to modulate (e.g., inhibit) the expression of the target genes in the cell.

[00283] In another embodiment, the invention features a method for modulating the expression of more than one target gene within a cell comprising: (a) synthesizing a siNA molecule of the invention, which can be chemically-modified or unmodified, wherein one of the siNA strands comprises a sequence complementary to RNA of the target gene and wherein the sense strand sequence of the siNA comprises a sequence identical or substantially similar to the sequences of the target RNAs; and (b) introducing the siNA molecule into a cell under conditions suitable to modulate (e.g., inhibit) the expression of the target genes in the cell.

[00284] In another embodiment, the invention features a method for modulating the expression of a target and corresponding modulators gene within a cell comprising: (a) synthesizing a siNA molecule of the invention, which can be chemically-modified or unmodified, wherein one of the siNA strands comprises a sequence complementary to RNA of the target and corresponding modulators gene, wherein the sense strand sequence of the siNA comprises a sequence identical or substantially similar to the sequences of the target RNAs; and (b) introducing the siNA molecule into a cell under conditions suitable to modulate (e.g., inhibit) the expression of the target and corresponding modulators in the cell.

[00285] In one embodiment, nucleic acid or polynucleotide (e.g., siNA) molecules of the invention are used as reagents in ex vivo applications. For example, siNA reagents are introduced into tissue or cells that are transplanted into a subject for therapeutic effect. The cells and/or tissue can be derived from an organism or subject that later receives the explant, or can be derived from another organism or subject prior to transplantation. The siNA molecules can be used to modulate the expression of one or

more genes in the cells or tissue, such that the cells or tissue obtain a desired phenotype or are able to perform a function when transplanted in vivo. In one embodiment, certain target cells from a patient are extracted. These extracted cells are contacted with siNAs targeting a specific nucleotide sequence within the cells under conditions suitable for uptake of the siNAs by these cells (e.g. using delivery reagents such as cationic lipids, liposomes and the like or using techniques such as electroporation to facilitate the delivery of siNAs into cells). The cells are then reintroduced back into the same patient or other patients.

[00286] In one embodiment, the invention features a method of modulating the expression of a target gene in a tissue explant comprising: (a) synthesizing a siNA molecule of the invention, which can be chemically-modified, wherein one of the siNA strands comprises a sequence complementary to RNA of the target gene; and (b) introducing the siNA molecule into a cell of the tissue explant derived from a particular organism under conditions suitable to modulate (e.g., inhibit) the expression of the target gene in the tissue explant. In another embodiment, the method further comprises introducing the tissue explant back into the organism the tissue was derived from or into another organism under conditions suitable to modulate (e.g., inhibit) the expression of the target gene in that organism.

[00287] In one embodiment, the invention features a method of modulating the expression of a target gene in a tissue explant comprising: (a) synthesizing a siNA molecule of the invention, which can be chemically-modified, wherein one of the siNA strands comprises a sequence complementary to RNA of the target gene and wherein the sense strand sequence of the siNA comprises a sequence identical or substantially similar to the sequence of the target RNA; and (b) introducing the siNA molecule into a cell of the tissue explant derived from a particular organism under conditions suitable to modulate (e.g., inhibit) the expression of the target gene in the tissue explant. In another embodiment, the method further comprises introducing the tissue explant back into the organism the tissue was derived from or into another organism under conditions suitable to modulate (e.g., inhibit) the expression of the target gene in that organism.

[00288] In another embodiment, the invention features a method of modulating the expression of more than one target gene in a tissue explant comprising: (a) synthesizing

siNA molecules of the invention, which can be chemically-modified, wherein one of the siNA strands comprises a sequence complementary to RNA of the target genes; and (b) introducing the siNA molecules into a cell of the tissue explant derived from a particular organism under conditions suitable to modulate (e.g., inhibit) the expression of the target genes in the tissue explant. In another embodiment, the method further comprises introducing the tissue explant back into the organism the tissue was derived from or into another organism under conditions suitable to modulate (e.g., inhibit) the expression of the target genes in that organism.

[00289] In one embodiment, the invention features a method of modulating the expression of a target gene in a subject or organism comprising: (a) synthesizing a siNA molecule of the invention, which can be chemically-modified, wherein one of the siNA strands comprises a sequence complementary to RNA of the target gene; and (b) introducing the siNA molecule into the subject or organism under conditions suitable to modulate (e.g., inhibit) the expression of the target gene in the subject or organism. The level of target protein or RNA can be determined using various methods well-known in the art.

[00290] In another embodiment, the invention features a method of modulating the expression of more than one target gene in a subject or organism comprising: (a) synthesizing siNA molecules of the invention, which can be chemically-modified, wherein one of the siNA strands comprises a sequence complementary to RNA of the target; and (b) introducing the siNA molecules into the subject or organism under conditions suitable to modulate (e.g., inhibit) the expression of the target genes in the subject or organism. The level of target protein or RNA can be determined as is known in the art.

[00291] In one embodiment, the invention features a method for modulating the expression of a target gene within a cell (e.g., a liver or adipose tissue cell) comprising: (a) synthesizing a siNA molecule of the invention, which can be chemically-modified, wherein the siNA comprises a single stranded sequence having complementarity to RNA of the target gene; and (b) introducing the siNA molecule into a cell under conditions suitable to modulate (e.g., inhibit) the expression of the PCSK9 gene in the cell.

[00292] In another embodiment, the invention features a method for modulating the expression of more than one target gene within a cell (e.g., a liver or adipose tissue cell) comprising: (a) synthesizing siNA molecules of the invention, which can be chemically-modified, wherein the siNA comprises a single stranded sequence having complementarity to RNA of the target gene; and (b) contacting the cell in vitro or in vivo with the siNA molecule under conditions suitable to modulate (e.g., inhibit) the expression of the target genes in the cell.

[00293] In one embodiment, the invention features a method of modulating the expression of a target gene in a tissue explant (e.g., liver or any other organ, tissue or cell as can be transplanted from one organism to another or back to the same organism from which the organ, tissue or cells is derived) comprising: (a) synthesizing a siNA molecule of the invention, which can be chemically-modified, wherein the siNA comprises a single stranded sequence having complementarity to RNA of the target gene; and (b) contacting a cell of the tissue explant derived from a particular subject or organism with the siNA molecule under conditions suitable to modulate (e.g., inhibit) the expression of the target gene in the tissue explant. In another embodiment, the method further comprises introducing the tissue explant back into the subject or organism the tissue was derived from or into another subject or organism under conditions suitable to modulate (e.g., inhibit) the expression of the target gene in that subject or organism.

[00294] In another embodiment, the invention features a method of modulating the expression of more than one target gene in a tissue explant (e.g., liver or any other organ, tissue or cell as can be transplanted from one organism to another or back to the same organism from which the organ, tissue or cell is derived) comprising: (a) synthesizing siNA molecules of the invention, which can be chemically-modified, wherein the siNA comprises a single stranded sequence having complementarity to RNA of the target gene; and (b) introducing the siNA molecules into a cell of the tissue explant derived from a particular subject or organism under conditions suitable to modulate the expression of the target genes in the tissue explant. In another embodiment, the method further comprises introducing the tissue explant back into the subject or organism the tissue was derived from or into another subject or organism under conditions suitable to modulate (e.g., inhibit) the expression of the target genes in that subject or organism.

[00295] In one embodiment, the invention features a method of modulating the expression of a target gene (e.g., PCSK9) in a tissue explant (e.g., skin, hair, lung, or any other tissue or cell as can be transplanted from one organism to another or back to the same organism from which the tissue or cell is derived) comprising: (a) synthesizing a siNA molecule of the invention, which can be chemically-modified, wherein the siNA comprises a single stranded sequence having complementarity to RNA of the target gene; and (b) contacting a cell of the tissue explant derived from a particular subject or organism with the siNA molecule under conditions suitable to modulate (e.g., inhibit) the expression of the PCSK9 target gene in the tissue explant. In another embodiment, the method further comprises introducing the tissue explant back into the subject or organism the tissue was derived from or into another subject or organism under conditions suitable to modulate (e.g., inhibit) the expression of the PCSK9 target gene in that subject or organism.

[00296] In another embodiment, the invention features a method of modulating the expression of more than one target gene (e.g., PCSK9) in a tissue explant (e.g., skin, hair, lung, or any other tissue or cell as can be transplanted from one organism to another or back to the same organism from which the tissue or cell is derived) comprising: (a) synthesizing siNA molecules of the invention, which can be chemically-modified, wherein the siNA comprises a single stranded sequence having complementarity to RNA of the target gene; and (b) introducing the siNA molecules into a cell of the tissue explant derived from a particular subject or organism under conditions suitable to modulate (e.g., inhibit) the expression of the target genes in the tissue explant. In another embodiment, the method further comprises introducing the tissue explant back into the subject or organism the tissue was derived from or into another subject or organism under conditions suitable to modulate (e.g., inhibit) the expression of the target genes in that subject or organism.

[00297] In one embodiment, the invention features a method of modulating the expression of a target gene in a subject or organism comprising: (a) synthesizing a siNA molecule of the invention, which can be chemically-modified, wherein the siNA comprises a single stranded sequence having complementarity to RNA of the target gene; and (b) introducing the siNA molecule into the subject or organism under

conditions suitable to modulate (e.g., inhibit) the expression of the target gene in the subject or organism.

[00298] In another embodiment, the invention features a method of modulating the expression of more than one target gene in a subject or organism comprising: (a) synthesizing siNA molecules of the invention, which can be chemically-modified, wherein the siNA comprises a single stranded sequence having complementarity to RNA of the target gene; and (b) introducing the siNA molecules into the subject or organism under conditions suitable to modulate (e.g., inhibit) the expression of the target genes in the subject or organism.

[00299] In one embodiment, the invention features a method of modulating the expression of a target gene in a subject or organism comprising contacting the subject or organism with a nucleic acid or polynucleotide (e.g., siNA) molecule of the invention under conditions suitable to modulate the expression of the target gene in the subject or organism.

[00300] In another embodiment, the invention features a method of modulating the expression of more than one target gene in a subject or organism comprising contacting the subject or organism with one or more nucleic acid or polynucleotide (e.g., siNA) molecules of the invention under conditions suitable to modulate (e.g., inhibit) the expression of the target genes in the subject or organism.

[00301] In one embodiment, the invention features a method of modulating the expression of a target gene (e.g., PCSK9) in a subject or organism comprising: (a) synthesizing a siNA molecule of the invention, which can be chemically-modified, wherein the siNA comprises a single stranded sequence having complementarity to RNA of the PCSK9 gene; and (b) introducing the siNA molecule into the subject or organism under conditions suitable to modulate (e.g., inhibit) the expression of the target gene in the subject or organism.

[00302] In another embodiment, the invention features a method of modulating the expression of more than one target gene (e.g., PCSK9) in a subject or organism comprising: (a) synthesizing siNA molecules of the invention, which can be chemically-modified, wherein the siNA comprises a single stranded sequence having

complementarity to RNA of the PCSK9 gene; and (b) introducing the siNA molecules into the subject or organism under conditions suitable to modulate (e.g., inhibit) the expression of the target genes in the subject or organism.

[00303] In one embodiment, the invention features a method of modulating the expression of a target gene (e.g., PCSK9) in a subject or organism comprising contacting the subject or organism with a nucleic acid or polynucleotide (e.g., siNA) molecule of the invention under conditions suitable to modulate (e.g., inhibit) the expression of the target gene in the subject or organism.

VII. Delivery of Nucleic Acid based RNAi Constructs

[00304] Suitable methods for nucleic acid delivery to effect RNAi according to the present invention are believed to include virtually any method by which a nucleic acid (e.g., DNA, RNA, including viral and nonviral vectors) can be introduced into an organelle, a cell, a tissue or an organism, as described herein or as would be known to one of ordinary skill in the art. Such methods include, but are not limited to, direct delivery of DNA such as by injection (U.S. Pat. Nos. 5,994,624; 5,981,274; 5,945,100; 5,780,448; 5,736,524; 5,702,932; 5,656,610; 5,589,466, and 5,580,859, each incorporated herein by reference), including microinjection (Harlan and Weintraub, 1985; U.S. Pat. No. 5,789,215, incorporated herein by reference); by electroporation (U.S. Pat. No. 5,384,253, incorporated herein by reference); by calcium phosphate precipitation (Graham and Van Der Eb, 1973; Chen and Okayama, 1987; Rippe et al., 1990); by using DEAE-dextran followed by polyethylene glycol (Gopal, 1985); by direct sonic loading (Fechheimer et al., 1987); by liposome mediated transfection (Nicolau and Sene, 1982; Fraley et al., 1979; Nicolau et al., 1987; Wong et al., 1980; Kaneda et al., 1989; Kato et al., 1991); by microprojectile bombardment (PCT Application Nos. WO 94/09699 and 95/06128; U.S. Pat. Nos. 5,610,042; 5,322,783; 5,563,055; 5,550,318; 5,538,877 and 5,538,880, each incorporated herein by reference); by agitation with silicon carbide fibers (Kaeppeler et al., 1990; U.S. Pat. Nos. 5,302,523 and 5,464,765, each incorporated herein by reference); by Agrobacterium-mediated transformation (U.S. Pat. Nos. 5,591,616 and 5,563,055, each incorporated herein by reference); or by PEG-mediated transformation of protoplasts (Omirulleh et al., 1993; U.S. Pat. Nos. 4,684,611 and 4,952,500, each incorporated herein by reference); by desiccation/inhibition-

mediated DNA uptake (Potrykus et al., 1985). Through the application of techniques such as these, organelle(s), cell(s), tissue(s) or organism(s) may be stably or transiently transformed.

[00305] There are a number of ways in which expression vectors may be introduced into cells to generate dsRNA. In certain embodiments of the invention, the expression vector comprises a virus or engineered vector derived from a viral genome, while in other embodiments, it is a nonviral vector. Other expression systems are also readily available.

[00306] It will be appreciated by those skilled in the art that effective use of RNAi in humans and other mammals for such purposes as prevention and therapy of infections and other diseases and conditions will be enhanced by efficient delivery of RNAi-inducing entities, e.g., siRNA, shRNA or RNAi inducing vectors ((i.e., vectors whose presence within a cell results in production of an siRNA or shRNA) to cells in which inhibition of a transcript is desired. For use in humans, it may be preferable to employ non-viral methods that facilitate intracellular uptake of RNAi-inducing entities such as siRNA, shRNA, or RNAi-inducing vectors (e.g., DNA vectors).

[00307] In accordance therewith, an embodiment of the invention provides compositions comprising any of a variety of non-viral delivery agents for enhanced delivery of siRNA, shRNA, and/or RNAi-inducing vectors to cells. While it is anticipated that the delivery agents described herein will primarily be used to enhance delivery of RNA or DNA rather than intact virus, their use for the latter purpose is not excluded.

[00308] As used herein, the broad concept of "delivery" includes transport of an RNAi-inducing entity such as an siRNA, shRNA, or RNAi-inducing vector from its site of entry into the body to the location of the cells in which it is to function, in addition to cellular uptake of the entity and any subsequent steps involved in making siRNA or shRNA available to the intracellular RNAi machinery (e.g., release of siRNA or shRNA from endosomes). In general, the delivery agents described herein serve as a vehicle or carrier for delivery of the RNA or vector, facilitate one or more steps in the process of

making the siRNA or shRNA available to the intracellular RNAi machinery, and/or help to protect or stabilize the RNAi-inducing entity within the body.

[00309] In one embodiment, the invention provides compositions comprising one or more RNAi-inducing entities targeted to a PCSK9 mRNA transcript and any of a variety of delivery agents including, but not limited to, cationic polymers, modified cationic polymers, peptide molecular transporters (including arginine or histidine-rich peptides), lipids (including cationic lipids, neutral lipids, and combinations thereof), liposomes, lipopolyplexes, non-cationic polymers, modified non-cationic polymers, chloroquine, bupivacaine, and surfactants suitable for introduction into a target tissue or cell. Certain of the delivery agents are modified to incorporate a moiety that increases delivery or increases the selective delivery of the one or more RNAi-inducing entity to cells in which it is desired to inhibit a particular transcript.

[00310] In certain embodiments of the invention a disease or condition, or a symptom thereof, is associated with, characterized by, or features aberrant PCSK9 mRNA expression, e.g., mutant PCSK9 or aberrant functional activity of a polypeptide encoded by the mRNA transcript. In preferred embodiments of the invention, administration of the RNAi-inducing composition inhibits expression of the target mRNA transcript, e.g., PCSK9 mRNA, thereby treating the disease. In certain embodiments, the administration of the RNAi-inducing compositions inhibits PCSK9 mRNA transcript thereby lowering LDL expression relative to before administration of the composition. Thus, in another embodiment, the invention provides a method of treating a disease or condition, mediated or related to or will respond to the levels of PCSK9 in a cell or tissue comprising the step of administering a composition comprising an RNAi-inducing entity, wherein the RNAi-inducing entity is targeted to the PCSK9 mRNA transcript and a delivery agent selected from the group consisting of cationic polymers, modified cationic polymers, peptide molecular transporters (including arginine or histidine-rich peptides), lipids (including cationic lipids, neutral lipids, and combinations thereof), liposomes, lipopolyplexes, non-cationic polymers, modified non-cationic polymers, chloroquine, bupivacaine, and surfactants suitable for introduction into the lung. In preferred embodiments of the invention the RNAi-inducing entity is an siRNA. In certain preferred embodiments the RNAi-inducing agent is shRNA, or RNAi-inducing vector.

The RNAi construct containing composition of the invention can be used to prevent, inhibit or treat hyperlipidemia, including hypercholesterolemia, type I hyperlipoproteinemia, type II hyperlipoproteinemia, type III hyperlipoproteinemia, type IV hyperlipoproteinemia, type V hyperlipoproteinemia, secondary hypertriglyceridemia, and familial lecithin cholesterol acyltransferase deficiency; and cardiovascular disease, such as coronary heart disease (CHD), cerebrovascular disease (CVD), aortic stenosis, peripheral vascular disease, atherosclerosis, arteriosclerosis, myocardial infarction (heart attack), cerebrovascular diseases (stroke), transient ischemic attacks (TIA), angina (stable and unstable), atrial fibrillation, arrhythmia, valvular disease, and/or congestive heart failure or any other trait. In certain embodiments, the compositions can be administered alone or in combination with other therapies.

[00311] In certain preferred embodiments of the invention the compositions provide enhanced delivery of RNAi-inducing entities to cardiac tissue or cells that express PCSK9 mRNA. However, the efficacy of the various delivery agents described herein is not limited to particular cell types. Therefore, various embodiments of the invention encompass delivery of RNAi-inducing entities to any cell, tissue or solid organ in the body. Various embodiments of the invention also encompass delivery of RNAi-inducing entities such as siRNA, shRNA, or RNAi-inducing vectors to cells in the walls of blood vessels, e.g., endothelial cells, smooth muscle cells, fibroblasts, macrophages, etc., and to cells in the blood itself, e.g., lymphocytes, neutrophils, etc.

[00312] (i) Cationic Polymers and Modified Cationic Polymers - Han, S. O., et al, Development of Biomaterials for gene therapy. Mol. Therapy 2:302-317 have reported that cationic polymer-based systems are efficient as carriers for DNA transfection. It is believed that the ability of cationic polymers to promote cellular uptake of DNA is thought to arise partly from their ability to bind to DNA and condense large plasmid DNA molecules into smaller DNA/polymer complexes for more efficient endocytosis. Some investigators have proposed that the DNA/cationic polymer complexes also act as bioadhesives because of their electrostatic interaction with negatively charged sialic acid residues of cell surface glycoproteins. See Soane, R. J. et al., Evaluation of the clearance characteristics of bioadhesive systems in humans. Int. J. Pharm. 178:55-65 (1999). As well, some cationic polymers apparently promote disruption of the endosomal membrane

and therefore release of DNA into the cytosol (Putnam, D., et al, Polymer-based gene delivery with low cytotoxicity by a unique balance of side-chain termini. Proc. Natl. Acad. Sci. USA 98:1200-1205.32 (2001)).

[00313] Consequently, an embodiment of the invention provides a composition comprising (i) an RNAi-inducing entity, wherein the nucleic acid or polynucleotide (*e.g.*, siNA) entity is targeted to a PCSK9 mRNA target transcript; and (ii) a cationic polymer. The invention further provides methods of inhibiting target transcripts by administering such compositions.

[00314] In general, a cationic polymer is a polymer that is positively charged at approximately physiological pH, *e.g.*, a pH ranging from approximately 7.0 to 7.6, preferably approximately 7.2 to 7.6, more preferably approximately 7.4. Such cationic polymers include, but are not limited to, polylysine (PLL), polyarginine (PLA), polyhistidine, polyethyleneimine (PEI) (Boussif et al, A versatile vector for gene and oligonucleotide transfer into cells in culture and in vivo: polyethylenimine. Proc. Natl. Acad. Sci. USA 92:7297-7301 (1995)), including linear PEI and low molecular weight PEI as described, for example, in (Kunath, et al. J Control Release 89(1): 113-25.76 (2003)), polyvinylpyrrolidone (PVP), and chitosan. It will be appreciated that certain of these polymers comprise primary amine groups, imine groups, guanidine groups, and/or imidazole groups. Preferred cationic polymers have relatively low toxicity and high DNA transfection efficiency. See US 20050008617, Compositions and methods for delivery of short interfering RNA and short hairpin RNA, to Chen Jianzhu et al.; ('617 patent application hereafter) filed September 29, 2003. The contents of each of the references cited are incorporated herein by reference in their entirety.

[00315] While not wishing to be bound by any theory, it is believed that cationic polymers such as PEI compact or condense DNA into positively charged particles capable of interacting with anionic proteoglycans at the cell surface and entering cells by endocytosis. Such polymers may possess the property of acting as a "proton sponge" that buffers the endosomal pH and protects DNA from degradation. Continuous proton influx also induces endosome osmotic swelling and rupture, which provides an escape mechanism for DNA particles to the cytoplasm. That these teachings apply equally to the RNAi-inducing entities and DNA vectors of the invention is clear from the teachings of

the '617 patent application. Furthermore, the inventors in the '617 application specifically demonstrated effective delivery of siRNA and DNA vectors providing a template for synthesis of shRNA to cells in mammalian subjects, resulting in inhibition of target transcripts using a variety of such agents. See also 85-87; U.S. Pat. No. 6,013,240 cited in the '617 application. Likewise, WO9602655 provide further information on PEI and other cationic polymers useful in the practice of the invention. According to certain embodiments of the invention the commercially available PEI reagent known as jetPEITM. (Qbiogene, Carlsbad, Calif.), a linear form of PEI (U.S. Pat. No. 6,013,240) may be used.

[00316] In certain embodiments, the RNAi-inducing entities of the invention may be delivered via technique known to one skilled in the art as “hydrodynamic transfection.” It is noted such techniques to deliver RNAi-inducing entities intravenously to solid organs and tissues within the body are known as is evident from the teachings for example, (see, e.g., McCaffrey et al, RNA interference in adult mice. *Nature* 418, 38-39 (2002), McCaffrey et al, Inhibition of hepatitis B virus in mice by RNA interference. *Nat Biotechnol* 21, 639-644 (2003).

[00317] A variety of additional cationic polymers may also be used. Large libraries of novel cationic polymers and oligomers from diacrylate and amine monomers have been developed and tested in DNA transfection. For example, Lynn et al, Accelerated discovery of synthetic transfection vectors: parallel synthesis and screening of a degradable polymer library. *J. Am. Chem. Soc.* 123:8155-8156 (2001) report the development of a library of 140 polymers from 7 diacrylate monomers and 20 amine monomers. Larger libraries can be produced using similar or identical methodology. Of the 140 members of this library, 70 were found sufficiently water-soluble (2 mg/ml, 25 mM acetate buffer, pH=5.0). Fifty-six of the 70 water-soluble polymers interacted with DNA as shown by electrophoretic mobility shift. Most importantly, two of the 56 polymers mediated DNA transfection into COS-7 cells. Transfection efficiencies of the novel polymers were 4-8 times higher than PEI and equal or better than Lipofectamine 2000. Thus, in another embodiment, the invention provides compositions comprising at least one siRNA molecule and a cationic polymer, wherein the cationic polymer is a poly(beta-amino ester –See Lyn supra), and methods of inhibiting target gene expression, PCSK9 mRNA

by administering such compositions. The above-mentioned poly(beta-amino esters) are further described in U.S. published patent application 20020131951, entitled "Biodegradable poly(beta-amino esters) and uses thereof", filed Sep. 19, 2002, by Langer et al. It is noted that the cationic polymers for use to facilitate delivery of RNAi-inducing entities may be modified so that they incorporate one or more residues other than the major monomeric subunit of which the polymer is comprised. For example, one or more alternate residues may be added to the end of a polymer, or polymers may be joined by a residue other than the major monomer of which the polymer is comprised.

[00318] Additional cationic polymers that may also be used to enhance delivery of RNAi-inducing entities include polyamidoamine (PAMAM) dendrimers, poly(2-dimethylamino)ethyl methacrylate (pDMAEMA), and its quaternary amine analog poly(2-trimethylamino)ethyl methacrylate (pTMAEMA), poly [alpha-(4-aminobutyl)-L-glycolic acid (PAGA), and poly (4-hydroxy-1-proline ester). See Han (2000) for further description of these agents. See the '617 application.

[00319] (ii) Peptide Molecular Transporters - In certain embodiments, the invention provides compositions comprising any one or more of the RNA-inducing entities described herein that are specific for PCSK9 mRNA in conjunction with a peptide molecular transporter. Investigators have shown that a variety of peptides are able to act as delivery agents for nucleic acids. (As used herein, a polypeptide is considered to be a "peptide" if it shorter than approximately 50 amino acids in length). For example, transcription factors, including HIV Tat protein (Frankel et al, Cellular uptake of the tat protein from human immunodeficiency virus. Cell 55:1189-1193, (1988)), VP22 protein of herpes simplex virus (Elliott, et al. Intercellular trafficking and protein delivery by a herpes virus structural protein. Cell 88:223-233 (1997), and Antennapedia protein of Drosophila (Joliot et al. Antennapedia homeobox peptide regulates neural morphogenesis. Proc. Natl. Acad. Sci. USA 88:1864-1868 (1999)), can penetrate the plasma membrane from the cell surface. The peptide segments responsible for membrane penetration consist of 11-34 amino acid residues, are highly enriched for arginine, and are often referred to as arginine rich peptides (ARPs) or penetratins. When covalently linked with much larger polypeptides, the ARPs are capable of transporting the fused polypeptide across the plasma membrane (Fawell et al, Tat-mediated delivery

of heterologous proteins into cells. Proc. Natl. Acad. Sci. USA 91:664-668 (1994); Schwarze et al. In vivo protein transduction: delivery of a biologically active protein. Science 285:1569-1572 (1999) and Derossi et al., Trojan peptides: the penetratin system for intracellular delivery. Trends Cell Biol. 8:84-87 (1998).

[00320] Likewise, when oligonucleotides were covalently linked to ARPs, they were much more rapidly taken up by cells. See references 49 & 50 in the '617 patent application. As well, recent studies, Futaki et al. Arginine-rich peptides. An abundant source of membrane-permeable peptides having potential as carriers for intracellular protein delivery. J. Biol. Chem. 276:5836-5840 (2001) have shown that a polymer of eight arginines is sufficient for this transmembrane transport. Like cationic polymers, ARPs are also positively charged and likely capable of binding RNA, suggesting that it is probably not necessary to covalently link siRNA or shRNA to ARPs.

[00321] Thus, in one embodiment, the invention provides compositions comprising at least one RNAi-inducing entity, wherein the RNAi-inducing entity is siRNA targeted to a PCSK9 mRNA transcript, and a peptide molecular transporter and methods of inhibiting target transcript expression by administering such compositions. Peptide molecular transporters include, but are not limited to, those described in references 46-51, 120, and 134-136 and variations thereof evident to one of ordinary skill in the art as detailed in the '617 patent application. Arginine-rich peptides include a peptide consisting of arginine residues only.

[00322] Preferred peptide molecular transporters are less than approximately 50 amino acids in length. In certain embodiments of the invention the peptide molecular transporter is a peptide having length between approximately 7 and 34 amino acids. Many of the preferred peptides are arginine-rich. In certain embodiments of the invention a peptide is arginine-rich if it includes at least 20%, at least 30%, or at least 40%, or at least 50%, or at least 60% or at least 70%, or at least 80%, or at least 90% arginine. In yet other embodiments of the invention the peptide molecular transporter is an arginine-rich peptide that includes between 6 and 20 arginine residues. In still another embodiment, the RNAi-inducing entity such as a siRNA and the peptide molecular transporter are covalently bound, whereas in other embodiments of the invention the

RNAi-inducing entity and the peptide molecular transporter are mixed together but are not covalently bound to one another.

[00323] In yet another embodiment, the invention features a histidine-rich peptide as the peptide molecular transporter. See Kichler et al., Histidine-rich amphipathic peptide antibiotics promote efficient delivery of DNA into mammalian cells. *Proc Natl Acad Sci USA*. 18;100(4):1564-8 (2003). The histidine-rich peptides may exhibit lengths and percentage of histidine residues as described for arginine-rich peptides. Thus, an embodiment of the invention provides compositions comprising at least one RNAi-inducing entity targeted to a PCSK9 mRNA transcript and a histidine-rich peptide and methods of inhibiting target transcript expression by administering such compositions.

[00324] Additional peptides or modified peptides that facilitate the delivery of RNAi-inducing entities to cells in a subject may also be used in the inventive compositions. See for example McKenzie et al, Potent New Class of Reductively Activated Peptide Gene Delivery Agents. *J. Biol. Chem.*, 275(14): 9970-9977 (2000), which describes a family of lysine-rich peptides. See also Park et al, Synthesis of Sulfhydryl Cross-Linking Poly(Ethylene Glycol Peptides and Glycopeptides as Carriers for Gene Delivery. *Bioconjugate Chem.*, 13: 232-239 (2002) which describes peptides that comprise sulfhydryl residues for formation of disulfide bonds may incorporate polyethylene glycol (PEG), which is believed to reduce nonspecific binding to serum proteins.

[00325] Glycopeptides that include moieties such as galactose or mannose residues may also be used to enhance the selective uptake of RNAi-inducing entities in accordance with the present invention. Such glycopeptides may also include sulfhydryl groups for formation of disulfide bonds, Parks et al. *supra*. The invention further encompasses administration of various agents that enhance the exit of nucleic acids from endocytic vesicles. Such agents include, for example chloroquine as detailed in Zhang et al. The in vivo use of chloroquine to promote non-viral gene delivery to the liver via the portal vein and bile duct. *J. Gene Med.*, 5:209-218, 2003 and bupivacaine as detailed in Satishchandran C. Characterization of a new class of DNA delivery complexes formed by the local anesthetic bupivacaine. *Biochimica et Biophysica Acta* 1468: 20-30 (2000). The exit-enhancing agents may be administered systemically, orally, and/or locally (e.g. at or

in close proximity to the desired site of action). They may be delivered together with RNAi-inducing entities or separately.

[00326] The invention encompasses modification of the other delivery agents described herein (e.g., polymeric delivery agents) to incorporate a peptide molecular transporter to facilitate transport of the delivery agent into cells.

C. Additional Polymeric Delivery Agents

[00327] In certain embodiments, the invention provides compositions comprising an RNAi-inducing entity and any of a variety of polymeric delivery agents, including modified polymers, in addition to those described above. The invention further provides methods of inhibiting expression of a PCSK9 mRNA transcript in a cell and methods of treating or preventing a disease or condition associated or mediated by PCSK9 mRNA or its encoded protein.

[00328] Suitable delivery agents include various agents that have been shown to enhance delivery of DNA to cells. These include modified versions of cationic polymers such as those mentioned above, e.g., poly(L-histidine)-graft-poly(L-lysine) polymers (Benns et al, pH-Sensitive Cationic Polymer Gene Delivery Vehicle: N-Ac-poly(L-histidine)-graft-poly(L-lysine) Comb Shaped Polymer, *Bioconj. Chem.* 11:637-645 (2000)), polyhistidine-PEG (Putnam et al, Polyhistidine-PEG: DNA nanocomposites for gene delivery, *Biomaterials* 24: 4425-4433 (2003)), folate-PEG-graft-polyethyleneimine (Benns et al, Optimization of factors influencing the transfection efficiency of folate-PEG-folate-graft polyethyleneimine, *J. Cont. Release* 79:255-269 (2002)), polyethylenimine-dextran sulfate (Tiyaboonchai et al, Formulation and characterization of DNA-polyethylenimine-dextran sulfate nanoparticles. *Eur. J. Pharm. Sci.* 19:191-202 (2003)). The polymers may be branched or linear and may be grafted or ungrafted.

[00329] In certain embodiments, the polymers form complexes with the RNAi-inducing entity, which are then administered to a subject. The complexes may be referred to as nanoparticles or nanocomposites. Any of the polymers may be modified to incorporate PEG or other hydrophilic polymers, which is useful to reduce complement activation and binding of other plasma proteins. Cationic polymers may be multiply modified. For example, a cationic polymer may be modified to incorporate a moiety that

reduces the negative charge of the polymer (e.g., imidazole) and may be further modified with a second moiety such as PEG.

[00330] More, a variety of polymers and polymer matrices distinct from the cationic polymers described above may also be used. Such polymers include a number of non-cationic polymers, i.e., polymers not having positive charge at physiological pH. Such polymers may have certain advantages, e.g., reduced cytotoxicity and, in some cases, FDA approval. A number of suitable polymers have been shown to enhance drug and gene delivery in other contexts. Such polymers include, for example, poly(lactide) (PLA), poly(glycolide) (PLG), and poly(DL-lactide-co-glycol-ide) (PLGA) (Panyam et al, Rapid endo-lysosomal escape of poly(DL-lactide-co-glycol-ide) nanoparticles: implications for drug and gene delivery, FASEB J., 16: 1217-1226 (2002)), which can be formulated into nanoparticles for delivery of inventive RNAi-inducing entities. Copolymers and combinations of the foregoing may also be used. In certain embodiments of the invention a cationic polymer is used to condense the siRNA, shRNA, microRNA or vector, and the condensed complex is protected by PLGA or another non-cationic polymer. Other polymers that may be used include noncondensing polymers such as polyvinyl alcohol, or poly(N-ethyl-4-vinylpyridium bromide, which may be complexed with Pluronic 85. Other polymers of use in the invention include combinations between cationic and non-cationic polymers. For example, poly(lactic-co-glycolic acid) (PLGA)-grafted poly(L-lysine) (Jeong et al., Poly(L-lysine)-g-poly(D, L-lactic-co-glycolic acid) micelles for low cytotoxic biodegradable gene delivery carriers. J Control Release, 82(1): 159-66 (2002)) and other combinations including PLA, PLG, or PLGA and any of the cationic polymers or modified cationic polymers such as those discussed above, may be used.

D. Modified Delivery Agents Incorporating Targeting Moieties

[00331] The present invention also encompasses modification of any of the delivery agents to incorporate a moiety that enhances delivery of the agent to cells and/or enhances the selective delivery of the agent to cells in which it is desired to inhibit a target transcript. Such an agent is referred to as a “delivery-enhancing agent” or a “targeting moiety”.

[00332] Any of a variety of targeting moieties may be used including, but not limited to, (i) antibodies or antibody fragments that specifically bind to a molecule expressed by a cell in which inhibition is desired, (e.g., a respiratory epithelial cell); (ii) ligands that specifically bind to a molecule expressed by a cell in which inhibition is desired.

[00333] In certain preferred embodiments of the invention binding of the antibody or ligand induces internalization of the bound complex. In certain embodiments of the invention the delivery enhancing agent (e.g., antibody, antibody fragment, or ligand), is conjugated to an RNAi-inducing vector (e.g., a DNA vector) to increase delivery or enhance selectivity. Methods for conjugating antibodies or ligands to nucleic acids or to the various delivery agents described herein are well known in the art. See e.g., "Cross-Linking", Pierce Chemical Technical Library, originally published in the 1994-95 Pierce Catalog and references cited therein and Wong S S, Chemistry of Protein Conjugation and Crosslinking, CRC Press Publishers, Boca Raton, 1991.

[00334] In one embodiment, the invention features a method for treating or preventing a disease, disorder, trait or condition related to PCSK9 gene expression or PCSK9 activity in a subject or organism comprising contacting the subject or organism with a siNA molecule of the invention under conditions suitable to modulate the expression of the target gene in the subject or organism.

[00335] In one embodiment, the invention features a method for treating or preventing one or more metabolic diseases, traits, or conditions in a subject or organism comprising contacting the subject or organism with a nucleic acid or polynucleotide (e.g., siNA) molecule of the invention under conditions suitable to modulate the expression of an inhibitor of PCSK9 gene expression in the subject or organism. In one embodiment, the inhibitor of PCSK9 gene expression is a miRNA.

[00336] In one embodiment, the invention features a method for treating or preventing one or more metabolic diseases, traits, or conditions in a subject or organism comprising contacting the subject or organism with a nucleic acid or polynucleotide (e.g., siNA) molecule of the invention under conditions suitable to modulate the expression of the PCSK9 target gene in the subject or organism whereby the treatment or prevention of the metabolic disease(s), trait(s), or condition(s) can be achieved.

[00337] In one embodiment, the invention features a method for treating or preventing one or more cardiovascular diseases, traits, or conditions in a subject or organism comprising contacting the subject or organism with a nucleic acid or polynucleotide (*e.g.*, siNA) molecule of the invention under conditions suitable to modulate the expression of the PCSK9 target gene in the subject or organism whereby the treatment or prevention of the cardiovascular disease(s), trait(s), or condition(s) can be achieved.

[00338] In one embodiment, the invention features a method for treating or preventing one or more cardiovascular diseases, traits, or conditions in a subject or organism comprising contacting the subject or organism with a nucleic acid or polynucleotide (*e.g.*, siNA) molecule of the invention under conditions suitable to modulate (*e.g.*, inhibit) the expression of an inhibitor of target gene expression in the subject or organism. In one embodiment, the inhibitor of target gene expression is a miRNA.

[00339] In one embodiment, the invention features a method for treating or preventing hypertension in a subject or organism comprising contacting the subject or organism with a nucleic acid or polynucleotide (*e.g.*, siNA) molecule of the invention under conditions suitable to modulate the expression of the PCSK9 gene in the subject or organism.

[00340] In one embodiment, the invention features a method for treating or preventing coronary thrombosis in a subject or organism comprising contacting the subject or organism with a nucleic acid or polynucleotide (*e.g.*, siNA) molecule of the invention under conditions suitable to modulate the expression of the PCSK9 in the subject or organism.

[00341] In one embodiment, the invention features a method for treating or preventing stroke in a subject or organism comprising contacting the subject or organism with a nucleic acid or polynucleotide (*e.g.*, siNA) molecule of the invention under conditions suitable to modulate the expression of the PCSK9 gene in the subject or organism.

[00342] In one embodiment, the invention features a method for treating or preventing lipid syndromes in a subject or organism comprising contacting the subject or organism with a nucleic acid or polynucleotide (*e.g.*, siNA) molecule of the invention under

conditions suitable to modulate the expression of the PCSK9 gene in the subject or organism.

[00343] In one embodiment, the invention features a method for treating or preventing hyperglycemia in a subject or organism comprising contacting the subject or organism with a nucleic acid or polynucleotide (*e.g.*, siNA) molecule of the invention under conditions suitable to modulate the expression of the PCSK9 gene in the subject or organism.

[00344] In one embodiment, the invention features a method for treating or preventing hypertriglyceridemia in a subject or organism comprising contacting the subject or organism with a nucleic acid or polynucleotide (*e.g.*, siNA) molecule of the invention under conditions suitable to modulate the expression of the PCSK9 gene in the subject or organism.

[00345] In one embodiment, the invention features a method for treating or preventing hyperlipidemia in a subject or organism comprising contacting the subject or organism with a nucleic acid or polynucleotide (*e.g.*, siNA) molecule of the invention under conditions suitable to modulate the expression of the PCSK9 gene in the subject or organism.

[00346] In one embodiment, the invention features a method for treating or preventing ischemia in a subject or organism comprising contacting the subject or organism with a nucleic acid or polynucleotide (*e.g.*, siNA) molecule of the invention under conditions suitable to modulate the expression of the PCSK9 gene in the subject or organism.

[00347] In one embodiment, the invention features a method for treating or preventing congestive heart failure in a subject or organism comprising contacting the subject or organism with a nucleic acid or polynucleotide (*e.g.*, siNA) molecule of the invention under conditions suitable to modulate the expression of the PCSK9 gene in the subject or organism.

[00348] In one embodiment, the invention features a method for treating or preventing myocardial infarction in a subject or organism comprising contacting the subject or

organism with a nucleic acid or polynucleotide (*e.g.*, siNA) molecule of the invention under conditions suitable to modulate the expression of the PCSK9 gene in the subject or organism.

[00349] In another embodiment, the invention features a method of modulating the expression of more than one target genes in a subject or organism comprising contacting the subject or organism with one or more nucleic acid or polynucleotide (*e.g.*, siNA) molecules of the invention features contacting the subject or organism with a siNA molecule of the invention via local administration to relevant tissues or cells, *e.g.*, liver, pancreas, small intestine, adipose tissue or such as cells and tissues involved in the disorder, trait or condition. In one embodiment, the invention features contacting the subject or organism with a siNA molecule of the invention via systemic administration (such as via intravenous, intramuscular, subcutaneous, or GI administration of siNA) to relevant tissues or cells, such as tissues or cells involved in the maintenance or development of the metabolic and/or cardiovascular disease, trait, or condition in a subject or organism. The siNA molecule of the invention can be formulated or conjugated as described herein or otherwise known in the art to target appropriate tissues or cells in the subject or organism. The siNA molecule can be combined with other therapeutic treatments and modalities as are known in the art for the treatment of or prevention of cardiovascular diseases, traits, disorders, or conditions in a subject or organism. In one embodiment the cardiovascular disease is selected from the group consisting of hypertension, coronary thrombosis, stroke, lipid syndromes, hyperglycemia, hypertriglyceridemia, hyperlipidemia, ischemia, congestive heart failure, and myocardial infarction.

[00350] In one embodiment, the siNA molecule or double stranded nucleic acid molecule of the invention is formulated as a composition described in U.S. Provisional patent application No. 60/678,531 and in related U.S. Provisional patent application No. 60/703,946, filed July 29, 2005, and U.S. Provisional patent application No. 60/737,024, filed November 15, 2005 (Vargeese et al.).

[00351] In any of the methods herein for modulating the expression of one or more PCSK9 targets or for treating or preventing PCSK9 related diseases, traits, conditions, or

phenotypes in a cell, subject, or organism, the nucleic acid or polynucleotide (*e.g.*, siNA) molecule of the invention modulates expression of one or more PCSK9 targets via RNA interference. In one embodiment, the RNA interference is RISC mediated cleavage of the PCSK9 target (*e.g.*, siRNA mediated RNA interference). In one embodiment, the RNA interference is translational inhibition of the PCSK9 target (*e.g.*, miRNA mediated RNA interference). In one embodiment, the RNA interference is transcriptional inhibition of the PCSK9 target (*e.g.*, siRNA mediated transcriptional silencing). In one embodiment, the RNA interference takes place in the cytoplasm. In one embodiment, the RNA interference takes place in the nucleus.

[00352] The nucleic acid or polynucleotide (*e.g.*, siNA) molecules of the invention can be designed to down regulate or inhibit target (*e.g.*, PCSK9) gene expression through RNAi targeting of a variety of nucleic acid molecules. In one embodiment, the siNA molecules of the invention are used to target various DNA corresponding to a target gene, for example via heterochromatic silencing or transcriptional inhibition. In one embodiment, the siNA molecules of the invention are used to target various RNAs corresponding to a target gene, for example via RNA target cleavage or translational inhibition. Non-limiting examples of such RNAs include messenger RNA (mRNA), non-coding RNA (ncRNA) or regulatory elements (see for example Mattick, 2005, Science, 309, 1527-1528 and Claverie, 2005, Science, 309, 1529-1530) which includes miRNA and other small RNAs, alternate RNA splice variants of target gene(s), post-transcriptionally modified RNA of target gene(s), pre-mRNA of target gene(s), and/or RNA templates. If alternate splicing produces a family of transcripts that are distinguished by usage of appropriate exons, the instant invention can be used to inhibit gene expression through the appropriate exons to specifically inhibit or to distinguish among the functions of gene family members. For example, a protein that contains an alternatively spliced transmembrane domain can be expressed in both membrane bound and secreted forms. Use of the invention to target the exon containing the transmembrane domain can be used to determine the functional consequences of pharmaceutical targeting of the membrane bound as opposed to the secreted form of the protein. Non-limiting examples of applications of the invention relating to targeting these RNA molecules include therapeutic pharmaceutical applications, cosmetic applications, veterinary applications, pharmaceutical discovery applications, molecular

diagnostic and gene function applications, and gene mapping, for example using single nucleotide polymorphism mapping with siNA molecules of the invention. Such applications can be implemented using known gene sequences or from partial sequences available from an expressed sequence tag (EST).

[00353] In another embodiment, the nucleic acid or polynucleotide (*e.g.*, siNA) molecules of the invention are used to target conserved sequences corresponding to a gene family or gene families such as PCSK9 family genes having homologous sequences (*e.g.*, all known PCSK9 isotypes, or select groupings of PCSK9 isotypes). As such, siNA molecules targeting multiple PCSK9 genes or RNA targets can provide increased therapeutic effect. In addition, by avoiding other PCSK9 isotypes, toxicity may be avoided.

[00354] In another embodiment, the acid or polynucleotide (*e.g.*, siNA) molecules of the invention are used to target conserved sequences corresponding to a gene family or gene families, such as PCSK9 family genes. For example, the invention features the targeting (cleavage or inhibition of expression or function) of more than one PCSK9 gene sequence using a single siNA molecule, by targeting the conserved sequences of the targeted receptor gene. As such, siNA molecules targeting multiple PCSK9 targets can provide increased therapeutic effect.

[00355] In one embodiment, nucleic acid or polynucleotide (*e.g.*, siNA) molecules can be used to characterize pathways of gene function in a variety of applications. For example, the present invention can be used to inhibit the activity of target gene(s) in a pathway to determine the function of uncharacterized gene(s) in gene function analysis, mRNA function analysis, or translational analysis. The invention can be used to determine potential target gene pathways involved in various diseases and conditions toward pharmaceutical development. The invention can be used to understand pathways of gene expression involved in, for example the progression and/or maintenance of metabolic and/or cardiovascular diseases, disorders, traits and conditions associated with, for example, PCSK9 gene expression or activity in a subject or organism. .

[00356] In one embodiment, nucleic acid or polynucleotide (*e.g.*, siNA) molecule(s) and/or methods of the invention are used to down regulate the expression of gene(s) that

encode RNA referred to by Genbank Accession, for example, target genes encoding RNA sequence(s) referred to herein by Genbank Accession number, for example, Genbank Accession Nos. shown in Table I or Genbank Accession Nos. shown in PCT/US03/05028, U.S. Provisional Patent Application No. 60/363,124, or USSN 10/923,536, all of which are incorporated by reference herein.

[00357] In one embodiment, the invention features a method for treating or preventing one or more metabolic diseases, traits, or conditions in a subject or organism comprising contacting the subject or organism with a nucleic acid or polynucleotide (*e.g.*, siNA) molecule of the invention under conditions suitable to modulate the expression of the target gene in the subject or organism whereby the treatment or prevention of the metabolic disease(s), trait(s), or condition(s) can be achieved. In one embodiment, the invention features contacting the subject or organism with a siNA molecule of the invention via local administration to relevant tissues or cells. In one embodiment, the invention features contacting the subject or organism with a siNA molecule of the invention via systemic administration (such as via intravenous, intramuscular, subcutaneous, or GI administration of siNA) to relevant tissues or cells, such as tissues or cells involved in the maintenance or development of the metabolic disease, trait, or condition in a subject or organism (*e.g.*, liver, pancreas, small intestine, adipose tissue or cells). The siNA molecule of the invention can be formulated or conjugated as described herein or otherwise known in the art to target appropriate tissues or cells in the subject or organism (*e.g.*, liver, pancreas, small intestine, adipose tissue or cells). The siNA molecule can be combined with other therapeutic treatments and modalities as are known in the art for the treatment of or prevention of metabolic diseases, traits, or conditions in a subject or organism. In one embodiment, the metabolic disease is selected from the group consisting of diabetes (*e.g.*, type I and/or type II diabetes), insulin resistance, obesity, or related conditions, including but not limited to sleep apnea, hiatal hernia, reflux esophagitis, osteoarthritis, gout, cancers associated with weight gain, gallstones, kidney stones, pulmonary hypertension, infertility, cardiovascular disease, above normal weight, and above normal lipid levels, uric acid levels, or oxalate levels.

[00358] In one embodiment, the invention features a method for weight loss in a subject or organism comprising contacting the subject or organism with a nucleic acid or

polynucleotide (*e.g.*, siNA) molecule of the invention under conditions suitable to modulate the expression of the target gene in the subject or organism whereby the weight loss can be achieved. In one embodiment, the invention features contacting the subject or organism with a siNA molecule of the invention via local administration to relevant tissues or cells, *e.g.*, liver, pancreas, small intestine, adipose tissue or cells. In one embodiment, the invention features contacting the subject or organism with a siNA molecule of the invention via systemic administration (such as via intravenous, intramuscular, subcutaneous, or GI administration of siNA) to relevant tissues or cells. The siNA molecule of the invention can be formulated or conjugated as described herein or otherwise known in the art to target appropriate tissues or cells in the subject or organism. The siNA molecule can be combined with other therapeutic treatments and modalities as are known in the art for weight loss in a subject or organism.

[00359] In one embodiment, the invention features a method for treating or preventing a cardiovascular disease, disorder, and/or condition in a subject or organism comprising contacting the subject or organism with a nucleic acid or polynucleotide (*e.g.*, siNA) molecule of the invention under conditions suitable to modulate the expression of the target gene (*e.g.*, PCSK9) in the subject or organism whereby the treatment or prevention of metabolic and/or cardiovascular, disease, disorder, and/or condition can be achieved. In one embodiment, the invention features contacting the subject or organism with a siNA molecule of the invention via local administration to relevant tissues or cells, such as tissues or cells affected by the metabolic and/or cardiovascular disease, disorder, or condition. In one embodiment, the invention features contacting the subject or organism with a siNA molecule of the invention via systemic administration (such as via intravenous or subcutaneous administration of siNA) to relevant tissues or cells, such as tissues or cells affected by the metabolic and/or cardiovascular disease, disorder, or condition. The siNA molecule of the invention can be formulated or conjugated as described herein or otherwise known in the art to target appropriate tissues or cells in the subject or organism. The siNA molecule can be combined with other therapeutic treatments and modalities as are known in the art for the treatment of or prevention of cardiovascular diseases, traits, disorders, or conditions in a subject or organism.

[00360] In one embodiment, the invention features a method for treating or preventing a disease, disorder, trait or condition related to gene expression in a subject or organism comprising contacting the subject or organism with a siNA molecule of the invention under conditions suitable to modulate the expression of the target gene (e.g., PCSK9) in the subject or organism. The reduction of gene expression and thus reduction in the level of the respective protein/RNA relieves, to some extent, the symptoms of the disease, disorder, trait or condition.

[00361] In one embodiment, the invention features a composition comprising PEG Interferon and one or more double stranded nucleic acid molecules or siNA molecules of the invention in a pharmaceutically acceptable carrier or diluent. In another embodiment, the invention features a composition comprising, but not limited to, (e.g., atorvastatin, simvastatin, pravastatin, fluvastatin, lovastatin), other PCSK9 inhibitors such as tocetrapib and JTT-705, and antihypertensive agents, such as Alpha1-Adrenergic Antagonists (e.g., Prazosin), Beta-Adrenergic Antagonists (e.g., Propranolol, Nadolol, Timolol, Metoprolol, Pindolol), Combined Alpha/Beta Adrenergic Antagonists (e.g., Labetalol), Adrenergic Neuron Blocking Agents (e.g., Guanethidine, Reserpine), CNS-Acting Antihypertensives (e.g., Clonidine, Methyldopa, Guanabenz), Anti-angiotensin II agents, including Angiotensin Converting Enzyme (ACE) Inhibitors (e.g., Captopril, Enalapril, Lisinopril) and Verapamil, Diltiazem, Nifedipine), Diuretics (e.g., Hydrochlorothiazide, Furosemide, Triamterene) and Direct Vasodilators (e.g., Hydralazine, Minoxidil, Nitroprusside, Diazoxide) and one or more double stranded nucleic acid molecules or siNA molecules of the invention in a pharmaceutically acceptable carrier or diluent.

[00362] In one embodiment, a method of treatment of the invention features administration of a double stranded nucleic acid molecule of the invention in combination with one or more other therapeutic modalities, including statins (e.g., atorvastatin, simvastatin, pravastatin, fluvastatin, lovastatin), other PCSK9 inhibitors such as tocetrapib and JTT-705, and antihypertensive agents, such as Alpha1-Adrenergic Antagonists (e.g., Prazosin), Beta-Adrenergic Antagonists (e.g., Propranolol, Nadolol, Timolol, Metoprolol, Pindolol), Combined Alpha/Beta Adrenergic Antagonists (e.g., Labetalol), Adrenergic Neuron Blocking Agents (e.g., Guanethidine, Reserpine), CNS-

Acting Antihypertensives (e.g., Clonidine, Methyldopa, Guanabenz), Anti-angiotensin II agents, including Angiotensin Converting Enzyme (ACE) Inhibitors (e.g., Captopril, Enalapril, Lisinopril) and Verapamil, Diltiazem, Nifedipine), Diuretics (e.g., Hydrochlorothiazide, Fhlorthalidone, Furosemide, Triamterene) and Direct Vasodialators (e.g., Hydralazine, Minoxidil, Nitroprusside, Diazoxide) and combinations thereof. In another embodiment, such combination therapies can be utilized in any of the embodiments herein.

[00363] In any of the methods of treatment or prevention of the invention, the nucleic acid or polynucleotide (e.g., siNA) can be administered to the subject as a course of treatment, for example administration at various time intervals, such as once per day over the course of treatment, once every two days over the course of treatment, once every three days over the course of treatment, once every four days over the course of treatment, once every five days over the course of treatment, once every six days over the course of treatment, once per week over the course of treatment, once every other week over the course of treatment, once per month over the course of treatment, etc. In one embodiment, the course of treatment is once every 1, 2, 3, 4, 5, 6, 7, 8, 9, or 10 weeks. In one embodiment, the course of treatment is from about one to about 52 weeks or longer (e.g., indefinitely). In one embodiment, the course of treatment is from about one to about 48 months or longer (e.g., indefinitely).

[00364] In one embodiment, a course of treatment or prevention involves an initial course of treatment, such as once every 1, 2, 3, 4, 5, 6, 7, 8, 9, 10 or more weeks for a fixed interval (e.g., 1x, 2x, 3x, 4x, 5x, 6x, 7x, 8x, 9x, 10x or more) followed by a maintenance course of treatment, such as once every 4, 6, 8, 10, 15, 20, 25, 30, 35, 40, or more weeks for an additional fixed interval (e.g., 1x, 2x, 3x, 4x, 5x, 6x, 7x, 8x, 9x, 10x or more). In any of the methods of treatment or prevention of the invention, the nucleic acid or polynucleotide (e.g., siNA) can be administered to the subject systemically as described herein or otherwise known in the art, either alone as a monotherapy or in combination with additional therapies described herein or as are known in the art. Systemic administration can include, for example, pulmonary (inhalation, nebulization etc.) intravenous, subcutaneous, intramuscular, catheterization, nasopharangeal, transdermal, or oral/gastrointestinal administration as is generally known in the art.

[00365] In any of the methods of treatment or prevention of the invention, the nucleic acid or polynucleotide (*e.g.*, siNA) can be administered to the subject locally or to local tissues as described herein or otherwise known in the art, either alone as a monotherapy or in combination with additional therapies as are known in the art. Local administration can include, for example, inhalation, nebulization, catheterization, implantation, direct injection, dermal/transdermal application, stenting, ear/eye drops, or portal vein administration to relevant tissues, or any other local administration technique, method or procedure, as is generally known in the art.

[00366] In one embodiment, the invention features a method comprising: (a) generating a library of nucleic acid or polynucleotide (*e.g.*, siNA) constructs having a predetermined complexity; and (b) assaying the siNA constructs of (a) above, under conditions suitable to determine RNAi target sites within the target RNA sequence. In one embodiment, the siNA molecules of (a) have strands of a fixed length, for example, about 23 nucleotides in length. In another embodiment, the siNA molecules of (a) are of differing length, for example having strands of about 15 to about 30 (*e.g.*, about 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, or 30) nucleotides in length. In one embodiment, the assay can comprise a reconstituted *in vitro* siNA assay as described herein. In another embodiment, the assay can comprise a cell culture system in which target RNA is expressed. In another embodiment, fragments of target RNA are analyzed for detectable levels of cleavage, for example by gel electrophoresis, northern blot analysis, or RNase protection assays, to determine the most suitable target site(s) within the target RNA sequence. The target RNA sequence can be obtained as is known in the art, for example, by cloning and/or transcription for *in vitro* systems, and by cellular expression in *in vivo* systems.

[00367] In one embodiment, the invention features a method comprising: (a) generating a randomized library of nucleic acid or polynucleotide (*e.g.*, siNA) constructs having a predetermined complexity, such as of 4^N , where N represents the number of base paired nucleotides in each of the siNA construct strands (*e.g.* for a siNA construct having 21 nucleotide sense and antisense strands with 19 base pairs, the complexity would be 4^{19}); and (b) assaying the siNA constructs of (a) above, under conditions suitable to determine RNAi target sites within the target RNA sequence. In another

embodiment, the siNA molecules of (a) have strands of a fixed length, for example about 23 nucleotides in length. In yet another embodiment, the nucleic acid or polynucleotide (*e.g.*, siNA) molecules of (a) are of differing length, for example having strands of about 15 to about 30 (*e.g.*, about 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, or 30) nucleotides in length. In one embodiment, the assay can comprise a reconstituted *in vitro* siNA assay as described in Example 6 herein. In another embodiment, the assay can comprise a cell culture system in which target RNA is expressed. In another embodiment, fragments of target RNA are analyzed for detectable levels of cleavage, for example, by gel electrophoresis, northern blot analysis, or RNase protection assays, to determine the most suitable target site(s) within the target RNA sequence. The target RNA sequence can be obtained as is known in the art, for example, by cloning and/or transcription for *in vitro* systems, and by cellular expression in *in vivo* systems.

[00368] In another embodiment, the invention features a method comprising: (a) analyzing the sequence of a RNA target encoded by a target gene; (b) synthesizing one or more sets of nucleic acid or polynucleotide (*e.g.*, siNA) molecules having sequence complementary to one or more regions of the RNA of (a); and (c) assaying the siNA molecules of (b) under conditions suitable to determine RNAi targets within the target RNA sequence. In one embodiment, the siNA molecules of (b) have strands of a fixed length, for example about 23 nucleotides in length. In another embodiment, the siNA molecules of (b) are of differing length, for example having strands of about 15 to about 30 (*e.g.*, about 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, or 30) nucleotides in length. In one embodiment, the assay can comprise a reconstituted *in vitro* siNA assay as described herein. In another embodiment, the assay can comprise a cell culture system in which target RNA is expressed. Fragments of target RNA are analyzed for detectable levels of cleavage, for example by gel electrophoresis, northern blot analysis, or RNase protection assays, to determine the most suitable target site(s) within the target RNA sequence. The target RNA sequence can be obtained as is known in the art, for example, by cloning and/or transcription for *in vitro* systems, and by expression in *in vivo* systems.

[00369] By "target site" is meant a sequence within a target RNA that is "targeted" for cleavage mediated by a siNA construct which contains sequences within its antisense region that are complementary to the target sequence.

[00370] By "detectable level of cleavage" is meant cleavage of target RNA (and formation of cleaved product RNAs) to an extent sufficient to discern cleavage products above the background of RNAs produced by random degradation of the target RNA. Production of cleavage products from 1-5% of the target RNA is sufficient to detect above the background for most methods of detection.

[00371] In one embodiment, the invention features a composition comprising a siNA molecule of the invention, which can be chemically-modified, in a pharmaceutically acceptable carrier or diluent. In another embodiment, the invention features a pharmaceutical composition comprising nucleic acid or polynucleotide (*e.g.*, siNA) molecules of the invention, which can be chemically-modified, targeting one or more genes in a pharmaceutically acceptable carrier or diluent. In another embodiment, the invention features a method for diagnosing a disease, trait, or condition in a subject comprising administering to the subject a composition of the invention under conditions suitable for the diagnosis of the disease, trait, or condition in the subject. In another embodiment, the invention features a method for treating or preventing a disease, trait, or condition, such as metabolic and/or cardiovascular diseases, traits, conditions, or disorders in a subject, comprising administering to the subject a composition of the invention under conditions suitable for the treatment or prevention of the disease, trait, or condition in the subject, alone or in conjunction with one or more other therapeutic compounds.

[00372] In another embodiment, the invention features a method for validating a target gene target, comprising: (a) synthesizing a siNA molecule of the invention, which can be chemically-modified, wherein one of the siNA strands includes a sequence complementary to RNA of a target gene; (b) introducing the siNA molecule into a cell, tissue, subject, or organism under conditions suitable for modulating expression of the target gene in the cell, tissue, subject, or organism; and (c) determining the function of the gene by assaying for any phenotypic change in the cell, tissue, subject, or organism.

[00373] In another embodiment, the invention features a method for validating a target comprising: (a) synthesizing a siNA molecule of the invention, which can be chemically-modified, wherein one of the siNA strands includes a sequence complementary to RNA of a target gene; (b) introducing the siNA molecule into a biological system under conditions suitable for modulating expression of the target gene in the biological system; and (c) determining the function of the gene by assaying for any phenotypic change in the biological system.

[00374] By “biological system” is meant, material, in a purified or unpurified form, from biological sources, including but not limited to human or animal, wherein the system comprises the components required for RNAi activity. The term “biological system” includes, for example, a cell, tissue, subject, or organism, or extract thereof. The term biological system also includes reconstituted RNAi systems that can be used in an in vitro setting.

[00375] By "phenotypic change" is meant any detectable change to a cell that occurs in response to contact or treatment with a nucleic acid or polynucleotide molecule of the invention (e.g., siNA). Such detectable changes include, but are not limited to, changes in shape, size, proliferation, motility, protein expression or RNA expression or other physical or chemical changes as can be assayed by methods known in the art. The detectable change can also include expression of reporter genes/molecules such as Green Florescent Protein (GFP) or various tags that are used to identify an expressed protein or any other cellular component that can be assayed.

[00376] In one embodiment, the invention features a kit containing a nucleic acid or polynucleotide (e.g., siNA) molecule of the invention, which can be chemically-modified, that can be used to modulate the expression of a target gene in a biological system, including, for example, in a cell, tissue, subject, or organism. In another embodiment, the invention features a kit containing more than one siNA molecule of the invention, which can be chemically-modified, that can be used to modulate the expression of more than one target gene in a biological system, including, for example, in a cell, tissue, subject, or organism.

[00377] In one embodiment, the invention features a cell containing one or more nucleic acid or polynucleotide (*e.g.*, siNA) molecules of the invention, which can be chemically-modified. In another embodiment, the cell containing a nucleic acid or polynucleotide (*e.g.*, siNA) molecule of the invention is a mammalian cell. In yet another embodiment, the cell containing a siNA molecule of the invention is a human cell.

[00378] In one embodiment, the synthesis of a nucleic acid or polynucleotide (*e.g.*, siNA) molecule of the invention, which can be chemically-modified, comprises: (a) synthesis of two complementary strands of the siNA molecule; (b) annealing the two complementary strands together under conditions suitable to obtain a double-stranded siNA molecule. In another embodiment, synthesis of the two complementary strands of the siNA molecule is by solid phase oligonucleotide synthesis. In yet another embodiment, synthesis of the two complementary strands of the siNA molecule is by solid phase tandem oligonucleotide synthesis.

[00379] In one embodiment, the invention features a method for synthesizing a nucleic acid or polynucleotide (*e.g.*, siNA) duplex molecule comprising: (a) synthesizing a first oligonucleotide sequence strand of the siNA molecule, wherein the first oligonucleotide sequence strand comprises a cleavable linker molecule that can be used as a scaffold for the synthesis of the second oligonucleotide sequence strand of the siNA; (b) synthesizing the second oligonucleotide sequence strand of siNA on the scaffold of the first oligonucleotide sequence strand, wherein the second oligonucleotide sequence strand further comprises a chemical moiety that can be used to purify the siNA duplex; (c) cleaving the linker molecule of (a) under conditions suitable for the two siNA oligonucleotide strands to hybridize and form a stable duplex; and (d) purifying the siNA duplex utilizing the chemical moiety of the second oligonucleotide sequence strand. In one embodiment, cleavage of the linker molecule in (c) above takes place during deprotection of the oligonucleotide, for example, under hydrolysis conditions using an alkylamine base such as methylamine. In one embodiment, the method of synthesis comprises solid phase synthesis on a solid support such as controlled pore glass (CPG) or polystyrene, wherein the first sequence of (a) is synthesized on a cleavable linker, such as a succinyl linker, using the solid support as a scaffold. The cleavable linker in (a)

used as a scaffold for synthesizing the second strand can comprise similar reactivity as the solid support derivatized linker, such that cleavage of the solid support derivatized linker and the cleavable linker of (a) takes place concomitantly. In another embodiment, the chemical moiety of (b) that can be used to isolate the attached oligonucleotide sequence comprises a trityl group, for example a dimethoxytrityl group, which can be employed in a trityl-on synthesis strategy as described herein. In yet another embodiment, the chemical moiety, such as a dimethoxytrityl group, is removed during purification, for example, using acidic conditions.

[00380] In a further embodiment, the method for nucleic acid or polynucleotide (*e.g.*, siNA) synthesis is a solution phase synthesis or hybrid phase synthesis wherein both strands of the siNA duplex are synthesized in tandem using a cleavable linker attached to the first sequence which acts a scaffold for synthesis of the second sequence. Cleavage of the linker under conditions suitable for hybridization of the separate siNA sequence strands results in formation of the double-stranded siNA molecule.

[00381] In another embodiment, the invention features a method for synthesizing a nucleic acid or polynucleotide (*e.g.*, siNA) duplex molecule comprising: (a) synthesizing one oligonucleotide sequence strand of the siNA molecule, wherein the sequence comprises a cleavable linker molecule that can be used as a scaffold for the synthesis of another oligonucleotide sequence; (b) synthesizing a second oligonucleotide sequence having complementarity to the first sequence strand on the scaffold of (a), wherein the second sequence comprises the other strand of the double-stranded siNA molecule and wherein the second sequence further comprises a chemical moiety than can be used to isolate the attached oligonucleotide sequence; (c) purifying the product of (b) utilizing the chemical moiety of the second oligonucleotide sequence strand under conditions suitable for isolating the full-length sequence comprising both siNA oligonucleotide strands connected by the cleavable linker and under conditions suitable for the two siNA oligonucleotide strands to hybridize and form a stable duplex. In one embodiment, cleavage of the linker molecule in (c) above takes place during deprotection of the oligonucleotide, for example, under hydrolysis conditions. In another embodiment, cleavage of the linker molecule in (c) above takes place after deprotection of the oligonucleotide. In another embodiment, the method of synthesis comprises solid

phase synthesis on a solid support such as controlled pore glass (CPG) or polystyrene, wherein the first sequence of (a) is synthesized on a cleavable linker, such as a succinyl linker, using the solid support as a scaffold. The cleavable linker in (a) used as a scaffold for synthesizing the second strand can comprise similar reactivity or differing reactivity as the solid support derivatized linker, such that cleavage of the solid support derivatized linker and the cleavable linker of (a) takes place either concomitantly or sequentially. In one embodiment, the chemical moiety of (b) that can be used to isolate the attached oligonucleotide sequence comprises a trityl group, for example a dimethoxytrityl group.

[00382] In another embodiment, the invention features a method for making a double-stranded nucleic acid or polynucleotide (*e.g.*, siNA) molecule in a single synthetic process comprising: (a) synthesizing an oligonucleotide having a first and a second sequence, wherein the first sequence is complementary to the second sequence, and the first oligonucleotide sequence is linked to the second sequence via a cleavable linker, and wherein a terminal 5'-protecting group, for example, a 5'-O-dimethoxytrityl group (5'-O-DMT) remains on the oligonucleotide having the second sequence; (b) deprotecting the oligonucleotide whereby the deprotection results in the cleavage of the linker joining the two oligonucleotide sequences; and (c) purifying the product of (b) under conditions suitable for isolating the double-stranded siNA molecule, for example using a trityl-on synthesis strategy as described herein.

[00383] In another embodiment, the method of synthesis of nucleic acid or polynucleotide (*e.g.*, siNA) molecules of the invention comprises the teachings of Scaringe et al., US Patent Nos. 5,889,136; 6,008,400; and 6,111,086, incorporated by reference herein in their entirety.

[00384] In one embodiment, the invention features nucleic acid or polynucleotide (*e.g.*, siNA) constructs that mediate RNAi against a target polynucleotide (*e.g.*, PCSK9 RNA or DNA), wherein the siNA construct comprises one or more chemical modifications, for example, one or more chemical modifications having any of Formulae I-VII or any combination thereof that increases the nuclease resistance of the siNA construct.

[00385] In another embodiment, the invention features a method for generating nucleic acid or polynucleotide (*e.g.*, siNA) molecules with increased nuclease resistance

comprising (a) introducing nucleotides having any of Formula I-VII or any combination thereof into a siNA molecule, and (b) assaying the siNA molecule of step (a) under conditions suitable for isolating siNA molecules having increased nuclease resistance.

[00386] In another embodiment, the invention features a method for generating nucleic acid or polynucleotide (*e.g.*, siNA) molecules with improved toxicologic profiles (*e.g.*, having attenuated or no immunstimulatory properties) comprising (a) introducing nucleotides having any of Formula I-VII (*e.g.*, siNA motifs referred to in Table IV) or any combination thereof into a siNA molecule, and (b) assaying the siNA molecule of step (a) under conditions suitable for isolating siNA molecules having improved toxicologic profiles.

[00387] In another embodiment, the invention features a method for generating nucleic acid or polynucleotide (*e.g.*, siNA) formulations with improved toxicologic profiles (*e.g.*, having attenuated or no immunstimulatory properties) comprising (a) generating a siNA formulation comprising a siNA molecule of the invention and a delivery vehicle or delivery particle as described herein or as otherwise known in the art, and (b) assaying the siNA formulation of step (a) under conditions suitable for isolating siNA formulations having improved toxicologic profiles.

[00388] In another embodiment, the invention features a method for generating nucleic acid or polynucleotide (*e.g.*, siNA) molecules that do not stimulate an interferon response (*e.g.*, no interferon response or attenuated interferon response) in a cell, subject, or organism, comprising (a) introducing nucleotides having any of Formula I-VII (*e.g.*, siNA motifs referred to in Table IV) or any combination thereof into a siNA molecule, and (b) assaying the siNA molecule of step (a) under conditions suitable for isolating siNA molecules that do not stimulate an interferon response.

[00389] In another embodiment, the invention features a method for generating nucleic acid or polynucleotide (*e.g.*, siNA) formulations that do not stimulate an interferon response (*e.g.*, no interferon response or attenuated interferon response) in a cell, subject, or organism, comprising (a) generating a siNA formulation comprising a siNA molecule of the invention and a delivery vehicle or delivery particle as described herein or as otherwise known in the art, and (b) assaying the siNA formulation of step (a) under

conditions suitable for isolating siNA formulations that do not stimulate an interferon response. In one embodiment, the interferon comprises interferon alpha.

[00390] In another embodiment, the invention features a method for generating nucleic acid or polynucleotide (*e.g.*, siNA) molecules that do not stimulate an inflammatory or proinflammatory cytokine response (*e.g.*, no cytokine response or attenuated cytokine response) in a cell, subject, or organism, comprising (a) introducing nucleotides having any of Formula I-VII (*e.g.*, siNA motifs referred to in Table IV) or any combination thereof into a siNA molecule, and (b) assaying the siNA molecule of step (a) under conditions suitable for isolating siNA molecules that do not stimulate a cytokine response. In one embodiment, the cytokine comprises an interleukin, such as interleukin-6 (IL-6) and/or tumor necrosis alpha (TNF- α).

[00391] In another embodiment, the invention features a method for generating nucleic acid or polynucleotide (*e.g.*, siNA) formulations that do not stimulate an inflammatory or proinflammatory cytokine response (*e.g.*, no cytokine response or attenuated cytokine response) in a cell, subject, or organism, comprising (a) generating a siNA formulation comprising a siNA molecule of the invention and a delivery vehicle or delivery particle as described herein or as otherwise known in the art, and (b) assaying the siNA formulation of step (a) under conditions suitable for isolating siNA formulations that do not stimulate a cytokine response. In one embodiment, the cytokine comprises an interleukin, such as interleukin-6 (IL-6) and/or tumor necrosis alpha (TNF- α).

[00392] In another embodiment, the invention features a method for generating nucleic acid or polynucleotide (*e.g.*, siNA) molecules that do not stimulate Toll-like Receptor (TLR) response (*e.g.*, no TLR response or attenuated TLR response) in a cell, subject, or organism, comprising (a) introducing nucleotides having any of Formula I-VII (*e.g.*, siNA motifs referred to in Table IV) or any combination thereof into a siNA molecule, and (b) assaying the siNA molecule of step (a) under conditions suitable for isolating siNA molecules that do not stimulate a TLR response. In one embodiment, the TLR comprises TLR3, TLR7, TLR8 and/or TLR9.

[00393] In another embodiment, the invention features a method for generating nucleic acid or polynucleotide (*e.g.*, siNA) formulations that do not stimulate a Toll-like

Receptor (TLR) response (e.g., no TLR response or attenuated TLR response) in a cell, subject, or organism, comprising (a) generating a siNA formulation comprising a siNA molecule of the invention and a delivery vehicle or delivery particle as described herein or as otherwise known in the art, and (b) assaying the siNA formulation of step (a) under conditions suitable for isolating siNA formulations that do not stimulate a TLR response. In one embodiment, the TLR comprises TLR3, TLR7, TLR8 and/or TLR9.

[00394] In one embodiment, a chemically modified nucleic acid or polynucleotide (e.g., siNA) molecule of the invention has an improved toxicologic profile compared to a corresponding siRNA molecule having no chemical modifications or fewer chemical modifications.

[00395] In one embodiment, the invention features a chemically synthesized double stranded short interfering nucleic acid or polynucleotide (e.g., siNA) molecule that directs cleavage of a target RNA via RNA interference (RNAi), wherein: (a) each strand of said siNA molecule is about 18 to about 38 nucleotides in length; (b) one strand of said siNA molecule comprises nucleotide sequence having sufficient complementarity to said target RNA for the siNA molecule to direct cleavage of the target RNA via RNA interference; and (c) wherein the nucleotide positions within said siNA molecule are chemically modified to reduce the immunostimulatory properties of the siNA molecule to a level below that of a corresponding unmodified siRNA molecule. Such siNA molecules are said to have an improved toxicologic profile compared to an unmodified or minimally modified siNA.

[00396] By "improved toxicologic profile", is meant that the chemically modified or formulated nucleic acid or polynucleotide (e.g., siNA) construct exhibits decreased toxicity in a cell, subject, or organism compared to an unmodified or unformulated siNA, or siNA molecule having fewer modifications or modifications that are less effective in imparting improved toxicology. Such siNA molecules are also considered to have "improved RNAi activity". In a non-limiting example, siNA molecules and formulations with improved toxicologic profiles are associated with reduced off target effects and/or immunostimulatory properties, such as a reduced, decreased or attenuated off target and/or immunostimulatory response in a cell, subject, or organism compared to an unmodified or unformulated siNA, or siNA molecule having fewer modifications or

modifications that are less effective in imparting improved toxicology. In one example, such an improved toxicologic profile can be characterized by abrogated or reduced off target effects. In another example, such an improved toxicologic profile can be characterized by abrogated or reduced immunostimulation, such as reduction or abrogation of induction of interferons (e.g., interferon alpha), inflammatory cytokines (e.g., interleukins such as IL-6, and/or TNF-alpha), and/or toll like receptors (e.g., TLR-3, TLR-7, TLR-8, and/or TLR-9). In one embodiment, a siNA molecule or formulation with an improved toxicological profile comprises no ribonucleotides. In one embodiment, a siNA molecule or formulation with an improved toxicological profile comprises less than 5 ribonucleotides (e.g., 1, 2, 3, or 4 ribonucleotides). In one embodiment, a siNA molecule or formulation with an improved toxicological profile comprises Stab 7, Stab 8, Stab 11, Stab 12, Stab 13, Stab 16, Stab 17, Stab 18, Stab 19, Stab 20, Stab 23, Stab 24, Stab 25, Stab 26, Stab 27, Stab 28, Stab 29, Stab 30, Stab 31, Stab 32, Stab 33, Stab 34, Stab 35, Stab 36 or any combination thereof (see Table IV). Herein, numeric Stab chemistries include both 2'-fluoro and 2'-OCF₃ versions of the chemistries shown in Table IV. For example, "Stab 7/8" refers to both Stab 7/8 and Stab 7F/8F etc. In one embodiment, a siNA molecule or formulation with an improved toxicological profile comprises a siNA molecule of the invention and a formulation as described in United States Patent Application Publication No. 20030077829, incorporated by reference herein in its entirety including the drawings.

[00397] In one embodiment, the level of immunostimulatory response associated with a given nucleic acid or polynucleotide (e.g., siNA) molecule can be measured as is described herein or as is otherwise known in the art, for example by determining the level of PKR/interferon response, proliferation, B-cell activation, and/or cytokine production in assays to quantitate the immunostimulatory response of particular siNA molecules (see, for example, Leifer et al., 2003, J Immunother. 26, 313-9; and U.S. Patent No. 5,968,909, incorporated in its entirety by reference). In one embodiment, the reduced immunostimulatory response is between about 10% and about 100% compared to an unmodified or minimally modified siRNA molecule, e.g., about 10%, 20%, 30%, 40%, 50%, 60%, 70%, 80%, 90% or 100% reduced immunostimulatory response. In one embodiment, the immunostimulatory response associated with a siNA molecule can be modulated by the degree of chemical modification. For example, a siNA molecule

having between about 10% and about 100%, (e.g., about 10%, 20%, 30%, 40%, 50%, 60%, 70%, 80%, 90% or 100% or at least about 10%, 20%, 30%, 40%, 50%, 60%, 70%, 80%, 90% or 100%) of the nucleotide positions in the siNA molecule modified can be selected to have a corresponding degree of immunostimulatory properties as described herein.

[00398] In one embodiment, the degree of reduced immunostimulatory response is selected for optimized RNAi activity. For example, retaining a certain degree of immunostimulation can be preferred to treat viral infection, where less than 100% reduction in immunostimulation may be preferred for maximal antiviral activity (e.g., about 10%, 20%, 30%, 40%, 50%, 60%, 70%, 80%, or 90% reduction in immunostimulation) whereas the inhibition of expression of an endogenous gene target may be preferred with nucleic acid or polynucleotide (e.g., siNA) molecules that possess minimal immunostimulatory properties to prevent non-specific toxicity or off target effects (e.g., about 90% to about 100% reduction in immunostimulation).

[00399] In one embodiment, the invention features a chemically synthesized double stranded nucleic acid or polynucleotide (e.g., siNA) molecule that directs cleavage of a target RNA via RNA interference (RNAi), wherein (a) each strand of said siNA molecule is about 18 to about 38 nucleotides in length; (b) one strand of said siNA molecule comprises nucleotide sequence having sufficient complementarity to said target RNA for the siNA molecule to direct cleavage of the target RNA via RNA interference; and (c) wherein one or more nucleotides of said siNA molecule are chemically modified to reduce the immunostimulatory properties of the siNA molecule to a level below that of a corresponding unmodified siNA molecule. In one embodiment, each strand comprises at least about 18 nucleotides that are complementary to the nucleotides of the other strand.

[00400] In another embodiment, the nucleic acid or polynucleotide (e.g., siNA) molecule comprising modified nucleotides to reduce the immunostimulatory properties of the siNA molecule comprises an antisense region having nucleotide sequence that is complementary to a nucleotide sequence of a target gene or a portion thereof and further comprises a sense region, wherein said sense region comprises a nucleotide sequence substantially similar to the nucleotide sequence of said target gene or portion thereof. In

one embodiment thereof, the antisense region and the sense region comprise about 18 to about 38 nucleotides, wherein said antisense region comprises at least about 18 nucleotides that are complementary to nucleotides of the sense region. In one embodiment thereof, the pyrimidine nucleotides in the sense region are 2'-O-methyl pyrimidine nucleotides. In another embodiment thereof, the purine nucleotides in the sense region are 2'-deoxy purine nucleotides. In yet another embodiment thereof, the pyrimidine nucleotides present in the sense region are 2'-deoxy-2'-fluoro pyrimidine nucleotides. In another embodiment thereof, the pyrimidine nucleotides of said antisense region are 2'-deoxy-2'-fluoro pyrimidine nucleotides. In yet another embodiment thereof, the purine nucleotides of said antisense region are 2'-O-methyl purine nucleotides. In still another embodiment thereof, the purine nucleotides present in said antisense region comprise 2'-deoxypurine nucleotides. In another embodiment, the antisense region comprises a phosphorothioate internucleotide linkage at the 3' end of said antisense region. In another embodiment, the antisense region comprises a glyceryl modification at a 3' end of said antisense region.

[00401] In other embodiments, the nucleic acid or polynucleotide (*e.g.*, siNA) molecule comprising modified nucleotides to reduce the immunostimulatory properties of the siNA molecule can comprise any of the structural features of siNA molecules described herein. In other embodiments, the siNA molecule comprising modified nucleotides to reduce the immunostimulatory properties of the siNA molecule can comprise any of the chemical modifications of siNA molecules described herein.

[00402] In one embodiment, the invention features a method for generating a chemically synthesized double stranded nucleic acid or polynucleotide (*e.g.*, siNA) molecule having chemically modified nucleotides to reduce the immunostimulatory properties of the siNA molecule, comprising (a) introducing one or more modified nucleotides in the siNA molecule, and (b) assaying the siNA molecule of step (a) under conditions suitable for isolating an siNA molecule having reduced immunostimulatory properties compared to a corresponding siNA molecule having unmodified nucleotides. Each strand of the siNA molecule is about 18 to about 38 nucleotides in length. One strand of the siNA molecule comprises nucleotide sequence having sufficient complementarity to the target RNA for the siNA molecule to direct cleavage of the target

RNA via RNA interference. In one embodiment, the reduced immunostimulatory properties comprise an abrogated or reduced induction of inflammatory or proinflammatory cytokines, such as interleukin-6 (IL-6) or tumor necrosis alpha (TNF- α), in response to the siNA being introduced in a cell, tissue, or organism. In another embodiment, the reduced immunostimulatory properties comprise an abrogated or reduced induction of Toll Like Receptors (TLRs), such as TLR3, TLR7, TLR8 or TLR9, in response to the siNA being introduced in a cell, tissue, or organism. In another embodiment, the reduced immunostimulatory properties comprise an abrogated or reduced induction of interferons, such as interferon alpha, in response to the siNA being introduced in a cell, tissue, or organism.

[00403] In one embodiment, the invention features nucleic acid or polynucleotide (*e.g.*, siNA) constructs that mediate RNAi against a target polynucleotide, wherein the siNA construct comprises one or more chemical modifications described herein that modulates the binding affinity between the sense and antisense strands of the siNA construct.

[00404] In another embodiment, the invention features a method for generating nucleic acid or polynucleotide (*e.g.*, siNA) molecules with increased binding affinity between the sense and antisense strands of the siNA molecule comprising (a) introducing nucleotides having any of Formula I-VII or any combination thereof into a siNA molecule, and (b) assaying the siNA molecule of step (a) under conditions suitable for isolating siNA molecules having increased binding affinity between the sense and antisense strands of the siNA molecule.

[00405] In one embodiment, the invention features nucleic acid or polynucleotide (*e.g.*, siNA) constructs that mediate RNAi against a target polynucleotide, wherein the siNA construct comprises one or more chemical modifications described herein that modulates the binding affinity between the antisense strand of the siNA construct and a complementary target RNA sequence within a cell.

[00406] In one embodiment, the invention features nucleic acid or polynucleotide (*e.g.*, siNA) constructs that mediate RNAi against a target polynucleotide, wherein the siNA construct comprises one or more chemical modifications described herein that modulates

the binding affinity between the antisense strand of the siNA construct and a complementary target DNA sequence within a cell.

[00407] In another embodiment, the invention features a method for generating nucleic acid or polynucleotide (*e.g.*, siNA) molecules with increased binding affinity between the antisense strand of the siNA molecule and a complementary target RNA sequence comprising (a) introducing nucleotides having any of Formula I-VII or any combination thereof into a siNA molecule, and (b) assaying the siNA molecule of step (a) under conditions suitable for isolating siNA molecules having increased binding affinity between the antisense strand of the siNA molecule and a complementary target RNA sequence.

[00408] In another embodiment, the invention features a method for generating nucleic acid or polynucleotide (*e.g.*, siNA) molecules with increased binding affinity between the antisense strand of the siNA molecule and a complementary target DNA sequence comprising (a) introducing nucleotides having any of Formula I-VII or any combination thereof into a siNA molecule, and (b) assaying the siNA molecule of step (a) under conditions suitable for isolating siNA molecules having increased binding affinity between the antisense strand of the siNA molecule and a complementary target DNA sequence.

[00409] In one embodiment, the invention features nucleic acid or polynucleotide (*e.g.*, siNA) constructs that mediate RNAi against a target polynucleotide, wherein the siNA construct comprises one or more chemical modifications described herein that modulate the polymerase activity of a cellular polymerase capable of generating additional endogenous siNA molecules having sequence homology to the chemically-modified siNA construct.

[00410] In another embodiment, the invention features a method for generating nucleic acid or polynucleotide (*e.g.*, siNA) molecules capable of mediating increased polymerase activity of a cellular polymerase capable of generating additional endogenous siNA molecules having sequence homology to a chemically-modified siNA molecule comprising (a) introducing nucleotides having any of Formula I-VII or any combination thereof into a siNA molecule, and (b) assaying the siNA molecule of step (a) under

conditions suitable for isolating siNA molecules capable of mediating increased polymerase activity of a cellular polymerase capable of generating additional endogenous siNA molecules having sequence homology to the chemically-modified siNA molecule.

[00411] In one embodiment, the invention features chemically-modified nucleic acid or polynucleotide (*e.g.*, siNA) constructs that mediate RNAi against a target polynucleotide in a cell, wherein the chemical modifications do not significantly effect the interaction of siNA with a target RNA molecule, DNA molecule and/or proteins or other factors that are essential for RNAi in a manner that would decrease the efficacy of RNAi mediated by such siNA constructs.

[00412] In another embodiment, the invention features a method for generating nucleic acid or polynucleotide (*e.g.*, siNA) molecules with improved RNAi specificity against polynucleotide targets comprising (a) introducing nucleotides having any of Formula I-VII or any combination thereof into a siNA molecule, and (b) assaying the siNA molecule of step (a) under conditions suitable for isolating siNA molecules having improved RNAi specificity. In one embodiment, improved specificity comprises having reduced off target effects compared to an unmodified siNA molecule. For example, introduction of terminal cap moieties at the 3'-end, 5'-end, or both 3' and 5'-ends of the sense strand or region of a siNA molecule of the invention can direct the siNA to have improved specificity by preventing the sense strand or sense region from acting as a template for RNAi activity against a corresponding target having complementarity to the sense strand or sense region.

[00413] In another embodiment, the invention features a method for generating nucleic acid or polynucleotide (*e.g.*, siNA) molecules with improved RNAi activity against a target polynucleotide comprising (a) introducing nucleotides having any of Formula I-VII or any combination thereof into a siNA molecule, and (b) assaying the siNA molecule of step (a) under conditions suitable for isolating siNA molecules having improved RNAi activity.

[00414] In yet another embodiment, the invention features a method for generating nucleic acid or polynucleotide (*e.g.*, siNA) molecules with improved RNAi activity

against a target RNA comprising (a) introducing nucleotides having any of Formula I-VII or any combination thereof into a siNA molecule, and (b) assaying the siNA molecule of step (a) under conditions suitable for isolating siNA molecules having improved RNAi activity against the target RNA.

[00415] In yet another embodiment, the invention features a method for generating nucleic acid or polynucleotide (*e.g.*, siNA) molecules with improved RNAi activity against a target DNA comprising (a) introducing nucleotides having any of Formula I-VII or any combination thereof into a siNA molecule, and (b) assaying the siNA molecule of step (a) under conditions suitable for isolating siNA molecules having improved RNAi activity against the target DNA.

[00416] In one embodiment, the invention features nucleic acid or polynucleotide (*e.g.*, siNA) constructs that mediate RNAi against a target polynucleotide, wherein the siNA construct comprises one or more chemical modifications described herein that modulates the cellular uptake of the siNA construct, such as cholesterol conjugation of the siNA.

[00417] In another embodiment, the invention features a method for generating nucleic acid or polynucleotide (*e.g.*, siNA) molecules against a target polynucleotide with improved cellular uptake comprising (a) introducing nucleotides having any of Formula I-VII or any combination thereof into a siNA molecule, and (b) assaying the siNA molecule of step (a) under conditions suitable for isolating siNA molecules having improved cellular uptake.

[00418] In one embodiment, the invention features nucleic acid or polynucleotide (*e.g.*, siNA) constructs that mediate RNAi against a target polynucleotide, wherein the siNA construct comprises one or more chemical modifications described herein that increases the bioavailability of the siNA construct, for example, by attaching polymeric conjugates such as polyethyleneglycol or equivalent conjugates that improve the pharmacokinetics of the siNA construct, or by attaching conjugates that target specific tissue types or cell types *in vivo*. Non-limiting examples of such conjugates are described in Vargeese et al., U.S. Serial No. 10/201,394 incorporated by reference herein.

[00419] In one embodiment, the invention features a method for generating nucleic acid or polynucleotide (*e.g.*, siNA) molecules of the invention with improved

bioavailability comprising: (a) introducing a conjugate into the structure of a siNA molecule, and (b) assaying the siNA molecule of step (a) under conditions suitable for isolating siNA molecules having improved bioavailability. Such conjugates can include ligands for cellular receptors, such as peptides derived from naturally occurring protein ligands; protein localization sequences, including cellular ZIP code sequences; antibodies; nucleic acid aptamers; vitamins and other co-factors, such as folate and N-acetylgalactosamine; polymers, such as polyethyleneglycol (PEG); phospholipids; cholesterol; cholesterol derivatives, polyamines, such as spermine or spermidine; and others.

[00420] In one embodiment, the invention features a double stranded short interfering nucleic acid or polynucleotide (*e.g.*, siNA) molecule that comprises a first nucleotide sequence complementary to a target RNA sequence or a portion thereof, and a second sequence having complementarity to said first sequence, wherein said second sequence is chemically modified in a manner that it can no longer act as a guide sequence for efficiently mediating RNA interference and/or be recognized by cellular proteins that facilitate RNAi. In one embodiment, the first nucleotide sequence of the siNA is chemically modified as described herein. In one embodiment, the first nucleotide sequence of the siNA is not modified (*e.g.*, is all RNA).

[00421] In one embodiment, the invention features a double stranded short interfering nucleic acid or polynucleotide (*e.g.*, siNA) molecule that comprises a first nucleotide sequence complementary to a target RNA sequence or a portion thereof, and a second sequence having complementarity to said first sequence, wherein the second sequence is designed or modified in a manner that prevents its entry into the RNAi pathway as a guide sequence or as a sequence that is complementary to a target nucleic acid (*e.g.*, RNA) sequence. In one embodiment, the first nucleotide sequence of the siNA is chemically modified as described herein. In one embodiment, the first nucleotide sequence of the siNA is not modified (*e.g.*, is all RNA). Such design or modifications are expected to enhance the activity of siNA and/or improve the specificity of siNA molecules of the invention. These modifications are also expected to minimize any off-target effects and/or associated toxicity.

[00422] In one embodiment, the invention features a double stranded short interfering nucleic acid or polynucleotide (*e.g.*, siNA) molecule that comprises a first nucleotide sequence complementary to a target RNA sequence or a portion thereof, and a second sequence having complementarity to said first sequence, wherein said second sequence is incapable of acting as a guide sequence for mediating RNA interference. In one embodiment, the first nucleotide sequence of the siNA is chemically modified as described herein. In one embodiment, the first nucleotide sequence of the siNA is not modified (*e.g.*, is all RNA).

[00423] In one embodiment, the invention features a double stranded short interfering nucleic acid or polynucleotide (*e.g.*, siNA) molecule that comprises a first nucleotide sequence complementary to a target RNA sequence or a portion thereof, and a second sequence having complementarity to said first sequence, wherein said second sequence does not have a terminal 5'-hydroxyl (5'-OH) or 5'-phosphate group.

[00424] In one embodiment, the invention features a double stranded short interfering nucleic acid or polynucleotide (*e.g.*, siNA) molecule that comprises a first nucleotide sequence complementary to a target RNA sequence or a portion thereof, and a second sequence having complementarity to said first sequence, wherein said second sequence comprises a terminal cap moiety at the 5'-end of said second sequence. In one embodiment, the terminal cap moiety comprises an inverted abasic, inverted deoxy abasic, inverted nucleotide moiety, a group shown in Figure 10, an alkyl or cycloalkyl group, a heterocycle, or any other group that prevents RNAi activity in which the second sequence serves as a guide sequence or template for RNAi.

[00425] In one embodiment, the invention features a double stranded short interfering nucleic acid or polynucleotide (*e.g.*, siNA) molecule that comprises a first nucleotide sequence complementary to a target RNA sequence or a portion thereof, and a second sequence having complementarity to said first sequence, wherein said second sequence comprises a terminal cap moiety at the 5'-end and 3'-end of said second sequence. In one embodiment, each terminal cap moiety individually comprises an inverted abasic, inverted deoxy abasic, inverted nucleotide moiety, a group shown in Figure 10, an alkyl or cycloalkyl group, a heterocycle, or any other group that prevents RNAi activity in which the second sequence serves as a guide sequence or template for RNAi.

[00426] In one embodiment, the invention features a method for generating nucleic acid or polynucleotide (*e.g.*, siNA) molecules of the invention with improved specificity for down regulating or inhibiting the expression of a target nucleic acid (*e.g.*, a DNA or RNA such as a gene or its corresponding RNA), comprising (a) introducing one or more chemical modifications into the structure of a siNA molecule, and (b) assaying the siNA molecule of step (a) under conditions suitable for isolating siNA molecules having improved specificity. In another embodiment, the chemical modification used to improve specificity comprises terminal cap modifications at the 5'-end, 3'-end, or both 5' and 3'-ends of the siNA molecule. The terminal cap modifications can comprise, for example, structures shown in Figure 10 (*e.g.* inverted deoxyabasic moieties), or any other chemical modification that renders a portion of the siNA molecule (*e.g.* the sense strand) incapable of mediating RNA interference against an off target nucleic acid sequence.

[00427] By "improved specificity" is meant that a modified nucleic acid or polynucleotide (*e.g.*, siNA) molecule of the invention has reduced or fewer off target effects compared to an unmodified or minimally modified siNA molecule. Off target effects can result from a siNA molecule that has RNAi or other biologic activity, such as via an immunostimulatory response, against unintended targets that can result in toxicity.

[00428] In one embodiment, a nucleic acid or polynucleotide (*e.g.*, siNA) molecule is designed such that only the antisense sequence of the siNA molecule can serve as a guide sequence for RISC mediated degradation of a corresponding target RNA sequence. This can be accomplished by rendering the sense sequence of the siNA inactive by introducing chemical modifications to the sense strand that preclude recognition of the sense strand as a guide sequence by RNAi machinery. In one embodiment, such chemical modifications comprise any chemical group at the 5'-end of the sense strand of the siNA, or any other group that serves to render the sense strand inactive as a guide sequence for mediating RNA interference. These modifications, for example, can result in a molecule where the 5'-end of the sense strand no longer has a free 5'-hydroxyl (5'-OH) or a free 5'-phosphate group (*e.g.*, phosphate, diphosphate, triphosphate, cyclic phosphate *etc.*). Non-limiting examples of such siNA constructs are described herein, such as "Stab 9/10", "Stab 7/8", "Stab 7/19", "Stab 17/22", "Stab 23/24", "Stab 24/25",

and “Stab 24/26” (e.g., any siNA having Stab 7, 9, 17, 23, or 24 sense strands) chemistries and variants thereof (see Table IV) wherein the 5'-end and 3'-end of the sense strand of the siNA do not comprise a hydroxyl group or phosphate group. Herein, numeric Stab chemistries include both 2'-fluoro and 2'-OCF₃, versions of the chemistries shown in Table IV. For example, “Stab 7/8” refers to both Stab 7/8 and Stab 7F/8F etc.

[00429] In one embodiment, the invention features a method for generating nucleic acid or polynucleotide (e.g., siNA) molecules of the invention with improved specificity for down regulating or inhibiting the expression of a target nucleic acid (e.g., a DNA or RNA such as a gene or its corresponding RNA), comprising introducing one or more chemical modifications into the structure of a siNA molecule that prevent a strand or portion of the siNA molecule from acting as a template or guide sequence for RNAi activity. In one embodiment, the inactive strand or sense region of the siNA molecule is the sense strand or sense region of the siNA molecule, i.e. the strand or region of the siNA that does not have complementarity to the target nucleic acid sequence. In one embodiment, such chemical modifications comprise any chemical group at the 5'-end of the sense strand or region of the siNA that does not comprise a 5'-hydroxyl (5'-OH) or 5'-phosphate group, or any other group that serves to render the sense strand or sense region inactive as a guide sequence for mediating RNA interference. Non-limiting examples of such siNA constructs are described herein, such as “Stab 9/10”, “Stab 7/8”, “Stab 7/19”, “Stab 17/22”, “Stab 23/24”, “Stab 24/25”, and “Stab 24/26” (e.g., any siNA having Stab 7, 9, 17, 23, or 24 sense strands) chemistries and variants thereof (see Table IV) wherein the 5'-end and 3'-end of the sense strand of the siNA do not comprise a hydroxyl group or phosphate group. Herein, numeric Stab chemistries include both 2'-fluoro and 2'-OCF₃ versions of the chemistries shown in Table IV. For example, “Stab 7/8” refers to both Stab 7/8 and Stab 7F/8F etc.

[00430] In one embodiment, the invention features a method for screening nucleic acid or polynucleotide (e.g., siNA) molecules that are active in mediating RNA interference against a target nucleic acid sequence comprising (a) generating a plurality of unmodified siNA molecules, (b) screening the siNA molecules of step (a) under conditions suitable for isolating siNA molecules that are active in mediating RNA

interference against the target nucleic acid sequence, and (c) introducing chemical modifications (e.g. chemical modifications as described herein or as otherwise known in the art) into the active siNA molecules of (b). In one embodiment, the method further comprises re-screening the chemically modified siNA molecules of step (c) under conditions suitable for isolating chemically modified siNA molecules that are active in mediating RNA interference against the target nucleic acid sequence.

[00431] In one embodiment, the invention features a method for screening chemically modified nucleic acid or polynucleotide (*e.g.*, siNA) molecules that are active in mediating RNA interference against a target nucleic acid sequence comprising (a) generating a plurality of chemically modified siNA molecules (*e.g.* siNA molecules as described herein or as otherwise known in the art), and (b) screening the siNA molecules of step (a) under conditions suitable for isolating chemically modified siNA molecules that are active in mediating RNA interference against the target nucleic acid sequence.

[00432] The term “ligand” refers to any compound or molecule, such as a drug, peptide, hormone, or neurotransmitter that is capable of interacting with another compound, such as a receptor, either directly or indirectly. The receptor that interacts with a ligand can be present on the surface of a cell or can alternately be an intercellular and/or intracellular receptor. Interaction of the ligand with the receptor can result in a biochemical reaction, or can simply be a physical interaction or association.

[00433] In another embodiment, the invention features a method for generating nucleic acid or polynucleotide (*e.g.*, siNA) molecules of the invention with improved bioavailability comprising: (a) introducing an excipient formulation to a siNA molecule, and (b) assaying the siNA molecule of step (a) under conditions suitable for isolating siNA molecules having improved bioavailability. Such excipients include polymers such as cyclodextrins, lipids, cationic lipids, polyamines, phospholipids, nanoparticles, receptors, ligands, and others.

[00434] In another embodiment, the invention features a method for generating nucleic acid or polynucleotide (*e.g.*, siNA) molecules of the invention with improved bioavailability comprising (a) introducing nucleotides having any of Formulae I-VII or any combination thereof into a siNA molecule, and (b) assaying the siNA molecule of

step (a) under conditions suitable for isolating siNA molecules having improved bioavailability.

[00435] In another embodiment, polyethylene glycol (PEG) can be covalently attached to nucleic acid or polynucleotide (*e.g.*, siNA) compounds of the present invention. The attached PEG can be any molecular weight, preferably from about 100 to about 50,000 daltons (Da).

[00436] The present invention can be used alone or as a component of a kit having at least one of the reagents necessary to carry out the *in vitro* or *in vivo* introduction of RNA to test samples and/or subjects. For example, preferred components of the kit include a nucleic acid or polynucleotide (*e.g.*, siNA) molecule of the invention and a vehicle that promotes introduction of the siNA into cells of interest as described herein (*e.g.*, using lipids and other methods of transfection known in the art, see for example Beigelman et al, US 6,395,713). The kit can be used for target validation, such as in determining gene function and/or activity, or in drug optimization, and in drug discovery (see for example Usman et al., USSN 60/402,996). Such a kit can also include instructions to allow a user of the kit to practice the invention.

[00437] The term "short interfering nucleic acid", "siNA", "short interfering RNA", "siRNA", "short interfering nucleic acid molecule", "short interfering oligonucleotide molecule", or "chemically-modified short interfering nucleic acid molecule", "nucleic acid" or "polynucleotide" as used herein refers to any nucleic acid molecule capable of inhibiting or down regulating gene expression or viral replication by mediating RNA interference "RNAi" or gene silencing in a sequence-specific manner. These terms can refer to both individual nucleic acid molecules, a plurality of such nucleic acid molecules, or pools of such nucleic acid molecules. The siNA can be a double-stranded nucleic acid molecule comprising self-complementary sense and antisense regions, wherein the antisense region comprises nucleotide sequence that is complementary to nucleotide sequence in a target nucleic acid molecule or a portion thereof and the sense region having nucleotide sequence corresponding to the target nucleic acid sequence or a portion thereof. The siNA can be assembled from two separate oligonucleotides, where one strand is the sense strand and the other is the antisense strand, wherein the antisense and sense strands are self-complementary (*i.e.*, each strand comprises nucleotide

sequence that is complementary to nucleotide sequence in the other strand; such as where the antisense strand and sense strand form a duplex or double stranded structure, for example wherein the double stranded region is about 15 to about 30, e.g., about 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29 or 30 base pairs; the antisense strand comprises nucleotide sequence that is complementary to nucleotide sequence in a target nucleic acid molecule or a portion thereof and the sense strand comprises nucleotide sequence corresponding to the target nucleic acid sequence or a portion thereof (e.g., about 15 to about 25 or more nucleotides of the siNA molecule are complementary to the target nucleic acid or a portion thereof). Alternatively, the siNA is assembled from a single oligonucleotide, where the self-complementary sense and antisense regions of the siNA are linked by means of a nucleic acid based or non-nucleic acid-based linker(s). The siNA can be a polynucleotide with a duplex, asymmetric duplex, hairpin or asymmetric hairpin secondary structure, having self-complementary sense and antisense regions, wherein the antisense region comprises nucleotide sequence that is complementary to nucleotide sequence in a separate target nucleic acid molecule or a portion thereof and the sense region having nucleotide sequence corresponding to the target nucleic acid sequence or a portion thereof. The siNA can be a circular single-stranded polynucleotide having two or more loop structures and a stem comprising self-complementary sense and antisense regions, wherein the antisense region comprises nucleotide sequence that is complementary to nucleotide sequence in a target nucleic acid molecule or a portion thereof and the sense region having nucleotide sequence corresponding to the target nucleic acid sequence or a portion thereof, and wherein the circular polynucleotide can be processed either in vivo or in vitro to generate an active siNA molecule capable of mediating RNAi. The siNA can also comprise a single stranded polynucleotide having nucleotide sequence complementary to nucleotide sequence in a target nucleic acid molecule or a portion thereof (for example, where such siNA molecule does not require the presence within the siNA molecule of nucleotide sequence corresponding to the target nucleic acid sequence or a portion thereof), wherein the single stranded polynucleotide can further comprise a terminal phosphate group, such as a 5'-phosphate (see for example Martinez et al., 2002, Cell., 110, 563-574 and Schwarz et al., 2002, Molecular Cell, 10, 537-568), or 5',3'-diphosphate. In certain embodiments, the siNA molecule of the invention comprises separate sense and

antisense sequences or regions, wherein the sense and antisense regions are covalently linked by nucleotide or non-nucleotide linker molecules as is known in the art, or are alternately non-covalently linked by ionic interactions, hydrogen bonding, van der Waals interactions, hydrophobic interactions, and/or stacking interactions. In certain embodiments, the siNA molecules of the invention comprise a nucleotide sequence that is complementary to a nucleotide sequence of a target gene. In another embodiment, the siNA molecule of the invention interacts with a nucleotide sequence of a target gene in a manner that causes inhibition of expression of the target gene. As used herein, siNA molecules need not be limited to those molecules containing only RNA, but further encompasses chemically-modified nucleotides and non-nucleotides. In certain embodiments, the short interfering nucleic acid molecules of the invention lack 2'-hydroxy (2'-OH) containing nucleotides. Applicant describes in certain embodiments short interfering nucleic acids that do not require the presence of nucleotides having a 2'-hydroxy group for mediating RNAi and as such, short interfering nucleic acid molecules of the invention optionally do not include any ribonucleotides (e.g., nucleotides having a 2'-OH group). Such siNA molecules that do not require the presence of ribonucleotides within the siNA molecule to support RNAi can however have an attached linker or linkers or other attached or associated groups, moieties, or chains containing one or more nucleotides with 2'-OH groups. Optionally, siNA molecules can comprise ribonucleotides at about 5, 10, 20, 30, 40, or 50% of the nucleotide positions. The modified short interfering nucleic acid molecules of the invention can also be referred to as short interfering modified oligonucleotides "siMON." As used herein, the term siNA is meant to be equivalent to other terms used to describe nucleic acid molecules that are capable of mediating sequence specific RNAi, for example short interfering RNA (siRNA), double-stranded RNA (dsRNA), micro-RNA (miRNA), short hairpin RNA (shRNA), short interfering oligonucleotide, short interfering nucleic acid, short interfering modified oligonucleotide, chemically-modified siRNA, post-transcriptional gene silencing RNA (ptgsRNA), and others. Non limiting examples of siNA molecules of the invention are shown in Figures 4-6, and Tables II and III herein. Such siNA molecules are distinct from other nucleic acid technologies known in the art that mediate inhibition of gene expression, such as ribozymes, antisense, triplex forming, aptamer, 2,5-A chimera, or decoy oligonucleotides.

[00438] By “RNA interference” or “RNAi” is meant a biological process of inhibiting or down regulating gene expression in a cell as is generally known in the art and which is mediated by short interfering nucleic acid molecules, see for example Zamore and Haley, 2005, *Science*, 309, 1519-1524; Vaughn and Martienssen, 2005, *Science*, 309, 1525-1526; Zamore et al., 2000, *Cell*, 101, 25-33; Bass, 2001, *Nature*, 411, 428-429; Elbashir et al., 2001, *Nature*, 411, 494-498; and Kreutzer et al., International PCT Publication No. WO 00/44895; Zernicka-Goetz et al., International PCT Publication No. WO 01/36646; Fire, International PCT Publication No. WO 99/32619; Plaetinck et al., International PCT Publication No. WO 00/01846; Mello and Fire, International PCT Publication No. WO 01/29058; Deschamps-Depaillette, International PCT Publication No. WO 99/07409; and Li et al., International PCT Publication No. WO 00/44914; Allshire, 2002, *Science*, 297, 1818-1819; Volpe et al., 2002, *Science*, 297, 1833-1837; Jenuwein, 2002, *Science*, 297, 2215-2218; and Hall et al., 2002, *Science*, 297, 2232-2237; Hutvagner and Zamore, 2002, *Science*, 297, 2056-60; McManus et al., 2002, *RNA*, 8, 842-850; Reinhart et al., 2002, *Gene & Dev.*, 16, 1616-1626; and Reinhart & Bartel, 2002, *Science*, 297, 1831). In addition, as used herein, the term RNAi is meant to be equivalent to other terms used to describe sequence specific RNA interference, such as post transcriptional gene silencing, translational inhibition, transcriptional inhibition, or epigenetics. For example, siNA molecules of the invention can be used to epigenetically silence genes at both the post-transcriptional level or the pre-transcriptional level. In a non-limiting example, epigenetic modulation of gene expression by siNA molecules of the invention can result from siNA mediated modification of chromatin structure or methylation patterns to alter gene expression (see, for example, Verdel et al., 2004, *Science*, 303, 672-676; Pal-Bhadra et al., 2004, *Science*, 303, 669-672; Allshire, 2002, *Science*, 297, 1818-1819; Volpe et al., 2002, *Science*, 297, 1833-1837; Jenuwein, 2002, *Science*, 297, 2215-2218; and Hall et al., 2002, *Science*, 297, 2232-2237). In another non-limiting example, modulation of gene expression by siNA molecules of the invention can result from siNA mediated cleavage of RNA (either coding or non-coding RNA) via RISC, or alternately, translational inhibition as is known in the art. In another embodiment, modulation of gene expression by siNA molecules of the invention can result from transcriptional inhibition (see for example Janowski et al., 2005, *Nature Chemical Biology*, 1, 216-222).

[00439] In one embodiment, a siNA molecule of the invention is a duplex forming oligonucleotide “DFO”, (see for example Figures 14-15 and Vaish et al., USSN 10/727,780 filed December 3, 2003 and International PCT Application No. US04/16390, filed May 24, 2004).

[00440] In one embodiment, a siNA molecule of the invention is a multifunctional siNA, (see for example Figures 16-28 and Jadhav et al., USSN 60/543,480 filed February 10, 2004 and International PCT Application No. US04/16390, filed May 24, 2004). In one embodiment, the multifunctional siNA of the invention can comprise sequence targeting, for example, two or more regions of PCSK9 RNA (see for example target sequences in Tables II and III). In one embodiment, the multifunctional siNA of the invention can comprise sequence targeting one or more PCSK9 coding or non-coding sequences. In one embodiment, the multifunctional siNA of the invention can comprise sequence targeting one or more PCSK9 RNA and one or more Torc2 coding or non-coding sequences.

[00441] By “asymmetric hairpin” as used herein is meant a linear siNA molecule comprising an antisense region, a loop portion that can comprise nucleotides or non-nucleotides, and a sense region that comprises fewer nucleotides than the antisense region to the extent that the sense region has enough complementary nucleotides to base pair with the antisense region and form a duplex with loop. For example, an asymmetric hairpin siNA molecule of the invention can comprise an antisense region having length sufficient to mediate RNAi in a cell or in vitro system (e.g. about 15 to about 30, or about 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, or 30 nucleotides) and a loop region comprising about 4 to about 12 (e.g., about 4, 5, 6, 7, 8, 9, 10, 11, or 12) nucleotides, and a sense region having about 3 to about 25 (e.g., about 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, or 25) nucleotides that are complementary to the antisense region. The asymmetric hairpin siNA molecule can also comprise a 5'-terminal phosphate group that can be chemically modified. The loop portion of the asymmetric hairpin siNA molecule can comprise nucleotides, non-nucleotides, linker molecules, or conjugate molecules as described herein.

[00442] By “asymmetric duplex” as used herein is meant a nucleic acid or polynucleotide (e.g., siNA) molecule having two separate strands comprising a sense

region and an antisense region, wherein the sense region comprises fewer nucleotides than the antisense region to the extent that the sense region has enough complementary nucleotides to base pair with the antisense region and form a duplex. For example, an asymmetric duplex siNA molecule of the invention can comprise an antisense region having length sufficient to mediate RNAi in a cell or in vitro system (e.g., about 15 to about 30, or about 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, or 30 nucleotides) and a sense region having about 3 to about 25 (e.g., about 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, or 25) nucleotides that are complementary to the antisense region.

[00443] By “RNAi inhibitor” is meant any molecule that can down regulate, reduce or inhibit RNA interference function or activity in a cell or organism. An RNAi inhibitor can down regulate, reduce or inhibit RNAi (e.g., RNAi mediated cleavage of a target polynucleotide, translational inhibition, or transcriptional silencing) by interaction with or interfering the function of any component of the RNAi pathway, including protein components such as RISC, or nucleic acid components such as miRNAs or siRNAs. A RNAi inhibitor can be a siNA molecule, an antisense molecule, an aptamer, or a small molecule that interacts with or interferes with the function of RISC, a miRNA, or a siRNA or any other component of the RNAi pathway in a cell or organism. By inhibiting RNAi (e.g., RNAi mediated cleavage of a target polynucleotide, translational inhibition, or transcriptional silencing), a RNAi inhibitor of the invention can be used to modulate (e.g, up-regulate or down regulate) the expression of a target gene. In one embodiment, a RNA inhibitor of the invention is used to up-regulate gene expression by interfering with (e.g., reducing or preventing) endogenous down-regulation or inhibition of gene expression through translational inhibition, transcriptional silencing, or RISC mediated cleavage of a polynucleotide (e.g., mRNA). By interfering with mechanisms of endogenous repression, silencing, or inhibition of gene expression, RNAi inhibitors of the invention can therefore be used to up-regulate gene expression for the treatment of diseases, traits, or conditions resulting from a loss of function. In one embodiment, the term “RNAi inhibitor” is used in place of the term “siNA” in the various embodiments herein, for example, with the effect of increasing gene expression for the treatment of loss of function diseases, traits, and/or conditions.

[00444] By “aptamer” or “nucleic acid aptamer” as used herein is meant a polynucleotide that binds specifically to a target molecule wherein the nucleic acid molecule has sequence that is distinct from sequence recognized by the target molecule in its natural setting. Alternately, an aptamer can be a nucleic acid molecule that binds to a target molecule where the target molecule does not naturally bind to a nucleic acid. The target molecule can be any molecule of interest. For example, the aptamer can be used to bind to a ligand-binding domain of a protein, thereby preventing interaction of the naturally occurring ligand with the protein. This is a non-limiting example and those in the art will recognize that other embodiments can be readily generated using techniques generally known in the art, see for example Gold et al., 1995, *Annu. Rev. Biochem.*, 64, 763; Brody and Gold, 2000, *J. Biotechnol.*, 74, 5; Sun, 2000, *Curr. Opin. Mol. Ther.*, 2, 100; Kusser, 2000, *J. Biotechnol.*, 74, 27; Hermann and Patel, 2000, *Science*, 287, 820; and Jayasena, 1999, *Clinical Chemistry*, 45, 1628. Aptamer molecules of the invention can be chemically modified as is generally known in the art or as described herein.

[00445] The term “antisense nucleic acid”, as used herein, refers to a nucleic acid molecule that binds to target RNA by means of RNA-RNA or RNA-DNA or RNA-PNA (protein nucleic acid; Egholm et al., 1993 *Nature* 365, 566) interactions and alters the activity of the target RNA (for a review, see Stein and Cheng, 1993 *Science* 261, 1004 and Woolf et al., US patent No. 5,849,902) by steric interaction or by RNase H mediated target recognition. Typically, antisense molecules are complementary to a target sequence along a single contiguous sequence of the antisense molecule. However, in certain embodiments, an antisense molecule can bind to substrate such that the substrate molecule forms a loop, and/or an antisense molecule can bind such that the antisense molecule forms a loop. Thus, the antisense molecule can be complementary to two (or even more) non-contiguous substrate sequences or two (or even more) non-contiguous sequence portions of an antisense molecule can be complementary to a target sequence or both. For a review of current antisense strategies, see Schmajuk et al., 1999, *J. Biol. Chem.*, 274, 21783-21789, Delihias et al., 1997, *Nature*, 15, 751-753, Stein et al., 1997, *Antisense N. A. Drug Dev.*, 7, 151, Crooke, 2000, *Methods Enzymol.*, 313, 3-45; Crooke, 1998, *Biotech. Genet. Eng. Rev.*, 15, 121-157, Crooke, 1997, *Ad. Pharmacol.*, 40, 1-49. In addition, antisense DNA or antisense modified with 2'-MOE and other

modifications as are known in the art can be used to target RNA by means of DNA-RNA interactions, thereby activating RNase H, which digests the target RNA in the duplex. The antisense oligonucleotides can comprise one or more RNase H activating region, which is capable of activating RNase H cleavage of a target RNA. Antisense DNA can be synthesized chemically or expressed via the use of a single stranded DNA expression vector or equivalent thereof. Antisense molecules of the invention can be chemically modified as is generally known in the art or as described herein.

[00446] By "modulate" is meant that the expression of the gene, or level of a RNA molecule or equivalent RNA molecules encoding one or more proteins or protein subunits, or activity of one or more proteins or protein subunits is up regulated or down regulated, such that expression, level, or activity is greater than or less than that observed in the absence of the modulator. For example, the term "modulate" can mean "inhibit," but the use of the word "modulate" is not limited to this definition.

[00447] By "inhibit", "down-regulate", or "reduce", it is meant that the expression of the gene, or level of RNA molecules or equivalent RNA molecules encoding one or more proteins or protein subunits, or activity of one or more proteins or protein subunits, is reduced below that observed in the absence of the nucleic acid or polynucleotide (e.g., siNA) molecules of the invention. In one embodiment, inhibition, down-regulation or reduction with an siNA molecule is below that level observed in the presence of an inactive or attenuated molecule. In another embodiment, inhibition, down-regulation, or reduction with siNA molecules is below that level observed in the presence of, for example, an siNA molecule with scrambled sequence or with mismatches. In another embodiment, inhibition, down-regulation, or reduction of gene expression with a nucleic acid molecule of the instant invention is greater in the presence of the nucleic acid molecule than in its absence. In one embodiment, inhibition, down regulation, or reduction of gene expression is associated with post transcriptional silencing, such as RNAi mediated cleavage of a target nucleic acid molecule (e.g. RNA) or inhibition of translation. In one embodiment, inhibition, down regulation, or reduction of gene expression is associated with pretranscriptional silencing, such as by alterations in DNA methylation patterns and DNA chromatin structure.

[00448] By "up-regulate", or "promote", it is meant that the expression of the gene, or level of RNA molecules or equivalent RNA molecules encoding one or more proteins or protein subunits, or activity of one or more proteins or protein subunits, is increased above that observed in the absence of the nucleic acid molecules (e.g., siNA) of the invention. In one embodiment, up-regulation or promotion of gene expression with an siNA molecule is above that level observed in the presence of an inactive or attenuated molecule. In another embodiment, up-regulation or promotion of gene expression with siNA molecules is above that level observed in the presence of, for example, an siNA molecule with scrambled sequence or with mismatches. In another embodiment, up-regulation or promotion of gene expression with a nucleic acid molecule of the instant invention is greater in the presence of the nucleic acid molecule than in its absence. In one embodiment, up-regulation or promotion of gene expression is associated with inhibition of RNA mediated gene silencing, such as RNAi mediated cleavage or silencing of a coding or non-coding RNA target that down regulates, inhibits, or silences the expression of the gene of interest to be up-regulated. The down regulation of gene expression can, for example, be induced by a coding RNA or its encoded protein, such as through negative feedback or antagonistic effects. The down regulation of gene expression can, for example, be induced by a non-coding RNA having regulatory control over a gene of interest, for example by silencing expression of the gene via translational inhibition, chromatin structure, methylation, RISC mediated RNA cleavage, or translational inhibition. As such, inhibition or down regulation of targets that down regulate, suppress, or silence a gene of interest can be used to up-regulate or promote expression of the gene of interest toward therapeutic use.

[00449] In one embodiment, a RNAi inhibitor of the invention is used to up regulate gene expression by inhibiting RNAi or gene silencing. For example, a RNAi inhibitor of the invention can be used to treat loss of function diseases and conditions by up-regulating gene expression, such as in instances of haploinsufficiency where one allele of a particular gene harbors a mutation (e.g., a frameshift, missense, or nonsense mutation) resulting in a loss of function of the protein encoded by the mutant allele. In such instances, the RNAi inhibitor can be used to up regulate expression of the protein encoded by the wild type or functional allele, thus correcting the haploinsufficiency by compensating for the mutant or null allele. In another embodiment, a siNA molecule of

the invention is used to down regulate expression of a toxic gain of function allele while a RNAi inhibitor of the invention is used concomitantly to up regulate expression of the wild type or functional allele, such as in the treatment of diseases, traits, or conditions herein or otherwise known in the art (see for example Rhodes et al., 2004, PNAS USA, 101:11147-11152 and Meisler et al. 2005, The Journal of Clinical Investigation, 115:2010-2017).

[00450] By "gene", or "target gene" or "target DNA", is meant a nucleic acid that encodes an RNA, for example, nucleic acid sequences including, but not limited to, structural genes encoding a polypeptide. A gene or target gene can also encode a functional RNA (fRNA) or non-coding RNA (ncRNA), such as small temporal RNA (stRNA), micro RNA (miRNA), small nuclear RNA (snRNA), short interfering RNA (siRNA), small nucleolar RNA (snoRNA), ribosomal RNA (rRNA), transfer RNA (tRNA) and precursor RNAs thereof. Such non-coding RNAs can serve as target nucleic acid molecules for siRNA mediated RNA interference in modulating the activity of fRNA or ncRNA involved in functional or regulatory cellular processes. Aberrant fRNA or ncRNA activity leading to disease can therefore be modulated by siRNA molecules of the invention. siRNA molecules targeting fRNA and ncRNA can also be used to manipulate or alter the genotype or phenotype of a subject, organism or cell, by intervening in cellular processes such as genetic imprinting, transcription, translation, or nucleic acid processing (e.g., transamination, methylation etc.). The target gene can be a gene derived from a cell, an endogenous gene, a transgene, or exogenous genes such as genes of a pathogen, for example a virus, which is present in the cell after infection thereof. The cell containing the target gene can be derived from or contained in any organism, for example a plant, animal, protozoan, virus, bacterium, or fungus. Non-limiting examples of plants include monocots, dicots, or gymnosperms. Non-limiting examples of animals include vertebrates or invertebrates. Non-limiting examples of fungi include molds or yeasts. For a review, see for example Snyder and Gerstein, 2003, Science, 300, 258-260.

[00451] By "non-canonical base pair" is meant any non-Watson Crick base pair, such as mismatches and/or wobble base pairs, including flipped mismatches, single hydrogen bond mismatches, trans-type mismatches, triple base interactions, and quadruple base interactions. Non-limiting examples of such non-canonical base pairs include, but are

not limited to, AC reverse Hoogsteen, AC wobble, AU reverse Hoogsteen, GU wobble, AA N7 amino, CC 2-carbonyl-amino(H1)-N3-amino(H2), GA sheared, UC 4-carbonyl-amino, UU imino-carbonyl, AC reverse wobble, AU Hoogsteen, AU reverse Watson Crick, CG reverse Watson Crick, GC N3-amino-amino N3, AA N1-amino symmetric, AA N7-amino symmetric, GA N7-N1 amino-carbonyl, GA+ carbonyl-amino N7-N1, GG N1-carbonyl symmetric, GG N3-amino symmetric, CC carbonyl-amino symmetric, CC N3-amino symmetric, UU 2-carbonyl-imino symmetric, UU 4-carbonyl-imino symmetric, AA amino-N3, AA N1-amino, AC amino 2-carbonyl, AC N3-amino, AC N7-amino, AU amino-4-carbonyl, AU N1-imino, AU N3-imino, AU N7-imino, CC carbonyl-amino, GA amino-N1, GA amino-N7, GA carbonyl-amino, GA N3-amino, GC amino-N3, GC carbonyl-amino, GC N3-amino, GC N7-amino, GG amino-N7, GG carbonyl-imino, GG N7-amino, GU amino-2-carbonyl, GU carbonyl-imino, GU imino-2-carbonyl, GU N7-imino, psiU imino-2-carbonyl, UC 4-carbonyl-amino, UC imino-carbonyl, UU imino-4-carbonyl, AC C2-H-N3, GA carbonyl-C2-H, UU imino-4-carbonyl 2 carbonyl-C5-H, AC amino(A) N3(C)-carbonyl, GC imino amino-carbonyl, Gpsi imino-2-carbonyl amino-2- carbonyl, and GU imino amino-2-carbonyl base pairs.

[00452] By “PCSK9” as used herein is meant, any Proprotein Convertase Subtilisin Kexin 9 or PCSK9 protein, peptide, or polypeptide having PCSK9 activity, such as encoded by PCSK9 Genbank Accession Nos. shown in **Table I**. The term PCSK9 also refers to nucleic acid sequences encoding or regulating the expression of any PCSK9 protein, peptide, or polypeptide having PCSK9 activity. The term “PCSK9” is also meant to include other PCSK9 encoding sequence, such as PCSK9 sequences derived from various subjects or organisms, including other PCSK9 isoforms, mutant PCSK9 genes, splice variants of PCSK9 genes, and PCSK9 gene polymorphisms.

[00453] By “PCSK9 pathway target” is meant any target involved in PCSK9 pathways of gene expression or activity, including such targets as ApoB, SREBP-1a, SREBP-2, calcium/calmodulin-dependent protein kinase 1D, fatty acid binding protein 5, proprotein convertase subtilisin/kexin 9), LXR target genes, metalloprotease domain 11, apoptosis-inhibitory 6, and F-box-only protein 3.

[00454] By “target” as used herein is meant, any target protein, peptide, or polypeptide, such as encoded by Genbank Accession Nos. herein (see **Table I**) and in USSN

10/923,536 and PCT/US03/05028, both incorporated by reference herein. The term “target” also refers to nucleic acid sequences or target polynucleotide sequence encoding any target protein, peptide, or polypeptide, such as proteins, peptides, or polypeptides encoded by sequences having Genbank Accession Nos. shown herein and/or in U.S. Provisional Patent Application No. 60/363,124, USSN 10/923,536 and/or USSN PCT/US03/05028. The target of interest can include target polynucleotide sequences, such as target DNA or target RNA. The term “target” is also meant to include other sequences, such as differing isoforms, mutant target genes, splice variants of target polynucleotides, target polymorphisms, and non-coding (e.g., ncRNA, miRNA, stRNA) or other regulatory polynucleotide sequences as described herein. Therefore, in various embodiments of the invention, a double stranded nucleic acid molecule of the invention (e.g., siNA) having complementarity to a target RNA can be used to inhibit or down regulate miRNA or other ncRNA activity. In one embodiment, inhibition of miRNA or ncRNA activity can be used to down regulate or inhibit gene expression (e.g., gene targets described herein or otherwise known in the art) or viral replication (e.g., viral targets described herein or otherwise known in the art) that is dependent on miRNA or ncRNA activity. In another embodiment, inhibition of miRNA or ncRNA activity by double stranded nucleic acid molecules of the invention (e.g. siNA) having complementarity to the miRNA or ncRNA can be used to up regulate or promote target gene expression (e.g., gene targets described herein or otherwise known in the art) where the expression of such genes is down regulated, suppressed, or silenced by the miRNA or ncRNA. Such up-regulation of gene expression can be used to treat diseases and conditions associated with a loss of function or haploinsufficiency as are generally known in the art.

[00455] By “pathway target” or “host target” is meant any target involved in pathways of gene expression or activity or cellular or host proteins or polynucleotides encoding such proteins or that are otherwise involved in PCSK9 expression and/or viral, bacterial, or other pathogenic infection and/or replication. For example, any given target can have related pathway or host targets that can include upstream, downstream, or modifier genes in a biologic pathway. These pathway and host target genes can provide additive or synergistic effects in the treatment of diseases, conditions, and traits herein.

- [00456] In one embodiment, the target is any target RNA or a portion thereof.
- [00457] In one embodiment, the target is any target DNA or a portion thereof.
- [00458] In one embodiment, the target is any target mRNA or a portion thereof.
- [00459] In one embodiment, the target is any target miRNA or a portion thereof.
- [00460] In one embodiment, the target is any target siRNA or a portion thereof.
- [00461] In one embodiment, the target is any target stRNA or a portion thereof.
- [00462] In one embodiment, the target is a target and or pathway target or a portion thereof.
- [00463] In one embodiment, the target is any (e.g., one or more) of target sequences described herein and/or in U.S. Provisional Patent Application No. 60/363,124, USSN 10/923,536 and/or PCT/US03/05028, or a portion thereof. In one embodiment, the target is any (e.g., one or more) of target sequences shown in Tables I, II, or III or a portion thereof. In another embodiment, the target is a siRNA, miRNA, shRNA or stRNA corresponding to any (e.g., one or more) target, upper strand, or lower strand sequence shown in Table II or Table III or a portion thereof. In another embodiment, the target is any siRNA, miRNA, shRNA or stRNA corresponding any (e.g., one or more) sequence corresponding to a sequence herein or described in U.S. Provisional Patent Application No. 60/363,124, USSN 10/923,536 and/or PCT/US03/05028.
- [00464] By “homologous sequence” is meant, a nucleotide sequence that is shared by one or more polynucleotide sequences, such as genes, gene transcripts and/or non-coding polynucleotides. For example, a homologous sequence can be a nucleotide sequence that is shared by two or more genes encoding related but different proteins, such as different members of a gene family, different protein epitopes, different protein isoforms or completely divergent genes, such as a cytokine and its corresponding receptors. A homologous sequence can be a nucleotide sequence that is shared by two or more non-coding polynucleotides, such as noncoding DNA or RNA, regulatory sequences, introns, and sites of transcriptional control or regulation. Homologous sequences can also include conserved sequence regions shared by more than one polynucleotide sequence.

Homology does not need to be perfect homology (e.g., 100%), as partially homologous sequences are also contemplated by the instant invention (e.g., 99%, 98%, 97%, 96%, 95%, 94%, 93%, 92%, 91%, 90%, 89%, 88%, 87%, 86%, 85%, 84%, 83%, 82%, 81%, 80% etc.).

[00465] By "conserved sequence region" is meant, a nucleotide sequence of one or more regions in a polynucleotide does not vary significantly between generations or from one biological system, subject, or organism to another biological system, subject, or organism. The polynucleotide can include both coding and non-coding DNA and RNA.

[00466] By "sense region" is meant a nucleotide sequence of a nucleic acid or polynucleotide (e.g., siNA) molecule having complementarity to an antisense region of the siNA molecule. In addition, the sense region of a siNA molecule can comprise a nucleic acid sequence having homology with a target nucleic acid sequence. In one embodiment, the sense region of the siNA molecule is referred to as the sense strand or passenger strand.

[00467] By "antisense region" is meant a nucleotide sequence of a nucleic acid or polynucleotide (e.g., siNA) molecule having complementarity to a target nucleic acid sequence. In addition, the antisense region of a siNA molecule can optionally comprise a nucleic acid sequence having complementarity to a sense region of the siNA molecule. In one embodiment, the antisense region of the siNA molecule is referred to as the antisense strand or guide strand.

[00468] By "target nucleic acid" or "target polynucleotide" is meant any nucleic acid sequence (e.g., any target and/or pathway target sequence) whose expression or activity is to be modulated. The target nucleic acid can be DNA or RNA. In one embodiment, a target nucleic acid of the invention is target RNA or DNA.

[00469] By "complementarity" is meant that a nucleic acid can form hydrogen bond(s) with another nucleic acid sequence by either traditional Watson-Crick or other non-traditional types as described herein. In one embodiment, a double stranded nucleic acid molecule of the invention, such as an siNA molecule, wherein each strand is between 15 and 30 nucleotides in length, comprises between about 10% and about 100% (e.g., about 10%, 20%, 30%, 40%, 50%, 60%, 70%, 80%, 90%, or 100%) complementarity between

the two strands of the double stranded nucleic acid molecule. In another embodiment, a double stranded nucleic acid molecule of the invention, such as an siNA molecule, where one strand is the sense strand and the other strand is the antisense strand, wherein each strand is between 15 and 30 nucleotides in length, comprises between at least about 10% and about 100% (e.g., at least about 10%, 20%, 30%, 40%, 50%, 60%, 70%, 80%, 90%, or 100%) complementarity between the nucleotide sequence in the antisense strand of the double stranded nucleic acid molecule and the nucleotide sequence of its corresponding target nucleic acid molecule, such as a target RNA or target mRNA or viral RNA. In one embodiment, a double stranded nucleic acid molecule of the invention, such as an siNA molecule, where one strand comprises nucleotide sequence that is referred to as the sense region and the other strand comprises a nucleotide sequence that is referred to as the antisense region, wherein each strand is between 15 and 30 nucleotides in length, comprises between about 10% and about 100% (e.g., about 10%, 20%, 30%, 40%, 50%, 60%, 70%, 80%, 90%, or 100%) complementarity between the sense region and the antisense region of the double stranded nucleic acid molecule. In reference to the nucleic molecules of the present invention, the binding free energy for a nucleic acid molecule with its complementary sequence is sufficient to allow the relevant function of the nucleic acid to proceed, e.g., RNAi activity. Determination of binding free energies for nucleic acid molecules is well known in the art (see, e.g., Turner et al., 1987, CSH Symp. Quant. Biol. LII pp.123-133; Frier et al., 1986, Proc. Nat. Acad. Sci. USA 83:9373-9377; Turner et al., 1987, J. Am. Chem. Soc. 109:3783-3785). A percent complementarity indicates the percentage of contiguous residues in a nucleic acid molecule that can form hydrogen bonds (e.g., Watson-Crick base pairing) with a second nucleic acid sequence (e.g., 5, 6, 7, 8, 9, or 10 nucleotides out of a total of 10 nucleotides in the first oligonucleotide being base paired to a second nucleic acid sequence having 10 nucleotides represents 50%, 60%, 70%, 80%, 90%, and 100% complementary respectively). In one embodiment, a siNA molecule of the invention has perfect complementarity between the sense strand or sense region and the antisense strand or antisense region of the siNA molecule. In one embodiment, a siNA molecule of the invention is perfectly complementary to a corresponding target nucleic acid molecule. "Perfectly complementary" means that all the contiguous residues of a nucleic acid sequence will hydrogen bond with the same number of contiguous residues in a

second nucleic acid sequence. In one embodiment, a siNA molecule of the invention comprises about 15 to about 30 or more (e.g., about 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, or 30 or more) nucleotides that are complementary to one or more target nucleic acid molecules or a portion thereof. In one embodiment, a siNA molecule of the invention has partial complementarity (i.e., less than 100% complementarity) between the sense strand or sense region and the antisense strand or antisense region of the siNA molecule or between the antisense strand or antisense region of the siNA molecule and a corresponding target nucleic acid molecule. For example, partial complementarity can include various mismatches or non-based paired nucleotides (e.g., 1, 2, 3, 4, 5 or more mismatches or non-based paired nucleotides) within the siNA structure which can result in bulges, loops, or overhangs that result between the between the sense strand or sense region and the antisense strand or antisense region of the siNA molecule or between the antisense strand or antisense region of the siNA molecule and a corresponding target nucleic acid molecule.

[00470] In one embodiment, a double stranded nucleic acid or polynucleotide (e.g., siNA) molecule of the invention has perfect complementarity between the sense strand or sense region and the antisense strand or antisense region of the nucleic acid molecule. In one embodiment, double stranded nucleic acid molecule of the invention, such as siNA molecule, is perfectly complementary to a corresponding target nucleic acid molecule.

[00471] In one embodiment, double stranded nucleic acid molecule of the invention, such as nucleic acid or polynucleotide (e.g., siNA) molecule, has partial complementarity (i.e., less than 100% complementarity) between the sense strand or sense region and the antisense strand or antisense region of the double stranded nucleic acid molecule or between the antisense strand or antisense region of the nucleic acid molecule and a corresponding target nucleic acid molecule. For example, partial complementarity can include various mismatches or non-base paired nucleotides (e.g., 1, 2, 3, 4, 5 or more mismatches or non-based paired nucleotides, such as nucleotide bulges) within the double stranded nucleic acid molecule, structure which can result in bulges, loops, or overhangs that result between the sense strand or sense region and the antisense strand or antisense region of the double stranded nucleic acid molecule or between the antisense

strand or antisense region of the double stranded nucleic acid molecule and a corresponding target nucleic acid molecule.

[00472] In one embodiment, double stranded nucleic acid molecule of the invention is a microRNA (miRNA). By “microRNA” or “miRNA”, a small double stranded RNA that regulates the expression of target messenger RNAs either by mRNA cleavage, translational repression/inhibition or heterochromatic silencing (see for example Ambros, 2004, *Nature*, 431, 350-355; Bartel, 2004, *Cell*, 116, 281-297; Cullen, 2004, *Virus Research*, 102, 3-9; He et al., 2004, *Nat. Rev. Genet.*, 5, 522-531; and Ying et al., 2004, *Gene*, 342, 25-28). In one embodiment, the microRNA of the invention, has partial complementarity (i.e., less than 100% complementarity) between the sense strand or sense region and the antisense strand or antisense region of the miRNA molecule or between the antisense strand or antisense region of the miRNA and a corresponding target nucleic acid molecule. For example, partial complementarity can include various mismatches or non-base paired nucleotides (e.g., 1, 2, 3, 4, 5 or more mismatches or non-based paired nucleotides, such as nucleotide bulges) within the double stranded nucleic acid molecule, structure which can result in bulges, loops, or overhangs that result between the sense strand or sense region and the antisense strand or antisense region of the miRNA or between the antisense strand or antisense region of the miRNA and a corresponding target nucleic acid molecule.

[00473] In one embodiment, nucleic acid or polynucleotide (e.g., siNA) molecules of the invention that down regulate or reduce PCSK9 gene expression are used for preventing or treating metabolic and/or cardiovascular and related diseases, disorders, conditions, or traits in a subject or organism as described herein or otherwise known in the art.

[00474] By “metabolic disease” is meant any disease or condition affecting metabolic pathways as in known in the art. Metabolic disease can result in an abnormal metabolic process, either congenital due to inherited enzyme abnormality (inborn errors of metabolism) or acquired due to disease of an endocrine organ or failure of a metabolically important organ such as the liver. In one embodiment, metabolic disease includes hyperlipidemia, hypercholesterolemia, cardiovascular disease, atherosclerosis, hypertension, diabetes (e.g., type I and/or type II diabetes), insulin resistance, and/or

obesity By “cardiovascular disease” is meant a disease or condition affecting the heart and vasculature, including but not limited to, coronary heart disease (CHD), cerebrovascular disease (CVD), aortic stenosis, peripheral vascular disease, atherosclerosis, arteriosclerosis, myocardial infarction (heart attack), cerebrovascular diseases (stroke), transient ischaemic attacks (TIA), angina (stable and unstable), atrial fibrillation, arrhythmia, valvular disease, congestive heart failure, hypercholesterolemia, type I hyperlipoproteinemia, type II hyperlipoproteinemia, type III hyperlipoproteinemia, type IV hyperlipoproteinemia, type V hyperlipoproteinemia, secondary hypertriglyceridemia, and familial lecithin cholesterol acyltransferase deficiency.

[00475] In one embodiment of the present invention, each sequence of a nucleic acid or polynucleotide (*e.g.*, siNA) molecule of the invention is independently about 15 to about 30 nucleotides in length, in specific embodiments about 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, or 30 nucleotides in length. In another embodiment, the siNA duplexes of the invention independently comprise about 15 to about 30 base pairs (*e.g.*, about 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, or 30). In another embodiment, one or more strands of the siNA molecule of the invention independently comprises about 15 to about 30 nucleotides (*e.g.*, about 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, or 30) that are complementary to a target nucleic acid molecule. In yet another embodiment, siNA molecules of the invention comprising hairpin or circular structures are about 35 to about 55 (*e.g.*, about 35, 40, 45, 50 or 55) nucleotides in length, or about 38 to about 44 (*e.g.*, about 38, 39, 40, 41, 42, 43, or 44) nucleotides in length and comprising about 15 to about 25 (*e.g.*, about 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, or 25) base pairs. Exemplary siNA molecules of the invention are shown in Tables II and III and/or Figures 4-5.

[00476] As used herein “cell” is used in its usual biological sense, and does not refer to an entire multicellular organism, *e.g.*, specifically does not refer to a human. The cell can be present in an organism, *e.g.*, birds, plants and mammals such as humans, cows, sheep, apes, monkeys, swine, dogs, and cats. The cell can be prokaryotic (*e.g.*, bacterial cell) or eukaryotic (*e.g.*, mammalian or plant cell). The cell can be of somatic or germ line origin, totipotent or pluripotent, dividing or non-dividing. The cell can also be derived from or can comprise a gamete or embryo, a stem cell, or a fully differentiated

cell. The cell can be an isolated cell, purified cell, or substantially purified cell as is generally recognized in the art.

[00477] The nucleic acid or polynucleotide (*e.g.*, siNA) molecules of the invention are added directly, or can be complexed with cationic lipids, packaged within liposomes, or otherwise delivered to target cells or tissues. The nucleic acid or nucleic acid complexes can be locally administered to relevant tissues *ex vivo*, or *in vivo* through local delivery to the lung, with or without their incorporation in biopolymers. In particular embodiments, the nucleic acid molecules of the invention comprise sequences shown in Tables II-III and/or Figures 4-5. Examples of such nucleic acid molecules consist essentially of sequences defined in these tables and figures. Furthermore, the chemically modified constructs described in Table IV and the lipid nanoparticle (LNP) formulations shown in Table VI can be applied to any siNA sequence or group of siNA sequences of the invention.

[00478] In another aspect, the invention provides mammalian cells containing one or more nucleic acid or polynucleotide (*e.g.*, siNA) molecules of this invention. The one or more siNA molecules can independently be targeted to the same or different sites within a target polynucleotide of the invention.

[00479] By "RNA" is meant a molecule comprising at least one ribonucleotide residue. By "ribonucleotide" is meant a nucleotide with a hydroxyl group at the 2' position of a β -D-ribofuranose moiety. The terms include double-stranded RNA, single-stranded RNA, isolated RNA such as partially purified RNA, essentially pure RNA, synthetic RNA, recombinantly produced RNA, as well as altered RNA that differs from naturally occurring RNA by the addition, deletion, substitution and/or alteration of one or more nucleotides. Such alterations can include addition of non-nucleotide material, such as to the end(s) of the siNA or internally, for example at one or more nucleotides of the RNA. Nucleotides in the RNA molecules of the instant invention can also comprise non-standard nucleotides, such as non-naturally occurring nucleotides or chemically synthesized nucleotides or deoxynucleotides. These altered RNAs can be referred to as analogs or analogs of naturally-occurring RNA.

[00480] By "subject" is meant an organism, which is a donor or recipient of explanted cells or the cells themselves. "Subject" also refers to an organism to which the nucleic acid molecules of the invention can be administered. A subject can be a mammal or mammalian cells, including a human or human cells. In one embodiment, the subject is an infant (e.g., subjects that are less than 1 month old, or 1, 2, 3, 4, 5, 6, 7, 8, 9 10, 11, or 12 months old). In one embodiment, the subject is a toddler (e.g., 1, 2, 3, 4, 5 or 6 years old). In one embodiment, the subject is a senior (e.g., anyone over the age of about 65 years of age).

[00481] By "chemical modification" as used herein is meant any modification of chemical structure of the nucleotides that differs from nucleotides of native siRNA or RNA. The term "chemical modification" encompasses the addition, substitution, or modification of native siRNA or RNA nucleosides and nucleotides with modified nucleosides and modified nucleotides as described herein or as is otherwise known in the art. Non-limiting examples of such chemical modifications include without limitation compositions having any of Formulae I, II, III, IV, V, VI, or VII herein, phosphorothioate internucleotide linkages, 2'-deoxyribonucleotides, 2'-O-methyl ribonucleotides, 2'-deoxy-2'-fluoro ribonucleotides, 4'-thio ribonucleotides, 2'-O-trifluoromethyl nucleotides, 2'-O-ethyl-trifluoromethoxy nucleotides, 2'-O-difluoromethoxy-ethoxy nucleotides (see for example USSN 10/981,966 filed November 5, 2004, incorporated by reference herein), FANA, "universal base" nucleotides, "acyclic" nucleotides, 5-C-methyl nucleotides, terminal glyceryl and/or inverted deoxy abasic residue incorporation, or a modification having any of Formulae I-VII herein. In one embodiment, the nucleic acid molecules of the invention (e.g, dsRNA, siNA etc.) are partially modified (e.g., about 5%, 10,%, 15%, 20%, 25%, 30%, 35%, 40%, 45%, 50%, 55%, 60%, 65%, 70%, 75%, 80%, 85%, 90%, or 95% modified) with chemical modifications. In another embodiment, the the nucleic acid molecules of the invention (e.g, dsRNA, siNA etc.) are completely modified (e.g., about 100% modified) with chemical modifications.

[00482] The term "phosphorothioate" as used herein refers to an internucleotide linkage having Formula I, wherein Z and/or W comprise a sulfur atom. Hence, the term

phosphorothioate refers to both phosphorothioate and phosphorodithioate internucleotide linkages.

[00483] The term “phosphonoacetate” as used herein refers to an internucleotide linkage having Formula I, wherein Z and/or W comprise an acetyl or protected acetyl group.

[00484] The term “thiophosphonoacetate” as used herein refers to an internucleotide linkage having Formula I, wherein Z comprises an acetyl or protected acetyl group and W comprises a sulfur atom or alternately W comprises an acetyl or protected acetyl group and Z comprises a sulfur atom.

[00485] The term “universal base” as used herein refers to nucleotide base analogs that form base pairs with each of the natural DNA/RNA bases with little discrimination between them. Non-limiting examples of universal bases include C-phenyl, C-naphthyl and other aromatic derivatives, inosine, azole carboxamides, and nitroazole derivatives such as 3-nitropyrrole, 4-nitroindole, 5-nitroindole, and 6-nitroindole as known in the art (see for example Loakes, 2001, Nucleic Acids Research, 29, 2437-2447).

[00486] The term “acyclic nucleotide” as used herein refers to any nucleotide having an acyclic ribose sugar, for example where any of the ribose carbons (C1, C2, C3, C4, or C5), are independently or in combination absent from the nucleotide.

[00487] The nucleic acid or polynucleotide (e.g., siNA) molecules of the instant invention, individually, or in combination or in conjunction with other drugs, can be used to for preventing or treating diseases, disorders, conditions, and traits described herein or otherwise known in the art, in a subject or organism. For example, the siNA molecules can be administered to a subject or can be administered to other appropriate cells evident to those skilled in the art, individually or in combination with one or more drugs under conditions suitable for the treatment.

[00488] The nucleic acid molecules of the instant invention, individually, or in combination or in conjunction with other drugs, can be used to for preventing or treating hyperlipidemia, hypercholesterolemia, cardiovascular disease, atherosclerosis, hypertension, diabetes (e.g., type I and/or type II diabetes), insulin resistance, obesity

conditions, disorders traits described herein or otherwise known in the art in a subject or organism.

[00489] For example, the nucleic acid or polynucleotide (*e.g.*, siNA) molecules can be administered to a subject or can be administered to other appropriate cells evident to those skilled in the art, individually or in combination with one or more drugs under conditions suitable for the treatment.

[00490] In one embodiment, the nucleic acid or polynucleotide (*e.g.*, siNA) molecules of the invention can be administered to a subject or can be administered to other appropriate cells evident to those skilled in the art, individually or in combination with one or more drugs under conditions suitable for the treatment.

[00491] In a further embodiment, the siNA molecules can be used in combination with other known treatments to prevent or treat diseases disorders or conditions, disorders and traits described herein in a subject or organism as are known in the art.

[00492] In a further embodiment, the siNA molecules can be used in combination with other known treatments to prevent or treat in a subject or organism. For example, the described molecules could be used in combination with one or more known compounds, treatments, or procedures to prevent or treat diseases, disorders, conditions, and traits described herein in a subject or organism as are known in the art.

[00493] In one embodiment, the invention features an expression vector comprising a nucleic acid sequence encoding at least one siNA molecule of the invention, in a manner which allows expression of the siNA molecule. For example, the vector can contain sequence(s) encoding both strands of a siNA molecule comprising a duplex. The vector can also contain sequence(s) encoding a single nucleic acid molecule that is self-complementary and thus forms a siNA molecule. Non-limiting examples of such expression vectors are described in Paul et al., 2002, Nature Biotechnology, 19, 505; Miyagishi and Taira, 2002, Nature Biotechnology, 19, 497; Lee et al., 2002, Nature Biotechnology, 19, 500; and Novina et al., 2002, Nature Medicine, advance online publication doi:10.1038/nm725.

[00494] In another embodiment, the invention features a mammalian cell, for example, a human cell, including an expression vector of the invention.

[00495] In yet another embodiment, the expression vector of the invention comprises a sequence for a siNA molecule having complementarity to a RNA molecule referred to by a Genbank Accession numbers, for example Genbank Accession Nos. described herein or in U.S. Provisional Patent Application No. 60/363,124, USSN 10/923,536 and/or PCT/US03/05028.

[00496] In one embodiment, an expression vector of the invention comprises a nucleic acid sequence encoding two or more siNA molecules, which can be the same or different.

[00497] In another aspect of the invention, siNA molecules that interact with target RNA molecules and down-regulate gene encoding target RNA molecules (for example target RNA molecules referred to by Genbank Accession numbers herein) are expressed from transcription units inserted into DNA or RNA vectors. The recombinant vectors can be DNA plasmids or viral vectors. siNA expressing viral vectors can be constructed based on, but not limited to, adeno-associated virus, retrovirus, adenovirus, or alphavirus. The recombinant vectors capable of expressing the siNA molecules can be delivered as described herein, and persist in target cells. Alternatively, viral vectors can be used that provide for transient expression of siNA molecules. Such vectors can be repeatedly administered as necessary. Once expressed, the siNA molecules bind and down-regulate gene function or expression via RNA interference (RNAi). Delivery of siNA expressing vectors can be systemic, such as by intravenous or intramuscular administration, by administration to target cells ex-planted from a subject followed by reintroduction into the subject, or by any other means that would allow for introduction into the desired target cell.

[00498] In an additional embodiment, the invention provides a method of validating a candidate protein as a suitable target for drug discovery by introducing into a cell or organism an siRNA as described herein and maintaining the cell or organism under conditions such that target-specific RNAi can occur, determining a characteristic or property of the cell or organism, and comparing the characteristic or property to a

suitable control, the comparison yielding information about whether the candidate protein is a suitable target for drug discovery.

[00499] An exemplary embodiment provides a method of assessing whether a gene product is a suitable target for drug discovery comprising: (a) introducing an RNAi construct of the invention which targets the mRNA of the gene for degradation into a cell or organism; (b) maintaining the cell or organism of (a) under conditions in which degradation of the mRNA occurs resulting in decreased expression of the gene; and (c) determining the effect of the decreased expression of the gene on the cell or organism, wherein if decreased expression has an effect, then the gene product is a target for drug discovery.

[00500] By "vectors" is meant any nucleic acid- and/or viral-based technique used to deliver a desired nucleic acid.

[00501] Other features and advantages of the invention will be apparent from the following description of the preferred embodiments thereof, and from the claims.

BRIEF DESCRIPTION OF THE DRAWINGS

[00502] **Figure 1** shows a non-limiting example of a scheme for the synthesis of siNA molecules. The complementary siNA sequence strands, strand 1 and strand 2, are synthesized in tandem and are connected by a cleavable linkage, such as a nucleotide succinate or abasic succinate, which can be the same or different from the cleavable linker used for solid phase synthesis on a solid support. The synthesis can be either solid phase or solution phase, in the example shown, the synthesis is a solid phase synthesis. The synthesis is performed such that a protecting group, such as a dimethoxytrityl group, remains intact on the terminal nucleotide of the tandem oligonucleotide. Upon cleavage and deprotection of the oligonucleotide, the two siNA strands spontaneously hybridize to form a siNA duplex, which allows the purification of the duplex by utilizing the properties of the terminal protecting group, for example by applying a trityl on purification method wherein only duplexes/oligonucleotides with the terminal protecting group are isolated.

[00503] Figure 2 shows a MALDI-TOF mass spectrum of a purified siNA duplex synthesized by a method of the invention. The two peaks shown correspond to the predicted mass of the separate siNA sequence strands. This result demonstrates that the siNA duplex generated from tandem synthesis can be purified as a single entity using a simple trityl-on purification methodology.

[00504] Figure 3 shows a non-limiting proposed mechanistic representation of target RNA degradation involved in RNAi. Double-stranded RNA (dsRNA), which is generated by RNA-dependent RNA polymerase (RdRP) from foreign single-stranded RNA, for example viral, transposon, or other exogenous RNA, activates the DICER enzyme that in turn generates siNA duplexes. Alternately, synthetic or expressed siNA can be introduced directly into a cell by appropriate means. An active siNA complex forms which recognizes a target RNA, resulting in degradation of the target RNA by the RISC endonuclease complex or in the synthesis of additional RNA by RNA-dependent RNA polymerase (RdRP), which can activate DICER and result in additional siNA molecules, thereby amplifying the RNAi response.

[00505] Figure 4A-F shows non-limiting examples of chemically-modified siNA constructs of the present invention. In the figure, N stands for any nucleotide (adenosine, guanosine, cytosine, uridine, or optionally thymidine, for example thymidine can be substituted in the overhanging regions designated by parenthesis (N N). Various modifications are shown for the sense and antisense strands of the siNA constructs. The (N N) nucleotide positions can be chemically modified as described herein (e.g., 2'-O-methyl, 2'-deoxy-2'-fluoro etc.) and can be either derived from a corresponding target nucleic acid sequence or not (see for example Figure 6C). Furthermore, the sequences shown in Figure 4 can optionally include a ribonucleotide at the 9th position from the 5'-end of the sense strand or the 11th position based on the 5'-end of the guide strand by counting 11 nucleotide positions in from the 5'-terminus of the guide strand (see Figure 6C).

[00506] Figure 4A: The sense strand comprises 21 nucleotides wherein the two terminal 3'-nucleotides are optionally base paired and wherein all nucleotides present are ribonucleotides except for (N N) nucleotides, which can comprise ribonucleotides, deoxynucleotides, universal bases, or other chemical modifications described herein.

The antisense strand comprises 21 nucleotides, optionally having a 3'-terminal glyceryl moiety wherein the two terminal 3'-nucleotides are optionally complementary to the target RNA sequence, and wherein all nucleotides present are ribonucleotides except for (N N) nucleotides, which can comprise ribonucleotides, deoxynucleotides, universal bases, or other chemical modifications described herein. A modified internucleotide linkage, such as a phosphorothioate, phosphorodithioate or other modified internucleotide linkage as described herein, shown as "s", optionally connects the (N N) nucleotides in the antisense strand.

[00507] Figure 4B: The sense strand comprises 21 nucleotides wherein the two terminal 3'-nucleotides are optionally base paired and wherein all pyrimidine nucleotides that may be present are 2'-deoxy-2'-fluoro modified nucleotides and all purine nucleotides that may be present are 2'-O-methyl modified nucleotides except for (N N) nucleotides, which can comprise ribonucleotides, deoxynucleotides, universal bases, or other chemical modifications described herein. The antisense strand comprises 21 nucleotides, optionally having a 3'-terminal glyceryl moiety and wherein the two terminal 3'-nucleotides are optionally complementary to the target RNA sequence, and wherein all pyrimidine nucleotides that may be present are 2'-deoxy-2'-fluoro modified nucleotides and all purine nucleotides that may be present are 2'-O-methyl modified nucleotides except for (N N) nucleotides, which can comprise ribonucleotides, deoxynucleotides, universal bases, or other chemical modifications described herein. A modified internucleotide linkage, such as a phosphorothioate, phosphorodithioate or other modified internucleotide linkage as described herein, shown as "s", optionally connects the (N N) nucleotides in the sense and antisense strand.

[00508] Figure 4C: The sense strand comprises 21 nucleotides having 5'- and 3'-terminal cap moieties wherein the two terminal 3'-nucleotides are optionally base paired and wherein all pyrimidine nucleotides that may be present are 2'-O-methyl or 2'-deoxy-2'-fluoro modified nucleotides except for (N N) nucleotides, which can comprise ribonucleotides, deoxynucleotides, universal bases, or other chemical modifications described herein. The antisense strand comprises 21 nucleotides, optionally having a 3'-terminal glyceryl moiety and wherein the two terminal 3'-nucleotides are optionally complementary to the target RNA sequence, and wherein all pyrimidine nucleotides that

may be present are 2'-deoxy-2'-fluoro modified nucleotides except for (N N) nucleotides, which can comprise ribonucleotides, deoxynucleotides, universal bases, or other chemical modifications described herein. A modified internucleotide linkage, such as a phosphorothioate, phosphorodithioate or other modified internucleotide linkage as described herein, shown as “s”, optionally connects the (N N) nucleotides in the antisense strand.

[00509] Figure 4D: The sense strand comprises 21 nucleotides having 5'- and 3'-terminal cap moieties wherein the two terminal 3'-nucleotides are optionally base paired and wherein all pyrimidine nucleotides that may be present are 2'-deoxy-2'-fluoro modified nucleotides except for (N N) nucleotides, which can comprise ribonucleotides, deoxynucleotides, universal bases, or other chemical modifications described herein and wherein and all purine nucleotides that may be present are 2'-deoxy nucleotides. The antisense strand comprises 21 nucleotides, optionally having a 3'-terminal glyceryl moiety and wherein the two terminal 3'-nucleotides are optionally complementary to the target RNA sequence, wherein all pyrimidine nucleotides that may be present are 2'-deoxy-2'-fluoro modified nucleotides and all purine nucleotides that may be present are 2'-O-methyl modified nucleotides except for (N N) nucleotides, which can comprise ribonucleotides, deoxynucleotides, universal bases, or other chemical modifications described herein. A modified internucleotide linkage, such as a phosphorothioate, phosphorodithioate or other modified internucleotide linkage as described herein, shown as “s”, optionally connects the (N N) nucleotides in the antisense strand.

[00510] Figure 4E: The sense strand comprises 21 nucleotides having 5'- and 3'-terminal cap moieties wherein the two terminal 3'-nucleotides are optionally base paired and wherein all pyrimidine nucleotides that may be present are 2'-deoxy-2'-fluoro modified nucleotides except for (N N) nucleotides, which can comprise ribonucleotides, deoxynucleotides, universal bases, or other chemical modifications described herein. The antisense strand comprises 21 nucleotides, optionally having a 3'-terminal glyceryl moiety and wherein the two terminal 3'-nucleotides are optionally complementary to the target RNA sequence, and wherein all pyrimidine nucleotides that may be present are 2'-deoxy-2'-fluoro modified nucleotides and all purine nucleotides that may be present are 2'-O-methyl modified nucleotides except for (N N) nucleotides, which can comprise

ribonucleotides, deoxynucleotides, universal bases, or other chemical modifications described herein. A modified internucleotide linkage, such as a phosphorothioate, phosphorodithioate or other modified internucleotide linkage as described herein, shown as “s”, optionally connects the (N N) nucleotides in the antisense strand.

[00511] Figure 4F: The sense strand comprises 21 nucleotides having 5'- and 3'-terminal cap moieties wherein the two terminal 3'-nucleotides are optionally base paired and wherein all pyrimidine nucleotides that may be present are 2'-deoxy-2'-fluoro modified nucleotides except for (N N) nucleotides, which can comprise ribonucleotides, deoxynucleotides, universal bases, or other chemical modifications described herein and wherein all purine nucleotides that may be present are 2'-deoxy nucleotides. The antisense strand comprises 21 nucleotides, optionally having a 3'-terminal glyceryl moiety and wherein the two terminal 3'-nucleotides are optionally complementary to the target RNA sequence, and having one 3'-terminal phosphorothioate internucleotide linkage and wherein all pyrimidine nucleotides that may be present are 2'-deoxy-2'-fluoro modified nucleotides and all purine nucleotides that may be present are 2'-deoxy nucleotides except for (N N) nucleotides, which can comprise ribonucleotides, deoxynucleotides, universal bases, or other chemical modifications described herein. A modified internucleotide linkage, such as a phosphorothioate, phosphorodithioate or other modified internucleotide linkage as described herein, shown as “s”, optionally connects the (N N) nucleotides in the antisense strand. The antisense strand of constructs A-F comprise sequence complementary to any target nucleic acid sequence of the invention. Furthermore, when a glyceryl moiety (L) is present at the 3'-end of the antisense strand for any construct shown in Figure 4 A-F, the modified internucleotide linkage is optional.

[00512] Figure 5A-F shows non-limiting examples of specific chemically-modified siNA sequences of the invention. A-F applies the chemical modifications described in Figure 4A-F to PCSK9 siNA sequence. Such chemical modifications can be applied to any PCSK9 sequence and/or PCSK9 polymorphism. Furthermore, the sequences shown in Figure 5 can optionally include a ribonucleotide at the 9th position from the 5'-end of the sense strand or the 11th position based on the 5'-end of the guide strand by counting 11 nucleotide positions in from the 5'-terminus of the guide strand (see Figure 6C). In

addition, the sequences shown in Figure 5 can optionally include terminal ribonucleotides at up to about 4 positions at the 5'-end of the antisense strand (e.g., about 1, 2, 3, or 4 terminal ribonucleotides at the 5'-end of the antisense strand) and/or cellular target sequence.

[00513] Figure 6A-C shows non-limiting examples of different siNA constructs of the invention.

[00514] The examples shown in Figure 6A (constructs 1, 2, and 3) have 19 representative base pairs; however, different embodiments of the invention include any number of base pairs described herein. Bracketed regions represent nucleotide overhangs, for example, comprising about 1, 2, 3, or 4 nucleotides in length, preferably about 2 nucleotides. Constructs 1 and 2 can be used independently for RNAi activity. Construct 2 can comprise a polynucleotide or non-nucleotide linker, which can optionally be designed as a biodegradable linker. In one embodiment, the loop structure shown in construct 2 can comprise a biodegradable linker that results in the formation of construct 1 in vivo and/or in vitro. In another example, construct 3 can be used to generate construct 2 under the same principle wherein a linker is used to generate the active siNA construct 2 in vivo and/or in vitro, which can optionally utilize another biodegradable linker to generate the active siNA construct 1 in vivo and/or in vitro. As such, the stability and/or activity of the siNA constructs can be modulated based on the design of the siNA construct for use in vivo or in vitro and/or in vitro.

[00515] The examples shown in Figure 6B represent different variations of double stranded nucleic acid molecule of the invention, such as microRNA, that can include overhangs, bulges, loops, and stem-loops resulting from partial complementarity. Such motifs having bulges, loops, and stem-loops are generally characteristics of miRNA. The bulges, loops, and stem-loops can result from any degree of partial complementarity, such as mismatches or bulges of about 1, 2, 3, 4, 5, 6, 7, 8, 9, 10 or more nucleotides in one or both strands of the double stranded nucleic acid molecule of the invention.

[00516] The example shown in Figure 6C represents a model double stranded nucleic acid molecule of the invention comprising a 19 base pair duplex of two 21 nucleotide sequences having dinucleotide 3'-overhangs. The top strand (1) represents the sense

strand (passenger strand), the middle strand (2) represents the antisense (guide strand), and the lower strand (3) represents a target polynucleotide sequence. The dinucleotide overhangs (NN) can comprise sequence derived from the target polynucleotide. For example, the 3'-(NN) sequence in the guide strand can be complementary to the 5'-[NN] sequence of the target polynucleotide. In addition, the 5'-(NN) sequence of the passenger strand can comprise the same sequence as the 5'-[NN] sequence of the target polynucleotide sequence. In other embodiments, the overhangs (NN) are not derived from the target polynucleotide sequence, for example where the 3'-(NN) sequence in the guide strand are not complementary to the 5'-[NN] sequence of the target polynucleotide and the 5'-(NN) sequence of the passenger strand can comprise different sequence from the 5'-[NN] sequence of the target polynucleotide sequence. In additional embodiments, any (NN) nucleotides are chemically modified, e.g., as 2'-O-methyl, 2'-deoxy-2'-fluoro, and/or other modifications herein. Furthermore, the passenger strand can comprise a ribonucleotide position N of the passenger strand. For the representative 19 base pair 21 mer duplex shown, position N can be 9 nucleotides in from the 3' end of the passenger strand. However, in duplexes of differing length, the position N is determined based on the 5'-end of the guide strand by counting 11 nucleotide positions in from the 5'-terminus of the guide strand and picking the corresponding base paired nucleotide in the passenger strand. Cleavage by Ago2 takes place between positions 10 and 11 as indicated by the arrow. In additional embodiments, there are two ribonucleotides, NN, at positions 10 and 11 based on the 5'-end of the guide strand by counting 10 and 11 nucleotide positions in from the 5'-terminus of the guide strand and picking the corresponding base paired nucleotides in the passenger strand.

[00517] Figure 7A-C is a diagrammatic representation of a scheme utilized in generating an expression cassette to generate siNA hairpin constructs.

[00518] Figure 7A: A DNA oligomer is synthesized with a 5'-restriction site (R1) sequence followed by a region having sequence identical (sense region of siNA) to a predetermined target sequence, wherein the sense region comprises, for example, about 19, 20, 21, or 22 nucleotides (N) in length, which is followed by a loop sequence of defined sequence (X), comprising, for example, about 3 to about 10 nucleotides.

[00519] Figure 7B: The synthetic construct is then extended by DNA polymerase to generate a hairpin structure having self-complementary sequence that will result in a siNA transcript having specificity for a target sequence and having self-complementary sense and antisense regions.

[00520] Figure 7C: The construct is heated (for example to about 95°C) to linearize the sequence, thus allowing extension of a complementary second DNA strand using a primer to the 3'-restriction sequence of the first strand. The double-stranded DNA is then inserted into an appropriate vector for expression in cells. The construct can be designed such that a 3'-terminal nucleotide overhang results from the transcription, for example, by engineering restriction sites and/or utilizing a poly-U termination region as described in Paul et al., 2002, Nature Biotechnology, 29, 505-508.

[00521] Figure 8A-C is a diagrammatic representation of a scheme utilized in generating an expression cassette to generate double-stranded siNA constructs.

[00522] Figure 8A: A DNA oligomer is synthesized with a 5'-restriction (R1) site sequence followed by a region having sequence identical (sense region of siNA) to a predetermined target sequence, wherein the sense region comprises, for example, about 19, 20, 21, or 22 nucleotides (N) in length, and which is followed by a 3'-restriction site (R2) which is adjacent to a loop sequence of defined sequence (X).

[00523] Figure 8B: The synthetic construct is then extended by DNA polymerase to generate a hairpin structure having self-complementary sequence.

[00524] Figure 8C: The construct is processed by restriction enzymes specific to R1 and R2 to generate a double-stranded DNA which is then inserted into an appropriate vector for expression in cells. The transcription cassette is designed such that a U6 promoter region flanks each side of the dsDNA which generates the separate sense and antisense strands of the siNA. Poly T termination sequences can be added to the constructs to generate U overhangs in the resulting transcript.

[00525] Figure 9A-E is a diagrammatic representation of a method used to determine target sites for siNA mediated RNAi within a particular target nucleic acid sequence, such as messenger RNA.

[00526] **Figure 9A:** A pool of siNA oligonucleotides are synthesized wherein the antisense region of the siNA constructs has complementarity to target sites across the target nucleic acid sequence, and wherein the sense region comprises sequence complementary to the antisense region of the siNA.

[00527] **Figure 9B-C:** (**Figure 9B**) The sequences are pooled and are inserted into vectors such that (**Figure 9C**) transfection of a vector into cells results in the expression of the siNA.

[00528] **Figure 9D:** Cells are sorted based on phenotypic change that is associated with modulation of the target nucleic acid sequence.

[00529] **Figure 9E:** The siNA is isolated from the sorted cells and is sequenced to identify efficacious target sites within the target nucleic acid sequence.

[00530] **Figure 10** shows non-limiting examples of different stabilization chemistries (1-10) that can be used, for example, to stabilize the 3'-end of siNA sequences of the invention, including (1) [3-3']-inverted deoxyribose; (2) deoxyribonucleotide; (3) [5'-3']-3'-deoxyribonucleotide; (4) [5'-3']-ribonucleotide; (5) [5'-3']-3'-O-methyl ribonucleotide; (6) 3'-glyceryl; (7) [3'-5']-3'-deoxyribonucleotide; (8) [3'-3']-deoxyribonucleotide; (9) [5'-2']-deoxyribonucleotide; and (10) [5-3']-dideoxyribonucleotide. In addition to modified and unmodified backbone chemistries indicated in the figure, these chemistries can be combined with different backbone modifications as described herein, for example, backbone modifications having Formula I. In addition, the 2'-deoxy nucleotide shown 5' to the terminal modifications shown can be another modified or unmodified nucleotide or non-nucleotide described herein, for example modifications having any of Formulae I-VII or any combination thereof.

[00531] **Figure 11** shows a non-limiting example of a strategy used to identify chemically modified siNA constructs of the invention that are nuclease resistant while preserving the ability to mediate RNAi activity. Chemical modifications are introduced into the siNA construct based on educated design parameters (e.g. introducing 2'-mofications, base modifications, backbone modifications, terminal cap modifications etc). The modified construct is tested in an appropriate system (e.g. human serum for nuclease resistance, shown, or an animal model for PK/delivery parameters). In parallel,

the siNA construct is tested for RNAi activity, for example in a cell culture system such as a luciferase reporter assay). Lead siNA constructs are then identified which possess a particular characteristic while maintaining RNAi activity, and can be further modified and assayed once again. This same approach can be used to identify siNA-conjugate molecules with improved pharmacokinetic profiles, delivery, and RNAi activity.

[00532] Figure 12 shows non-limiting examples of phosphorylated siNA molecules of the invention, including linear and duplex constructs and asymmetric derivatives thereof.

[00533] Figure 13 shows non-limiting examples of chemically modified terminal phosphate groups of the invention.

[00534] Figure 14A shows a non-limiting example of methodology used to design self complementary DFO constructs utilizing palindrome and/or repeat nucleic acid sequences that are identified in a target nucleic acid sequence. (i) A palindrome or repeat sequence is identified in a nucleic acid target sequence. (ii) A sequence is designed that is complementary to the target nucleic acid sequence and the palindrome sequence. (iii) An inverse repeat sequence of the non-palindrome/repeat portion of the complementary sequence is appended to the 3'-end of the complementary sequence to generate a self complementary DFO molecule comprising sequence complementary to the nucleic acid target. (iv) The DFO molecule can self-assemble to form a double stranded oligonucleotide. Figure 14B shows a non-limiting representative example of a duplex forming oligonucleotide sequence. Figure 14C shows a non-limiting example of the self assembly schematic of a representative duplex forming oligonucleotide sequence. Figure 14D shows a non-limiting example of the self assembly schematic of a representative duplex forming oligonucleotide sequence followed by interaction with a target nucleic acid sequence resulting in modulation of gene expression.

[00535] Figure 15 shows a non-limiting example of the design of self complementary DFO constructs utilizing palindrome and/or repeat nucleic acid sequences that are incorporated into the DFO constructs that have sequence complementary to any target nucleic acid sequence of interest. Incorporation of these palindrome/repeat sequences allow the design of DFO constructs that form duplexes in which each strand is capable of mediating modulation of target gene expression, for example by RNAi. First, the target

sequence is identified. A complementary sequence is then generated in which nucleotide or non-nucleotide modifications (shown as X or Y) are introduced into the complementary sequence that generate an artificial palindrome (shown as XYXYXY in the Figure). An inverse repeat of the non-palindrome/repeat complementary sequence is appended to the 3'-end of the complementary sequence to generate a self complementary DFO comprising sequence complementary to the nucleic acid target. The DFO can self-assemble to form a double stranded oligonucleotide.

[00536] **Figure 16** shows non-limiting examples of multifunctional siNA molecules of the invention comprising two separate polynucleotide sequences that are each capable of mediating RNAi directed cleavage of differing target nucleic acid sequences. Figure 16A shows a non-limiting example of a multifunctional siNA molecule having a first region that is complementary to a first target nucleic acid sequence (complementary region 1) and a second region that is complementary to a second target nucleic acid sequence (complementary region 2), wherein the first and second complementary regions are situated at the 3'-ends of each polynucleotide sequence in the multifunctional siNA. The dashed portions of each polynucleotide sequence of the multifunctional siNA construct have complementarity with regard to corresponding portions of the siNA duplex, but do not have complementarity to the target nucleic acid sequences. Figure 16B shows a non-limiting example of a multifunctional siNA molecule having a first region that is complementary to a first target nucleic acid sequence (complementary region 1) and a second region that is complementary to a second target nucleic acid sequence (complementary region 2), wherein the first and second complementary regions are situated at the 5'-ends of each polynucleotide sequence in the multifunctional siNA. The dashed portions of each polynucleotide sequence of the multifunctional siNA construct have complementarity with regard to corresponding portions of the siNA duplex, but do not have complementarity to the target nucleic acid sequences.

[00537] **Figure 17** shows non-limiting examples of multifunctional siNA molecules of the invention comprising a single polynucleotide sequence comprising distinct regions that are each capable of mediating RNAi directed cleavage of differing target nucleic acid sequences.

[00538] **Figure 17A** shows a non-limiting example of a multifunctional siNA molecule having a first region that is complementary to a first target nucleic acid sequence (complementary region 1) and a second region that is complementary to a second target nucleic acid sequence (complementary region 2), wherein the second complementary region is situated at the 3'-end of the polynucleotide sequence in the multifunctional siNA. The dashed portions of each polynucleotide sequence of the multifunctional siNA construct have complementarity with regard to corresponding portions of the siNA duplex, but do not have complementarity to the target nucleic acid sequences.

[00539] **Figure 17B** shows a non-limiting example of a multifunctional siNA molecule having a first region that is complementary to a first target nucleic acid sequence (complementary region 1) and a second region that is complementary to a second target nucleic acid sequence (complementary region 2), wherein the first complementary region is situated at the 5'-end of the polynucleotide sequence in the multifunctional siNA. The dashed portions of each polynucleotide sequence of the multifunctional siNA construct have complementarity with regard to corresponding portions of the siNA duplex, but do not have complementarity to the target nucleic acid sequences. In one embodiment, these multifunctional siNA constructs are processed in vivo or in vitro to generate multifunctional siNA constructs as shown in **Figure 16**.

[00540] **Figure 18** shows non-limiting examples of multifunctional siNA molecules of the invention comprising two separate polynucleotide sequences that are each capable of mediating RNAi directed cleavage of differing target nucleic acid sequences and wherein the multifunctional siNA construct further comprises a self complementary, palindrome, or repeat region, thus enabling shorter bifunctional siNA constructs that can mediate RNA interference against differing target nucleic acid sequences.

[00541] **Figure 18A** shows a non-limiting example of a multifunctional siNA molecule having a first region that is complementary to a first target nucleic acid sequence (complementary region 1) and a second region that is complementary to a second target nucleic acid sequence (complementary region 2), wherein the first and second complementary regions are situated at the 3'-ends of each polynucleotide sequence in the multifunctional siNA, and wherein the first and second complementary regions further comprise a self complementary, palindrome, or repeat region. The dashed portions of

each polynucleotide sequence of the multifunctional siNA construct have complementarity with regard to corresponding portions of the siNA duplex, but do not have complementarity to the target nucleic acid sequences.

[00542] **Figure 18B** shows a non-limiting example of a multifunctional siNA molecule having a first region that is complementary to a first target nucleic acid sequence (complementary region 1) and a second region that is complementary to a second target nucleic acid sequence (complementary region 2), wherein the first and second complementary regions are situated at the 5'-ends of each polynucleotide sequence in the multifunctional siNA, and wherein the first and second complementary regions further comprise a self complementary, palindrome, or repeat region. The dashed portions of each polynucleotide sequence of the multifunctional siNA construct have complementarity with regard to corresponding portions of the siNA duplex, but do not have complementarity to the target nucleic acid sequences.

[00543] **Figure 19** shows non-limiting examples of multifunctional siNA molecules of the invention comprising a single polynucleotide sequence comprising distinct regions that are each capable of mediating RNAi directed cleavage of differing target nucleic acid sequences and wherein the multifunctional siNA construct further comprises a self complementary, palindrome, or repeat region, thus enabling shorter bifunctional siNA constructs that can mediate RNA interference against differing target nucleic acid sequences.

[00544] **Figure 19A** shows a non-limiting example of a multifunctional siNA molecule having a first region that is complementary to a first target nucleic acid sequence (complementary region 1) and a second region that is complementary to a second target nucleic acid sequence (complementary region 2), wherein the second complementary region is situated at the 3'-end of the polynucleotide sequence in the multifunctional siNA, and wherein the first and second complementary regions further comprise a self complementary, palindrome, or repeat region. The dashed portions of each polynucleotide sequence of the multifunctional siNA construct have complementarity with regard to corresponding portions of the siNA duplex, but do not have complementarity to the target nucleic acid sequences.

[00545] **Figure 19B** shows a non-limiting example of a multifunctional siNA molecule having a first region that is complementary to a first target nucleic acid sequence (complementary region 1) and a second region that is complementary to a second target nucleic acid sequence (complementary region 2), wherein the first complementary region is situated at the 5'-end of the polynucleotide sequence in the multifunctional siNA, and wherein the first and second complementary regions further comprise a self complementary, palindrome, or repeat region. The dashed portions of each polynucleotide sequence of the multifunctional siNA construct have complementarity with regard to corresponding portions of the siNA duplex, but do not have complementarity to the target nucleic acid sequences. In one embodiment, these multifunctional siNA constructs are processed *in vivo* or *in vitro* to generate multifunctional siNA constructs as shown in **Figure 18**.

[00546] **Figure 20** shows a non-limiting example of how multifunctional siNA molecules of the invention can target two separate target nucleic acid molecules, such as separate RNA molecules encoding differing proteins (e.g., any of PCSK9 targets herein), for example, a cytokine and its corresponding receptor, differing viral strains, a virus and a cellular protein involved in viral infection or replication, or differing proteins involved in a common or divergent biologic pathway that is implicated in the maintenance of progression of disease. Each strand of the multifunctional siNA construct comprises a region having complementarity to separate target nucleic acid molecules. The multifunctional siNA molecule is designed such that each strand of the siNA can be utilized by the RISC complex to initiate RNA interference mediated cleavage of its corresponding target. These design parameters can include destabilization of each end of the siNA construct (see for example Schwarz et al., 2003, Cell, 115, 199-208). Such destabilization can be accomplished for example by using guanosine-cytidine base pairs, alternate base pairs (e.g., wobbles), or destabilizing chemically modified nucleotides at terminal nucleotide positions as is known in the art.

[00547] **Figure 21** shows a non-limiting example of how multifunctional siNA molecules of the invention can target two separate target nucleic acid sequences within the same target nucleic acid molecule, such as alternate coding regions of a RNA, coding and non-coding regions of a RNA, or alternate splice variant regions of a RNA. Each

strand of the multifunctional siNA construct comprises a region having complementarity to the separate regions of the target nucleic acid molecule. The multifunctional siNA molecule is designed such that each strand of the siNA can be utilized by the RISC complex to initiate RNA interference mediated cleavage of its corresponding target region. These design parameters can include destabilization of each end of the siNA construct (see for example Schwarz et al., 2003, Cell, 115, 199-208). Such destabilization can be accomplished for example by using guanosine-cytidine base pairs, alternate base pairs (e.g., wobbles), or destabilizing chemically modified nucleotides at terminal nucleotide positions as is known in the art.

[00548] Figure 22(A-H) shows non-limiting examples of tethered multifunctional siNA constructs of the invention. In the examples shown, a linker (e.g., nucleotide or non-nucleotide linker) connects two siNA regions (e.g., two sense, two antisense, or alternately a sense and an antisense region together. Separate sense (or sense and antisense) sequences corresponding to a first target sequence and second target sequence are hybridized to their corresponding sense and/or antisense sequences in the multifunctional siNA. In addition, various conjugates, ligands, aptamers, polymers or reporter molecules can be attached to the linker region for selective or improved delivery and/or pharmacokinetic properties.

[00549] Figure 23 shows a non-limiting example of various dendrimer based multifunctional siNA designs.

[00550] Figure 24 shows a non-limiting example of various supramolecular multifunctional siNA designs.

[00551] Figure 25 shows a non-limiting example of a dicer enabled multifunctional siNA design using a 30 nucleotide precursor siNA construct. A 30 base pair duplex is cleaved by Dicer into 22 and 8 base pair products from either end (8 b.p. fragments not shown). For ease of presentation the overhangs generated by dicer are not shown – but can be compensated for. Three targeting sequences are shown. The required sequence identity overlapped is indicated by grey boxes. The N's of the parent 30 b.p. siNA are suggested sites of 2'-OH positions to enable Dicer cleavage if this is tested in stabilized chemistries. Note that processing of a 30mer duplex by Dicer RNase III does not give a

precise 22+8 cleavage, but rather produces a series of closely related products (with 22+8 being the primary site). Therefore, processing by Dicer will yield a series of active siNAs.

[00552] Figure 26 shows a non-limiting example of a dicer enabled multifunctional siNA design using a 40 nucleotide precursor siNA construct. A 40 base pair duplex is cleaved by Dicer into 20 base pair products from either end. For ease of presentation the overhangs generated by dicer are not shown – but can be compensated for. Four targeting sequences are shown. The target sequences having homology are enclosed by boxes. This design format can be extended to larger RNAs. If chemically stabilized siNAs are bound by Dicer, then strategically located ribonucleotide linkages can enable designer cleavage products that permit our more extensive repertoire of multifunctional designs. For example cleavage products not limited to the Dicer standard of approximately 22-nucleotides can allow multifunctional siNA constructs with a target sequence identity overlap ranging from, for example, about 3 to about 15 nucleotides.

[00553] Figure 27 shows a non-limiting example of additional multifunctional siNA construct designs of the invention. In one example, a conjugate, ligand, aptamer, label, or other moiety is attached to a region of the multifunctional siNA to enable improved delivery or pharmacokinetic profiling.

[00554] Figure 28 shows a non-limiting example of additional multifunctional siNA construct designs of the invention. In one example, a conjugate, ligand, aptamer, label, or other moiety is attached to a region of the multifunctional siNA to enable improved delivery or pharmacokinetic profiling.

[00555] Figure 29 shows a non-limiting example of a cholesterol linked phosphoramidite that can be used to synthesize cholesterol conjugated siNA molecules of the invention. An example is shown with the cholesterol moiety linked to the 5'-end of the sense strand of a siNA molecule.

[00556] Figures 30A and 30B shows a non-limiting example of a Western Blot analysis of PCSK9 silencing in cells stably expressing PCSK9 and control cell line.

[00557] Figure 31 shows a non-limiting example of diI-LDL uptake (fluorescently labeled LDL particles) by control cell line and PCSK9-expressing cell line.

[00558] Figure 32 shows a non-limiting example of in vitro screening of PCSK9 ShRNA plasmids in Hepa1-6 cells.

[00559] Figure 33 shows a non-limiting example of data confirming that Ad-PCSK9ShRNA mediated knockdown of mouse PCSK9 in Hepa1-6 cells.

[00560] Figure 34A shows a non-limiting example of Adenovirus-mediated inhibition of PCSK9 in liver of C57BL6 mice.

[00561] Figure 34B shows a non-limiting example of hepatic knockdown on plasma LDL level.

[00562] Figure 35 shows a non-limiting example of Hepatic knockdown of PCSK9 on Body weight.

DETAILED DESCRIPTION OF THE INVENTION

Mechanism of Action of Nucleic Acid Molecules of the Invention

[00563] The discussion that follows discusses the proposed mechanism of RNA interference mediated by short interfering RNA as is presently known, and is not meant to be limiting and is not an admission of prior art. Applicant demonstrates herein that chemically-modified short interfering nucleic acids possess similar or improved capacity to mediate RNAi as do siRNA molecules and are expected to possess improved stability and activity in vivo; therefore, this discussion is not meant to be limiting only to siRNA and can be applied to siNA as a whole. By "improved capacity to mediate RNAi" or "improved RNAi activity" is meant to include RNAi activity measured in vitro and/or in vivo where the RNAi activity is a reflection of both the ability of the siNA to mediate RNAi and the stability of the siNAs of the invention. In this invention, the product of these activities can be increased in vitro and/or in vivo compared to an all RNA siRNA or a siNA containing a plurality of ribonucleotides. In some cases, the activity or stability

of the siRNA molecule can be decreased (i.e., less than ten-fold), but the overall activity of the siRNA molecule is enhanced in vitro and/or in vivo.

[00564] RNA interference refers to the process of sequence specific post-transcriptional gene silencing in animals mediated by short interfering RNAs (siRNAs) (Fire et al., 1998, *Nature*, 391, 806). The corresponding process in plants is commonly referred to as post-transcriptional gene silencing or RNA silencing and is also referred to as quelling in fungi. The process of post-transcriptional gene silencing is thought to be an evolutionarily-conserved cellular defense mechanism used to prevent the expression of foreign genes which is commonly shared by diverse flora and phyla (Fire et al., 1999, *Trends Genet.*, 15, 358). Such protection from foreign gene expression may have evolved in response to the production of double-stranded RNAs (dsRNAs) derived from viral infection or the random integration of transposon elements into a host genome via a cellular response that specifically destroys homologous single-stranded RNA or viral genomic RNA. The presence of dsRNA in cells triggers the RNAi response through a mechanism that has yet to be fully characterized. This mechanism appears to be different from the interferon response that results from dsRNA-mediated activation of protein kinase PKR and 2', 5'-oligoadenylate synthetase resulting in non-specific cleavage of mRNA by ribonuclease L.

[00565] The presence of long dsRNAs in cells stimulates the activity of a ribonuclease III enzyme referred to as Dicer. Dicer is involved in the processing of the dsRNA into short pieces of dsRNA known as short interfering RNAs (siRNAs) (Bernstein et al., 2001, *Nature*, 409, 363). Short interfering RNAs derived from Dicer activity are typically about 21 to about 23 nucleotides in length and comprise about 19 base pair duplexes. Dicer has also been implicated in the excision of 21- and 22-nucleotide small temporal RNAs (stRNAs) from precursor RNA of conserved structure that are implicated in translational control (Hutvagner et al., 2001, *Science*, 293, 834). The RNAi response also features an endonuclease complex containing a siRNA, commonly referred to as an RNA-induced silencing complex (RISC), which mediates cleavage of single-stranded RNA having sequence homologous to the siRNA. Cleavage of the target RNA takes place in the middle of the region complementary to the guide sequence of the siRNA duplex (Elbashir et al., 2001, *Genes Dev.*, 15, 188). In addition, RNA interference can

also involve small RNA (e.g., micro-RNA or miRNA) mediated gene silencing, presumably through cellular mechanisms that regulate chromatin structure and thereby prevent transcription of target gene sequences (see for example Allshire, 2002, *Science*, 297, 1818-1819; Volpe et al., 2002, *Science*, 297, 1833-1837; Jenuwein, 2002, *Science*, 297, 2215-2218; and Hall et al., 2002, *Science*, 297, 2232-2237). As such, siRNA molecules of the invention can be used to mediate gene silencing via interaction with RNA transcripts or alternately by interaction with particular gene sequences, wherein such interaction results in gene silencing either at the transcriptional level or post-transcriptional level.

[00566] RNAi has been studied in a variety of systems. Fire et al., 1998, *Nature*, 391, 806, were the first to observe RNAi in *C. elegans*. Wianny and Goetz, 1999, *Nature Cell Biol.*, 2, 70, describe RNAi mediated by dsRNA in mouse embryos. Hammond et al., 2000, *Nature*, 404, 293, describe RNAi in *Drosophila* cells transfected with dsRNA. Elbashir et al., 2001, *Nature*, 411, 494, describe RNAi induced by introduction of duplexes of synthetic 21-nucleotide RNAs in cultured mammalian cells including human embryonic kidney and HeLa cells. Recent work in *Drosophila* embryonic lysates has revealed certain requirements for siRNA length, structure, chemical composition, and sequence that are essential to mediate efficient RNAi activity. These studies have shown that 21 nucleotide siRNA duplexes are most active when containing two 2-nucleotide 3'-terminal nucleotide overhangs. Furthermore, substitution of one or both siRNA strands with 2'-deoxy or 2'-O-methyl nucleotides abolishes RNAi activity, whereas substitution of 3'-terminal siRNA nucleotides with deoxy nucleotides was shown to be tolerated. Mismatch sequences in the center of the siRNA duplex were also shown to abolish RNAi activity. In addition, these studies also indicate that the position of the cleavage site in the target RNA is defined by the 5'-end of the siRNA guide sequence rather than the 3'-end (Elbashir et al., 2001, *EMBO J.*, 20, 6877). Other studies have indicated that a 5'-phosphate on the target-complementary strand of a siRNA duplex is required for siRNA activity and that ATP is utilized to maintain the 5'-phosphate moiety on the siRNA (Nykanen et al., 2001, *Cell*, 107, 309); however, siRNA molecules lacking a 5'-phosphate are active when introduced exogenously, suggesting that 5'-phosphorylation of siRNA constructs may occur in vivo.

Duplex Forming Oligonucleotides (DFO) of the Invention

[00567] In one embodiment, the invention features siNA molecules comprising duplex forming oligonucleotides (DFO) that can self-assemble into double stranded oligonucleotides. The duplex forming oligonucleotides of the invention can be chemically synthesized or expressed from transcription units and/or vectors. The DFO molecules of the instant invention provide useful reagents and methods for a variety of therapeutic, diagnostic, agricultural, veterinary, target validation, genomic discovery, genetic engineering and pharmacogenomic applications.

[00568] Applicant demonstrates herein that certain oligonucleotides, referred to herein for convenience but not limitation as duplex forming oligonucleotides or DFO molecules, are potent mediators of sequence specific regulation of gene expression. The oligonucleotides of the invention are distinct from other nucleic acid sequences known in the art (e.g., siRNA, miRNA, stRNA, shRNA, antisense oligonucleotides etc.) in that they represent a class of linear polynucleotide sequences that are designed to self-assemble into double stranded oligonucleotides, where each strand in the double stranded oligonucleotides comprises a nucleotide sequence that is complementary to a target nucleic acid molecule. Nucleic acid molecules of the invention can thus self assemble into functional duplexes in which each strand of the duplex comprises the same polynucleotide sequence and each strand comprises a nucleotide sequence that is complementary to a target nucleic acid molecule.

[00569] Generally, double stranded oligonucleotides are formed by the assembly of two distinct oligonucleotide sequences where the oligonucleotide sequence of one strand is complementary to the oligonucleotide sequence of the second strand; such double stranded oligonucleotides are assembled from two separate oligonucleotides, or from a single molecule that folds on itself to form a double stranded structure, often referred to in the field as hairpin stem-loop structure (e.g., shRNA or short hairpin RNA). These double stranded oligonucleotides known in the art all have a common feature in that each strand of the duplex has a distinct nucleotide sequence.

[00570] Distinct from the double stranded nucleic acid molecules known in the art, the applicants have developed a novel, potentially cost effective and simplified method of

forming a double stranded nucleic acid molecule starting from a single stranded or linear oligonucleotide. The two strands of the double stranded oligonucleotide formed according to the instant invention have the same nucleotide sequence and are not covalently linked to each other. Such double-stranded oligonucleotides molecules can be readily linked post-synthetically by methods and reagents known in the art and are within the scope of the invention. In one embodiment, the single stranded oligonucleotide of the invention (the duplex forming oligonucleotide) that forms a double stranded oligonucleotide comprises a first region and a second region, where the second region includes a nucleotide sequence that is an inverted repeat of the nucleotide sequence in the first region, or a portion thereof, such that the single stranded oligonucleotide self assembles to form a duplex oligonucleotide in which the nucleotide sequence of one strand of the duplex is the same as the nucleotide sequence of the second strand. Non-limiting examples of such duplex forming oligonucleotides are illustrated in Figures 14 and 15. These duplex forming oligonucleotides (DFOs) can optionally include certain palindrome or repeat sequences where such palindrome or repeat sequences are present in between the first region and the second region of the DFO.

[00571] In one embodiment, the invention features a duplex forming oligonucleotide (DFO) molecule, wherein the DFO comprises a duplex forming self complementary nucleic acid sequence that has nucleotide sequence complementary to a target nucleic acid sequence. The DFO molecule can comprise a single self complementary sequence or a duplex resulting from assembly of such self complementary sequences.

[00572] In one embodiment, a duplex forming oligonucleotide (DFO) of the invention comprises a first region and a second region, wherein the second region comprises a nucleotide sequence comprising an inverted repeat of nucleotide sequence of the first region such that the DFO molecule can assemble into a double stranded oligonucleotide. Such double stranded oligonucleotides can act as a short interfering nucleic acid (siNA) to modulate gene expression. Each strand of the double stranded oligonucleotide duplex formed by DFO molecules of the invention can comprise a nucleotide sequence region that is complementary to the same nucleotide sequence in a target nucleic acid molecule (e.g., PCSK9 target RNA).

[00573] In one embodiment, the invention features a single stranded DFO that can assemble into a double stranded oligonucleotide. The applicant has surprisingly found that a single stranded oligonucleotide with nucleotide regions of self complementarity can readily assemble into duplex oligonucleotide constructs. Such DFOs can assemble into duplexes that can inhibit gene expression in a sequence specific manner. The DFO molecules of the invention comprise a first region with nucleotide sequence that is complementary to the nucleotide sequence of a second region and where the sequence of the first region is complementary to a target nucleic acid (e.g., RNA). The DFO can form a double stranded oligonucleotide wherein a portion of each strand of the double stranded oligonucleotide comprises a sequence complementary to a target nucleic acid sequence.

[00574] In one embodiment, the invention features a double stranded oligonucleotide, wherein the two strands of the double stranded oligonucleotide are not covalently linked to each other, and wherein each strand of the double stranded oligonucleotide comprises a nucleotide sequence that is complementary to the same nucleotide sequence in a target nucleic acid molecule or a portion thereof (e.g., PCSK9 RNA target). In another embodiment, the two strands of the double stranded oligonucleotide share an identical nucleotide sequence of at least about 15, preferably at least about 16, 17, 18, 19, 20, or 21 nucleotides.

[00575] In one embodiment, a DFO molecule of the invention comprises a structure having Formula DFO-I:

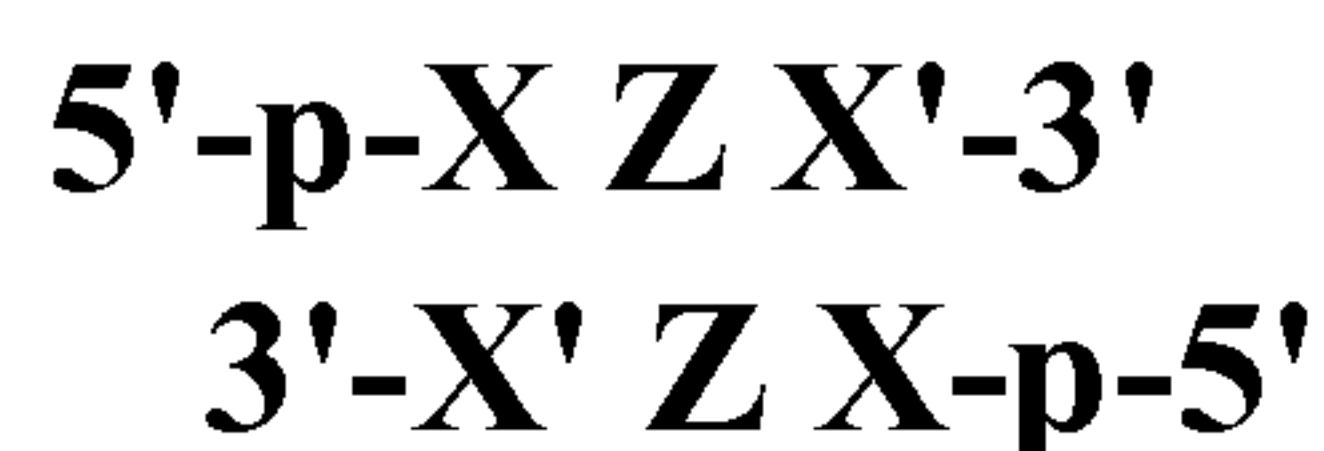


wherein Z comprises a palindromic or repeat nucleic acid sequence optionally with one or more modified nucleotides (e.g., nucleotide with a modified base, such as 2-amino purine, 2-amino-1,6-dihydro purine or a universal base), for example of length about 2 to about 24 nucleotides in even numbers (e.g., about 2, 4, 6, 8, 10, 12, 14, 16, 18, 20, or 22 or 24 nucleotides), X represents a nucleic acid sequence, for example of length of about 1 to about 21 nucleotides (e.g., about 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, or 21 nucleotides), X' comprises a nucleic acid sequence, for example of length about 1 and about 21 nucleotides (e.g., about 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13,

14, 15, 16, 17, 18, 19, 20 or 21 nucleotides) having nucleotide sequence complementarity to sequence X or a portion thereof, p comprises a terminal phosphate group that can be present or absent, and wherein sequence X and Z, either independently or together, comprise nucleotide sequence that is complementary to a target nucleic acid sequence or a portion thereof and is of length sufficient to interact (e.g., base pair) with the target nucleic acid sequence or a portion thereof (e.g., PCSK9 RNA target). For example, X independently can comprise a sequence from about 12 to about 21 or more (e.g., about 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, or more) nucleotides in length that is complementary to nucleotide sequence in a target RNA or a portion thereof. In another non-limiting example, the length of the nucleotide sequence of X and Z together, when X is present, that is complementary to the target RNA or a portion thereof (e.g., PCSK9 RNA target) is from about 12 to about 21 or more nucleotides (e.g., about 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, or more). In yet another non-limiting example, when X is absent, the length of the nucleotide sequence of Z that is complementary to the PCSK9 target RNA or a portion thereof is from about 12 to about 24 or more nucleotides (e.g., about 12, 14, 16, 18, 20, 22, 24, or more). In one embodiment X, Z and X' are independently oligonucleotides, where X and/or Z comprises a nucleotide sequence of length sufficient to interact (e.g., base pair) with a nucleotide sequence in the target RNA or a portion thereof (e.g., PCSK9 RNA target). In one embodiment, the lengths of oligonucleotides X and X' are identical. In another embodiment, the lengths of oligonucleotides X and X' are not identical. In another embodiment, the lengths of oligonucleotides X and Z, or Z and X', or X, Z and X' are either identical or different.

[00576] When a sequence is described in this specification as being of “sufficient” length to interact (i.e., base pair) with another sequence, it is meant that the length is such that the number of bonds (e.g., hydrogen bonds) formed between the two sequences is enough to enable the two sequence to form a duplex under the conditions of interest. Such conditions can be in vitro (e.g., for diagnostic or assay purposes) or in vivo (e.g., for therapeutic purposes). It is a simple and routine matter to determine such lengths.

[00577] In one embodiment, the invention features a double stranded oligonucleotide construct having Formula DFO-I(a):



wherein Z comprises a palindromic or repeat nucleic acid sequence or palindromic or repeat-like nucleic acid sequence with one or more modified nucleotides (e.g., nucleotides with a modified base, such as 2-amino purine, 2-amino-1,6-dihydro purine or a universal base), for example of length about 2 to about 24 nucleotides in even numbers (e.g., about 2, 4, 6, 8, 10, 12, 14, 16, 18, 20, 22 or 24 nucleotides), X represents a nucleic acid sequence, for example of length about 1 to about 21 nucleotides (e.g., about 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, or 21 nucleotides), X' comprises a nucleic acid sequence, for example of length about 1 to about 21 nucleotides (e.g., about 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20 or 21 nucleotides) having nucleotide sequence complementarity to sequence X or a portion thereof, p comprises a terminal phosphate group that can be present or absent, and wherein each X and Z independently comprises a nucleotide sequence that is complementary to a target nucleic acid sequence or a portion thereof (e.g., PCSK9 RNA target) and is of length sufficient to interact with the target nucleic acid sequence of a portion thereof (e.g., PCSK9 RNA target). For example, sequence X independently can comprise a sequence from about 12 to about 21 or more nucleotides (e.g., about 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, or more) in length that is complementary to a nucleotide sequence in a target RNA or a portion thereof (e.g., PCSK9 RNA target). In another non-limiting example, the length of the nucleotide sequence of X and Z together (when X is present) that is complementary to the target or a portion thereof is from about 12 to about 21 or more nucleotides (e.g., about 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, or more). In yet another non-limiting example, when X is absent, the length of the nucleotide sequence of Z that is complementary to the target RNA or a portion thereof is from about 12 to about 24 or more nucleotides (e.g., about 12, 14, 16, 18, 20, 22, 24 or more). In one embodiment X, Z and X' are independently oligonucleotides, where X and/or Z comprises a nucleotide sequence of length sufficient to interact (e.g., base pair) with nucleotide sequence in the target RNA or a portion thereof (e.g., PCSK9 RNA target). In one embodiment, the lengths of oligonucleotides X and X' are identical. In another embodiment, the lengths of oligonucleotides X and X' are not identical. In another embodiment, the lengths of

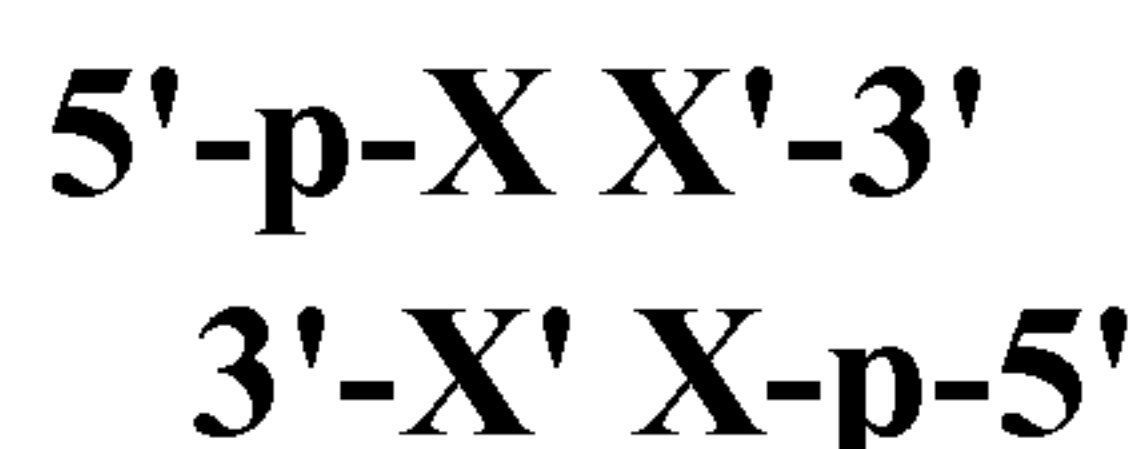
oligonucleotides X and Z or Z and X' or X, Z and X' are either identical or different. In one embodiment, the double stranded oligonucleotide construct of Formula DFO-I(a) includes one or more, specifically 1, 2, 3 or 4, mismatches, to the extent such mismatches do not significantly diminish the ability of the double stranded oligonucleotide to inhibit target gene expression.

[00578] In one embodiment, a DFO molecule of the invention comprises structure having Formula DFO-II:



wherein each X and X' are independently oligonucleotides of length about 12 nucleotides to about 21 nucleotides, wherein X comprises, for example, a nucleic acid sequence of length about 12 to about 21 nucleotides (e.g., about 12, 13, 14, 15, 16, 17, 18, 19, 20 or 21 nucleotides), X' comprises a nucleic acid sequence, for example of length about 12 to about 21 nucleotides (e.g., about 12, 13, 14, 15, 16, 17, 18, 19, 20, or 21 nucleotides) having nucleotide sequence complementarity to sequence X or a portion thereof, p comprises a terminal phosphate group that can be present or absent, and wherein X comprises a nucleotide sequence that is complementary to a target nucleic acid sequence (e.g., PCSK9 RNA) or a portion thereof and is of length sufficient to interact (e.g., base pair) with the target nucleic acid sequence of a portion thereof. In one embodiment, the length of oligonucleotides X and X' are identical. In another embodiment the length of oligonucleotides X and X' are not identical. In one embodiment, length of the oligonucleotides X and X' are sufficient to form a relatively stable double stranded oligonucleotide.

[00579] In one embodiment, the invention features a double stranded oligonucleotide construct having Formula DFO-II(a):



wherein each X and X' are independently oligonucleotides of length about 12 nucleotides to about 21 nucleotides, wherein X comprises a nucleic acid sequence, for

example of length about 12 to about 21 nucleotides (e.g., about 12, 13, 14, 15, 16, 17, 18, 19, 20 or 21 nucleotides), X' comprises a nucleic acid sequence, for example of length about 12 to about 21 nucleotides (e.g., about 12, 13, 14, 15, 16, 17, 18, 19, 20 or 21 nucleotides) having nucleotide sequence complementarity to sequence X or a portion thereof, p comprises a terminal phosphate group that can be present or absent, and wherein X comprises nucleotide sequence that is complementary to a target nucleic acid sequence or a portion thereof (e.g., PCSK9 RNA target) and is of length sufficient to interact (e.g., base pair) with the target nucleic acid sequence (e.g., PCSK9 RNA) or a portion thereof. In one embodiment, the lengths of oligonucleotides X and X' are identical. In another embodiment, the lengths of oligonucleotides X and X' are not identical. In one embodiment, the lengths of the oligonucleotides X and X' are sufficient to form a relatively stable double stranded oligonucleotide. In one embodiment, the double stranded oligonucleotide construct of Formula DFO-II(a) includes one or more, specifically 1, 2, 3 or 4, mismatches, to the extent such mismatches do not significantly diminish the ability of the double stranded oligonucleotide to inhibit target gene expression.

[00580] In one embodiment, the invention features a DFO molecule having Formula DFO-I(b):



where Z comprises a palindromic or repeat nucleic acid sequence optionally including one or more non-standard or modified nucleotides (e.g., nucleotide with a modified base, such as 2-amino purine or a universal base) that can facilitate base-pairing with other nucleotides. Z can be, for example, of length sufficient to interact (e.g., base pair) with nucleotide sequence of a target nucleic acid (e.g., PCSK9 RNA) molecule, preferably of length of at least 12 nucleotides, specifically about 12 to about 24 nucleotides (e.g., about 12, 14, 16, 18, 20, 22 or 24 nucleotides). p represents a terminal phosphate group that can be present or absent.

[00581] In one embodiment, a DFO molecule having any of Formula DFO-I, DFO-I(a), DFO-I(b), DFO-II(a) or DFO-II can comprise chemical modifications as described

herein without limitation, such as, for example, nucleotides having any of Formulae I-VII, stabilization chemistries as described in Table IV, or any other combination of modified nucleotides and non-nucleotides as described in the various embodiments herein.

[00582] In one embodiment, the palindrome or repeat sequence or modified nucleotide (e.g., nucleotide with a modified base, such as 2-amino purine or a universal base) in Z of DFO constructs having Formula DFO-I, DFO-I(a) and DFO-I(b), comprises chemically modified nucleotides that are able to interact with a portion of the target nucleic acid sequence (e.g., modified base analogs that can form Watson Crick base pairs or non-Watson Crick base pairs).

[00583] In one embodiment, a DFO molecule of the invention, for example a DFO having Formula DFO-I or DFO-II, comprises about 15 to about 40 nucleotides (e.g., about 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, or 40 nucleotides). In one embodiment, a DFO molecule of the invention comprises one or more chemical modifications. In a non-limiting example, the introduction of chemically modified nucleotides and/or non-nucleotides into nucleic acid molecules of the invention provides a powerful tool in overcoming potential limitations of in vivo stability and bioavailability inherent to unmodified RNA molecules that are delivered exogenously. For example, the use of chemically modified nucleic acid molecules can enable a lower dose of a particular nucleic acid molecule for a given therapeutic effect since chemically modified nucleic acid molecules tend to have a longer half-life in serum or in cells or tissues. Furthermore, certain chemical modifications can improve the bioavailability and/or potency of nucleic acid molecules by not only enhancing half-life but also facilitating the targeting of nucleic acid molecules to particular organs, cells or tissues and/or improving cellular uptake of the nucleic acid molecules. Therefore, even if the activity of a chemically modified nucleic acid molecule is reduced in vitro as compared to a native/unmodified nucleic acid molecule, for example when compared to an unmodified RNA molecule, the overall activity of the modified nucleic acid molecule can be greater than the native or unmodified nucleic acid molecule due to improved stability, potency, duration of effect, bioavailability and/or delivery of the molecule.

Multifunctional or Multi-targeted siNA molecules of the Invention

[00584] In one embodiment, the invention features siNA molecules comprising multifunctional short interfering nucleic acid (multifunctional siNA) molecules that modulate the expression of one or more target genes in a biologic system, such as a cell, tissue, or organism. The multifunctional short interfering nucleic acid (multifunctional siNA) molecules of the invention can target more than one region of the PCSK9 target nucleic acid sequence or can target sequences of more than one distinct target nucleic acid molecules (*e.g.*, PCSK9 or PCSK9 pathway RNA and/or DNA targets). The multifunctional siNA molecules of the invention can be chemically synthesized or expressed from transcription units and/or vectors. The multifunctional siNA molecules of the instant invention provide useful reagents and methods for a variety of human applications, therapeutic, diagnostic, agricultural, veterinary, target validation, genomic discovery, genetic engineering and pharmacogenomic applications.

[00585] Applicant demonstrates herein that certain oligonucleotides, referred to herein for convenience but not limitation as multifunctional short interfering nucleic acid or multifunctional siNA molecules, are potent mediators of sequence specific regulation of gene expression. The multifunctional siNA molecules of the invention are distinct from other nucleic acid sequences known in the art (*e.g.*, siRNA, miRNA, stRNA, shRNA, antisense oligonucleotides, etc.) in that they represent a class of polynucleotide molecules that are designed such that each strand in the multifunctional siNA construct comprises a nucleotide sequence that is complementary to a distinct nucleic acid sequence in one or more target nucleic acid molecules. A single multifunctional siNA molecule (generally a double-stranded molecule) of the invention can thus target more than one (*e.g.*, 2, 3, 4, 5, or more) differing target nucleic acid target molecules. Nucleic acid molecules of the invention can also target more than one (*e.g.*, 2, 3, 4, 5, or more) region of the same target nucleic acid sequence. As such multifunctional siNA molecules of the invention are useful in down regulating or inhibiting the expression of one or more target nucleic acid molecules. For example, a multifunctional siNA molecule of the invention can target (*e.g.*, have complementarity to) nucleic acid molecules selected from the group consisting of PCSK9, H6PD, PTP-1B, apoAI/CI/II/AIV, CETP, ApoB, SREBP-1a, SREBP-2, calcium/calmodulin-dependent

protein kinase 1D, fatty acid binding protein 5, proprotein convertase subtilisin/kexin 9, LXR target genes, metalloprotease domain 11, apoptosis-inhibitory 6, F-box-only protein 3 or any combination thereof. By reducing or inhibiting expression of more than one target nucleic acid molecule with one multifunctional siNA construct, multifunctional siNA molecules of the invention represent a class of potent therapeutic agents that can provide simultaneous inhibition of multiple targets within a disease (e.g., angiogenic) related pathway. Such simultaneous inhibition can provide synergistic therapeutic treatment strategies without the need for separate preclinical and clinical development efforts or complex regulatory approval process.

[00586] Use of multifunctional siNA molecules that target more than one region of a target nucleic acid molecule (e.g., messenger RNA) is expected to provide potent inhibition of gene expression. For example, a single multifunctional siNA construct of the invention can target both conserved and variable regions of a target nucleic acid molecule (e.g., PCSK9 RNA or DNA), thereby allowing down regulation or inhibition of different splice variants encoded by a single gene, or allowing for targeting of both coding and non-coding regions of a target nucleic acid molecule.

[00587] Generally, double stranded oligonucleotides are formed by the assembly of two distinct oligonucleotides where the oligonucleotide sequence of one strand is complementary to the oligonucleotide sequence of the second strand; such double stranded oligonucleotides are generally assembled from two separate oligonucleotides (e.g., siRNA). Alternately, a duplex can be formed from a single molecule that folds on itself (e.g., shRNA or short hairpin RNA). These double stranded oligonucleotides are known in the art to mediate RNA interference and all have a common feature wherein only one nucleotide sequence region (guide sequence or the antisense sequence) has complementarity to a target nucleic acid sequence, and the other strand (sense sequence) comprises nucleotide sequence that is homologous to the target nucleic acid sequence. Generally, the antisense sequence is retained in the active RISC complex and guides the RISC to the target nucleotide sequence by means of complementary base-pairing of the antisense sequence with the target sequence for mediating sequence-specific RNA interference. It is known in the art that in some cell culture systems, certain types of unmodified siRNAs can exhibit “off target” effects. It is hypothesized that this off-target

effect involves the participation of the sense sequence instead of the antisense sequence of the siRNA in the RISC complex (see for example Schwarz et al., 2003, Cell, 115:199-208). In this instance the sense sequence is believed to direct the RISC complex to a sequence (off-target sequence) that is distinct from the intended target sequence, resulting in the inhibition of the off-target sequence. In these double stranded nucleic acid molecules, each strand is complementary to a distinct target nucleic acid sequence. However, the off-targets that are affected by these dsRNAs are not entirely predictable and are non-specific.

[00588] Distinct from the double stranded nucleic acid molecules known in the art, the applicants have developed a novel, potentially cost effective and simplified method of down regulating or inhibiting the expression of more than one target nucleic acid sequence using a single multifunctional siNA construct. The multifunctional siNA molecules of the invention are designed to be double-stranded or partially double stranded, such that a portion of each strand or region of the multifunctional siNA is complementary to a target nucleic acid sequence of choice. As such, the multifunctional siNA molecules of the invention are not limited to targeting sequences that are complementary to each other, but rather to any two differing target nucleic acid sequences. Multifunctional siNA molecules of the invention are designed such that each strand or region of the multifunctional siNA molecule, that is complementary to a given target nucleic acid sequence, is of suitable length (e.g., from about 16 to about 28 nucleotides in length, preferably from about 18 to about 28 nucleotides in length) for mediating RNA interference against the target nucleic acid sequence. The complementarity between the target nucleic acid sequence and a strand or region of the multifunctional siNA must be sufficient (at least about 8 base pairs) for cleavage of the target nucleic acid sequence by RNA interference. Multifunctional siNA of the invention is expected to minimize off-target effects seen with certain siRNA sequences, such as those described in (Schwarz et al., supra).

[00589] It has been reported that dsRNAs of length between 29 base pairs and 36 base pairs (Tuschl et al., International PCT Publication No. WO 02/44321) do not mediate RNAi. One reason these dsRNAs are inactive may be the lack of turnover or dissociation of the strand that interacts with the target RNA sequence, such that the RISC

complex is not able to efficiently interact with multiple copies of the target RNA resulting in a significant decrease in the potency and efficiency of the RNAi process. Applicant has surprisingly found that the multifunctional siNAs of the invention can overcome this hurdle and are capable of enhancing the efficiency and potency of RNAi process. As such, in certain embodiments of the invention, multifunctional siNAs of length of about 29 to about 36 base pairs can be designed such that, a portion of each strand of the multifunctional siNA molecule comprises a nucleotide sequence region that is complementary to a target nucleic acid of length sufficient to mediate RNAi efficiently (e.g., about 15 to about 23 base pairs) and a nucleotide sequence region that is not complementary to the target nucleic acid. By having both complementary and non-complementary portions in each strand of the multifunctional siNA, the multifunctional siNA can mediate RNA interference against a target nucleic acid sequence without being prohibitive to turnover or dissociation (e.g., where the length of each strand is too long to mediate RNAi against the respective target nucleic acid sequence). Furthermore, design of multifunctional siNA molecules of the invention with internal overlapping regions allows the multifunctional siNA molecules to be of favorable (decreased) size for mediating RNA interference and of size that is well suited for use as a therapeutic agent (e.g., wherein each strand is independently from about 18 to about 28 nucleotides in length). Non-limiting examples are illustrated in Figures 16-28.

[00590] In one embodiment, a multifunctional siNA molecule of the invention comprises a first region and a second region, where the first region of the multifunctional siNA comprises a nucleotide sequence complementary to a nucleic acid sequence of a first target nucleic acid molecule, and the second region of the multifunctional siNA comprises nucleic acid sequence complementary to a nucleic acid sequence of a second target nucleic acid molecule. In one embodiment, a multifunctional siNA molecule of the invention comprises a first region and a second region, where the first region of the multifunctional siNA comprises nucleotide sequence complementary to a nucleic acid sequence of the first region of a target nucleic acid molecule, and the second region of the multifunctional siNA comprises nucleotide sequence complementary to a nucleic acid sequence of a second region of a the target nucleic acid molecule. In another embodiment, the first region and second region of the multifunctional siNA can comprise separate nucleic acid sequences that share some degree of complementarity (e.g., from

about 1 to about 10 complementary nucleotides). In certain embodiments, multifunctional siNA constructs comprising separate nucleic acid sequences can be readily linked post-synthetically by methods and reagents known in the art and such linked constructs are within the scope of the invention. Alternately, the first region and second region of the multifunctional siNA can comprise a single nucleic acid sequence having some degree of self complementarity, such as in a hairpin or stem-loop structure. Non-limiting examples of such double stranded and hairpin multifunctional short interfering nucleic acids are illustrated in Figures 16 and 17 respectively. These multifunctional short interfering nucleic acids (multifunctional siNAs) can optionally include certain overlapping nucleotide sequence where such overlapping nucleotide sequence is present in between the first region and the second region of the multifunctional siNA (see for example Figures 18 and 19).

[00591] In one embodiment, the invention features a multifunctional short interfering nucleic acid (multifunctional siNA) molecule, wherein each strand of the multifunctional siNA independently comprises a first region of nucleic acid sequence that is complementary to a distinct target nucleic acid sequence and the second region of nucleotide sequence that is not complementary to the target sequence. The target nucleic acid sequence of each strand is in the same target nucleic acid molecule or different target nucleic acid molecules.

[00592] In another embodiment, the multifunctional siNA comprises two strands, where: (a) the first strand comprises a region having sequence complementarity to a target nucleic acid sequence (complementary region 1) and a region having no sequence complementarity to the target nucleotide sequence (non-complementary region 1); (b) the second strand of the multifunction siNA comprises a region having sequence complementarity to a target nucleic acid sequence that is distinct from the target nucleotide sequence complementary to the first strand nucleotide sequence (complementary region 2), and a region having no sequence complementarity to the target nucleotide sequence of complementary region 2 (non-complementary region 2); (c) the complementary region 1 of the first strand comprises a nucleotide sequence that is complementary to a nucleotide sequence in the non-complementary region 2 of the second strand and the complementary region 2 of the second strand comprises a

nucleotide sequence that is complementary to a nucleotide sequence in the non-complementary region 1 of the first strand. The target nucleic acid sequence of complementary region 1 and complementary region 2 is in the same target nucleic acid molecule or different target nucleic acid molecules.

[00593] In another embodiment, the multifunctional siNA comprises two strands, where: (a) the first strand comprises a region having sequence complementarity to a target nucleic acid sequence derived from a gene (*e.g.*, a first PCSK9 gene) (complementary region 1) and a region having no sequence complementarity to the target nucleotide sequence of complementary region 1 (non-complementary region 1); (b) the second strand of the multifunction siNA comprises a region having sequence complementarity to a target nucleic acid sequence derived from a gene (*e.g.*, a second PCSK9 gene) that is distinct from the gene of complementary region 1 (complementary region 2), and a region having no sequence complementarity to the target nucleotide sequence of complementary region 2 (non-complementary region 2); (c) the complementary region 1 of the first strand comprises a nucleotide sequence that is complementary to a nucleotide sequence in the non-complementary region 2 of the second strand and the complementary region 2 of the second strand comprises a nucleotide sequence that is complementary to a nucleotide sequence in the non-complementary region 1 of the first strand.

[00594] In another embodiment, the multifunctional siNA comprises two strands, where: (a) the first strand comprises a region having sequence complementarity to a target nucleic acid sequence derived from a gene (*e.g.*, PCSK9 gene) (complementary region 1) and a region having no sequence complementarity to the target nucleotide sequence of complementary region 1 (non-complementary region 1); (b) the second strand of the multifunction siNA comprises a region having sequence complementarity to a second target nucleic acid sequence distinct from the first target nucleic acid sequence of complementary region 1 (complementary region 2), provided, however, that the target nucleic acid sequence for complementary region 1 and target nucleic acid sequence for complementary region 2 are both derived from the same gene, and a region having no sequence complementarity to the target nucleotide sequence of complementary region 2 (non-complementary region 2); (c) the complementary region 1 of the first strand

comprises a nucleotide sequence that is complementary to a nucleotide sequence in the non-complementary region 2 of the second strand and the complementary region 2 of the second strand comprises a nucleotide sequence that is complementary to nucleotide sequence in the non-complementary region 1 of the first strand.

[00595] In one embodiment, the invention features a multifunctional short interfering nucleic acid (multifunctional siNA) molecule, wherein the multifunctional siNA comprises two complementary nucleic acid sequences in which the first sequence comprises a first region having nucleotide sequence complementary to nucleotide sequence within a first target nucleic acid molecule, and in which the second sequence comprises a first region having nucleotide sequence complementary to a distinct nucleotide sequence within the same target nucleic acid molecule. Preferably, the first region of the first sequence is also complementary to the nucleotide sequence of the second region of the second sequence, and where the first region of the second sequence is complementary to the nucleotide sequence of the second region of the first sequence.

[00596] In one embodiment, the invention features a multifunctional short interfering nucleic acid (multifunctional siNA) molecule, wherein the multifunctional siNA comprises two complementary nucleic acid sequences in which the first sequence comprises a first region having a nucleotide sequence complementary to a nucleotide sequence within a first target nucleic acid molecule, and in which the second sequence comprises a first region having a nucleotide sequence complementary to a distinct nucleotide sequence within a second target nucleic acid molecule. Preferably, the first region of the first sequence is also complementary to the nucleotide sequence of the second region of the second sequence, and where the first region of the second sequence is complementary to the nucleotide sequence of the second region of the first sequence.

[00597] In one embodiment, the invention features a multifunctional siNA molecule comprising a first region and a second region, where the first region comprises a nucleic acid sequence having about 18 to about 28 nucleotides complementary to a nucleic acid sequence within a first target nucleic acid molecule, and the second region comprises nucleotide sequence having about 18 to about 28 nucleotides complementary to a distinct nucleic acid sequence within a second target nucleic acid molecule.

[00598] In one embodiment, the invention features a multifunctional siNA molecule comprising a first region and a second region, where the first region comprises nucleic acid sequence having about 18 to about 28 nucleotides complementary to a nucleic acid sequence within a target nucleic acid molecule, and the second region comprises nucleotide sequence having about 18 to about 28 nucleotides complementary to a distinct nucleic acid sequence within the same target nucleic acid molecule.

[00599] In one embodiment, the invention features a double stranded multifunctional short interfering nucleic acid (multifunctional siNA) molecule, wherein one strand of the multifunctional siNA comprises a first region having nucleotide sequence complementary to a first target nucleic acid sequence, and the second strand comprises a first region having a nucleotide sequence complementary to a second target nucleic acid sequence. The first and second target nucleic acid sequences can be present in separate target nucleic acid molecules or can be different regions within the same target nucleic acid molecule. As such, multifunctional siNA molecules of the invention can be used to target the expression of different genes, splice variants of the same gene, both mutant and conserved regions of one or more gene transcripts, or both coding and non-coding sequences of the same or differing genes or gene transcripts.

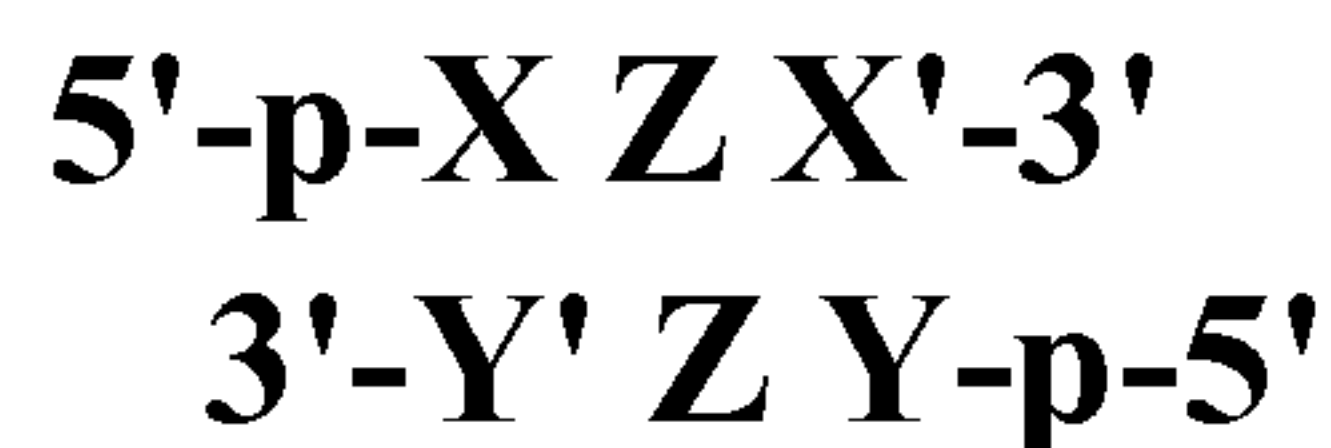
[00600] In one embodiment, a target nucleic acid molecule of the invention encodes a single protein. In another embodiment, a target nucleic acid molecule encodes more than one protein (e.g., 1, 2, 3, 4, 5 or more proteins). As such, a multifunctional siNA construct of the invention can be used to down regulate or inhibit the expression of several proteins. For example, a multifunctional siNA molecule comprising a region in one strand having nucleotide sequence complementarity to a first target nucleic acid sequence derived from a gene encoding one protein and the second strand comprising a region with nucleotide sequence complementarity to a second target nucleic acid sequence present in target nucleic acid molecules derived from genes encoding two or more proteins (e.g., two differing proteins) selected from the group consisting of PCSK9, H6PD, PTP-1B, apoAI/CIII/AIV, CETP, ApoB, SREBP-1a, SREBP-2, calcium/calmodulin-dependent protein kinase 1D, fatty acid binding protein 5, proprotein convertase subtilisin/kexin 9, LXR target genes, metalloprotease domain 11, apoptosis-inhibitory 6, F-box-only protein 3 or any combination thereof, which can be used to

down regulate, inhibit, or shut down a particular biologic pathway by targeting multiple PCSK9 or PCSK9 genes.

[00601] In one embodiment the invention takes advantage of conserved nucleotide sequences present in different PCSK9 variants. By designing multifunctional siNAs in a manner where one strand includes a sequence that is complementary to a target nucleic acid sequence conserved among various isoforms and the other strand includes sequence that is complementary to a target nucleic acid sequence, such as any of PCSK9, H6PD, PTP-1B, apoAI/CIII/AIV, CETP, ApoB, SREBP-1a, SREBP-2, calcium/calmodulin-dependent protein kinase 1D, fatty acid binding protein 5, proprotein convertase subtilisin/kexin 9, LXR target genes, metalloprotease domain 11, apoptosis-inhibitory 6, F-box-only protein 3, it is possible to selectively and effectively modulate or inhibit a biological pathway or multiple genes in a biological pathway (e.g., hyperlipidemia and/or insulin resistance) using a single multifunctional siNA.

[00602] In one embodiment, a multifunctional short interfering nucleic acid (multifunctional siNA) of the invention comprises a first region and a second region, wherein the first region comprises nucleotide sequence complementary to a first PCSK9 RNA of a first PCSK9 target and the second region comprises nucleotide sequence complementary to a second PCSK9 RNA of a second PCSK9 target. In one embodiment, the first and second regions can comprise nucleotide sequence complementary to shared or conserved RNA sequences of differing PCSK9 target sites within the same PCSK9 target sequence or shared amongst different PCSK9 target sequences.

[00603] In one embodiment, a double stranded multifunctional siNA molecule of the invention comprises a structure having Formula MF-I:

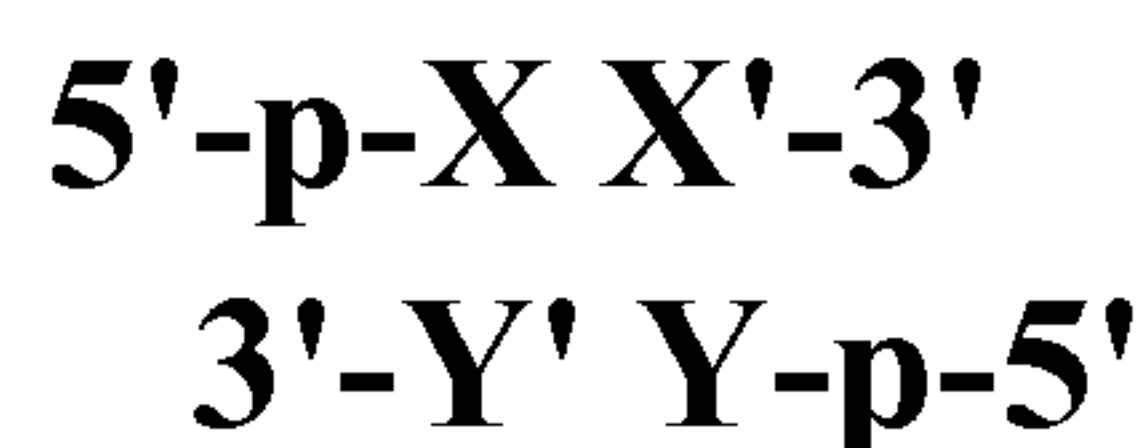


wherein each 5'-p-XZX'-3' and 5'-p-YZY'-3' are independently an oligonucleotide of length of about 20 nucleotides to about 300 nucleotides, preferably of about 20 to about 200 nucleotides, about 20 to about 100 nucleotides, about 20 to about 40 nucleotides,

about 20 to about 40 nucleotides, about 24 to about 38 nucleotides, or about 26 to about 38 nucleotides; XZ comprises a nucleic acid sequence that is complementary to a first target nucleic acid sequence; YZ is an oligonucleotide comprising nucleic acid sequence that is complementary to a second target nucleic acid sequence; Z comprises nucleotide sequence of length about 1 to about 24 nucleotides (e.g., about 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, or 24 nucleotides) that is self complimentary; X comprises nucleotide sequence of length about 1 to about 100 nucleotides, preferably about 1 to about 21 nucleotides (e.g., about 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, or 21 nucleotides) that is complementary to nucleotide sequence present in region Y'; Y comprises nucleotide sequence of length about 1 to about 100 nucleotides, preferably about 1 to about 21 nucleotides (e.g., about 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20 or 21 nucleotides) that is complementary to nucleotide sequence present in region X'; each p comprises a terminal phosphate group that is independently present or absent; each XZ and YZ is independently of length sufficient to stably interact (i.e., base pair) with the first and second target nucleic acid sequence, respectively, or a portion thereof. For example, each sequence X and Y can independently comprise sequence from about 12 to about 21 or more nucleotides in length (e.g., about 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, or more) that is complementary to a target nucleotide sequence in different target nucleic acid molecules, such as target RNAs or a portion thereof. In another non-limiting example, the length of the nucleotide sequence of X and Z together that is complementary to the first target nucleic acid sequence or a portion thereof is from about 12 to about 21 or more nucleotides (e.g., about 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, or more). In another non-limiting example, the length of the nucleotide sequence of Y and Z together, that is complementary to the second target nucleic acid sequence or a portion thereof is from about 12 to about 21 or more nucleotides (e.g., about 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, or more). In one embodiment, the first target nucleic acid sequence and the second target nucleic acid sequence are present in the same target nucleic acid molecule (e.g., PCSK9 target RNA or PCSK9 pathway target RNA such as PCSK9, H6PD, PTP-1B, apoAI/CIII/AIV, CETP, ApoB, SREBP-1a, SREBP-2, calcium/calmodulin-dependent protein kinase 1D, fatty acid binding protein 5, proprotein convertase subtilisin/kexin 9, LXR target genes, metalloprotease domain 11, apoptosis-inhibitory 6, and/or F-box-only

protein 3 RNA). In another embodiment, the first target nucleic acid sequence and the second target nucleic acid sequence are present in different target nucleic acid molecules (e.g., PCSK9 target RNA and PCSK9 pathway target RNA). In one embodiment, Z comprises a palindrome or a repeat sequence. In one embodiment, the lengths of oligonucleotides X and X' are identical. In another embodiment, the lengths of oligonucleotides X and X' are not identical. In one embodiment, the lengths of oligonucleotides Y and Y' are identical. In another embodiment, the lengths of oligonucleotides Y and Y' are not identical. In one embodiment, the double stranded oligonucleotide construct of Formula I(a) includes one or more, specifically 1, 2, 3 or 4, mismatches, to the extent such mismatches do not significantly diminish the ability of the double stranded oligonucleotide to inhibit target gene expression.

[00604] In one embodiment, a multifunctional siNA molecule of the invention comprises a structure having Formula MF-II:



wherein each 5'-p-XX'-3' and 5'-p-YY'-3' are independently an oligonucleotide of length of about 20 nucleotides to about 300 nucleotides, preferably about 20 to about 200 nucleotides, about 20 to about 100 nucleotides, about 20 to about 40 nucleotides, about 20 to about 40 nucleotides, about 24 to about 38 nucleotides, or about 26 to about 38 nucleotides; X comprises a nucleic acid sequence that is complementary to a first target nucleic acid sequence; Y is an oligonucleotide comprising nucleic acid sequence that is complementary to a second target nucleic acid sequence; X comprises a nucleotide sequence of length about 1 to about 100 nucleotides, preferably about 1 to about 21 nucleotides (e.g., about 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, or 21 nucleotides) that is complementary to nucleotide sequence present in region Y'; Y comprises nucleotide sequence of length about 1 to about 100 nucleotides, preferably about 1 to about 21 nucleotides (e.g., about 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20 or 21 nucleotides) that is complementary to nucleotide sequence present in region X'; each p comprises a terminal phosphate group that is independently present or absent; each X and Y independently is of length sufficient to stably interact

(i.e., base pair) with the first and second target nucleic acid sequence, respectively, or a portion thereof. For example, each sequence X and Y can independently comprise sequence from about 12 to about 21 or more nucleotides in length (e.g., about 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, or more) that is complementary to a target nucleotide sequence in different target nucleic acid molecules, such as target RNAs or a portion thereof. In one embodiment, the first target nucleic acid sequence and the second target nucleic acid sequence are present in the same target nucleic acid molecule (e.g., PCSK9 target RNA or PCSK9 pathway target RNA such as H6PD, PTP-1B, apoAI/CIII/AIV, and/or CETP RNA). In another embodiment, the first target nucleic acid sequence and the second target nucleic acid sequence are present in different target nucleic acid molecules (e.g., PCSK9 target RNA and PCSK9 pathway target RNA, such as H6PD, PTP-1B, apoAI/CIII/AIV, and/or CETP RNA). In one embodiment, Z comprises a palindrome or a repeat sequence. In one embodiment, the lengths of oligonucleotides X and X' are identical. In another embodiment, the lengths of oligonucleotides X and X' are not identical. In one embodiment, the lengths of oligonucleotides Y and Y' are identical. In another embodiment, the lengths of oligonucleotides Y and Y' are not identical. In one embodiment, the double stranded oligonucleotide construct of Formula I(a) includes one or more, specifically 1, 2, 3 or 4, mismatches, to the extent such mismatches do not significantly diminish the ability of the double stranded oligonucleotide to inhibit target gene expression.

[00605] In one embodiment, a multifunctional siNA molecule of the invention comprises a structure having Formula MF-III:



wherein each X, X', Y, and Y' is independently an oligonucleotide of length of about 15 nucleotides to about 50 nucleotides, preferably about 18 to about 40 nucleotides, or about 19 to about 23 nucleotides; X comprises nucleotide sequence that is complementary to nucleotide sequence present in region Y'; X' comprises nucleotide sequence that is complementary to nucleotide sequence present in region Y; each X and X' is independently of length sufficient to stably interact (i.e., base pair) with a first and a

second target nucleic acid sequence, respectively, or a portion thereof; W represents a nucleotide or non-nucleotide linker that connects sequences Y' and Y; and the multifunctional siNA directs cleavage of the first and second target sequence via RNA interference. In one embodiment, the first target nucleic acid sequence and the second target nucleic acid sequence are present in the same target nucleic acid molecule (e.g., PCSK9 target RNA or PCSK9 pathway target RNA such as PCSK9, H6PD, PTP-1B, apoAI/CIII/AIV, CETP, ApoB, SREBP-1a, SREBP-2, calcium/calmodulin-dependent protein kinase 1D, fatty acid binding protein 5, proprotein convertase subtilisin/kexin 9, LXR target genes, metalloprotease domain 11, apoptosis-inhibitory 6, and/or F-box-only protein 3 RNA). In another embodiment, the first target nucleic acid sequence and the second target nucleic acid sequence are present in different target nucleic acid molecules (e.g., PCSK9 target RNA and PCSK9 pathway target RNA such as PCSK9, H6PD, PTP-1B, apoAI/CIII/AIV, CETP, ApoB, SREBP-1a, SREBP-2, calcium/calmodulin-dependent protein kinase 1D, fatty acid binding protein 5, proprotein convertase subtilisin/kexin 9, LXR target genes, metalloprotease domain 11, apoptosis-inhibitory 6, and/or F-box-only protein 3 RNA). In one embodiment, region W connects the 3'-end of sequence Y' with the 3'-end of sequence Y. In one embodiment, region W connects the 3'-end of sequence Y' with the 5'-end of sequence Y. In one embodiment, region W connects the 5'-end of sequence Y' with the 5'-end of sequence Y. In one embodiment, region W connects the 5'-end of sequence Y' with the 3'-end of sequence Y. In one embodiment, a terminal phosphate group is present at the 5'-end of sequence X. In one embodiment, a terminal phosphate group is present at the 5'-end of sequence X'. In one embodiment, a terminal phosphate group is present at the 5'-end of sequence Y. In one embodiment, a terminal phosphate group is present at the 5'-end of sequence Y'. In one embodiment, W connects sequences Y and Y' via a biodegradable linker. In one embodiment, W further comprises a conjugate, label, aptamer, ligand, lipid, or polymer.

[00606] In one embodiment, a multifunctional siNA molecule of the invention comprises a structure having Formula MF-IV:



wherein each X, X', Y, and Y' is independently an oligonucleotide of length of about 15 nucleotides to about 50 nucleotides, preferably about 18 to about 40 nucleotides, or about 19 to about 23 nucleotides; X comprises nucleotide sequence that is complementary to nucleotide sequence present in region Y'; X' comprises nucleotide sequence that is complementary to nucleotide sequence present in region Y; each Y and Y' is independently of length sufficient to stably interact (i.e., base pair) with a first and a second target nucleic acid sequence, respectively, or a portion thereof; W represents a nucleotide or non-nucleotide linker that connects sequences Y' and Y; and the multifunctional siNA directs cleavage of the first and second target sequence via RNA interference. In one embodiment, the first target nucleic acid sequence and the second target nucleic acid sequence are present in the same target nucleic acid molecule (e.g., PCSK9, H6PD, PTP-1B, apoAI/CIII/AIV, CETP, ApoB, SREBP-1a, SREBP-2, calcium/calmodulin-dependent protein kinase 1D, fatty acid binding protein 5, proprotein convertase subtilisin/kexin 9, LXR target genes, metalloprotease domain 11, apoptosis-inhibitory 6, and/or F-box-only protein 3 RNA). In another embodiment, the first target nucleic acid sequence and the second target nucleic acid sequence are present in different target nucleic acid molecules (e.g., PCSK9 target RNA and PCSK9 pathway target RNA, such as PCSK9, H6PD, PTP-1B, apoAI/CIII/AIV, CETP, ApoB, SREBP-1a, SREBP-2, calcium/calmodulin-dependent protein kinase 1D, fatty acid binding protein 5, proprotein convertase subtilisin/kexin 9, LXR target genes, metalloprotease domain 11, apoptosis-inhibitory 6, and/or F-box-only protein 3 RNA). In one embodiment, region W connects the 3'-end of sequence Y' with the 3'-end of sequence Y. In one embodiment, region W connects the 3'-end of sequence Y' with the 5'-end of sequence Y. In one embodiment, region W connects the 5'-end of sequence Y' with the 5'-end of sequence Y. In one embodiment, region W connects the 5'-end of sequence Y' with the 3'-end of sequence Y. In one embodiment, a terminal phosphate group is present at the 5'-end of sequence X. In one embodiment, a terminal phosphate group is present at the 5'-end of sequence X'. In one embodiment, a terminal phosphate group is present at the 5'-end of sequence Y. In one embodiment, a terminal phosphate group is present at the 5'-end of sequence Y'. In one embodiment, W connects sequences Y and Y' via a biodegradable linker. In one embodiment, W further comprises a conjugate, label, aptamer, ligand, lipid, or polymer.

[00607] In one embodiment, a multifunctional siNA molecule of the invention comprises a structure having Formula MF-V:



wherein each X, X', Y, and Y' is independently an oligonucleotide of length of about 15 nucleotides to about 50 nucleotides, preferably about 18 to about 40 nucleotides, or about 19 to about 23 nucleotides; X comprises nucleotide sequence that is complementary to nucleotide sequence present in region Y'; X' comprises nucleotide sequence that is complementary to nucleotide sequence present in region Y; each X, X', Y, or Y' is independently of length sufficient to stably interact (i.e., base pair) with a first, second, third, or fourth target nucleic acid sequence, respectively, or a portion thereof; W represents a nucleotide or non-nucleotide linker that connects sequences Y' and Y; and the multifunctional siNA directs cleavage of the first, second, third, and/or fourth target sequence via RNA interference. In one embodiment, the first, second, third and fourth target nucleic acid sequence are all present in the same target nucleic acid molecule (e.g., PCSK9 target RNA or PCSK9 pathway target RNA such as PCSK9, H6PD, PTP-1B, apoAI/CIII/AIV, CETP, ApoB, SREBP-1a, SREBP-2, calcium/calmodulin-dependent protein kinase 1D, fatty acid binding protein 5, proprotein convertase subtilisin/kexin 9, LXR target genes, metalloprotease domain 11, apoptosis-inhibitory 6, and/or F-box-only protein 3 RNA). In another embodiment, the first, second, third and fourth target nucleic acid sequence are independently present in different target nucleic acid molecules (e.g., PCSK9 target RNA and PCSK9 pathway target RNA such as PCSK9, H6PD, PTP-1B, apoAI/CIII/AIV, CETP, ApoB, SREBP-1a, SREBP-2, calcium/calmodulin-dependent protein kinase 1D, fatty acid binding protein 5, proprotein convertase subtilisin/kexin 9, LXR target genes, metalloprotease domain 11, apoptosis-inhibitory 6, and/or F-box-only protein 3 RNA). In one embodiment, region W connects the 3'-end of sequence Y' with the 3'-end of sequence Y. In one embodiment, region W connects the 3'-end of sequence Y' with the 5'-end of sequence Y. In one embodiment, region W connects the 5'-end of sequence Y' with the 5'-end of sequence Y. In one embodiment, region W connects the 5'-end of sequence Y' with the 3'-end of sequence Y. In one embodiment, a terminal phosphate group is present at the 5'-end of sequence X. In one embodiment, a

terminal phosphate group is present at the 5'-end of sequence X'. In one embodiment, a terminal phosphate group is present at the 5'-end of sequence Y. In one embodiment, a terminal phosphate group is present at the 5'-end of sequence Y'. In one embodiment, W connects sequences Y and Y' via a biodegradable linker. In one embodiment, W further comprises a conjugate, label, aptamer, ligand, lipid, or polymer.

[00608] In one embodiment, regions X and Y of multifunctional siNA molecule of the invention (e.g., having any of Formula MF-I - MF-V), are complementary to different target nucleic acid sequences that are portions of the same target nucleic acid molecule. In one embodiment, such target nucleic acid sequences are at different locations within the coding region of a RNA transcript. In one embodiment, such target nucleic acid sequences comprise coding and non-coding regions of the same RNA transcript. In one embodiment, such target nucleic acid sequences comprise regions of alternately spliced transcripts or precursors of such alternately spliced transcripts.

[00609] In one embodiment, a multifunctional siNA molecule having any of Formula MF-I - MF-V can comprise chemical modifications as described herein without limitation, such as, for example, nucleotides having any of Formulae I-VII described herein, stabilization chemistries as described in Table IV, or any other combination of modified nucleotides and non-nucleotides as described in the various embodiments herein.

[00610] In one embodiment, the palindrome or repeat sequence or modified nucleotide (e.g., nucleotide with a modified base, such as 2-amino purine or a universal base) in Z of multifunctional siNA constructs having Formula MF-I or MF-II comprises chemically modified nucleotides that are able to interact with a portion of the target nucleic acid sequence (e.g., modified base analogs that can form Watson Crick base pairs or non-Watson Crick base pairs).

[00611] In one embodiment, a multifunctional siNA molecule of the invention, for example each strand of a multifunctional siNA having MF-I – MF-V, independently comprises about 15 to about 40 nucleotides (e.g., about 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, or 40 nucleotides). In one embodiment, a multifunctional siNA molecule of the invention comprises one or more

chemical modifications. In a non-limiting example, the introduction of chemically modified nucleotides and/or non-nucleotides into nucleic acid molecules of the invention provides a powerful tool in overcoming potential limitations of in vivo stability and bioavailability inherent to unmodified RNA molecules that are delivered exogenously. For example, the use of chemically modified nucleic acid molecules can enable a lower dose of a particular nucleic acid molecule for a given therapeutic effect since chemically modified nucleic acid molecules tend to have a longer half-life in serum or in cells or tissues. Furthermore, certain chemical modifications can improve the bioavailability and/or potency of nucleic acid molecules by not only enhancing half-life but also facilitating the targeting of nucleic acid molecules to particular organs, cells or tissues and/or improving cellular uptake of the nucleic acid molecules. Therefore, even if the activity of a chemically modified nucleic acid molecule is reduced in vitro as compared to a native/unmodified nucleic acid molecule, for example when compared to an unmodified RNA molecule, the overall activity of the modified nucleic acid molecule can be greater than the native or unmodified nucleic acid molecule due to improved stability, potency, duration of effect, bioavailability and/or delivery of the molecule.

[00612] In another embodiment, the invention features multifunctional siNAs, wherein the multifunctional siNAs are assembled from two separate double-stranded siNAs, with one of the ends of each sense strand is tethered to the end of the sense strand of the other siNA molecule, such that the two antisense siNA strands are annealed to their corresponding sense strand that are tethered to each other at one end (see Figure 22). The tethers or linkers can be nucleotide-based linkers or non-nucleotide based linkers as generally known in the art and as described herein.

[00613] In one embodiment, the invention features a multifunctional siNA, wherein the multifunctional siNA is assembled from two separate double-stranded siNAs, with the 5'-end of one sense strand of the siNA is tethered to the 5'- end of the sense strand of the other siNA molecule, such that the 5'-ends of the two antisense siNA strands, annealed to their corresponding sense strand that are tethered to each other at one end, point away (in the opposite direction) from each other (see Figure 22 (A)). The tethers or linkers can be nucleotide-based linkers or non-nucleotide based linkers as generally known in the art and as described herein.

[00614] In one embodiment, the invention features a multifunctional siNA, wherein the multifunctional siNA is assembled from two separate double-stranded siNAs, with the 3'-end of one sense strand of the siNA is tethered to the 3'- end of the sense strand of the other siNA molecule, such that the 5'-ends of the two antisense siNA strands, annealed to their corresponding sense strand that are tethered to each other at one end, face each other (see Figure 22 (B)). The tethers or linkers can be nucleotide-based linkers or non-nucleotide based linkers as generally known in the art and as described herein.

[00615] In one embodiment, the invention features a multifunctional siNA, wherein the multifunctional siNA is assembled from two separate double-stranded siNAs, with the 5'-end of one sense strand of the siNA is tethered to the 3'- end of the sense strand of the other siNA molecule, such that the 5'-end of the one of the antisense siNA strands annealed to their corresponding sense strand that are tethered to each other at one end, faces the 3'-end of the other antisense strand (see Figure 22 (C-D)). The tethers or linkers can be nucleotide-based linkers or non-nucleotide based linkers as generally known in the art and as described herein.

[00616] In one embodiment, the invention features a multifunctional siNA, wherein the multifunctional siNA is assembled from two separate double-stranded siNAs, with the 5'-end of one antisense strand of the siNA is tethered to the 3'- end of the antisense strand of the other siNA molecule, such that the 5'-end of the one of the sense siNA strands annealed to their corresponding antisense sense strand that are tethered to each other at one end, faces the 3'-end of the other sense strand (see Figure 22 (G-H)). In one embodiment, the linkage between the 5'-end of the first antisense strand and the 3'-end of the second antisense strand is designed in such a way as to be readily cleavable (e.g., biodegradable linker) such that the 5'end of each antisense strand of the multifunctional siNA has a free 5'-end suitable to mediate RNA interference-based cleavage of the target RNA. The tethers or linkers can be nucleotide-based linkers or non-nucleotide based linkers as generally known in the art and as described herein.

[00617] In one embodiment, the invention features a multifunctional siNA, wherein the multifunctional siNA is assembled from two separate double-stranded siNAs, with the 5'-end of one antisense strand of the siNA is tethered to the 5'- end of the antisense strand of the other siNA molecule, such that the 3'-end of the one of the sense siNA

strands annealed to their corresponding antisense sense strand that are tethered to each other at one end, faces the 3'-end of the other sense strand (see Figure 22 (E)). In one embodiment, the linkage between the 5'-end of the first antisense strand and the 5'-end of the second antisense strand is designed in such a way as to be readily cleavable (e.g., biodegradable linker) such that the 5'-end of each antisense strand of the multifunctional siNA has a free 5'-end suitable to mediate RNA interference-based cleavage of the target RNA. The tethers or linkers can be nucleotide-based linkers or non-nucleotide based linkers as generally known in the art and as described herein.

[00618] In one embodiment, the invention features a multifunctional siNA, wherein the multifunctional siNA is assembled from two separate double-stranded siNAs, with the 3'-end of one antisense strand of the siNA is tethered to the 3'- end of the antisense strand of the other siNA molecule, such that the 5'-end of the one of the sense siNA strands annealed to their corresponding antisense sense strand that are tethered to each other at one end, faces the 3'-end of the other sense strand (see Figure 22 (F)). In one embodiment, the linkage between the 5'-end of the first antisense strand and the 5'-end of the second antisense strand is designed in such a way as to be readily cleavable (e.g., biodegradable linker) such that the 5'-end of each antisense strand of the multifunctional siNA has a free 5'-end suitable to mediate RNA interference-based cleavage of the target RNA. The tethers or linkers can be nucleotide-based linkers or non-nucleotide based linkers as generally known in the art and as described herein.

[00619] In any of the above embodiments, a first target nucleic acid sequence or second target nucleic acid sequence can independently comprise PCSK9, H6PD, PTP-1B, apoAI/CIII/AIV, CETP, ApoB, SREBP-1a, SREBP-2, calcium/calmodulin-dependent protein kinase 1D, fatty acid binding protein 5, proprotein convertase subtilisin/kexin 9, LXR target genes, metalloprotease domain 11, apoptosis-inhibitory 6, and/or F-box-only protein 3 RNA, DNA or a portion thereof. In one embodiment, the first target nucleic acid sequence is a PCSK9 target RNA, DNA or a portion thereof and the second PCSK9 RNA, DNA, or a portion thereof. In one embodiment, the first target nucleic acid sequence is a polynucleotide coding or non-coding sequence involved in disease processes associated with PCSK9 such as PCSK9, H6PD, PTP-1B, apoAI/CIII/AIV, CETP, ApoB, SREBP-1a, SREBP-2, calcium/calmodulin-dependent

protein kinase 1D, fatty acid binding protein 5, proprotein convertase subtilisin/kexin 9, LXR target genes, metalloprotease domain 11, apoptosis-inhibitory 6, and/or F-box-only protein 3 RNA of a portion thereof.

Synthesis of Nucleic Acid Molecules

[00620] Synthesis of nucleic acids greater than 100 nucleotides in length is difficult using automated methods, and the therapeutic cost of such molecules is prohibitive. In this invention, small nucleic acid motifs ("small" refers to nucleic acid motifs no more than 100 nucleotides in length, preferably no more than 80 nucleotides in length, and most preferably no more than 50 nucleotides in length; e.g., individual siNA oligonucleotide sequences or siNA sequences synthesized in tandem) are preferably used for exogenous delivery. The simple structure of these molecules increases the ability of the nucleic acid to invade targeted regions of protein and/or RNA structure. Exemplary molecules of the instant invention are chemically synthesized, and others can similarly be synthesized.

[00621] Oligonucleotides (e.g., certain modified oligonucleotides or portions of oligonucleotides lacking ribonucleotides) are synthesized using protocols known in the art, for example as described in Caruthers et al., 1992, Methods in Enzymology 211, 3-19, Thompson et al., International PCT Publication No. WO 99/54459, Wincott et al., 1995, Nucleic Acids Res. 23, 2677-2684, Wincott et al., 1997, Methods Mol. Bio., 74, 59, Brennan et al., 1998, Biotechnol Bioeng., 61, 33-45, and Brennan, U.S. Pat. No. 6,001,311. All of these references are incorporated herein by reference. The synthesis of oligonucleotides makes use of common nucleic acid protecting and coupling groups, such as dimethoxytrityl at the 5'-end, and phosphoramidites at the 3'-end. In a non-limiting example, small scale syntheses are conducted on a 394 Applied Biosystems, Inc. synthesizer using a 0.2 μ mol scale protocol with a 2.5 min coupling step for 2'-O-methylated nucleotides and a 45 second coupling step for 2'-deoxy nucleotides or 2'-deoxy-2'-fluoro nucleotides. Table V outlines the amounts and the contact times of the reagents used in the synthesis cycle. Alternatively, syntheses at the 0.2 μ mol scale can be performed on a 96-well plate synthesizer, such as the instrument produced by Protogene (Palo Alto, CA) with minimal modification to the cycle. A 33-fold excess (60 μ L of 0.11 M = 6.6 μ mol) of 2'-O-methyl phosphoramidite and a 105-fold excess of S-

ethyl tetrazole (60 μ L of 0.25 M = 15 μ mol) can be used in each coupling cycle of 2'-O-methyl residues relative to polymer-bound 5'-hydroxyl. A 22-fold excess (40 μ L of 0.11 M = 4.4 μ mol) of deoxy phosphoramidite and a 70-fold excess of S-ethyl tetrazole (40 μ L of 0.25 M = 10 μ mol) can be used in each coupling cycle of deoxy residues relative to polymer-bound 5'-hydroxyl. Average coupling yields on the 394 Applied Biosystems, Inc. synthesizer, determined by colorimetric quantitation of the trityl fractions, are typically 97.5-99%. Other oligonucleotide synthesis reagents for the 394 Applied Biosystems, Inc. synthesizer include the following: detritylation solution is 3% TCA in methylene chloride (ABI); capping is performed with 16% N-methyl imidazole in THF (ABI) and 10% acetic anhydride/10% 2,6-lutidine in THF (ABI); and oxidation solution is 16.9 mM I₂, 49 mM pyridine, 9% water in THF (PerSeptive Biosystems, Inc.). Burdick & Jackson Synthesis Grade acetonitrile is used directly from the reagent bottle. S-Ethyltetrazole solution (0.25 M in acetonitrile) is made up from the solid obtained from American International Chemical, Inc. Alternately, for the introduction of phosphorothioate linkages, Beaucage reagent (3H-1,2-Benzodithiol-3-one 1,1-dioxide, 0.05 M in acetonitrile) is used.

[00622] Deprotection of the DNA-based oligonucleotides is performed as follows: the polymer-bound trityl-on oligoribonucleotide is transferred to a 4 mL glass screw top vial and suspended in a solution of 40% aqueous methylamine (1 mL) at 65 °C for 10 minutes. After cooling to -20 °C, the supernatant is removed from the polymer support. The support is washed three times with 1.0 mL of EtOH:MeCN:H₂O/3:1:1, vortexed and the supernatant is then added to the first supernatant. The combined supernatants, containing the oligoribonucleotide, are dried to a white powder. In one embodiment, the nucleic acid molecules of the invention are synthesized, deprotected, and analyzed according to methods described in US 6,995,259, US 6,686,463, US 6,673,918, US 6,649,751, US 6,989,442, and USSN 10/190,359, all incorporated by reference herein in their entirety.

[00623] The method of synthesis used for RNA including certain siNA molecules of the invention follows the procedure as described in Usman et al., 1987, J. Am. Chem. Soc., 109, 7845; Scaringe et al., 1990, Nucleic Acids Res., 18, 5433; and Wincott et al., 1995, Nucleic Acids Res. 23, 2677-2684 Wincott et al., 1997, Methods Mol. Bio., 74,

59, and makes use of common nucleic acid protecting and coupling groups, such as dimethoxytrityl at the 5'-end, and phosphoramidites at the 3'-end. In a non-limiting example, small scale syntheses are conducted on a 394 Applied Biosystems, Inc. synthesizer using a 0.2 μmol scale protocol with a 7.5 min coupling step for alkylsilyl protected nucleotides and a 2.5 min coupling step for 2'-O-methylated nucleotides. Table V outlines the amounts and the contact times of the reagents used in the synthesis cycle. Alternatively, syntheses at the 0.2 μmol scale can be done on a 96-well plate synthesizer, such as the instrument produced by Protogene (Palo Alto, CA) with minimal modification to the cycle. A 33-fold excess (60 μL of 0.11 M = 6.6 μmol) of 2'-O-methyl phosphoramidite and a 75-fold excess of S-ethyl tetrazole (60 μL of 0.25 M = 15 μmol) can be used in each coupling cycle of 2'-O-methyl residues relative to polymer-bound 5'-hydroxyl. A 66-fold excess (120 μL of 0.11 M = 13.2 μmol) of alkylsilyl (ribo) protected phosphoramidite and a 150-fold excess of S-ethyl tetrazole (120 μL of 0.25 M = 30 μmol) can be used in each coupling cycle of ribo residues relative to polymer-bound 5'-hydroxyl. Average coupling yields on the 394 Applied Biosystems, Inc. synthesizer, determined by colorimetric quantitation of the trityl fractions, are typically 97.5-99%. Other oligonucleotide synthesis reagents for the 394 Applied Biosystems, Inc. synthesizer include the following: detritylation solution is 3% TCA in methylene chloride (ABI); capping is performed with 16% N-methyl imidazole in THF (ABI) and 10% acetic anhydride/10% 2,6-lutidine in THF (ABI); oxidation solution is 16.9 mM I₂, 49 mM pyridine, 9% water in THF (PerSeptive Biosystems, Inc.). Burdick & Jackson Synthesis Grade acetonitrile is used directly from the reagent bottle. S-Ethyltetrazole solution (0.25 M in acetonitrile) is made up from the solid obtained from American International Chemical, Inc. Alternately, for the introduction of phosphorothioate linkages, Beaucage reagent (3H-1,2-Benzodithiol-3-one 1,1-dioxide 0.05 M in acetonitrile) is used.

[00624] Deprotection of the RNA is performed using either a two-pot or one-pot protocol. For the two-pot protocol, the polymer-bound trityl-on oligoribonucleotide is transferred to a 4 mL glass screw top vial and suspended in a solution of 40% aq. methylamine (1 mL) at 65 °C for 10 min. After cooling to -20 °C, the supernatant is removed from the polymer support. The support is washed three times with 1.0 mL of EtOH:MeCN:H₂O/3:1:1, vortexed and the supernatant is then added to the first

supernatant. The combined supernatants, containing the oligoribonucleotide, are dried to a white powder. The base deprotected oligoribonucleotide is resuspended in anhydrous TEA/HF/NMP solution (300 μ L of a solution of 1.5 mL N-methylpyrrolidinone, 750 μ L TEA and 1 mL TEA•3HF to provide a 1.4 M HF concentration) and heated to 65 °C. After 1.5 h, the oligomer is quenched with 1.5 M NH₄HCO₃. In one embodiment, the nucleic acid molecules of the invention are synthesized, deprotected, and analyzed according to methods described in US 6,995,259, US 6,686,463, US 6,673,918, US 6,649,751, US 6,989,442, and USSN 10/190,359, all incorporated by reference herein in their entirety.

[00625] Alternatively, for the one-pot protocol, the polymer-bound trityl-on oligoribonucleotide is transferred to a 4 mL glass screw top vial and suspended in a solution of 33% ethanolic methylamine/DMSO: 1/1 (0.8 mL) at 65 °C for 15 minutes. The vial is brought to room temperature TEA•3HF (0.1 mL) is added and the vial is heated at 65 °C for 15 minutes. The sample is cooled at -20 °C and then quenched with 1.5 M NH₄HCO₃.

[00626] For purification of the trityl-on oligomers, the quenched NH₄HCO₃ solution is loaded onto a C-18 containing cartridge that had been prewashed with acetonitrile followed by 50 mM TEAA. After washing the loaded cartridge with water, the RNA is detritylated with 0.5% TFA for 13 minutes. The cartridge is then washed again with water, salt exchanged with 1 M NaCl and washed with water again. The oligonucleotide is then eluted with 30% acetonitrile.

[00627] The average stepwise coupling yields are typically >98% (Wincott et al., 1995 Nucleic Acids Res. 23, 2677-2684). Those of ordinary skill in the art will recognize that the scale of synthesis can be adapted to be larger or smaller than the example described above including but not limited to 96-well format.

[00628] Alternatively, the nucleic acid molecules of the present invention can be synthesized separately and joined together post-synthetically, for example, by ligation (Moore et al., 1992, Science 256, 9923; Draper et al., International PCT publication No. WO 93/23569; Shabarova et al., 1991, Nucleic Acids Research 19, 4247; Bellon et al.,

1997, Nucleosides & Nucleotides, 16, 951; Bellon et al., 1997, Bioconjugate Chem. 8, 204), or by hybridization following synthesis and/or deprotection.

[00629] The siNA molecules of the invention can also be synthesized via a tandem synthesis methodology as described in Example 1 herein, wherein both siNA strands are synthesized as a single contiguous oligonucleotide fragment or strand separated by a cleavable linker which is subsequently cleaved to provide separate siNA fragments or strands that hybridize and permit purification of the siNA duplex. The linker can be a polynucleotide linker or a non-nucleotide linker. The tandem synthesis of siNA as described herein can be readily adapted to both multiwell/multiplate synthesis platforms such as 96 well or similarly larger multi-well platforms. The tandem synthesis of siNA as described herein can also be readily adapted to large scale synthesis platforms employing batch reactors, synthesis columns and the like.

[00630] A siNA molecule can also be assembled from two distinct nucleic acid strands or fragments wherein one fragment includes the sense region and the second fragment includes the antisense region of the RNA molecule.

[00631] The nucleic acid molecules of the present invention can be modified extensively to enhance stability by modification with nuclease resistant groups, for example, 2'-amino, 2'-C-allyl, 2'-fluoro, 2'-O-methyl, 2'-H (for a review see Usman and Cedergren, 1992, TIBS 17, 34; Usman et al., 1994, Nucleic Acids Symp. Ser. 31, 163). siNA constructs can be purified by gel electrophoresis using general methods or can be purified by high pressure liquid chromatography (HPLC; see Wincott et al., supra, the totality of which is hereby incorporated herein by reference) and re-suspended in water.

[00632] In another aspect of the invention, siNA molecules of the invention are expressed from transcription units inserted into DNA or RNA vectors. The recombinant vectors can be DNA plasmids or viral vectors. siNA expressing viral vectors can be constructed based on, but not limited to, adeno-associated virus, retrovirus, adenovirus, or alphavirus. The recombinant vectors capable of expressing the siNA molecules can be delivered as described herein, and persist in target cells. Alternatively, viral vectors can be used that provide for transient expression of siNA molecules.

Optimizing Activity of the nucleic acid molecule of the invention.

[00633] Chemically synthesizing nucleic acid molecules with modifications (base, sugar and/or phosphate) can prevent their degradation by serum ribonucleases, which can increase their potency (see e.g., Eckstein et al., International Publication No. WO 92/07065; Perrault et al., 1990 Nature 344, 565; Pieken et al., 1991, Science 253, 314; Usman and Cedergren, 1992, Trends in Biochem. Sci. 17, 334; Usman et al., International Publication No. WO 93/15187; and Rossi et al., International Publication No. WO 91/03162; Sproat, U.S. Pat. No. 5,334,711; Gold et al., U.S. Pat. No. 6,300,074; and Burgin et al., *supra*; all of which are incorporated by reference herein). All of the above references describe various chemical modifications that can be made to the base, phosphate and/or sugar moieties of the nucleic acid molecules described herein. Modifications that enhance their efficacy in cells, and removal of bases from nucleic acid molecules to shorten oligonucleotide synthesis times and reduce chemical requirements are desired.

[00634] There are several examples in the art describing sugar, base and phosphate modifications that can be introduced into nucleic acid molecules with significant enhancement in their nuclease stability and efficacy. For example, oligonucleotides are modified to enhance stability and/or enhance biological activity by modification with nuclease resistant groups, for example, 2'-amino, 2'-C-allyl, 2'-fluoro, 2'-O-methyl, 2'-O-allyl, 2'-H, nucleotide base modifications (for a review see Usman and Cedergren, 1992, TIBS. 17, 34; Usman et al., 1994, Nucleic Acids Symp. Ser. 31, 163; Burgin et al., 1996, Biochemistry, 35, 14090). Sugar modification of nucleic acid molecules have been extensively described in the art (see Eckstein et al., International Publication PCT No. WO 92/07065; Perrault et al. Nature, 1990, 344, 565-568; Pieken et al. Science, 1991, 253, 314-317; Usman and Cedergren, Trends in Biochem. Sci., 1992, 17, 334-339; Usman et al. International Publication PCT No. WO 93/15187; Sproat, U.S. Pat. No. 5,334,711 and Beigelman et al., 1995, J. Biol. Chem., 270, 25702; Beigelman et al., International PCT publication No. WO 97/26270; Beigelman et al., U.S. Pat. No. 5,716,824; Usman et al., U.S. Pat. No. 5,627,053; Woolf et al., International PCT Publication No. WO 98/13526; Thompson et al., USSN 60/082,404 which was filed on April 20, 1998; Karpeisky et al., 1998, Tetrahedron Lett., 39, 1131; Earnshaw and Gait, 1998, Biopolymers (Nucleic Acid Sciences), 48, 39-55; Verma and Eckstein, 1998, Annu. Rev. Biochem., 67, 99-134; and Burlina et al., 1997, Bioorg. Med. Chem., 5,

1999-2010; all of the references are hereby incorporated in their totality by reference herein). Such publications describe general methods and strategies to determine the location of incorporation of sugar, base and/or phosphate modifications and the like into nucleic acid molecules without modulating catalysis, and are incorporated by reference herein. In view of such teachings, similar modifications can be used as described herein to modify the siNA nucleic acid molecules of the instant invention so long as the ability of siNA to promote RNAi in cells is not significantly inhibited.

[00635] In one embodiment, a nucleic acid molecule of the invention is chemically modified as described in US 20050020521, incorporated by reference herein in its entirety.

[00636] While chemical modification of oligonucleotide internucleotide linkages with phosphorothioate, phosphorodithioate, and/or 5'-methylphosphonate linkages improves stability, excessive modifications can cause some toxicity or decreased activity. Therefore, when designing nucleic acid molecules, the amount of these internucleotide linkages should be minimized. The reduction in the concentration of these linkages should lower toxicity, resulting in increased efficacy and higher specificity of these molecules.

[00637] Short interfering nucleic acid (siNA) molecules having chemical modifications that maintain or enhance activity are provided. Such a nucleic acid is also generally more resistant to nucleases than an unmodified nucleic acid. Accordingly, the in vitro and/or in vivo activity should not be significantly lowered. In cases in which modulation is the goal, therapeutic nucleic acid molecules delivered exogenously should optimally be stable within cells until translation of the target RNA has been modulated long enough to reduce the levels of the undesirable protein. This period of time varies between hours to days depending upon the disease state. Improvements in the chemical synthesis of RNA and DNA (Wincott et al., 1995, Nucleic Acids Res. 23, 2677; Caruthers et al., 1992, Methods in Enzymology 211, 3-19 (incorporated by reference herein)) have expanded the ability to modify nucleic acid molecules by introducing nucleotide modifications to enhance their nuclease stability, as described above.

[00638] In one embodiment, nucleic acid molecules of the invention include one or more (e.g., about 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, or more) G-clamp nucleotides. A G-clamp nucleotide is a modified cytosine analog wherein the modifications confer the ability to hydrogen bond both Watson-Crick and Hoogsteen faces of a complementary guanine within a duplex, see for example Lin and Matteucci, 1998, J. Am. Chem. Soc., 120, 8531-8532. A single G-clamp analog substitution within an oligonucleotide can result in substantially enhanced helical thermal stability and mismatch discrimination when hybridized to complementary oligonucleotides. The inclusion of such nucleotides in nucleic acid molecules of the invention results in both enhanced affinity and specificity to nucleic acid targets, complementary sequences, or template strands. In another embodiment, nucleic acid molecules of the invention include one or more (e.g., about 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, or more) LNA "locked nucleic acid" nucleotides such as a 2', 4'-C methylene bicyclo nucleotide (see for example Wengel et al., International PCT Publication No. WO 00/66604 and WO 99/14226).

[00639] In another embodiment, the invention features conjugates and/or complexes of siNA molecules of the invention. Such conjugates and/or complexes can be used to facilitate delivery of siNA molecules into a biological system, such as a cell. The conjugates and complexes provided by the instant invention can impart therapeutic activity by transferring therapeutic compounds across cellular membranes, altering the pharmacokinetics, and/or modulating the localization of nucleic acid molecules of the invention. The present invention encompasses the design and synthesis of novel conjugates and complexes for the delivery of molecules, including, but not limited to, small molecules, lipids, cholesterol, phospholipids, nucleosides, nucleotides, nucleic acids, antibodies, toxins, negatively charged polymers and other polymers, for example proteins, peptides, hormones, carbohydrates, polyethylene glycols, or polyamines, across cellular membranes. In general, the transporters described are designed to be used either individually or as part of a multi-component system, with or without degradable linkers. These compounds are expected to improve delivery and/or localization of nucleic acid molecules of the invention into a number of cell types originating from different tissues, in the presence or absence of serum (see Sullenger and Cech, U.S. Pat. No. 5,854,038). Conjugates of the molecules described herein can be attached to biologically active

molecules via linkers that are biodegradable, such as biodegradable nucleic acid linker molecules.

[00640] The term "biodegradable linker" as used herein, refers to a nucleic acid or non-nucleic acid linker molecule that is designed as a biodegradable linker to connect one molecule to another molecule, for example, a biologically active molecule to a siNA molecule of the invention or the sense and antisense strands of a siNA molecule of the invention. The biodegradable linker is designed such that its stability can be modulated for a particular purpose, such as delivery to a particular tissue or cell type. The stability of a nucleic acid-based biodegradable linker molecule can be modulated by using various chemistries, for example combinations of ribonucleotides, deoxyribonucleotides, and chemically-modified nucleotides, such as 2'-O-methyl, 2'-fluoro, 2'-amino, 2'-O-amino, 2'-C-allyl, 2'-O-allyl, and other 2'-modified or base modified nucleotides. The biodegradable nucleic acid linker molecule can be a dimer, trimer, tetramer or longer nucleic acid molecule, for example, an oligonucleotide of about 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, or 20 nucleotides in length, or can comprise a single nucleotide with a phosphorus-based linkage, for example, a phosphoramidate or phosphodiester linkage. The biodegradable nucleic acid linker molecule can also comprise nucleic acid backbone, nucleic acid sugar, or nucleic acid base modifications.

[00641] The term "biodegradable" as used herein, refers to degradation in a biological system, for example, enzymatic degradation or chemical degradation.

[00642] The term "biologically active molecule" as used herein refers to compounds or molecules that are capable of eliciting or modifying a biological response in a system. Non-limiting examples of biologically active siNA molecules either alone or in combination with other molecules contemplated by the instant invention include therapeutically active molecules such as antibodies, cholesterol, hormones, antivirals, peptides, proteins, chemotherapeutics, small molecules, vitamins, co-factors, nucleosides, nucleotides, oligonucleotides, enzymatic nucleic acids, antisense nucleic acids, triplex forming oligonucleotides, 2,5-A chimeras, siNA, dsRNA, allozymes, aptamers, decoys and analogs thereof. Biologically active molecules of the invention also include molecules capable of modulating the pharmacokinetics and/or

pharmacodynamics of other biologically active molecules, for example, lipids and polymers such as polyamines, polyamides, polyethylene glycol and other polyethers.

[00643] The term "phospholipid" as used herein, refers to a hydrophobic molecule comprising at least one phosphorus group. For example, a phospholipid can comprise a phosphorus-containing group and saturated or unsaturated alkyl group, optionally substituted with OH, COOH, oxo, amine, or substituted or unsubstituted aryl groups.

[00644] Therapeutic nucleic acid molecules (e.g., siNA molecules) delivered exogenously optimally are stable within cells until reverse transcription of the RNA has been modulated long enough to reduce the levels of the RNA transcript. The nucleic acid molecules are resistant to nucleases in order to function as effective intracellular therapeutic agents. Improvements in the chemical synthesis of nucleic acid molecules described in the instant invention and in the art have expanded the ability to modify nucleic acid molecules by introducing nucleotide modifications to enhance their nuclease stability as described above.

[00645] In yet another embodiment, siNA molecules having chemical modifications that maintain or enhance enzymatic activity of proteins involved in RNAi are provided. Such nucleic acids are also generally more resistant to nucleases than unmodified nucleic acids. Thus, in vitro and/or in vivo the activity should not be significantly lowered.

[00646] Use of the nucleic acid-based molecules of the invention will lead to better treatments by affording the possibility of combination therapies (e.g., multiple siNA molecules targeted to different genes; nucleic acid molecules coupled with known small molecule modulators; or intermittent treatment with combinations of molecules, including different motifs and/or other chemical or biological molecules). The treatment of subjects with siNA molecules can also include combinations of different types of nucleic acid molecules, such as enzymatic nucleic acid molecules (ribozymes), allozymes, antisense, 2,5-A oligoadenylate, decoys, and aptamers.

[00647] In another aspect a siNA molecule of the invention comprises one or more 5' and/or a 3'- cap structure, for example, on only the sense siNA strand, the antisense siNA strand, or both siNA strands.

[00648] By "cap structure" is meant chemical modifications, which have been incorporated at either terminus of the oligonucleotide (see, for example, Adamic et al., U.S. Pat. No. 5,998,203, incorporated by reference herein). These terminal modifications protect the nucleic acid molecule from exonuclease degradation, and may help in delivery and/or localization within a cell. The cap may be present at the 5'-terminus (5'-cap) or at the 3'-terminal (3'-cap) or may be present on both termini. In non-limiting examples, the 5'-cap includes, but is not limited to, glyceryl, inverted deoxy abasic residue (moiety); 4',5'-methylene nucleotide; 1-(beta-D-erythrofuransyl) nucleotide, 4'-thio nucleotide; carbocyclic nucleotide; 1,5-anhydrohexitol nucleotide; L-nucleotides; alpha-nucleotides; modified base nucleotide; phosphorodithioate linkage; threo-pentofuransyl nucleotide; acyclic 3',4'-seco nucleotide; acyclic 3,4-dihydroxybutyl nucleotide; acyclic 3,5-dihydroxypentyl nucleotide, 3'-3'-inverted nucleotide moiety; 3'-3'-inverted abasic moiety; 3'-2'-inverted nucleotide moiety; 3'-2'-inverted abasic moiety; 1,4-butanediol phosphate; 3'-phosphoramidate; hexylphosphate; aminohexyl phosphate; 3'-phosphate; 3'-phosphorothioate; phosphorodithioate; or bridging or non-bridging methylphosphonate moiety. Non-limiting examples of cap moieties are shown in Figure 10.

[00649] Non-limiting examples of the 3'-cap include, but are not limited to, glyceryl, inverted deoxy abasic residue (moiety), 4', 5'-methylene nucleotide; 1-(beta-D-erythrofuransyl) nucleotide; 4'-thio nucleotide, carbocyclic nucleotide; 5'-amino-alkyl phosphate; 1,3-diamino-2-propyl phosphate; 3-aminopropyl phosphate; 6-aminohexyl phosphate; 1,2-aminododecyl phosphate; hydroxypropyl phosphate; 1,5-anhydrohexitol nucleotide; L-nucleotide; alpha-nucleotide; modified base nucleotide; phosphorodithioate; threo-pentofuransyl nucleotide; acyclic 3',4'-seco nucleotide; 3,4-dihydroxybutyl nucleotide; 3,5-dihydroxypentyl nucleotide, 5'-5'-inverted nucleotide moiety; 5'-5'-inverted abasic moiety; 5'-phosphoramidate; 5'-phosphorothioate; 1,4-butanediol phosphate; 5'-amino; bridging and/or non-bridging 5'-phosphoramidate, phosphorothioate and/or phosphorodithioate, bridging or non bridging methylphosphonate and 5'-mercapto moieties (for more details see Beaucage and Iyer, 1993, Tetrahedron 49, 1925; incorporated by reference herein).

[00650] By the term "non-nucleotide" is meant any group or compound which can be incorporated into a nucleic acid chain in the place of one or more nucleotide units, including either sugar and/or phosphate substitutions, and allows the remaining bases to exhibit their enzymatic activity. The group or compound is abasic in that it does not contain a commonly recognized nucleotide base, such as adenosine, guanine, cytosine, uracil or thymine and therefore lacks a base at the 1'-position.

[00651] An "alkyl" group refers to a saturated aliphatic hydrocarbon, including straight-chain, branched-chain, and cyclic alkyl groups. Preferably, the alkyl group has 1 to 12 carbons. More preferably, it is a lower alkyl of from 1 to 7 carbons, more preferably 1 to 4 carbons. The alkyl group can be substituted or unsubstituted. When substituted the substituted group(s) is preferably, hydroxyl, cyano, alkoxy, =O, =S, NO₂ or N(CH₃)₂, amino, or SH. The term also includes alkenyl groups that are unsaturated hydrocarbon groups containing at least one carbon-carbon double bond, including straight-chain, branched-chain, and cyclic groups. Preferably, the alkenyl group has 1 to 12 carbons. More preferably, it is a lower alkenyl of from 1 to 7 carbons, more preferably 1 to 4 carbons. The alkenyl group may be substituted or unsubstituted. When substituted the substituted group(s) is preferably, hydroxyl, cyano, alkoxy, =O, =S, NO₂, halogen, N(CH₃)₂, amino, or SH. The term "alkyl" also includes alkynyl groups that have an unsaturated hydrocarbon group containing at least one carbon-carbon triple bond, including straight-chain, branched-chain, and cyclic groups. Preferably, the alkynyl group has 1 to 12 carbons. More preferably, it is a lower alkynyl of from 1 to 7 carbons, more preferably 1 to 4 carbons. The alkynyl group may be substituted or unsubstituted. When substituted the substituted group(s) is preferably, hydroxyl, cyano, alkoxy, =O, =S, NO₂ or N(CH₃)₂, amino or SH.

[00652] Such alkyl groups can also include aryl, alkylaryl, carbocyclic aryl, heterocyclic aryl, amide and ester groups. An "aryl" group refers to an aromatic group that has at least one ring having a conjugated pi electron system and includes carbocyclic aryl, heterocyclic aryl and biaryl groups, all of which may be optionally substituted. The preferred substituent(s) of aryl groups are halogen, trihalomethyl, hydroxyl, SH, OH, cyano, alkoxy, alkyl, alkenyl, alkynyl, and amino groups. An "alkylaryl" group refers to an alkyl group (as described above) covalently joined to an aryl group (as described

above). Carbocyclic aryl groups are groups wherein the ring atoms on the aromatic ring are all carbon atoms. The carbon atoms are optionally substituted. Heterocyclic aryl groups are groups having from 1 to 3 heteroatoms as ring atoms in the aromatic ring and the remainder of the ring atoms are carbon atoms. Suitable heteroatoms include oxygen, sulfur, and nitrogen, and include furanyl, thienyl, pyridyl, pyrrolyl, N-lower alkyl pyrrolo, pyrimidyl, pyrazinyl, imidazolyl and the like, all optionally substituted. An "amide" refers to an -C(O)-NH-R, where R is either alkyl, aryl, alkylaryl or hydrogen. An "ester" refers to an -C(O)-OR', where R is either alkyl, aryl, alkylaryl or hydrogen.

[00653] By "nucleotide" as used herein is as recognized in the art to include natural bases (standard), and modified bases well known in the art. Such bases are generally located at the 1' position of a nucleotide sugar moiety. Nucleotides generally comprise a base, sugar and a phosphate group. The nucleotides can be unmodified or modified at the sugar, phosphate and/or base moiety, (also referred to interchangeably as nucleotide analogs, modified nucleotides, non-natural nucleotides, non-standard nucleotides and other; see, for example, Usman and McSwiggen, *supra*; Eckstein et al., International PCT Publication No. WO 92/07065; Usman et al., International PCT Publication No. WO 93/15187; Uhlman & Peyman, *supra*, all are hereby incorporated by reference herein). There are several examples of modified nucleic acid bases known in the art as summarized by Limbach et al., 1994, *Nucleic Acids Res.* 22, 2183. Some of the non-limiting examples of base modifications that can be introduced into nucleic acid molecules include, inosine, purine, pyridin-4-one, pyridin-2-one, phenyl, pseudouracil, 2, 4, 6-trimethoxy benzene, 3-methyl uracil, dihydrouridine, naphthyl, aminophenyl, 5-alkylcytidines (e.g., 5-methylcytidine), 5-alkyluridines (e.g., ribothymidine), 5-halouridine (e.g., 5-bromouridine) or 6-azapyrimidines or 6-alkylpyrimidines (e.g. 6-methyluridine), propyne, and others (Burgin et al., 1996, *Biochemistry*, 35, 14090; Uhlman & Peyman, *supra*). By "modified bases" in this aspect is meant nucleotide bases other than adenine, guanine, cytosine and uracil at 1' position or their equivalents.

[00654] In one embodiment, the invention features modified siNA molecules, with phosphate backbone modifications comprising one or more phosphorothioate, phosphorodithioate, methylphosphonate, phosphotriester, morpholino, amidate carbamate, carboxymethyl, acetamidate, polyamide, sulfonate, sulfonamide, sulfamate,

formacetal, thioformacetal, and/or alkylsilyl, substitutions. For a review of oligonucleotide backbone modifications, see Hunziker and Leumann, 1995, Nucleic Acid Analogues: Synthesis and Properties, in Modern Synthetic Methods, VCH, 331-417, and Mesmaeker et al., 1994, Novel Backbone Replacements for Oligonucleotides, in Carbohydrate Modifications in Antisense Research, ACS, 24-39.

[00655] By "abasic" is meant sugar moieties lacking a nucleobase or having a hydrogen atom (H) or other other non-nucleobase chemical groups in place of a nucleobase at the 1' position of the sugar moiety, see for example Adamic et al., U.S. Pat. No. 5,998,203. In one embodiment, an abasic moiety of the invention is a ribose, deoxyribose, or dideoxyribose sugar. .

[00656] By "unmodified nucleoside" is meant one of the bases adenine, cytosine, guanine, thymine, or uracil joined to the 1' carbon of β -D-ribo-furanose.

[00657] By "modified nucleoside" is meant any nucleotide base which contains a modification in the chemical structure of an unmodified nucleotide base, sugar and/or phosphate. Non-limiting examples of modified nucleotides are shown by Formulae I-VII and/or other modifications described herein.

[00658] In connection with 2'-modified nucleotides as described for the present invention, by "amino" is meant 2'-NH₂ or 2'-O- NH₂, which can be modified or unmodified. Such modified groups are described, for example, in Eckstein et al., U.S. Pat. No. 5,672,695 and Matulic-Adamic et al., U.S. Pat. No. 6,248,878, which are both incorporated by reference in their entireties.

[00659] Various modifications to nucleic acid siNA structure can be made to enhance the utility of these molecules. Such modifications will enhance shelf-life, half-life in vitro, stability, and ease of introduction of such oligonucleotides to the target site, e.g., to enhance penetration of cellular membranes, and confer the ability to recognize and bind to targeted cells.

Administration of Nucleic Acid Molecules

[00660] A siNA molecule of the invention can be adapted for use to prevent or treat diseases, traits, disorders, and/or conditions described herein or otherwise known in the art to be related to PCSK9 gene or PCSK9 pathway gene expression, and/or any other trait, disease, disorder or condition that is related to or will respond to the levels of PCSK9 polynucleotides or proteins expressed therefrom in a cell or tissue, alone or in combination with other therapies. In one embodiment, the siNA molecules of the invention and formulations or compositions thereof are administered to a cell, subject, or organism as is described herein and as is generally known in the art.

[00661] In one embodiment, a nucleic acid or polynucleotide (*e.g.*, siNA) composition of the invention can comprise a delivery vehicle, including liposomes, for administration to a subject, carriers and diluents and their salts, and/or can be present in pharmaceutically acceptable formulations. Methods for the delivery of nucleic acid molecules are described in Akhtar et al., 1992, Trends Cell Bio., 2, 139; Delivery Strategies for Antisense Oligonucleotide Therapeutics, ed. Akhtar, 1995, Maurer et al., 1999, Mol. Membr. Biol., 16, 129-140; Hofland and Huang, 1999, Handb. Exp. Pharmacol., 137, 165-192; and Lee et al., 2000, ACS Symp. Ser., 752, 184-192, all of which are incorporated herein by reference. Beigelman et al., U.S. Pat. No. 6,395,713 and Sullivan et al., PCT WO 94/02595 further describe the general methods for delivery of nucleic acid molecules. These protocols can be utilized for the delivery of virtually any nucleic acid molecule. Nucleic acid molecules can be administered to cells by a variety of methods known to those of skill in the art, including, but not restricted to, encapsulation in liposomes, by iontophoresis, or by incorporation into other vehicles, such as biodegradable polymers, hydrogels, cyclodextrins (see for example Gonzalez et al., 1999, Bioconjugate Chem., 10, 1068-1074; Wang et al., International PCT publication Nos. WO 03/47518 and WO 03/46185), poly(lactic-co-glycolic)acid (PLGA) and PLCA microspheres (see for example US Patent 6,447,796 and US Patent Application Publication No. US 2002130430), biodegradable nanocapsules, and bioadhesive microspheres, or by proteinaceous vectors (O'Hare and Normand, International PCT Publication No. WO 00/53722). In another embodiment, the nucleic acid molecules of the invention can also be formulated or complexed with polyethyleneimine and derivatives thereof, such as polyethyleneimine-polyethyleneglycol-N-acetylgalactosamine (PEI-PEG-GAL) or polyethyleneimine-

polyethyleneglycol-tri-N-acetylgalactosamine (PEI-PEG-triGAL) derivatives. In one embodiment, the nucleic acid molecules of the invention are formulated as described in United States Patent Application Publication No. 20030077829, incorporated by reference herein in its entirety.

[00662] In one embodiment, a nucleic acid or polynucleotide (*e.g.*, siNA) molecule of the invention is formulated as a composition described in U.S. Provisional patent application No. 60/678,531 and in related U.S. Provisional patent application No. 60/703,946, filed July 29, 2005, U.S. Provisional patent application No. 60/737,024, filed November 15, 2005, and USSN 11/353,630, filed February 14, 2006 (Vargeese et al.), all of which are incorporated by reference herein in their entirety. Such siNA formulations are generally referred to as “lipid nucleic acid particles” (LNP). In one embodiment, a siNA molecule of the invention is formulated with one or more LNP compositions described herein in Table VI (see also USSN 11/353,630 and 11/586,102 incorporated by reference herein).

[00663] In one embodiment, the nucleic acid or polynucleotide (*e.g.*, siNA) molecules of the invention and formulations or compositions thereof are administered to lung tissues and cells as is described in US 2006/0062758; US 2006/0014289; and US 2004/0077540.

[00664] In one embodiment, a nucleic acid or polynucleotide (*e.g.*, siNA) molecule of the invention is complexed with membrane disruptive agents such as those described in U.S. Patent Application Publication No. 20010007666, incorporated by reference herein in its entirety including the drawings. In another embodiment, the membrane disruptive agent or agents and the siNA molecule are also complexed with a cationic lipid or helper lipid molecule, such as those lipids described in U.S. Patent No. 6,235,310, incorporated by reference herein in its entirety including the drawings.

[00665] In one embodiment, a nucleic acid or polynucleotide (*e.g.*, siNA) molecule of the invention is complexed with delivery systems as described in U.S. Patent Application Publication No. 2003077829 and International PCT Publication Nos. WO 00/03683 and WO 02/087541, all incorporated by reference herein in their entirety including the drawings.

[00666] In one embodiment, a nucleic acid or polynucleotide (*e.g.*, siNA) molecule of the invention is complexed with delivery systems as is generally described in U.S. Patent Application Publication Nos. US-20050287551; US-20050164220; US-20050191627; US-20050118594; US-20050153919; US-20050085486; and US-20030158133; all incorporated by reference herein in their entirety including the drawings.

[00667] In one embodiment, the nucleic acid molecules of the invention are administered to skeletal tissues (*e.g.*, bone, cartilage, tendon, ligament) or bone metastatic tumors via atelocollagen complexation or conjugation (see for example Takeshita et al., 2005, PNAS, 102, 12177-12182). Therefore, in one embodiment, the instant invention features one or more dsRNA molecules as a composition complexed with atelocollagen. In another embodiment, the instant invention features one or more siRNA molecules conjugated to atelocollagen via a linker as described herein or otherwise known in the art.

[00668] In one embodiment, the nucleic acid or polynucleotide (*e.g.*, siRNA) molecules of the invention and formulations thereof (*e.g.*, LNP formulations of double stranded nucleic acid molecules of the invention) are administered via pulmonary delivery, such as by inhalation of an aerosol or spray dried formulation administered by an inhalation device or nebulizer, providing rapid local uptake of the nucleic acid molecules into relevant pulmonary tissues. Solid particulate compositions containing respirable dry particles of micronized nucleic acid compositions can be prepared by grinding dried or lyophilized nucleic acid compositions, and then passing the micronized composition through, for example, a 400 mesh screen to break up or separate out large agglomerates. A solid particulate composition comprising the nucleic acid compositions of the invention can optionally contain a dispersant which serves to facilitate the formation of an aerosol as well as other therapeutic compounds. A suitable dispersant is lactose, which can be blended with the nucleic acid compound in any suitable ratio, such as a 1 to 1 ratio by weight.

[00669] Aerosols of liquid particles comprising a nucleic acid composition of the invention can be produced by any suitable means, such as with a nebulizer (see for example US 4,501,729). Nebulizers are commercially available devices which transform solutions or suspensions of an active ingredient into a therapeutic aerosol mist either by

means of acceleration of a compressed gas, typically air or oxygen, through a narrow venturi orifice or by means of ultrasonic agitation. Suitable formulations for use in nebulizers comprise the active ingredient in a liquid carrier in an amount of up to 40% w/w preferably less than 20% w/w of the formulation. The carrier is typically water or a dilute aqueous alcoholic solution, preferably made isotonic with body fluids by the addition of, for example, sodium chloride or other suitable salts. Optional additives include preservatives if the formulation is not prepared sterile, for example, methyl hydroxybenzoate, anti-oxidants, flavorings, volatile oils, buffering agents and emulsifiers and other formulation surfactants. The aerosols of solid particles comprising the active composition and surfactant can likewise be produced with any solid particulate aerosol generator. Aerosol generators for administering solid particulate therapeutics to a subject produce particles which are respirable, as explained above, and generate a volume of aerosol containing a predetermined metered dose of a therapeutic composition at a rate suitable for human administration.

[00670] In one embodiment, a solid particulate aerosol generator of the invention is an insufflator. Suitable formulations for administration by insufflation include finely comminuted powders which can be delivered by means of an insufflator. In the insufflator, the powder, e.g., a metered dose thereof effective to carry out the treatments described herein, is contained in capsules or cartridges, typically made of gelatin or plastic, which are either pierced or opened in situ and the powder delivered by air drawn through the device upon inhalation or by means of a manually-operated pump. The powder employed in the insufflator consists either solely of the active ingredient or of a powder blend comprising the active ingredient, a suitable powder diluent, such as lactose, and an optional surfactant. The active ingredient typically comprises from 0.1 to 100 w/w of the formulation. A second type of illustrative aerosol generator comprises a metered dose inhaler. Metered dose inhalers are pressurized aerosol dispensers, typically containing a suspension or solution formulation of the active ingredient in a liquified propellant. During use these devices discharge the formulation through a valve adapted to deliver a metered volume to produce a fine particle spray containing the active ingredient. Suitable propellants include certain chlorofluorocarbon compounds, for example, dichlorodifluoromethane, trichlorofluoromethane, dichlorotetrafluoroethane and mixtures thereof. The formulation can additionally contain one or more co-solvents,

for example, ethanol, emulsifiers and other formulation surfactants, such as oleic acid or sorbitan trioleate, anti-oxidants and suitable flavoring agents. Other methods for pulmonary delivery are described in, for example US Patent Application No. 20040037780, and US Patent Nos. 6,592,904; 6,582,728; 6,565,885, all incorporated by reference herein.

[00671] In one embodiment, the nucleic acid or polynucleotide (*e.g.*, siNA) and LNP compositions and formulations provided herein for use in pulmonary delivery further comprise one or more surfactants. Suitable surfactants or surfactant components for enhancing the uptake of the compositions of the invention include synthetic and natural as well as full and truncated forms of surfactant protein A, surfactant protein B, surfactant protein C, surfactant protein D and surfactant Protein E, di-saturated phosphatidylcholine (other than dipalmitoyl), dipalmitoylphosphatidylcholine, phosphatidylcholine, phosphatidylglycerol, phosphatidylinositol, phosphatidylethanolamine, phosphatidylserine; phosphatidic acid, ubiquinones, lysophosphatidylethanolamine, lysophosphatidylcholine, palmitoyl-lysophosphatidylcholine, dehydroepiandrosterone, dolichols, sulfatidic acid, glycerol-3-phosphate, dihydroxyacetone phosphate, glycerol, glycerol-3-phosphocholine, dihydroxyacetone, palmitate, cytidine diphosphate (CDP) diacylglycerol, CDP choline, choline, choline phosphate; as well as natural and artificial lamellar bodies which are the natural carrier vehicles for the components of surfactant, omega-3 fatty acids, polyenic acid, polyenoic acid, lecithin, palmitinic acid, non-ionic block copolymers of ethylene or propylene oxides, polyoxypropylene, monomeric and polymeric, polyoxyethylene, monomeric and polymeric, poly (vinyl amine) with dextran and/or alkanoyl side chains, Brij 35, Triton X-100 and synthetic surfactants ALEC, Exosurf, Survan and Atovaquone, among others. These surfactants may be used either as single or part of a multiple component surfactant in a formulation, or as covalently bound additions to the 5' and/or 3' ends of the nucleic acid component of a pharmaceutical composition herein.

[00672] The composition of the present invention may be administered into the respiratory system as a formulation including particles of respirable size, *e.g.* particles of a size sufficiently small to pass through the nose, mouth and larynx upon inhalation and through the bronchi and alveoli of the lungs. In general, respirable particles range from

about 0.5 to 10 microns in size. Particles of non-respirable size which are included in the aerosol tend to deposit in the throat and be swallowed, and the quantity of non-respirable particles in the aerosol is thus minimized. For nasal administration, a particle size in the range of 10-500 um is preferred to ensure retention in the nasal cavity.

[00673] In one embodiment, the nucleic acid or polynucleotide (*e.g.*, siNA) molecules of the invention and formulations or compositions thereof are administered to the liver as is generally known in the art (see for example Wen et al., 2004, *World J Gastroenterol.*, 10, 244-9; Murao et al., 2002, *Pharm Res.*, 19, 1808-14; Liu et al., 2003, *Gene Ther.*, 10, 180-7; Hong et al., 2003, *J Pharm Pharmacol.*, 54, 51-8; Herrmann et al., 2004, *Arch Virol.*, 149, 1611-7; and Matsuno et al., 2003, *Gene Ther.*, 10, 1559-66).

[00674] In one embodiment, a nucleic acid or polynucleotide (*e.g.*, siNA) molecule of the invention is administered iontophoretically, for example to a particular organ or compartment (*e.g.*, the eye, back of the eye, heart, liver, kidney, bladder, prostate, tumor, CNS etc.). Non-limiting examples of iontophoretic delivery nucleic acids by neurons. As an example of local administration of nucleic acids to nerve cells, Sommer et al., 1998, *Antisense Nuc. Acid Drug Dev.*, 8, 75, describe a study in which a 15mer phosphorothioate antisense nucleic acid molecule to c-fos is administered to rats via microinjection into the brain. Antisense molecules labeled with tetramethylrhodamine-isothiocyanate (TRITC) or fluorescein isothiocyanate (FITC) were taken up by exclusively by neurons thirty minutes post-injection. A diffuse cytoplasmic staining and nuclear staining was observed in these cells. As an example of systemic administration of nucleic acid to nerve cells, Epa et al., 2000, *Antisense Nuc. Acid Drug Dev.*, 10, 469, describe an in vivo mouse study in which beta-cyclodextrin-adamantane-oligonucleotide conjugates were used to target the p75 neurotrophin receptor in neuronally differentiated PC12 cells. Following a two week course of IP administration, pronounced uptake of p75 neurotrophin receptor antisense was observed in dorsal root ganglion (DRG) cells. In addition, a marked and consistent down-regulation of p75 was observed in DRG neurons. Additional approaches to the targeting of nucleic acid to neurons are described in Broaddus et al., 1998, *J. Neurosurg.*, 88(4), 734; Karle et al., 1997, *Eur. J. Pharmacol.*, 340(2/3), 153; Bannai et al., 1998, *Brain Research*, 784(1,2), 304; Rajakumar et al., 1997, *Synapse*, 26(3), 199; Wu-pong et al., 1999, *BioPharm*, 12(1), 32; Bannai et al.,

1998, Brain Res. Protoc., 3(1), 83; Simantov et al., 1996, Neuroscience, 74(1), 39. Nucleic acid molecules of the invention are therefore amenable to delivery to and uptake by cells that express repeat expansion allelic variants for modulation of RE gene expression. The delivery of nucleic acid molecules of the invention, targeting RE is provided by a variety of different strategies. Traditional approaches to CNS delivery that can be used include, but are not limited to, intrathecal and intracerebroventricular administration, implantation of catheters and pumps, direct injection or perfusion at the site of injury or lesion, injection into the brain arterial system, or by chemical or osmotic opening of the blood-brain barrier. Other approaches can include the use of various transport and carrier systems, for example though the use of conjugates and biodegradable polymers. Furthermore, gene therapy approaches, for example as described in Kaplitt et al., US 6,180,613 and Davidson, WO 04/013280, can be used to express nucleic acid molecules in the CNS.

[00675] In one embodiment, nucleic acid molecules of the invention are administered to the central nervous system (CNS) or peripheral nervous system (PNS). Experiments have demonstrated the efficient in vivo uptake of nucleic acids by neurons. As an example of local administration of nucleic acids to nerve cells, Sommer et al., 1998, Antisense Nuc. Acid Drug Dev., 8, 75, describe a study in which a 15mer phosphorothioate antisense nucleic acid molecule to c-fos is administered to rats via microinjection into the brain. Antisense molecules labeled with tetramethylrhodamine-isothiocyanate (TRITC) or fluorescein isothiocyanate (FITC) were taken up by exclusively by neurons thirty minutes post-injection. A diffuse cytoplasmic staining and nuclear staining was observed in these cells. As an example of systemic administration of nucleic acid to nerve cells, Epa et al., 2000, Antisense Nuc. Acid Drug Dev., 10, 469, describe an in vivo mouse study in which beta-cyclodextrin-adamantane-oligonucleotide conjugates were used to target the p75 neurotrophin receptor in neuronally differentiated PC12 cells. Following a two week course of IP administration, pronounced uptake of p75 neurotrophin receptor antisense was observed in dorsal root ganglion (DRG) cells. In addition, a marked and consistent down-regulation of p75 was observed in DRG neurons. Additional approaches to the targeting of nucleic acid to neurons are described in Broaddus et al., 1998, J. Neurosurg., 88(4), 734; Karle et al., 1997, Eur. J. Pharmacol., 340(2/3), 153; Bannai et al., 1998, Brain Research, 784(1,2), 304; Rajakumar et al.,

1997, Synapse, 26(3), 199; Wu-pong et al., 1999, BioPharm, 12(1), 32; Bannai et al., 1998, Brain Res. Protoc., 3(1), 83; Simantov et al., 1996, Neuroscience, 74(1), 39. Nucleic acid molecules of the invention are therefore amenable to delivery to and uptake by cells in the CNS and/or PNS.

[00676] The delivery of nucleic acid molecules of the invention to the CNS is provided by a variety of different strategies. Traditional approaches to CNS delivery that can be used include, but are not limited to, intrathecal and intracerebroventricular administration, implantation of catheters and pumps, direct injection or perfusion at the site of injury or lesion, injection into the brain arterial system, or by chemical or osmotic opening of the blood-brain barrier. Other approaches can include the use of various transport and carrier systems, for example though the use of conjugates and biodegradable polymers. Furthermore, gene therapy approaches, for example as described in Kaplitt et al., US 6,180,613 and Davidson, WO 04/013280, can be used to express nucleic acid molecules in the CNS.

[00677] In one embodiment, nucleic acid or polynucleotide (*e.g.*, siNA) compounds and compositions of the invention are administered either systemically or locally about every 1-50 weeks (*e.g.*, about every 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, or 50 weeks), alone or in combination with other compounds and/or therapies herein. In one embodiment, siNA compounds and compositions of the invention are administered systemically (*e.g.*, via intravenous, subcutaneous, intramuscular, infusion, pump, implant etc.) about every 1-50 weeks (*e.g.*, about every 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, or 50 weeks), alone or in combination with other compounds and/or therapies described herein and/or otherwise known in the art.

[00678] In one embodiment, a nucleic acid or polynucleotide (*e.g.*, siNA) molecule of the invention is administered iontophoretically, for example to a particular organ or compartment (*e.g.*, lung, nasopharynx, skin, follicle, the eye, back of the eye, heart, liver, kidney, bladder, prostate, tumor, CNS etc.). Non-limiting examples of iontophoretic

delivery are described in, for example, WO 03/043689 and WO 03/030989, which are incorporated by reference in their entireties herein.

[00679] In one embodiment, the nucleic acid or polynucleotide (*e.g.*, siNA) molecules of the invention and formulations or compositions thereof are administered to the liver as is generally known in the art (see for example Wen et al., 2004, *World J Gastroenterol.*, 10, 244-9; Murao et al., 2002, *Pharm Res.*, 19, 1808-14; Liu et al., 2003, *Gene Ther.*, 10, 180-7; Hong et al., 2003, *J Pharm Pharmacol.*, 54, 51-8; Herrmann et al., 2004, *Arch Virol.*, 149, 1611-7; and Matsuno et al., 2003, *Gene Ther.*, 10, 1559-66).

[00680] In one embodiment, the invention features the use of methods to deliver the nucleic acid molecules of the instant invention to hematopoietic cells, including monocytes and lymphocytes. These methods are described in detail by Hartmann et al., 1998, *J. Pharmacol. Exp. Ther.*, 285(2), 920-928; Kronenwett et al., 1998, *Blood*, 91(3), 852-862; Fillion and Phillips, 1997, *Biochim. Biophys. Acta.*, 1329(2), 345-356; Ma and Wei, 1996, *Leuk. Res.*, 20(11/12), 925-930; and Bongartz et al., 1994, *Nucleic Acids Research*, 22(22), 4681-8. Such methods, as described above, include the use of free oligonucleotide, cationic lipid formulations, liposome formulations including pH sensitive liposomes and immunoliposomes, and bioconjugates including oligonucleotides conjugated to fusogenic peptides, for the transfection of hematopoietic cells with oligonucleotides.

[00681] In one embodiment, the nucleic acid or polynucleotide (*e.g.*, siNA) molecules of the invention and formulations or compositions thereof are administered directly or topically (*e.g.*, locally) to the dermis or follicles as is generally known in the art (see for example Brand, 2001, *Curr. Opin. Mol. Ther.*, 3, 244-8; Regnier et al., 1998, *J. Drug Target*, 5, 275-89; Kanikkannan, 2002, *BioDrugs*, 16, 339-47; Wraight et al., 2001, *Pharmacol. Ther.*, 90, 89-104; and Preat and Dujardin, 2001, *STP PharmaSciences*, 11, 57-68). In one embodiment, the siNA molecules of the invention and formulations or compositions thereof are administered directly or topically using a hydroalcoholic gel formulation comprising an alcohol (*e.g.*, ethanol or isopropanol), water, and optionally including additional agents such as isopropyl myristate and carbomer 980.

[00682] In one embodiment, delivery systems of the invention include, for example, aqueous and nonaqueous gels, creams, multiple emulsions, microemulsions, liposomes, ointments, aqueous and nonaqueous solutions, lotions, aerosols, hydrocarbon bases and powders, and can contain excipients such as solubilizers, permeation enhancers (e.g., fatty acids, fatty acid esters, fatty alcohols and amino acids), and hydrophilic polymers (e.g., polycarbophil and polyvinylpyrrolidone). In one embodiment, the pharmaceutically acceptable carrier is a liposome or a transdermal enhancer. Examples of liposomes which can be used in this invention include the following: (1) CellFectin, 1:1.5 (M/M) liposome formulation of the cationic lipid N,N_I,N_{II},N_{III}-tetramethyl-N,N_I,N_{II},N_{III}-tetrapalmit-y-spermine and dioleoyl phosphatidylethanolamine (DOPE) (GIBCO BRL); (2) Cytofectin GSV, 2:1 (M/M) liposome formulation of a cationic lipid and DOPE (Glen Research); (3) DOTAP (N-[1-(2,3-dioleoyloxy)-N,N,N-tri-methyl-ammoniummethylsulfate) (Boehringer Mannheim); and (4) Lipofectamine, 3:1 (M/M) liposome formulation of the polycationic lipid DOSPA and the neutral lipid DOPE (GIBCO BRL).

[00683] In one embodiment, delivery systems of the invention include patches, tablets, suppositories, pessaries, gels and creams, and can contain excipients such as solubilizers and enhancers (e.g., propylene glycol, bile salts and amino acids), and other vehicles (e.g., polyethylene glycol, fatty acid esters and derivatives, and hydrophilic polymers such as hydroxypropylmethylcellulose and hyaluronic acid).

[00684] In one embodiment, a nucleic acid or polynucleotide (e.g., siNA) molecule of the invention is administered iontophoretically, for example to the dermis or to other relevant tissues. Non-limiting examples of iontophoretic delivery are described in, for example, WO 03/043689 and WO 03/030989, which are incorporated by reference in their entireties herein.

[00685] In one embodiment, nucleic acid or polynucleotide (e.g., siNA) molecules of the invention are formulated or complexed with polyethylenimine (e.g., linear or branched PEI) and/or polyethylenimine derivatives, including for example grafted PEIs such as galactose PEI, cholesterol PEI, antibody derivatized PEI, and polyethylene glycol PEI (PEG-PEI) derivatives thereof (see for example Ogris et al., 2001, AAPA PharmSci, 3, 1-11; Furgeson et al., 2003, Bioconjugate Chem., 14, 840-847; Kunath et al., 2002, Pharmaceutical Research, 19, 810-817; Choi et al., 2001, Bull. Korean Chem.

Soc., 22, 46-52; Bettinger et al., 1999, Bioconjugate Chem., 10, 558-561; Peterson et al., 2002, Bioconjugate Chem., 13, 845-854; Erbacher et al., 1999, Journal of Gene Medicine Preprint, 1, 1-18; Godbey et al., 1999., PNAS USA, 96, 5177-5181; Godbey et al., 1999, Journal of Controlled Release, 60, 149-160; Diebold et al., 1999, Journal of Biological Chemistry, 274, 19087-19094; Thomas and Klibanov, 2002, PNAS USA, 99, 14640-14645; and Sagara, US 6,586,524, incorporated by reference herein.

[00686] In one embodiment, a nucleic acid or polynucleotide (*e.g.*, siNA) molecule of the invention comprises a bioconjugate, for example a nucleic acid conjugate as described in Vargeese et al., USSN 10/427,160, filed April 30, 2003; US 6,528,631; US 6,335,434; US 6, 235,886; US 6,153,737; US 5,214,136; US 5,138,045, all incorporated by reference herein.

[00687] Thus, the invention features a pharmaceutical composition comprising one or more nucleic acid(s) of the invention in an acceptable carrier, such as a stabilizer, buffer, and the like. The polynucleotides of the invention can be administered (*e.g.*, RNA, DNA or protein) and introduced to a subject by any standard means, with or without stabilizers, buffers, and the like, to form a pharmaceutical composition. When it is desired to use a liposome delivery mechanism, standard protocols for formation of liposomes can be followed. The compositions of the present invention can also be formulated and used as creams, gels, sprays, oils and other suitable compositions for topical, dermal, or transdermal administration as is known in the art.

[00688] The present invention also includes pharmaceutically acceptable formulations of the compounds described. These formulations include salts of the above compounds, *e.g.*, acid addition salts, for example, salts of hydrochloric, hydrobromic, acetic acid, and benzene sulfonic acid.

[00689] A pharmacological composition, formulation, or salt refers to a composition or formulation in a form suitable for administration, *e.g.*, systemic or local administration, into a cell or subject, including for example a human. Suitable forms, in part, depend upon the use or the route of entry, for example oral, transdermal, or by injection. Such forms should not prevent the composition or formulation from reaching a target cell (*i.e.*, a cell to which the negatively charged nucleic acid is desirable for delivery). For

example, pharmacological compositions injected into the blood stream should be soluble. Other factors are known in the art, and include considerations such as toxicity and forms that prevent the composition or formulation from exerting its effect.

[00690] In one embodiment, nucleic acid or polynucleotide (*e.g.*, siNA) molecules of the invention are administered to a subject by systemic administration in a pharmaceutically acceptable composition, formulation or salt. By "systemic administration" is meant *in vivo* systemic absorption or accumulation of drugs in the blood stream followed by distribution throughout the entire body. Administration routes that lead to systemic absorption include, without limitation: intravenous, subcutaneous, portal vein, intraperitoneal, inhalation, nebulization, oral, intrapulmonary and intramuscular. Each of these administration routes exposes the siNA molecules of the invention to an accessible diseased tissue (*e.g.*, lung). The rate of entry of a drug into the circulation has been shown to be a function of molecular weight or size. The use of a liposome or other drug carrier comprising the compounds of the instant invention can potentially localize the drug, for example, in certain tissue types, such as the tissues of the reticular endothelial system (RES). A liposome formulation that can facilitate the association of drug with the surface of cells, such as, lymphocytes and macrophages is also useful. This approach can provide enhanced delivery of the drug to target cells by taking advantage of the specificity of macrophage and lymphocyte immune recognition of abnormal cells.

[00691] By "pharmaceutically acceptable formulation," "pharmaceutically acceptable composition" or "pharmaceutically acceptable salt" is meant, a composition, formulation, or salt that allows for the effective distribution of the nucleic acid molecules of the instant invention in the physical location most suitable for their desired activity. Non-limiting examples of agents suitable for formulation with the nucleic acid molecules of the instant invention include: P-glycoprotein inhibitors (such as Pluronic P85),; biodegradable polymers, such as poly (DL-lactide-co-glycolide) microspheres for sustained release delivery (Emerich, DF et al, 1999, Cell Transplant, 8, 47-58); and loaded nanoparticles, such as those made of polybutylcyanoacrylate. Other non-limiting examples of delivery strategies for the nucleic acid molecules of the instant invention include material described in Boado et al., 1998, J. Pharm. Sci., 87, 1308-1315; Tyler et

al., 1999, FEBS Lett., 421, 280-284; Pardridge et al., 1995, PNAS USA., 92, 5592-5596; Boado, 1995, Adv. Drug Delivery Rev., 15, 73-107; Aldrian-Herrada et al., 1998, Nucleic Acids Res., 26, 4910-4916; and Tyler et al., 1999, PNAS USA., 96, 7053-7058. Non-limiting examples of pharmaceutically acceptable salts include, but are not limited to, (a) salts formed with cations such as sodium, potassium, ammonium, magnesium, calcium, polyamines such as spermine and spermidine, etc.; (b) acid addition salts formed with inorganic acids, for example hydrochloric acid, hydrobromic acid, sulfuric acid, phosphoric acid, nitric acid and the like; (c) salts formed with organic acids such as, for example, acetic acid, oxalic acid, tartaric acid, succinic acid maleic acid, fumaric acid, gluconic acid, citric acid, malic acid, ascorbic acid, benzoic acid, tannic acid, palmitic acid, alginic acid, polyglutamic acid, naphthalenesulfonic acid, methanesulfonic acid, p-toluenesulfonic acid, naphthalene disulfonic acid, polygalacturonic acid, and the like; and (d) salts formed from elemental anions such as chlorine, bromine, and iodine.

[00692] The invention also features the use of a composition comprising surface-modified liposomes containing poly (ethylene glycol) lipids (PEG-modified, or long-circulating liposomes or stealth liposomes) and nucleic acid or polynucleotide (*e.g.*, siNA) molecules of the invention. These formulations offer a method for increasing the accumulation of drugs (*e.g.*, siNA) in target tissues. This class of drug carriers resists opsonization and elimination by the mononuclear phagocytic system (MPS or RES), thereby enabling longer blood circulation times and enhanced tissue exposure for the encapsulated drug (Lasic et al. Chem. Rev. 1995, 95, 2601-2627; Ishiwata et al., Chem. Pharm. Bull. 1995, 43, 1005-1011). Such liposomes have been shown to accumulate selectively in tumors, presumably by extravasation and capture in the neovascularized target tissues (Lasic et al., Science 1995, 267, 1275-1276; Oku et al., 1995, Biochim. Biophys. Acta, 1238, 86-90). The long-circulating liposomes enhance the pharmacokinetics and pharmacodynamics of DNA and RNA, particularly compared to conventional cationic liposomes which are known to accumulate in tissues of the MPS (Liu et al., J. Biol. Chem. 1995, 270, 24864-24870; Choi et al., International PCT Publication No. WO 96/10391; Ansell et al., International PCT Publication No. WO 96/10390; Holland et al., International PCT Publication No. WO 96/10392). Long-circulating liposomes are also likely to protect drugs from nuclease degradation to a

greater extent compared to cationic liposomes, based on their ability to avoid accumulation in metabolically aggressive MPS tissues such as the liver and spleen.

[00693] In one embodiment, a liposomal formulation of the invention comprises a double stranded nucleic acid or polynucleotide (*e.g.*, siNA) molecule of the invention formulated or complexed with compounds and compositions described in US 6,858,224; 6,534,484; 6,287,591; 6,835,395; 6,586,410; 6,858,225; 6,815,432; US 6,586,001; 6,120,798; US 6,977,223; US 6,998,115; 5,981,501; 5,976,567; 5,705,385; US 2006/0019912; US 2006/0019258; US 2006/0008909; US 2005/0255153; US 2005/0079212; US 2005/0008689; US 2003/0077829, US 2005/0064595, US 2005/0175682, US 2005/0118253; US 2004/0071654; US 2005/0244504; US 2005/0265961 and US 2003/0077829, all of which are incorporated by reference herein in their entirety.

[00694] The present invention also includes compositions prepared for storage or administration that include a pharmaceutically or therapeutically effective amount of the desired compounds in a pharmaceutically acceptable carrier or diluent. Acceptable carriers or diluents for therapeutic use are well known in the pharmaceutical art, and are described, for example, in Remington's Pharmaceutical Sciences, Mack Publishing Co. (A.R. Gennaro edit. 1985), hereby incorporated by reference herein. For example, preservatives, stabilizers, dyes and flavoring agents can be provided. These include sodium benzoate, sorbic acid and esters of p-hydroxybenzoic acid. In addition, antioxidants and suspending agents can be used.

[00695] A pharmaceutically effective dose is that dose required to prevent, inhibit the occurrence, or treat (alleviate a symptom to some extent, preferably all of the symptoms) of a disease state. The pharmaceutically effective dose depends on the type of disease, the composition used, the route of administration, the type of mammal being treated, the physical characteristics of the specific mammal under consideration, concurrent medication, and other factors that those skilled in the medical arts will recognize. Generally, an amount between 0.1 mg/kg and 100 mg/kg body weight/day of active ingredients is administered dependent upon potency of the negatively charged polymer.

[00696] A therapeutically effective amount refers to the amount present in the composition that is needed to provide the desired level of drug in the subject to be treated to give the anticipated physiological response. "Therapeutically effective amounts" are those amounts effective to produce beneficial results in the recipient animal or patient.

[00697] The nucleic acid or polynucleotide (*e.g.*, siNA) molecules of the invention and formulations thereof can be administered orally, topically, parenterally, by inhalation or spray, or rectally in dosage unit formulations containing conventional non-toxic pharmaceutically acceptable carriers, adjuvants and/or vehicles. The term parenteral as used herein includes percutaneous, subcutaneous, intravascular (*e.g.*, intravenous), intramuscular, or intrathecal injection or infusion techniques and the like. In addition, there is provided a pharmaceutical formulation comprising a nucleic acid molecule of the invention and a pharmaceutically acceptable carrier. One or more nucleic acid molecules of the invention can be present in association with one or more non-toxic pharmaceutically acceptable carriers and/or diluents and/or adjuvants, and if desired other active ingredients. The pharmaceutical compositions containing nucleic acid molecules of the invention can be in a form suitable for oral use, for example, as tablets, troches, lozenges, aqueous or oily suspensions, dispersible powders or granules, emulsion, hard or soft capsules, or syrups or elixirs.

[00698] Compositions intended for oral use can be prepared according to any method known to the art for the manufacture of pharmaceutical compositions and such compositions can contain one or more such sweetening agents, flavoring agents, coloring agents or preservative agents in order to provide pharmaceutically elegant and palatable preparations. Tablets contain the active ingredient in admixture with non-toxic pharmaceutically acceptable excipients that are suitable for the manufacture of tablets. These excipients can be, for example, inert diluents; such as calcium carbonate, sodium carbonate, lactose, calcium phosphate or sodium phosphate; granulating and disintegrating agents, for example, corn starch, or alginic acid; binding agents, for example starch, gelatin or acacia; and lubricating agents, for example magnesium stearate, stearic acid or talc. The tablets can be uncoated or they can be coated by known techniques. In some cases such coatings can be prepared by known techniques to delay disintegration and absorption in the gastrointestinal tract and thereby provide a sustained

action over a longer period. For example, a time delay material such as glyceryl monostearate or glyceryl distearate can be employed.

[00699] Formulations for oral use can also be presented as hard gelatin capsules wherein the active ingredient is mixed with an inert solid diluent, for example, calcium carbonate, calcium phosphate or kaolin, or as soft gelatin capsules wherein the active ingredient is mixed with water or an oil medium, for example peanut oil, liquid paraffin or olive oil.

[00700] Aqueous suspensions contain the active materials in a mixture with excipients suitable for the manufacture of aqueous suspensions. Such excipients are suspending agents, for example sodium carboxymethylcellulose, methylcellulose, hydropropyl-methylcellulose, sodium alginate, polyvinylpyrrolidone, gum tragacanth and gum acacia; dispersing or wetting agents can be a naturally-occurring phosphatide, for example, lecithin, or condensation products of an alkylene oxide with fatty acids, for example polyoxyethylene stearate, or condensation products of ethylene oxide with long chain aliphatic alcohols, for example heptadecaethyleneoxycetanol, or condensation products of ethylene oxide with partial esters derived from fatty acids and a hexitol such as polyoxyethylene sorbitol monooleate, or condensation products of ethylene oxide with partial esters derived from fatty acids and hexitol anhydrides, for example polyethylene sorbitan monooleate. The aqueous suspensions can also contain one or more preservatives, for example ethyl, or n-propyl p-hydroxybenzoate, one or more coloring agents, one or more flavoring agents, and one or more sweetening agents, such as sucrose or saccharin.

[00701] Oily suspensions can be formulated by suspending the active ingredients in a vegetable oil, for example arachis oil, olive oil, sesame oil or coconut oil, or in a mineral oil such as liquid paraffin. The oily suspensions can contain a thickening agent, for example beeswax, hard paraffin or cetyl alcohol. Sweetening agents and flavoring agents can be added to provide palatable oral preparations. These compositions can be preserved by the addition of an anti-oxidant such as ascorbic acid.

[00702] Dispersible powders and granules suitable for preparation of an aqueous suspension by the addition of water provide the active ingredient in admixture with a

dispersing or wetting agent, suspending agent and one or more preservatives. Suitable dispersing or wetting agents or suspending agents are exemplified by those already mentioned above. Additional excipients, for example sweetening, flavoring and coloring agents, can also be present.

[00703] Pharmaceutical compositions of the invention can also be in the form of oil-in-water emulsions. The oily phase can be a vegetable oil or a mineral oil or mixtures of these. Suitable emulsifying agents can be naturally-occurring gums, for example gum acacia or gum tragacanth, naturally-occurring phosphatides, for example soy bean, lecithin, and esters or partial esters derived from fatty acids and hexitol, anhydrides, for example sorbitan monooleate, and condensation products of the said partial esters with ethylene oxide, for example polyoxyethylene sorbitan monooleate. The emulsions can also contain sweetening and flavoring agents.

[00704] Syrups and elixirs can be formulated with sweetening agents, for example glycerol, propylene glycol, sorbitol, glucose or sucrose. Such formulations can also contain a demulcent, a preservative and flavoring and coloring agents. The pharmaceutical compositions can be in the form of a sterile injectable aqueous or oleaginous suspension. This suspension can be formulated according to the known art using those suitable dispersing or wetting agents and suspending agents that have been mentioned above. The sterile injectable preparation can also be a sterile injectable solution or suspension in a non-toxic parentally acceptable diluent or solvent, for example as a solution in 1,3-butanediol. Among the acceptable vehicles and solvents that can be employed are water, Ringer's solution and isotonic sodium chloride solution. In addition, sterile, fixed oils are conventionally employed as a solvent or suspending medium. For this purpose, any bland fixed oil can be employed including synthetic mono-or diglycerides. In addition, fatty acids such as oleic acid find use in the preparation of injectables.

[00705] The nucleic acid molecules of the invention can also be administered in the form of suppositories, e.g., for rectal administration of the drug. These compositions can be prepared by mixing the drug with a suitable non-irritating excipient that is solid at ordinary temperatures but liquid at the rectal temperature and will therefore melt in the

rectum to release the drug. Such materials include cocoa butter and polyethylene glycols.

[00706] Nucleic acid molecules of the invention can be administered parenterally in a sterile medium. The drug, depending on the vehicle and concentration used, can either be suspended or dissolved in the vehicle. Advantageously, adjuvants such as local anesthetics, preservatives and buffering agents can be dissolved in the vehicle.

[00707] Dosage levels of the order of from about 0.1 mg to about 140 mg per kilogram of body weight per day are useful in the treatment of the above-indicated conditions (about 0.5 mg to about 7 g per subject per day). The amount of active ingredient that can be combined with the carrier materials to produce a single dosage form varies depending upon the host treated and the particular mode of administration. Dosage unit forms generally contain between from about 1 mg to about 500 mg of an active ingredient.

[00708] It is understood that the specific dose level for any particular subject depends upon a variety of factors including the activity of the specific compound employed, the age, body weight, general health, sex, diet, time of administration, route of administration, and rate of excretion, drug combination and the severity of the particular disease undergoing therapy.

[00709] For administration to non-human animals, the composition can also be added to the animal feed or drinking water. It can be convenient to formulate the animal feed and drinking water compositions so that the animal takes in a therapeutically appropriate quantity of the composition along with its diet. It can also be convenient to present the composition as a premix for addition to the feed or drinking water.

[00710] The nucleic acid molecules of the present invention can also be administered to a subject in combination with other therapeutic compounds to increase the overall therapeutic effect. The use of multiple compounds to treat an indication can increase the beneficial effects while reducing the presence of side effects.

[00711] In one embodiment, the invention comprises compositions suitable for nucleic acid or polynucleotide (*e.g.*, siNA) molecules of the invention to specific cell types. For example, the asialoglycoprotein receptor (ASGPr) (Wu and Wu, 1987, J. Biol. Chem.

262, 4429-4432) is unique to hepatocytes and binds branched galactose-terminal glycoproteins, such as asialoorosomucoid (ASOR). In another example, the folate receptor is overexpressed in many cancer cells. Binding of such glycoproteins, synthetic glycoconjugates, or folates to the receptor takes place with an affinity that strongly depends on the degree of branching of the oligosaccharide chain, for example, triantennary structures are bound with greater affinity than biantennary or monoantennary chains (Baenziger and Fiete, 1980, *Cell*, 22, 611-620; Connolly et al., 1982, *J. Biol. Chem.*, 257, 939-945). Lee and Lee, 1987, *Glycoconjugate J.*, 4, 317-328, obtained this high specificity through the use of N-acetyl-D-galactosamine as the carbohydrate moiety, which has higher affinity for the receptor, compared to galactose. This "clustering effect" has also been described for the binding and uptake of mannosyl-terminating glycoproteins or glycoconjugates (Ponpipom et al., 1981, *J. Med. Chem.*, 24, 1388-1395). The use of galactose, galactosamine, or folate based conjugates to transport exogenous compounds across cell membranes can provide a targeted delivery approach to, for example, the treatment of liver disease, cancers of the liver, or other cancers. The use of bioconjugates can also provide a reduction in the required dose of therapeutic compounds required for treatment. Furthermore, therapeutic bioavailability, pharmacodynamics, and pharmacokinetic parameters can be modulated through the use of nucleic acid bioconjugates of the invention. Non-limiting examples of such bioconjugates are described in Vargeese et al., USSN 10/201,394, filed August 13, 2001; and Matulic-Adamic et al., USSN 60/362,016, filed March 6, 2002.

[00712] Alternatively, certain nucleic acid or polynucleotide (*e.g.*, siNA) molecules of the instant invention can be expressed within cells from eukaryotic promoters (*e.g.*, Izant and Weintraub, 1985, *Science*, 229, 345; McGarry and Lindquist, 1986, *Proc. Natl. Acad. Sci. USA* 83, 399; Scanlon et al., 1991, *Proc. Natl. Acad. Sci. USA*, 88, 10591-5; Kashani-Sabet et al., 1992, *Antisense Res. Dev.*, 2, 3-15; Dropulic et al., 1992, *J. Virol.*, 66, 1432-41; Weerasinghe et al., 1991, *J. Virol.*, 65, 5531-4; Ojwang et al., 1992, *Proc. Natl. Acad. Sci. USA*, 89, 10802-6; Chen et al., 1992, *Nucleic Acids Res.*, 20, 4581-9; Sarver et al., 1990 *Science*, 247, 1222-1225; Thompson et al., 1995, *Nucleic Acids Res.*, 23, 2259; Good et al., 1997, *Gene Therapy*, 4, 45. Those skilled in the art realize that any nucleic acid can be expressed in eukaryotic cells from the appropriate DNA/RNA vector. The activity of such nucleic acids can be augmented by their release from the

primary transcript by a enzymatic nucleic acid (Draper et al., PCT WO 93/23569, and Sullivan et al., PCT WO 94/02595; Ohkawa et al., 1992, Nucleic Acids Symp. Ser., 27, 15-6; Taira et al., 1991, Nucleic Acids Res., 19, 5125-30; Ventura et al., 1993, Nucleic Acids Res., 21, 3249-55; Chowrira et al., 1994, J. Biol. Chem., 269, 25856.

[00713] In another aspect of the invention, RNA molecules of the present invention can be expressed from transcription units (see for example Couture et al., 1996, TIG., 12, 510) inserted into DNA or RNA vectors. The recombinant vectors can be DNA plasmids or viral vectors. Nucleic acid or polynucleotide (*e.g.*, siNA) expressing viral vectors can be constructed based on, but not limited to, adeno-associated virus, retrovirus, adenovirus, or alphavirus. In another embodiment, pol III based constructs are used to express nucleic acid molecules of the invention (see for example Thompson, U.S. Pats. Nos. 5,902,880 and 6,146,886). The recombinant vectors capable of expressing the siNA molecules can be delivered as described above, and persist in target cells. Alternatively, viral vectors can be used that provide for transient expression of nucleic acid molecules. Such vectors can be repeatedly administered as necessary. Once expressed, the siNA molecule interacts with the target mRNA and generates an RNAi response. Delivery of siNA molecule expressing vectors can be systemic, such as by intravenous or intra-muscular administration, by administration to target cells ex-planted from a subject followed by reintroduction into the subject, or by any other means that would allow for introduction into the desired target cell (for a review see Couture et al., 1996, TIG., 12, 510).

[00714] In one aspect the invention features an expression vector comprising a nucleic acid sequence encoding at least one nucleic acid or polynucleotide (*e.g.*, siNA) molecule of the instant invention. The expression vector can encode one or both strands of a siNA duplex, or a single self-complementary strand that self hybridizes into a siNA duplex. The nucleic acid sequences encoding the siNA molecules of the instant invention can be operably linked in a manner that allows expression of the siNA molecule (see for example Paul et al., 2002, Nature Biotechnology, 19, 505; Miyagishi and Taira, 2002, Nature Biotechnology, 19, 497; Lee et al., 2002, Nature Biotechnology, 19, 500; and Novina et al., 2002, Nature Medicine, advance online publication doi:10.1038/nm725).

[00715] In another aspect, the invention features an expression vector comprising: a) a transcription initiation region (e.g., eukaryotic pol I, II or III initiation region); b) a transcription termination region (e.g., eukaryotic pol I, II or III termination region); and c) a nucleic acid sequence encoding at least one of the siNA molecules of the instant invention, wherein said sequence is operably linked to said initiation region and said termination region in a manner that allows expression and/or delivery of the siNA molecule. The vector can optionally include an open reading frame (ORF) for a protein operably linked on the 5' side or the 3'-side of the sequence encoding the siNA of the invention; and/or an intron (intervening sequences).

[00716] Transcription of the siNA molecule sequences can be driven from a promoter for eukaryotic RNA polymerase I (pol I), RNA polymerase II (pol II), or RNA polymerase III (pol III). Transcripts from pol II or pol III promoters are expressed at high levels in all cells; the levels of a given pol II promoter in a given cell type depends on the nature of the gene regulatory sequences (enhancers, silencers, etc.) present nearby. Prokaryotic RNA polymerase promoters are also used, providing that the prokaryotic RNA polymerase enzyme is expressed in the appropriate cells (Elroy-Stein and Moss, 1990, Proc. Natl. Acad. Sci. U S A, 87, 6743-7; Gao and Huang 1993, Nucleic Acids Res., 21, 2867-72; Lieber et al., 1993, Methods Enzymol., 217, 47-66; Zhou et al., 1990, Mol. Cell. Biol., 10, 4529-37). Several investigators have demonstrated that nucleic acid molecules expressed from such promoters can function in mammalian cells (e.g. Kashani-Sabet et al., 1992, Antisense Res. Dev., 2, 3-15; Ojwang et al., 1992, Proc. Natl. Acad. Sci. U S A, 89, 10802-6; Chen et al., 1992, Nucleic Acids Res., 20, 4581-9; Yu et al., 1993, Proc. Natl. Acad. Sci. U S A, 90, 6340-4; L'Huillier et al., 1992, EMBO J., 11, 4411-8; Lisiewicz et al., 1993, Proc. Natl. Acad. Sci. U. S. A, 90, 8000-4; Thompson et al., 1995, Nucleic Acids Res., 23, 2259; Sullenger & Cech, 1993, Science, 262, 1566). More specifically, transcription units such as the ones derived from genes encoding U6 small nuclear (snRNA), transfer RNA (tRNA) and adenovirus VA RNA are useful in generating high concentrations of desired RNA molecules such as siNA in cells (Thompson et al., supra; Couture and Stinchcomb, 1996, supra; Noonberg et al., 1994, Nucleic Acid Res., 22, 2830; Noonberg et al., U.S. Pat. No. 5,624,803; Good et al., 1997, Gene Ther., 4, 45; Beigelman et al., International PCT Publication No. WO 96/18736. The above siNA transcription units can be incorporated into a variety of

vectors for introduction into mammalian cells, including but not restricted to, plasmid DNA vectors, viral DNA vectors (such as adenovirus or adeno-associated virus vectors), or viral RNA vectors (such as retroviral or alphavirus vectors) (for a review see Couture and Stinchcomb, 1996, *supra*).

[00717] In another aspect the invention features an expression vector comprising a nucleic acid sequence encoding at least one of the nucleic acid or polynucleotide (*e.g.*, siNA) molecules of the invention in a manner that allows expression of that siNA molecule. The expression vector comprises in one embodiment; a) a transcription initiation region; b) a transcription termination region; and c) a nucleic acid sequence encoding at least one strand of the siNA molecule, wherein the sequence is operably linked to the initiation region and the termination region in a manner that allows expression and/or delivery of the siNA molecule.

[00718] In another embodiment the expression vector comprises: a) a transcription initiation region; b) a transcription termination region; c) an open reading frame; and d) a nucleic acid sequence encoding at least one strand of a nucleic acid or polynucleotide (*e.g.*, siNA) molecule, wherein the sequence is operably linked to the 3'-end of the open reading frame and wherein the sequence is operably linked to the initiation region, the open reading frame and the termination region in a manner that allows expression and/or delivery of the siNA molecule. In yet another embodiment, the expression vector comprises: a) a transcription initiation region; b) a transcription termination region; c) an intron; and d) a nucleic acid sequence encoding at least one siNA molecule, wherein the sequence is operably linked to the initiation region, the intron and the termination region in a manner which allows expression and/or delivery of the nucleic acid molecule.

[00719] In another embodiment, the expression vector comprises: a) a transcription initiation region; b) a transcription termination region; c) an intron; d) an open reading frame; and e) a nucleic acid sequence encoding at least one strand of a nucleic acid or polynucleotide (*e.g.*, siNA) molecule, wherein the sequence is operably linked to the 3'-end of the open reading frame and wherein the sequence is operably linked to the initiation region, the intron, the open reading frame and the termination region in a manner which allows expression and/or delivery of the siNA molecule.

[00720] The present invention also covers methods for delivering short hairpin RNA to target cells. The methods include the step of administering to an animal an effective amount of a preparation that includes a suitable delivery vehicle which includes within its interior a plasmid DNA encoding an shRNA in accordance with the present invention and a pharmaceutically acceptable carrier.

[00721] Thus in one aspect, the invention provides a composition comprising: (i) an RNAi-inducing entity, wherein the RNAi-inducing entity is targeted to a mRNA target transcript; and (ii) a delivery agent selected from the group consisting of: cationic polymers, modified cationic polymers, peptide molecular transporters, surfactants suitable for introduction into the lung, liposomes, non-cationic polymers, modified non-cationic polymers, bupivacaine, and chloroquine. In certain embodiments of the invention the delivery agent incorporates a targeting or delivery-enhancing moiety to enhance delivery or specificity of delivery to a cell of interest. In various embodiments of the invention the RNAi-inducing entity can be an siRNA, shRNA, or RNAi-inducing vector.

PCSK9 Biology and Biochemistry

[00722] Proprotein convertase subtilisin-kexin type 9 (hereinafter called “PCSK9”), also known as neural apoptosis- regulated convertase 1 (“NARC-1”), is a proteinase K-like subtilase identified as the 9th member of the secretory subtilase family; see Seidah et al., 2003 PNAS 100:928-933. PCSK9 is expressed in cells capable of proliferation and differentiation including, for example, hepatocytes, kidney mesenchymal cells, intestinal ileum, and colon epithelia as well as embryonic brain telencephalon neurons; Seidah et al., supra.

[00723] The PCSK9 gene (or NARC-1 gene) encodes the NARC-1 protein or polypeptide. It is a recently-discovered member of the subtilisin family of serine proteases, and is regulated by the cholesterol status of a cell. The protein sequence for human PCSK9, which is ~22-kb long with 12 exons encoding a 692 amino acid protein, can be found, for example, at Deposit No. NP_777596.2. PCSK9 is disclosed and/or claimed in several patent publications including, but not limited to the following: PCT Publication Nos. WO 01/31007, WO 01/57081, WO 02/14358, WO 01/98468, WO

02/102993, WO 02/102994, WO 02/46383, WO 02/90526, WO 01/77137, and WO 01/34768; US Publication Nos. US 2004/0009553 and US 2003/0119038, and European Publication Nos. EP 1 440 981, EP 1 067 182, and EP 1 471 152. The sequence of the NARC-1 gene has been described in patent applications WO 01/57081 and WO 02/14358, and partly characterized in Seidah et al (2003).

[00724] Its NARC-1 acronym reflects the fact that its mRNA was upregulated when apoptosis was induced in neuronal primary cultures and that it is similar to 8 other subtilase-like proteinases, called proprotein convertases (see for example International PCT Publication No. WO 01/57081). PCs are involved in the processing (and activation) of precursors of hormones, receptors, surface glycoproteins, etc, which transit through the secretory pathway. Seven of them, PC1/3, PC2, furin, PC4, PACE4, PC5/6, and PC7/LPC, recognize basic sites and belong to the kexin subfamily. The eighth member, SKI-1/S1P, is classified in the pyrolysins subfamily of subtilases. It has been involved in the processing of endoplasmic reticulum (ER)-anchored transcription factors such as sterol regulatory element (SRE)-binding proteins (SREBPs) and thus plays a key role in cholesterol homeostasis. When cellular cholesterol is low, SREBPs are relocated from the ER to the Golgi apparatus, where SKI-1/S1P cleaves in their luminal loop. A second cleavage by the metalloprotease S2P in their first transmembrane domain liberates the cytosolic N-terminal region that goes to the nucleus and activates target genes. SREBP-1c, the isoform that is dominant in liver, regulates the lipogenic pathway, whereas SREBP-2 preferentially activates genes of cholesterol metabolism.

[00725] The gene for PCSK9 localizes to human chromosome 1p33-p34.3; Seidah et al., supra. PCSK9 is expressed in cells capable of proliferation and differentiation including, for example, hepatocytes, kidney mesenchymal cells, intestinal ileum, and colon epithelia as well as embryonic brain telencephalon neurons; Seidah et al., supra.

[00726] Original synthesis of PCSK9 is in the form of an inactive enzyme precursor, or zymogen, of ~ 72-kDa which undergoes autocatalytic, intramolecular processing in the endoplasmic reticulum ("ER") to activate its functionality. This internal processing event has been reported to occur at the SSVFAQ ↓ SIPWNL158 motif rendering the first three N-terminal residues Ser-Ile-Pro (Benjannet et al., 2004 J. Biol. Chem. 279:48865-48875), and has been reported as a requirement of exit from the

ER; Benjannet et al., supra; Seidah et al., supra. The cleaved protein is then secreted. The cleaved peptide remains associated with the activated and secreted enzyme; supra.

[00727] PCSK9 has been ascribed a role in the differentiation of hepatic and neuronal cells (Seidah et al., supra.), is highly expressed in embryonic liver, and has been strongly implicated in cholesterol homeostasis. Recent studies seem to suggest a specific role in cholesterol biosynthesis or uptake. In a study of cholesterol-fed rats, Maxwell et al. found that PCSK9 was down regulated in a similar manner as three other genes involved in cholesterol biosynthesis, Maxwell et al., 2003 J. Lipid Res. 44:2109-2119. Interestingly, as well, the expression of PCSK9 was regulated by sterol regulatory element-binding proteins (“SREBP”), as seen with other genes involved in cholesterol metabolism; supra. These findings were later supported by a study of PCSK9 transcriptional regulation which demonstrated that such regulation was quite typical of other genes implicated in lipoprotein metabolism; Dubuc et al., 2004 Arterioscler. Thromb. Vasc. Biol. 24:1454-1459. PCSK9 expression was up regulated by statins in a manner attributed to the cholesterol-lowering effects of the drugs; supra. More, the PCSK9 promoters possessed two conserved sites involved in cholesterol regulation, a sterol regulatory element and an Sp1 site; supra. Adenoviral expression of PCSK9 has been shown to lead to a notable time-dependent increase in circulating LDL (Benjannet et al., 2004 J. Biol. Chem. 279:48865-48875). More, mice deleted of the PCSK9 gene have increased levels of hepatic LDL receptors and more rapidly clear LDL from the plasma; Rashid et al., 2005 Proc. Natl. Acad. Sci. USA 102:5374-5379.

[00728] A number of mutations in the gene PCSK9 have also been conclusively associated with autosomal dominant hypercholesterolemia (“ADH”), an inherited metabolism disorder characterized by marked elevations of low density lipoprotein (“LDL”) particles in the plasma which can lead to premature cardiovascular failure; see Abifadel et al., 2003 Nature Genetics 34:154-156; Timms et al., 2004 Hum. Genet. 114:349-353; Leren, 2004 Clin. Genet. 65:419-422. A later-published study on the S127R mutation of Abifadel et al., supra, reported that patients carrying such a mutation exhibited higher total cholesterol and ApoB100 in the plasma attributed to (1) an overproduction of ApoB100-containing lipoproteins, such as low density lipoprotein (“LDL”), very low density lipoprotein (“VLDL”) and intermediate density lipoprotein

(“IDL”), and (2) an associated reduction in clearance or conversion of said lipoproteins; Ouguerram et al., 2004 *Arterioscler. Thromb. Vasc. Biol.* 24:1448-1453.

[00729] PCSK9 is highly expressed in embryonic liver. It then decreases in the adult liver but significantly increases after hepatectomy. The transcript is also detected transiently in specific areas such as the telencephalon, skin, kidney, intestine, and cerebellum. It has been hypothesized that PCSK9 may be expressed preferentially in progenitor cells and play a role in hepatic and neuronal differentiation. In human, the PCSK9 gene is approximately 22 kilobases long and comprises 12 exons encoding a 692 amino acid protein. Located on chromosome 1p32, PCSK9 was identified recently as the third locus involved in autosomal dominant hypercholesterolemia (ADH), characterized by high levels of low-density lipoprotein (LDL) cholesterol, xanthomas, and a high frequency of coronary artery diseases. The majority of familial hypercholesterolemia cases are attributable to mutations in the genes encoding the LDL receptor (LDLR) and apolipoprotein B (apoB), the main component of LDL particles. By genetic analyses of several French families, 2 exonic PCSK9 mutations, S127R and F216L, were associated with haplotypes segregating with the disease. This work was confirmed recently by the identification of a new PCSK9 mutation, D374Y, in a large Utah kindred and 2 Japanese polymorphisms, intron 1/C(-161)T and I474V, all associated with abnormally high levels of LDL-cholesterol. (see Dubuc *et al.*, 2004, *Arteriosclerosis, Thrombosis, and Vascular Biology*, 24:1454).

[00730] Based upon the current understanding of PCSK9 function, the modulation of PCSK9 and other related genes is instrumental in the development of new therapeutics for metabolic disease. As such, modulation of PCSK9 using small interfering nucleic acid (siNA) mediated RNAi represents a novel approach to the treatment and study of diseases and conditions related to PCSK9 activity and/or gene expression including hyperlipidemia, hypercholesterolemia, cardiovascular disease, atherosclerosis, hypertension, diabetes (e.g., type I and/or type II diabetes), insulin resistance, obesity and/or other disease states, conditions, or traits associated with PCSK9 gene expression or activity in a subject or organism.

Examples:

[00731] The following are non-limiting examples showing the selection, isolation, synthesis and activity of nucleic acids of the instant invention.

Example 1: Tandem synthesis of siNA constructs

[00732] Exemplary siNA molecules of the invention are synthesized in tandem using a cleavable linker, for example, a succinyl-based linker. Tandem synthesis as described herein is followed by a one-step purification process that provides RNAi molecules in high yield. This approach is highly amenable to siNA synthesis in support of high throughput RNAi screening, and can be readily adapted to multi-column or multi-well synthesis platforms.

[00733] After completing a tandem synthesis of a siNA oligo and its complement in which the 5'-terminal dimethoxytrityl (5'-O-DMT) group remains intact (trityl on synthesis), the oligonucleotides are deprotected as described above. Following deprotection, the siNA sequence strands are allowed to spontaneously hybridize. This hybridization yields a duplex in which one strand has retained the 5'-O-DMT group while the complementary strand comprises a terminal 5'-hydroxyl. The newly formed duplex behaves as a single molecule during routine solid-phase extraction purification (Trityl-On purification) even though only one molecule has a dimethoxytrityl group. Because the strands form a stable duplex, this dimethoxytrityl group (or an equivalent group, such as other trityl groups or other hydrophobic moieties) is all that is required to purify the pair of oligos, for example, by using a C18 cartridge.

[00734] Standard phosphoramidite synthesis chemistry is used up to the point of introducing a tandem linker, such as an inverted deoxy abasic succinate or glyceryl succinate linker (see Figure 1) or an equivalent cleavable linker. A non-limiting example of linker coupling conditions that can be used includes a hindered base such as diisopropylethylamine (DIPA) and/or DMAP in the presence of an activator reagent such as Bromotripyrrolidinophosphoniumhexafluorophosphate (PyBrOP). After the linker is coupled, standard synthesis chemistry is utilized to complete synthesis of the second sequence leaving the terminal the 5'-O-DMT intact. Following synthesis, the resulting oligonucleotide is deprotected according to the procedures described herein and quenched with a suitable buffer, for example with 50mM NaOAc or 1.5M NH₄H₂CO₃.

[00735] Purification of the siNA duplex can be readily accomplished using solid phase extraction, for example, using a Waters C18 SepPak 1g cartridge conditioned with 1 column volume (CV) of acetonitrile, 2 CV H₂O, and 2 CV 50mM NaOAc. The sample is loaded and then washed with 1 CV H₂O or 50mM NaOAc. Failure sequences are eluted with 1 CV 14% ACN (Aqueous with 50mM NaOAc and 50mM NaCl). The column is then washed, for example with 1 CV H₂O followed by on-column detritylation, for example by passing 1 CV of 1% aqueous trifluoroacetic acid (TFA) over the column, then adding a second CV of 1% aqueous TFA to the column and allowing to stand for approximately 10 minutes. The remaining TFA solution is removed and the column washed with H₂O followed by 1 CV 1M NaCl and additional H₂O. The siNA duplex product is then eluted, for example, using 1 CV 20% aqueous CAN.

[00736] Figure 2 provides an example of MALDI-TOF mass spectrometry analysis of a purified siNA construct in which each peak corresponds to the calculated mass of an individual siNA strand of the siNA duplex. The same purified siNA provides three peaks when analyzed by capillary gel electrophoresis (CGE), one peak presumably corresponding to the duplex siNA, and two peaks presumably corresponding to the separate siNA sequence strands. Ion exchange HPLC analysis of the same siNA construct only shows a single peak. Testing of the purified siNA construct using a luciferase reporter assay described below demonstrated the same RNAi activity compared to siNA constructs generated from separately synthesized oligonucleotide sequence strands.

Example 2: Identification of potential siNA target sites in any RNA sequence

[00737] The sequence of an RNA target of interest, such as a human mRNA transcript (e.g., any of sequences referred to herein by GenBank Accession Number), is screened for target sites, for example by using a computer folding algorithm. In a non-limiting example, the sequence of a gene or RNA gene transcript derived from a database, such as GenBank, is used to generate siNA targets having complementarity to the target. Such sequences can be obtained from a database, or can be determined experimentally as known in the art. Target sites that are known, for example, those target sites determined to be effective target sites based on studies with other nucleic acid molecules, for example ribozymes or antisense, or those targets known to be associated with a disease,

trait, or condition such as those sites containing mutations or deletions, can be used to design siNA molecules targeting those sites. Various parameters can be used to determine which sites are the most suitable target sites within the target RNA sequence. These parameters include but are not limited to secondary or tertiary RNA structure, the nucleotide base composition of the target sequence, the degree of homology between various regions of the target sequence, or the relative position of the target sequence within the RNA transcript. Based on these determinations, any number of target sites within the RNA transcript can be chosen to screen siNA molecules for efficacy, for example by using in vitro RNA cleavage assays, cell culture, or animal models. In a non-limiting example, anywhere from 1 to 1000 target sites are chosen within the transcript based on the size of the siNA construct to be used. High throughput screening assays can be developed for screening siNA molecules using methods known in the art, such as with multi-well or multi-plate assays to determine efficient reduction in target gene expression.

Example 3: Selection of nucleic acid or polynucleotide (e.g., siNA) molecule target sites in a RNA

[00738] The following non-limiting steps can be used to carry out the selection of siNAs targeting a given gene sequence or transcript.

1. The target sequence is parsed *in silico* into a list of all fragments or subsequences of a particular length, for example 23 nucleotide fragments, contained within the target sequence. This step is typically carried out using a custom Perl script, but commercial sequence analysis programs such as Oligo, MacVector, or the GCG Wisconsin Package can be employed as well.
2. In some instances the siNAs correspond to more than one target sequence; such would be the case for example in targeting different transcripts of the same gene, targeting different transcripts of more than one gene, or for targeting both the human gene and an animal homolog. In this case, a subsequence list of a particular length is generated for each of the targets, and then the lists are compared to find matching sequences in each list. The subsequences are then ranked according to the number of target sequences that contain the given subsequence; the goal is to find subsequences that are present in most

or all of the target sequences. Alternately, the ranking can identify subsequences that are unique to a target sequence, such as a mutant target sequence. Such an approach would enable the use of siNA to target specifically the mutant sequence and not effect the expression of the normal sequence.

3. In some instances the siNA subsequences are absent in one or more sequences while present in the desired target sequence; such would be the case if the siNA targets a gene with a paralogous family member that is to remain untargeted. As in case 2 above, a subsequence list of a particular length is generated for each of the targets, and then the lists are compared to find sequences that are present in the target gene but are absent in the untargeted paralog.
4. The ranked siNA subsequences can be further analyzed and ranked according to GC content. A preference can be given to sites containing 30-70% GC, with a further preference to sites containing 40-60% GC.
5. The ranked siNA subsequences can be further analyzed and ranked according to self-folding and internal hairpins. Weaker internal folds are preferred; strong hairpin structures are to be avoided.
6. The ranked siNA subsequences can be further analyzed and ranked according to whether they have runs of GGG or CCC in the sequence. GGG (or even more Gs) in either strand can make oligonucleotide synthesis problematic and can potentially interfere with RNAi activity, so it is avoided whenever better sequences are available. CCC is searched in the target strand because that will place GGG in the antisense strand.
7. The ranked siNA subsequences can be further analyzed and ranked according to whether they have the dinucleotide UU (uridine dinucleotide) on the 3'-end of the sequence, and/or AA on the 5'-end of the sequence (to yield 3' UU on the antisense sequence). These sequences allow one to design siNA molecules with terminal TT thymidine dinucleotides.
8. Four or five target sites are chosen from the ranked list of subsequences as described above. For example, in subsequences having 23 nucleotides, the right 21 nucleotides of each chosen 23-mer subsequence are then designed and synthesized for the upper (sense)

strand of the siNA duplex, while the reverse complement of the left 21 nucleotides of each chosen 23-mer subsequence are then designed and synthesized for the lower (antisense) strand of the siNA duplex (see **Table II**). If terminal TT residues are desired for the sequence (as described in paragraph 7), then the two 3' terminal nucleotides of both the sense and antisense strands are replaced by TT prior to synthesizing the oligos.

9. The siNA molecules are screened in an *in vitro*, cell culture or animal model system to identify the most active siNA molecule or the most preferred target site within the target RNA sequence.
10. Other design considerations can be used when selecting target nucleic acid sequences, see, for example, Reynolds *et al.*, 2004, *Nature Biotechnology Advanced Online Publication*, 1 February 2004, doi:10.1038/nbt936 and Ui-Tei *et al.*, 2004, *Nucleic Acids Research*, 32, doi:10.1093/nar/gkh247.

[00739] In an alternate approach, a pool of siNA constructs specific to a target sequence is used to screen for target sites in cells expressing target RNA, such as cultured Jurkat, HeLa, A549 or 293T cells. The general strategy used in this approach is shown in **Figure 9**. Cells expressing the target RNA are transfected with the pool of siNA constructs and cells that demonstrate a phenotype associated with target inhibition are sorted. The pool of siNA constructs can be expressed from transcription cassettes inserted into appropriate vectors (see for example **Figure 7** and **Figure 8**). The siNA from cells demonstrating a positive phenotypic change (*e.g.*, decreased proliferation, decreased target mRNA levels or decreased target protein expression), are sequenced to determine the most suitable target site(s) within the target target RNA sequence. In one embodiment, siNA molecules of the invention are selected using the following methodology. The following guidelines were compiled to predict hyper-active siNAs that contain chemical modifications described herein. These rules emerged from a comparative analysis of hyper-active (>75% knockdown of target mRNA levels) and inactive (<75% knockdown of target mRNA levels) siNAs against several different targets. A total of 242 siNA sequences were analyzed. Thirty-five siNAs out of 242 siNAs were grouped into hyper-active and the remaining siNAs were grouped into inactive groups. The hyper-active siNAs clearly showed a preference for certain bases at particular nucleotide positions within the siNA sequence. For example, A or U

nucleobase was overwhelmingly present at position 19 of the sense strand in hyper-active siNAs and opposite was true for inactive siNAs. There was also a pattern of a A/U rich (3 out of 5 bases as A or U) region between positions 15-19 and G/C rich region between positions 1-5 (3 out of 5 bases as G or C) of the sense strand in hyperactive siNAs. As shown in Table VII, 12 such patterns were identified that were characteristics of hyper-active siNAs. It is to be noted that not every pattern was present in each hyper-active siNA. Thus, to design an algorithm for predicting hyper-active siNAs, a different score was assigned for each pattern. Depending on how frequently such patterns occur in hyper-active siNAs versus inactive siNAs, the design parameters were assigned a score with the highest being 10. If a certain nucleobase is not preferred at a position, then a negative score was assigned. For example, at positions 9 and 13 of the sense strand, a G nucleotide was not preferred in hyper-active siNAs and therefore they were given score of -3(minus 3). The differential score for each pattern is given in Table VII. The pattern # 4 was given a maximum score of -100. This is mainly to weed out any sequence that contains string of 4Gs or 4Cs as they can be highly incompatible for synthesis and can allow sequences to self-aggregate, thus rendering the siNA inactive. Using this algorithm, the highest score possible for any siNA is 66. As there are numerous siNA sequences possible against any given target of reasonable size (~1000 nucleotides), this algorithm is useful to generate hyper-active siNAs.

[00740] In one embodiment, rules 1-11 shown in Table VII are used to generate active siNA molecules of the invention. In another embodiment, rules 1-12 shown in Table VII are used to generate active siNA molecules of the invention.

Example 4: PCSK9 siNA design

[0107] siNA target sites were chosen by analyzing sequences of the PCSK9 RNA target and optionally prioritizing the target sites on the basis of the rules presented in Example 3 above, and alternately on the basis of folding (structure of any given sequence analyzed to determine siNA accessibility to the target), or by using a library of siNA molecules as described in Example 3, or alternately by using an in vitro siNA system as described in Example 6 herein. siNA molecules were designed that could bind each target and are selected using the algorithm above and are optionally individually analyzed by computer folding to assess whether the siNA molecule can interact with the

target sequence. Varying the length of the siNA molecules can be chosen to optimize activity. Generally, a sufficient number of complementary nucleotide bases are chosen to bind to, or otherwise interact with, the target RNA, but the degree of complementarity can be modulated to accommodate siNA duplexes or varying length or base composition. By using such methodologies, siNA molecules can be designed to target sites within any known RNA sequence, for example those RNA sequences corresponding to the any gene transcript.

[00741] Target sequences are analysed to generate targets from which double stranded siNA are designed (Table II). To generate synthetic siNA constructs, the algorithm described in Example 3 is utilized to pick active double stranded constructs and chemically modified versions thereof. For example, in Table II, the target sequence is shown, along with the upper (sense strand) and lower (antisense strand) of the siNA duplex. Multifunctional siNAs are designed by searching for homologous sites between different target sequences (e.g., from about 5 to about 15 nucleotide regions of shared homology) and allowing for non-canonical base pairs (e.g. G:U wobble base pairing) or mismatched base pairs.

[00742] Chemically modified siNA constructs were designed as described herein (see for example **Table V**) to provide nuclease stability for systemic administration in vivo and/or improved pharmacokinetic, localization, and delivery properties while preserving the ability to mediate RNAi activity. Chemical modifications as described herein are introduced synthetically using synthetic methods described herein and those generally known in the art. The synthetic siNA constructs are then assayed for nuclease stability in serum and/or cellular/tissue extracts (e.g. liver extracts). The synthetic siNA constructs are also tested in parallel for RNAi activity using an appropriate assay, such as a luciferase reporter assay as described herein or another suitable assay that can quantify RNAi activity. Synthetic siNA constructs that possess both nuclease stability and RNAi activity can be further modified and re-evaluated in stability and activity assays. The chemical modifications of the stabilized active siNA constructs can then be applied to any siNA sequence targeting any chosen RNA and used, for example, in target screening assays to pick lead siNA compounds for therapeutic development (see for example Figure 11).

Example 5: Chemical Synthesis, Purification, and Analysis of siNA

[00743] siNA molecules can be designed to interact with various sites in the RNA message, for example, target sequences within the RNA sequences described herein. The sequence of one strand of the siNA molecule(s) is complementary to the target site sequences described above. The siNA molecules can be chemically synthesized using methods described herein. Inactive siNA molecules that are used as control sequences can be synthesized by scrambling the sequence of the siNA molecules such that it is not complementary to the target sequence. Generally, siNA constructs can be synthesized using solid phase oligonucleotide synthesis methods as described herein (see for example Usman et al., US Patent Nos. 5,804,683; 5,831,071; 5,998,203; 6,117,657; 6,353,098; 6,362,323; 6,437,117; 6,469,158; Scaringe et al., US Patent Nos. 6,111,086; 6,008,400; 6,111,086 all incorporated by reference herein in their entirety).

[00744] In a non-limiting example, RNA oligonucleotides are synthesized in a stepwise fashion using the phosphoramidite chemistry as is known in the art. Standard phosphoramidite chemistry involves the use of nucleosides comprising any of 5'-O-dimethoxytrityl, 2'-O-tert-butyldimethylsilyl, 3'-O-2-Cyanoethyl N,N-diisopropylphosphoroamidite groups, and exocyclic amine protecting groups (e.g. N6-benzoyl adenosine, N4 acetyl cytidine, and N2-isobutyryl guanosine). Alternately, 2'-O-Silyl Ethers can be used in conjunction with acid-labile 2'-O-orthoester protecting groups in the synthesis of RNA as described by Scaringe supra. Differing 2' chemistries can require different protecting groups, for example 2'-deoxy-2'-amino nucleosides can utilize N-phthaloyl protection as described by Usman et al., US Patent 5,631,360, incorporated by reference herein in its entirety).

[00745] During solid phase synthesis, each nucleotide is added sequentially (3'- to 5'-direction) to the solid support-bound oligonucleotide. The first nucleoside at the 3'-end of the chain is covalently attached to a solid support (e.g., controlled pore glass or polystyrene) using various linkers. The nucleotide precursor, a ribonucleoside phosphoramidite, and activator are combined resulting in the coupling of the second nucleoside phosphoramidite onto the 5'-end of the first nucleoside. The support is then washed and any unreacted 5'-hydroxyl groups are capped with a capping reagent such as acetic anhydride to yield inactive 5'-acetyl moieties. The trivalent phosphorus linkage is

then oxidized to a more stable phosphate linkage. At the end of the nucleotide addition cycle, the 5'-O-protecting group is cleaved under suitable conditions (e.g., acidic conditions for trityl-based groups and fluoride for silyl-based groups). The cycle is repeated for each subsequent nucleotide.

[00746] Modification of synthesis conditions can be used to optimize coupling efficiency, for example by using differing coupling times, differing reagent/phosphoramidite concentrations, differing contact times, differing solid supports and solid support linker chemistries depending on the particular chemical composition of the siNA to be synthesized. Deprotection and purification of the siNA can be performed as is generally described in Usman et al., US 5,831,071, US 6,353,098, US 6,437,117, and Bellon et al., US 6,054,576, US 6,162,909, US 6,303,773, or Scaringe supra, incorporated by reference herein in their entireties. Additionally, deprotection conditions can be modified to provide the best possible yield and purity of siNA constructs. For example, applicant has observed that oligonucleotides comprising 2'-deoxy-2'-fluoro nucleotides can degrade under inappropriate deprotection conditions. Such oligonucleotides are deprotected using aqueous methylamine at about 35°C for 30 minutes. If the 2'-deoxy-2'-fluoro containing oligonucleotide also comprises ribonucleotides, after deprotection with aqueous methylamine at about 35°C for 30 minutes, TEA-HF is added and the reaction maintained at about 65°C for an additional 15 minutes. The deprotected single strands of siNA are purified by anion exchange to achieve a high purity while maintaining high yields. To form the siNA duplex molecule the single strands are combined in equal molar ratios in a saline solution to form the duplex. The duplex siNA is concentrated and desalted by tangential filtration prior to lyophilization.

Manufacture of siNA compositions

[00747] In a non-limiting example, for each siNA composition, the two individual, complementary strands of the siNA are synthesized separately using solid phase synthesis, then purified separately by ion exchange chromatography. The complementary strands are annealed to form the double strand (duplex). The duplex is then ultrafiltered and lyophilized to form the solid siNA composition (e.g.,

pharmaceutical composition). A non-limiting example of the manufacturing process is shown in the flow diagram in Table VIII.

Solid Phase Synthesis

[00748] The single strand oligonucleotides are synthesized using phosphoramidite chemistry on an automated solid-phase synthesizer, such as an Amersham Pharmacia AKTA Oligopilot (e.g., Oligopilot or Oligopilot 100 plus). An adjustable synthesis column is packed with solid support derivatized with the first nucleoside residue. Synthesis is initiated by detritylation of the acid labile 5'-O-dimethoxytrityl group to release the 5'-hydroxyl. Phosphoramidite and a suitable activator in acetonitrile are delivered simultaneously to the synthesis column resulting in coupling of the amidite to the 5'-hydroxyl. The column is then washed with acetonitrile. Iodine is pumped through the column to oxidize the phosphite triester linkage P(III) to its phosphotriester P(V) analog. Unreacted 5'-hydroxyl groups are capped using reagents such as acetic anhydride in the presence of 2,6-lutidine and N-methylimidazole. The elongation cycle resumes with the detritylation step for the next phosphoramidite incorporation. This process is repeated until the desired sequence has been synthesized. The synthesis concludes with the removal of the terminal dimethoxytrityl group.

Cleavage and Deprotection

[00749] On completion of the synthesis, the solid-support and associated oligonucleotide are transferred to a filter funnel, dried under vacuum, and transferred to a reaction vessel. Aqueous base is added and the mixture is heated to effect cleavage of the succinyl linkage, removal of the cyanoethyl phosphate protecting group, and deprotection of the exocyclic amine protection.

[00750] The following process is performed on single strands that do not contain ribonucleotides: After treating the solid support with the aqueous base, the mixture is filtered under vacuum to separate the solid support from the deprotected crude synthesis material. The solid support is then rinsed with water which is combined with the filtrate. The resultant basic solution is neutralized with acid to provide a solution of the crude single strand.

[00751] The following process is performed on single strands that contain ribonucleotides: After treating the solid support with the aqueous base, the mixture is filtered under vacuum to separate the solid support from the deprotected crude synthesis material. The solid support is then rinsed with dimethylsulfoxide (DMSO) which is combined with the filtrate. The mixture is cooled, fluoride reagent such as triethylamine trihydrofluoride is added, and the solution is heated. The reaction is quenched with suitable buffer to provide a solution of crude single strand.

Anion Exchange Purification

[00752] The solution of each crude single strand is purified using chromatographic purification. The product is eluted using a suitable buffer gradient. Fractions are collected in closed sanitized containers, analyzed by HPLC, and the appropriate fractions are combined to provide a pool of product which is analyzed for purity (HPLC), identity (HPLC), and concentration (UV A260).

Annealing

[00753] Based on the analysis of the pools of product, equal molar amounts (calculated using the theoretical extinction coefficient) of the sense and antisense oligonucleotide strands are transferred to a reaction vessel. The solution is mixed and analyzed for purity of duplex by chromatographic methods. If the analysis indicates an excess of either strand, then additional non-excess strand is titrated until duplexing is complete. When analysis indicates that the target product purity has been achieved, the material is transferred to the tangential flow filtration (TFF) system for concentration and desalting.

Ultrafiltration

[00754] The annealed product solution is concentrated using a TFF system containing an appropriate molecular weight cut-off membrane. Following concentration, the product solution is desalted via diafiltration using WFI quality water until the conductivity of the filtrate is that of water.

Lyophilization

[00755] The concentrated solution is transferred to sanitized trays in a shelf lyophilizer. The product is then freeze-dried to a powder. The trays are removed from the lyophilizer and transferred to a class 100 Laminar Air Flow (LAF) hood for packaging.

Packaging Drug Substance

[00756] The lyophilizer trays containing the freeze-dried product are opened in a class 100 LAF hood. The product is transferred to sanitized containers of appropriate size, which are then sealed and labeled.

Drug Substance Container Closure System

[00757] Lyophilized drug substance is bulk packaged in sanitized Nalgene containers with sanitized caps. The bottle size used is dependent upon the quantity of material to be placed within it. After filling, each bottle is additionally sealed at the closure with polyethylene tape.

Analytical Methods and Specifications

Raw Material and In-Process Methods

[00758] Raw materials are tested for identity prior to introduction into the drug substance manufacturing process. Critical raw materials, those incorporated into the drug substance molecule, are tested additionally using a purity test or an assay test as appropriate. In-process samples are tested at key control points in the manufacturing process to monitor and assure the quality of the final drug substance.

Drug Substance Analytical Methods and Specifications

[00759] Controls incorporating analytical methods and acceptance criteria for oligonucleotides are established prior to clinical testing of bulk siNA compositions. The following test methods and acceptance criteria reflect examples of these controls. Table IX summarizes examples of material specifications for siNA pharmaceutical compositions.

Summary of Analytical Methods

Identification (ID) Tests

[00760] ID Oligonucleotide Main Peak: The identity of the drug substance is established using a chromatographic method. The data used for this determination is generated by one of the HPLC test methods (see Purity Tests). The peak retention times of the drug substance sample and the standard injections are compared. Drug substance identity is supported by a favorable comparison of the main peak retention times.

[00761] Molecular Weight: The identity of the drug substance is established using a spectroscopic method. A sample of drug substance is prepared for analysis by precipitation with aqueous ammonium acetate. The molecular weight of the drug substance is determined by mass spectrometry. The test is controlled to within a set number of atomic mass units from the theoretical molecular weight.

[00762] Melting Temperature: This method supports the identity of the drug substance by measurement of the melting temperature (T_m) of the double stranded drug substance. A sample in solution is heated while monitoring the ultraviolet (UV) absorbance of the solution. The T_m is marked by the inflection point of the absorbance curve as the absorbance increases due to the dissociation of the duplex into single strands.

Assay Tests

[00763] Oligonucleotide Content: This assay determines the total oligonucleotide content in the drug substance. The oligonucleotide absorbs UV light with a local maximum at 260 nm. The oligonucleotide species present consist of the double stranded siRNA product and other minor related oligonucleotide substances from the manufacturing process, including residual single strands. A sample of the drug substance is accurately weighed, dissolved, and diluted volumetrically in water. The absorbance is measured in a quartz cell using a UV spectrophotometer. The total oligonucleotide assay value is calculated using the experimentally determined molar absorptivity of the working standard and reported in micrograms of sodium oligonucleotide per milligram of solid drug substance.

[00764] Purity Tests: Purity will be measured using one or more chromatographic methods. Depending on the separation and the number of nucleic acid analogs of the

drug substance present, orthogonal separation methods may be employed to monitor purity of the API. Separation may be achieved by the following means:

[00765] SAX-HPLC: an ion exchange interaction between the oligonucleotide phosphodiester and a strong anion exchange HPLC column using a buffered salt gradient to perform the separation.

[00766] RP-HPLC: a partitioning interaction between the oligonucleotide and a hydrophobic reversed-phase HPLC column using an aqueous buffer versus organic solvent gradient to perform the separation.

[00767] Capillary Gel Electrophoresis (CGE): an electrophoretic separation by molecular sieving in a buffer solution within a gel filled capillary. Separation occurs as an electrical field is applied, causing anionic oligonucleotides to separate by molecular size as they migrate through the gel matrix. In all separation methods, peaks elute generally in order of oligonucleotide length and are detected by UV at 260 nm.

Other Tests

[00768] Physical Appearance: The drug substance sample is visually examined. This test determines that the material has the character of a lyophilized solid, identifies the color of the solid, and determines whether any visible contaminants are present.

[00769] Bacterial Endotoxins Test: Bacterial endotoxin testing is performed by the Limulus Amebocyte Lysate (LAL) assay using the kinetic turbidimetric method in a 96-well plate. Endotoxin limits for the drug substance will be set appropriately such that when combined with the excipients, daily allowable limits for endotoxin in the administered drug product are not exceeded.

[00770] Aerobic Bioburden: Aerobic bioburden is performed by a contract laboratory using a method based on USP chapter <61>.

[00771] Acetonitrile content: Residual acetonitrile analysis is performed by a contract laboratory using gas chromatography (GC). Acetonitrile is the major organic solvent used in the upstream synthesis step although several other organic reagents are employed in synthesis. Subsequent purification process steps typically remove solvents in the drug

substances. Other solvents may be monitored depending on the outcome of process development work. Solvents will be limited within ICH limits.

[00772] Water content: Water content is determined by volumetric Karl Fischer (KF) titration using a solid evaporator unit (oven). Water is typically present in nucleic acid drug substances as several percent of the composition by weight, and therefore, will be monitored.

[00773] pH: The pH of reconstituted drug substance will be monitored to ensure suitability for human injection.

[00774] Ion Content: Testing for sodium, chloride, and phosphate will be performed by a contract laboratory using standard atomic absorption and ion chromatographic methods. General monitoring of ions will be performed to ensure that the osmolality of the drug product incorporating the drug substances will be within an acceptable physiological range.

[00775] Metals Content: Testing for pertinent metals is performed by a contract laboratory using a standard method of analysis, Inductively Coupled Plasma (ICP) spectroscopy.

Example 6: RNAi *in vitro* assay to assess siNA activity

[00776] An *in vitro* assay that recapitulates RNAi in a cell-free system is used to evaluate siNA constructs targeting target RNA targets. The assay comprises the system described by Tuschl *et al.*, 1999, *Genes and Development*, 13, 3191-3197 and Zamore *et al.*, 2000, *Cell*, 101, 25-33 adapted for use with a target RNA. A *Drosophila* extract derived from syncytial blastoderm is used to reconstitute RNAi activity *in vitro*. Target RNA is generated via *in vitro* transcription from an appropriate target expressing plasmid using T7 RNA polymerase or via chemical synthesis as described herein. Sense and antisense siNA strands (for example 20 uM each) are annealed by incubation in buffer (such as 100 mM potassium acetate, 30 mM HEPES-KOH, pH 7.4, 2 mM magnesium acetate) for 1 minute at 90°C followed by 1 hour at 37°C, then diluted in lysis buffer (for example 100 mM potassium acetate, 30 mM HEPES-KOH at pH 7.4, 2mM magnesium acetate). Annealing can be monitored by gel electrophoresis on an agarose gel in TBE

buffer and stained with ethidium bromide. The *Drosophila* lysate is prepared using zero to two-hour-old embryos from Oregon R flies collected on yeasted molasses agar that are dechorionated and lysed. The lysate is centrifuged and the supernatant isolated. The assay comprises a reaction mixture containing 50% lysate [vol/vol], RNA (10-50 pM final concentration), and 10% [vol/vol] lysis buffer containing siNA (10 nM final concentration). The reaction mixture also contains 10 mM creatine phosphate, 10 ug/ml creatine phosphokinase, 100 μ M GTP, 100 μ M UTP, 100 μ M CTP, 500 μ M ATP, 5 mM DTT, 0.1 U/uL RNasin (Promega), and 100 μ M of each amino acid. The final concentration of potassium acetate is adjusted to 100 mM. The reactions are pre-assembled on ice and preincubated at 25° C for 10 minutes before adding RNA, then incubated at 25° C for an additional 60 minutes. Reactions are quenched with 4 volumes of 1.25 x Passive Lysis Buffer (Promega). Target RNA cleavage is assayed by RT-PCR analysis or other methods known in the art and are compared to control reactions in which siNA is omitted from the reaction.

[00777] Alternately, internally-labeled target RNA for the assay is prepared by in vitro transcription in the presence of [α -32P] CTP, passed over a G50 Sephadex column by spin chromatography and used as target RNA without further purification. Optionally, target RNA is 5'-32P-end labeled using T4 polynucleotide kinase enzyme. Assays are performed as described above and target RNA and the specific RNA cleavage products generated by RNAi are visualized on an autoradiograph of a gel. The percentage of cleavage is determined by PHOSPHOR IMAGER® (autoradiography) quantitation of bands representing intact control RNA or RNA from control reactions without siNA and the cleavage products generated by the assay.

[00778] In one embodiment, this assay is used to determine target sites in the target RNA target for siNA mediated RNAi cleavage, wherein a plurality of siNA constructs are screened for RNAi mediated cleavage of the target RNA target, for example, by analyzing the assay reaction by electrophoresis of labeled target RNA, or by northern blotting, as well as by other methodology well known in the art.

Example 7: Nucleic acid inhibition of PCSK9 target RNA

[00779] siNA molecules targeted to human PCSK9 RNA are designed and synthesized as described above. These nucleic acid molecules can be tested for cleavage activity in vivo, for example, using the following procedure. The target sequences and the nucleotide location within the PCSK9 RNA are given in Tables II and III.

[00780] Two formats are used to test the efficacy of siNAs targeting PCSK9. First, the reagents are tested in cell culture using HepG2, Jurkat, HeLa, A549 or 293T cells, to determine the extent of RNA and protein inhibition. siNA reagents (e.g.; see **Tables II and III**) are selected against the PCSK9 target as described herein. RNA inhibition is measured after delivery of these reagents by a suitable transfection agent to, for example, HepG2, Jurkat, HeLa, A549 or 293T cells. Relative amounts of target RNA are measured versus actin using real-time PCR monitoring of amplification (eg., ABI 7700 TAQMAN®). A comparison is made to a mixture of oligonucleotide sequences made to unrelated targets or to a randomized siNA control with the same overall length and chemistry, but randomly substituted at each position. Primary and secondary lead reagents are chosen for the target and optimization performed. After an optimal transfection agent concentration is chosen, a RNA time-course of inhibition is performed with the lead siNA molecule. In addition, a cell-plating format can be used to determine RNA inhibition.

[00781] In addition, a cell-plating format can be used to determine RNA inhibition. This system is described in Rice et al., US 5,874,565 and US 6,127,116, both incorporated by reference herein.

Delivery of siNA to Cells

[00782] Cells (e.g., HepG2, Jurkat, HeLa, A549 or 293T) are seeded, for example, at 1×10^5 cells per well of a six-well dish in EGM-2 (BioWhittaker) the day before transfection. siNA (final concentration, for example 20nM) and cationic lipid (e.g., final concentration 2 μ g/ml) are complexed in EGM basal media (Bio Whittaker) at 37°C for 30 minutes in polystyrene tubes. Following vortexing, the complexed siNA is added to each well and incubated for the times indicated. For initial optimization experiments, cells are seeded, for example, at 1×10^3 in 96 well plates and siNA complex added as described. Efficiency of delivery of siNA to cells is determined using a fluorescent siNA

complexed with lipid. Cells in 6-well dishes are incubated with siNA for 24 hours, rinsed with PBS and fixed in 2% paraformaldehyde for 15 minutes at room temperature. Uptake of siNA is visualized using a fluorescent microscope.

TAQMAN® (real-time PCR monitoring of amplification) and Lightcycler quantification of mRNA

[00783] Total RNA is prepared from cells following siNA delivery, for example, using Qiagen RNA purification kits for 6-well or Rneasy extraction kits for 96-well assays. For TAQMAN® analysis (real-time PCR monitoring of amplification), dual-labeled probes are synthesized with the reporter dye, FAM or JOE, covalently linked at the 5'-end and the quencher dye TAMRA conjugated to the 3'-end. One-step RT-PCR amplifications are performed on, for example, an ABI PRISM 7700 Sequence Detector using 50 µl reactions consisting of 10 µl total RNA, 100 nM forward primer, 900 nM reverse primer, 100 nM probe, 1X TaqMan PCR reaction buffer (PE-Applied Biosystems), 5.5 mM MgCl₂, 300 µM each dATP, dCTP, dGTP, and dTTP, 10U RNase Inhibitor (Promega), 1.25U AMPLITAQ GOLD® (DNA polymerase) (PE-Applied Biosystems) and 10U M-MLV Reverse Transcriptase (Promega). The thermal cycling conditions can consist of 30 minutes at 48°C, 10 minutes at 95°C, followed by 40 cycles of 15 seconds at 95°C and 1 minute at 60°C. Quantitation of mRNA levels is determined relative to standards generated from serially diluted total cellular RNA (300, 100, 33, 11 ng/reaction) and normalizing to β-actin or GAPDH mRNA in parallel TAQMAN® reactions (real-time PCR monitoring of amplification). For each gene of interest an upper and lower primer and a fluorescently labeled probe are designed. Real time incorporation of SYBR Green I dye into a specific PCR product can be measured in glass capillary tubes using a lightcycler. A standard curve is generated for each primer pair using control cRNA. Values are represented as relative expression to GAPDH in each sample.

Western blotting

[00784] Nuclear extracts can be prepared using a standard micro preparation technique (see for example Andrews and Faller, 1991, Nucleic Acids Research, 19, 2499). Protein extracts from supernatants are prepared, for example using TCA precipitation. An equal volume of 20% TCA is added to the cell supernatant, incubated on ice for 1 hour and

pelleted by centrifugation for 5 minutes. Pellets are washed in acetone, dried and resuspended in water. Cellular protein extracts are run on a 10% Bis-Tris NuPage (nuclear extracts) or 4-12% Tris-Glycine (supernatant extracts) polyacrylamide gel and transferred onto nitro-cellulose membranes. Non-specific binding can be blocked by incubation, for example, with 5% non-fat milk for 1 hour followed by primary antibody for 16 hour at 4°C. Following washes, the secondary antibody is applied, for example (1:10,000 dilution) for 1 hour at room temperature and the signal detected with SuperSignal reagent (Pierce).

Example 8: Models useful to evaluate the down-regulation of PCSK9 gene expression

[00785] Evaluating the efficacy of siNA molecules of the invention in animal models is an important prerequisite to human clinical trials. Various animal models of metabolic and cardiovascular diseases, conditions, or disorders as are known in the art can be adapted for use for pre-clinical evaluation of the efficacy of nucleic acid compositions of the invention in modulating target (e.g., PCSK9) gene expression toward therapeutic or research use. Goldberg, 2004, *J Clin Invest.* 114(5): 613–615, provides a review of animal models useful for evaluating therapeutic agents for metabolic disease, including hyperlipidemia and diabetes. These models and others can be utilized to evaluate the efficacy of siNA molecules and compositions of molecules of the invention. As such, cells treated with nucleic acid molecules of the invention in treating metabolic and related diseases and conditions. (e.g., siNA) targeting PCSK9 RNA would be expected to have decreased PCSK9 expression capacity compared to matched control nucleic acid molecules having a scrambled or inactive sequence. In a non-limiting example, human T cells are cultured and PCSK9 expression is quantified, for example, by time-resolved immuno fluorometric assay. PCSK9 messenger-RNA expression is quantitated with RT-PCR in cultured cells. Untreated cells are compared to cells treated with siNA molecules transfected with a suitable reagent, for example, a cationic lipid such as lipofectamine, and PCSK9 protein and RNA levels are quantitated. Dose response assays are then performed to establish dose dependent inhibition of PCSK9 expression. In a non-limiting example, cell culture experiments are adapted as described in Curiel et al., 2003, *Nature Medicine*, 9, 562.

Example 9: RNAi mediated inhibition of PCSK9 gene expression

In vitro siNA mediated inhibition of PCSK9 RNA

[00786] siNA constructs are tested for efficacy in reducing PCSK9 RNA expression in cells, (e.g., HepG2 cells). Cells are plated approximately 24 hours before transfection in 96-well plates at 5,000-7,500 cells/well, 100 μ l/well, such that at the time of transfection cells are 70-90% confluent. For transfection, annealed siNAs are mixed with the transfection reagent (Lipofectamine 2000, Invitrogen) in a volume of 50 μ l/well and incubated for 20 minutes at room temperature. The siNA transfection mixtures are added to cells to give a final siNA concentration of 25 nM in a volume of 150 μ l. Each siNA transfection mixture is added to 3 wells for triplicate siNA treatments. Cells are incubated at 37° for 24 hours in the continued presence of the siNA transfection mixture. At 24 hours, RNA is prepared from each well of treated cells. The supernatants with the transfection mixtures are first removed and discarded, then the cells are lysed and RNA prepared from each well. Target gene expression following treatment is evaluated by RT-PCR for the target gene and for a control gene (36B4, an RNA polymerase subunit) for normalization. The triplicate data is averaged and the standard deviations determined for each treatment. Normalized data are graphed and the percent reduction of target mRNA by active siNAs in comparison to their respective inverted control siNAs is determined.

Vector-mediated knockdown of PCSK9 RNA expression

[00787] Two stable cell lines, one expressing a control vector pcDNA and the other expressing wild-type PCSK9, were established to characterize the effects of siRNA knockdown of PCSK9 on LDL receptor levels. The effects of siRNA specific to PCSK9 were analyzed by Western blotting (**Figure 30**) and by measuring uptake of fluorescently labeled LDL particles (**Figure 31**).

[00788] Referring to **Figure 30A**, the data show that the PCSK9 protein is only detected in the cells stably expressing PCSK9 whereas the control cell line has undetectable levels of PCSK9 (compare lanes 3 and 6). Treating these cells with a mixture of siRNAs targeting PCSK9 resulted in specific knockdown of PCSK9 protein in the PCSK9-expressing stable cell line (lane 5); in comparison the non-targeting siRNAs had no effect on PCSK9 levels (lane 4).

[00789] Further, the data show that the specific knockdown of PCSK9 by siRNA leads to an increase in LDL receptor levels in the wild-type PCSK9 stable cell line (**Figure 30B**), but not in the control pcDNA stable cell line. In addition, the levels of LDL receptor were significant and easily detected in the control cell lines. In contrast, the PCSK9-expressing stable cell line exhibited a much reduced (almost undetectable) level of LDL receptor protein (compare lanes 3 and 6 in **Figure 30B**). This result was consistent with the diI-LDL uptake assay data shown in **Figure 30**.

[00790] Referring to **Figure 31**, lanes 1-3 demonstrate that the levels of diI-LDL uptake were not significantly affected by siRNA treatment in the control stable cell line; however, there was observed an overall low level of diI-LDL uptake in the PCSK9-expressing stable cell line (lanes 4 & 6). Specific knockdown of PCSK9 by siRNA thus resulted in an increase in diI-LDL uptake, consistent with an increase in LDL receptor levels.

[00791] PCSK9 was knocked down by a Reverse Transfection method described. A siPORT amine reagent (1.5 ul, from Ambion) was mixed with 10 ul of OptiMEM (reduced serum, from Gibco) at room temperature for 10 min (reaction A). In a separate reaction (reaction B) 1 ul of a 10 uM stock of siRNAs specific to PCSK9 (each siRNA is 3.3 uM) is mixed with 10 ul of OptiMEM (reduced serum) at room temperature for 10 min. The three double-stranded siRNAs sequences specific to PCSK9 listed below are ordered from Prologo.

[00792] RNAi Duplex #1. GUGCUC AACUGCCAAGGGATT (SEQ ID NO: 597)

CACGAGUUGACGGUUCCCUAA (SEQ ID NO: 598)

[00793] RNAi Duplex #2. CAGCAAGUGUGACAGUCAUTT (SEQ ID NO: 599)

GUCGUUCACACUGUCAGUGAA (SEQ ID NO: 600)

[00794] RNAi Duplex #3. CACGGUACCGGGCGGAUGATT (SEQ ID NO: 601)

GUGCCAUGGCCCGCCUACUAA (SEQ ID NO: 602)

[00795] A control siRNA sequence (non-targeting siRNA #1-siCONTROL Non-Targeting siRNA #1 D-001210-01-05) was ordered from Dharmacon.

[00796] Reactions A & B were combined and 22.5 ul of this mixture and was added to each well containing 20,000 cells in a 96 well plate. Cell media (100 ul of DMEM, high glucose media supplemented with sodium pyruvate and glutamine, no antibiotics, 5% fetal bovine serum) was added to each well. After 48 hours the cells were harvested to measure PCSK9 protein and LDL-receptor protein levels by Western blots.

Protocol for the dI-LDL uptake Assay:

[00797] LDL-uptake is an art-recognized method to to provide a functional readout of LDL-receptor levels. Cells were plated at a density of 20,000 cells/well in a 96 well polyD-lysine coated plate (Fisher catalog # 3665) and the siRNA transfection mixture was added to the cells, as described above. After 48 hr, the cell media was removed and the cells were washed with OptiMEM (Gibco). Mixture A (100 ul), which is DMEM media containing lipoprotein deficient serum (from IntraCel) and dI-LDL (at a concentration of 10 ug/ml), was added to the cells. To measure non-specific binding, 100 ul of mixture A that also contained 400 ug/ml unlabeled LDL was added to control wells. The plates were incubated at 37 degrees for 6 hr. The cells were washed quickly in TBS (BioRad, # 170-6435) containing 2 mg/ml BSA (from Sigma, catalog # A3059). The wash step was repeated, but this time the wash buffer was incubated for 2 min with the cells. Finally, the cells were washed twice (but quickly) with TBS (without BSA). The cells were lysed in 100 ul RIPA buffer (from TekNova, # R3792) and the lysate was transferred to a black plate (MicroFluor2 Black "U" bottom plates from Fisher). The fluorescence was measured in the plate using an excitation wavelength of 520 nm and an emission wavelength of 580 nm. The total total cellular protein in each well was measured using a BCA Protein Assay (from Pierce, catalog # 23225) and the fluorescence units were normalized to total protein. The amount of specific LDL uptake is the difference between the total counts measured (in the absence of unlabeled LDL) and the counts measured in the presence of unlabeled LDL (non-specific background fluorescence).

Experimental protocols:

[00798] ShRNA design: 6ShRNA sequences for mouse PCSK9 were designed at Rosetta using a proprietary algorithm. The 19mer sequence of Sh2 starting from nucleotide 1566 (NM_153565) is SEQ ID NO: 909, GCACATGCTTCATGTCACA, and the Sh6 sequence starting from nucleotide 2402 is: SEQ ID NO: 910, GTGGCAAGAGTCCATGACA. The forward and reverse oligos of the ShRNA cassettes were synthesized by Integrated DNA technologies.

[00799] Sh2:

[00800] Forward:

[00801] ATCCCCGCACATGCTTCATGTCACATTCAAGAGATGTGACATGA
AGCATGTGCTTTTTGGA (SEQ ID NO: 605)

[00802] Reverse:

[00803] AGCTTCCAAAAAGCACATGCTTCATGTCACATCTCTTGAATGTGAC
ATGAAGCATGTGCGGG (SEQ ID NO: 606)

[00804] Sh6:

[00805] Forward:

[00806] GATCCCCGTGGCAAGAGTCCATGACATTCAAGAGATGTCATGGACT
CTTGCCACTTTTTGGA (SEQ ID NO: 607)

[00807] Reverse:

[00808] AGCTTCCAAAAAGTGGCAAGAGTCCATGACATCTCTTGAATGTCAT
GGACTCTTGCCACGGG (SEQ ID NO: 608)

[00809] ShRNA plasmid generation and screening: The forward and reverse oligos of the ShRNA cassettes were annealed and ligated into a Gateway entry vector pENTR1AH1 containing the human H1 promoter. To evaluate the potency of these ShRNAs in vitro, the six pENTR1SH1-PCSK9ShRNA plasmids were transfected into mouse hepatoma cells Hepa 1-6 using Lipofectamine 2000 transfection reagent, and the cells were harvested at 48 hours post transfection. Total RNA were prepared for real time

PCR analysis. The relative mRNA expression levels were normalized to expression of beta Actin. The taqman primer and probe sets for mouse PCSK9 and beta ACTB were purchased from Applied Biosystems.

[00810] Recombinant adenovirus preparation and in vitro knockdown confirmation: In order to generate the recombinant adenovirus vectors expressing ShRNAs for mPCSK9, selected mPCSK9 Sh2 and Sh6 were recombined into Gateway-based pAd-Block-iT DEST vector (Invitrogen, Carlsbad, CA). Recombinant adenovirus were produced in HEK293 cells and purified by two rounds of CsCl density gradient ultracentrifugation. The purified virus were desalted by dialysis and concentrated over CentriPrep YM-50 column and stored in -80C before use. To confirm that Ad-mPCSK9Sh2 and Ad-mPCSK9Sh6 was functional to downregulate PCSK9 at mRNA levels in vitro, Hepa1-6 cells were transduced with the two ShRNA adenovirus at different MOIs (multiplicity of infection), and cells were harvested at 72 hour post transduction for downstream total RNA isolation and real time PCR analysis.

[00811] Animal Protocols: All animal protocols used in this study were approved by Merck Institutional Animal care and Use Committee. Male C57BL6 mice were purchased from Taconic Farm, and were individually housed. 9 week old mice were divided into experimental groups of equivalent average body weight at the beginning of the study. Mice were injected with 2.5×10^9 PFU/mouse Ad-Cont, Ad-PCSK9Sh2 or Ad-PCSK9Sh6 in a final volume of 100ul saline via the tail vein. Body weight and food intake were measured 4 and 8 days post injection. At day 4 and day 8 post-injection, animals were euthanized under fed condition for blood and tissue collection.

[00812] LDL measurement: Fresh plasma from mice in this study was treated with lipase inhibitors, filtered through a 0.65uM filter tubes, and then applied to the RoboFLAN, a FPLC analyzer. Referring to **Figure 32**, 6 PCSK9 ShRNA in pENTR1AH1 vector were transfected into Hepa 1-6 cells expressing mouse PCSK9 endogenously. The pENTR1A vector containing H1 promoter and termination sequences were used as vector control. Efficacy of the 6 PCSK9 ShRNA in knocking down PCSK9 was assayed by real-time PCR analysis. As shown, mPCSK9Sh2 and mPCSK9Sh6 down-regulated PCSK9 mRNA expression level by about 80% relative to the control as determined by real-time PCR analysis. Data points represent mean $\pm$ SD (n=3).

[00813] Referring to **Figure 33**, Ad-PCSK9Sh2 and Ad-PCSK9Sh6 were transduced into Hepal-6 cells at different MOIs to confirm the efficacy of adenoviral mediated ShRNA knockdown. 72 hour post injection, cells were harvested and subject to real-time PCR analysis. PCSK9Sh2 and Sh6 reduced PCSK9 mRNA in a viral-dose dependent manner. At MOI 400, Ad-PCSK9sh2 and Ad-PCSK9Sh6 reduced PCSK9 mRNA by ~90%. Data points represent mean $\pm$ SD (n=4).

[00814] To assess the effects of PCSK9 inhibition in liver, recombinant adenoviral vectors expressing the control vector, PCSK9Sh2 or PCSK9sh6 were injected intravenously into male C57/BL6 mice. It has been well documented in the literature that adenovirus-mediated expression by this approach is liver specific (Zinn et al Gene Ther. 1998, 5: 798; Dentin et al Diabetes 2006, 55: 2159; Ragozin et al. Arterioscler Thromb Vasc Biol. 2005, 25:1433).

[00815] Referring to **Figure 34**, C57BL/6 mice were injected with 2.5×10^9 pfu recombinant adenovirus expressing PCSK9 Sh2, PCSK9 Sh9 or empty vector. Four days and eight days after injection, mice were euthanized under fed condition for blood and tissue collection.

[00816] As shown in **Figure 34A**, four days after injection, PCSK9 level was reduced by about 55% by Ad-PCSK9Sh2 and about 65% by Ad-PCSK9-Sh6. Eight day after injection, PCSK9 level was reduced ~50% by Ad-PCSK9Sh2 and ~65% by Ad-PCSK9-Sh6. Data points represent mean $\pm$ SD (n=5).

[00817] Referring to **Figure 34B**, lipoprotein profile of mouse plasma was measured by RoboFlan. The LDL level in mice treated with adenovirus expressing PCSK9 Sh2 or Sh6 were similar to the adenoviral control group. Eight days after transduction, hepatic knockdown of PCSK9 by Ad-PCSK9ShRNA6 resulted in about 80% reduction of LDL, while no change of LDL level in mice treated with adenovirus expressing PCSK9Sh2 was observed. Data points represent mean $\pm$ SEM (n=5). While not being bound by any hypothesis, it is believed that the difference of LDL level between mice treated with adenovirus expressing PCSK9Sh2 and Sh6 on day 8 could be partially explained by the difference of knockdown at mRNA levels.

[00818] Referring to **Figure 35** legend, body weight and food intake were monitored on day 4 and day 8 after adenovirus transduction. As shown, no statistically significant change of body weight and food intake (not shown) in mice treated with adenovirus expressing PCSK9sh2 or PCSK9sh6 compared with mice treated with control adenovirus at day 4 and day 8 post-injection was observed. Data points represent mean $\pm$ SEM (n=5).

Summary of shRNA Construct Sequences:

[00819] SEQ ID NO: 597 – Sense Strand of shRNA Construct/Duplex #1.

[00820] SEQ ID NO: 598 – Anti-sense Strand of shRNA Construct/Duplex #1 that is complimentary to a portion of the PCSK9 mRNA.

[00821] SEQ ID NO: 599 – Sense Strand of shRNA Construct/Duplex #2.

[00822] SEQ ID NO: 600 – Anti-sense Strand of shRNA Construct/Duplex #2 that is complimentary to a portion of the PCSK9 mRNA.

[00823] SEQ ID NO: 601 – Sense Strand of shRNA Construct/Duplex #3.

[00824] SEQ ID NO: 60 – Anti-sense Strand of shRNA Construct/Duplex #3 that is complimentary to a portion of the PCSK9 mRNA.

[00825] SEQ ID NO: 603 – amino acid sequence –human PCSK9 encoded by PCSK9 mRNA targeted by the RNAi constructs of the invention.

[00826] SEQ ID NO: 604- mRNA sequence encoding the amino acid sequence as set forth in SEQ ID NO: 597.

[00827] SEQ ID NO: 605 – Forward oligonucleotide for PCSK9-sh2 (Generated from GenBank Accession number NM_153565).

[00828] SEQ ID NO: 606 – Reverse oligonucleotide for PCSK9-sh2 (Generated from GenBank Accession number NM_153565).

[00829] SEQ ID NO: 607 – Forward oligonucleotide for PCSK9-sh6 (Generated from GenBank Accession number NM_153565).

[00830] SEQ ID NO: 608 – Reverse oligonucleotide for PCSK9-sh6 (Generated from GenBank Accession number NM_153565).

Example 10: Indications

[00831] Particular conditions and disease states that can be associated with gene expression modulation include, but are not limited to metabolic and cardiovascular diseases, conditions, or disorders as described herein or otherwise known in the art, and any other diseases, conditions or disorders that are related to or will respond to the levels of a target (e.g., target PCSK9 protein or target PCSK9 polynucleotide) in a cell or tissue, alone or in combination with other therapies. Non-limiting examples of such diseases and conditions include hypercholesterolemia, dyslipidemia, cardiovascular disease, atherosclerosis, hypertension, diabetes (e.g., type I and/or type II diabetes), insulin resistance, obesity, and/or metabolic syndrome, type I hyperlipoproteinemia, type II hyperlipoproteinemia, type III hyperlipoproteinemia, type IV hyperlipoproteinemia, type V hyperlipoproteinemia, secondary hypertriglyceridemia, and familial lecithin cholesterol acyltransferase deficiency; and cardiovascular disease, such as coronary heart disease (CHD), cerebrovascular disease (CVD), aortic stenosis, peripheral vascular disease, atherosclerosis, arteriosclerosis, myocardial infarction (heart attack), cerebrovascular diseases (stroke), transient ischaemic attacks (TIA), angina (stable and unstable), atrial fibrillation, arrhythmia, valvular disease, and/or congestive heart failure or any other trait, disease or condition that is related to or will respond to the levels of PCSK9 in a cell or tissue, alone or in combination with other therapies.

[00832] Non-limiting examples of compounds that can be used in combination with RNAi constructs of the invention include but are not limited to statins (e.g., atorvastatin, simvastatin, pravastatin, fluvastatin, lovastatin), other PCSK9 inhibitors, such as torcetrapib and JTT-705, and antihypertensive agents, such as Alpha1-Adrenergic Antagonists (e.g., Prazosin), Beta-Adrenergic Antagonists (e.g., Propranolol, Nadolol, Timolol, Metoprolol, Pindolol), Combined Alpha/Beta-Adrenergic Antagonists (e.g., Labetalol), Adrenergic Neuron Blocking Agents (e.g., Guanethidine, Reserpine), CNS-

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CECI EST LE TOME 1 DE 2
CONTENANT LES PAGES 1 À 288

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JUMBO APPLICATIONS/PATENTS

THIS SECTION OF THE APPLICATION/PATENT CONTAINS MORE THAN ONE VOLUME

THIS IS VOLUME 1 OF 2
CONTAINING PAGES 1 TO 288

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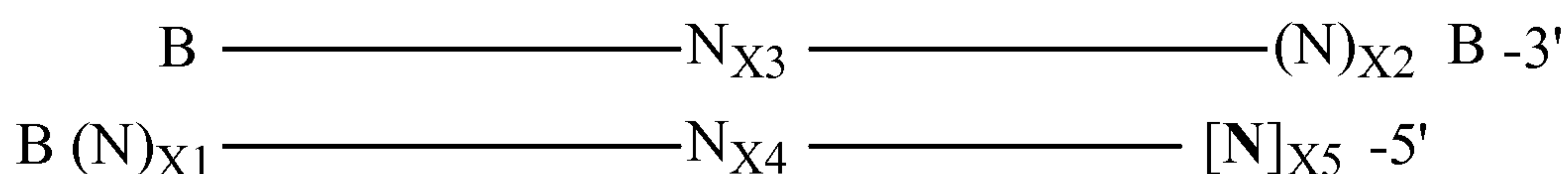
NOM DU FICHER / FILE NAME :

NOTE POUR LE TOME / VOLUME NOTE:

CLAIMS

What we claim is:

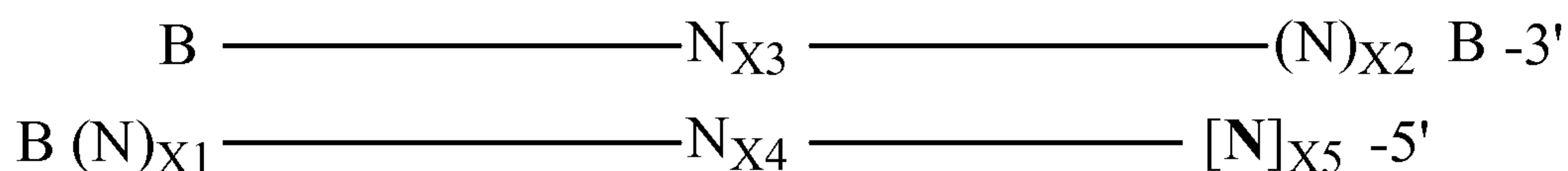
1. A double stranded nucleic acid molecule having structure SIX comprising a sense strand and an antisense strand:



SIX

wherein the upper strand is the sense strand and the lower strand is the antisense strand of the double stranded nucleic acid molecule; said antisense strand comprises sequence complementary to a Proprotein Convertase Subtilisin Kexin 9 (PCSK9) RNA; each N is independently a nucleotide which can be unmodified or chemically modified; each B is a terminal cap moiety that can be present or absent; (N) represents non-base paired or overhanging nucleotides which can be unmodified or chemically modified; [N] represents nucleotide positions that are ribonucleotides; X1 and X2 are independently integers from about 0 to about 4; X3 is an integer from about 9 to about 30; X4 is an integer from about 11 to about 30, provided that the sum of X4 and X5 is about 17-36; X5 is an integer from about 1 to about 6; and

- (a) any pyrimidine nucleotides present in the antisense strand are 2'-deoxy-2'-fluoro nucleotides; any purine nucleotides present in the antisense strand other than the purines nucleotides in the [N] nucleotide positions, are independently 2'-O-methyl nucleotides, 2'-deoxyribonucleotides or a combination of 2'-deoxyribonucleotides and 2'-O-methyl nucleotides;
 - (b) any pyrimidine nucleotides present in the sense strand are 2'-deoxy-2'-fluoro nucleotides; any purine nucleotides present in the sense strand are independently 2'-deoxyribonucleotides, 2'-O-methyl nucleotides or a combination of 2'-deoxyribonucleotides and 2'-O-methyl nucleotides; and
 - (c) any (N) nucleotides are optionally 2'-O-methyl, 2'-deoxy-2'-fluoro, or deoxyribonucleotides.
2. A double stranded nucleic acid molecule having structure SX comprising a sense strand and an antisense strand:

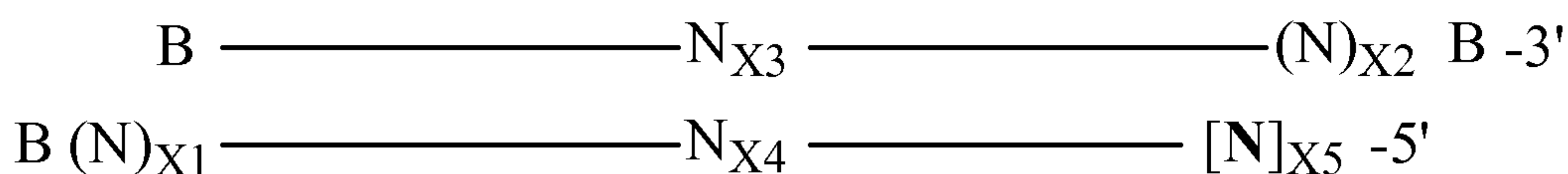


SX

wherein the upper strand is the sense strand and the lower strand is the antisense strand of the double stranded nucleic acid molecule; said antisense strand comprises sequence complementary to a Proprotein Convertase Subtilisin Kexin 9 (PCSK9) RNA; each N is independently a nucleotide which can be unmodified or chemically modified; each B is a terminal cap moiety that can be present or absent; (N) represents non-base paired or overhanging nucleotides which can be unmodified or chemically modified; [N] represents nucleotide positions that are ribonucleotides; X1 and X2 are independently integers from about 0 to about 4; X3 is an integer from about 9 to about 30; X4 is an integer from about 11 to about 30, provided that the sum of X4 and X5 is about 17-36; X5 is an integer from about 1 to about 6; and

- (a) any pyrimidine nucleotides present in the antisense strand are 2'-deoxy-2'-fluoro nucleotides; any purine nucleotides present in the antisense strand other than the purines nucleotides in the [N] nucleotide positions, are 2'-O-methyl nucleotides;
- (b) any pyrimidine nucleotides present in the sense strand are ribonucleotides; any purine nucleotides present in the sense strand are ribonucleotides; and
- (c) any (N) nucleotides are optionally 2'-O-methyl, 2'-deoxy-2'-fluoro, or deoxyribonucleotides.

3. A double stranded nucleic acid molecule having structure SXI comprising a sense strand and an antisense strand:



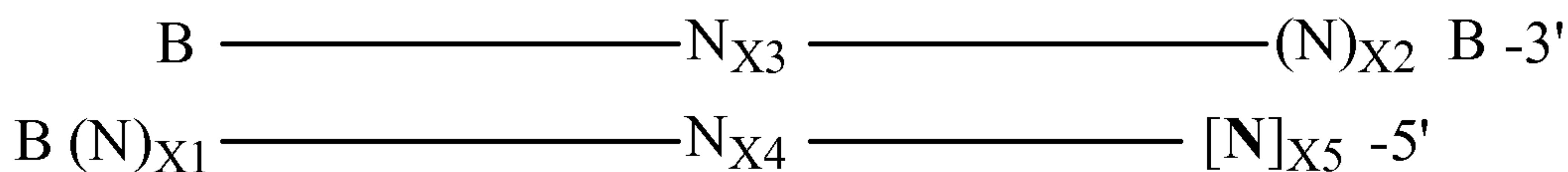
SXI

wherein the upper strand is the sense strand and the lower strand is the antisense strand of the double stranded nucleic acid molecule; said antisense strand comprises sequence complementary to a Proprotein Convertase Subtilisin Kexin 9 (PCSK9)

RNA; each N is independently a nucleotide which can be unmodified or chemically modified; each B is a terminal cap moiety that can be present or absent; (N) represents non-base paired or overhanging nucleotides which can be unmodified or chemically modified; [N] represents nucleotide positions that are ribonucleotides; X1 and X2 are independently integers from about 0 to about 4; X3 is an integer from about 9 to about 30; X4 is an integer from about 11 to about 30, provided that the sum of X4 and X5 is about 17-36; X5 is an integer from about 1 to about 6; and

- (a) any pyrimidine nucleotides present in the antisense strand are 2'-deoxy-2'-fluoro nucleotides; any purine nucleotides present in the antisense strand other than the purines nucleotides in the [N] nucleotide positions, are 2'-O-methyl nucleotides;
- (b) any pyrimidine nucleotides present in the sense strand are 2'-deoxy-2'-fluoro nucleotides; any purine nucleotides present in the sense strand are ribonucleotides; and
- (c) any (N) nucleotides are optionally 2'-O-methyl, 2'-deoxy-2'-fluoro, or deoxyribonucleotides.

4. A double stranded nucleic acid molecule having structure SXII comprising a sense strand and an antisense strand:

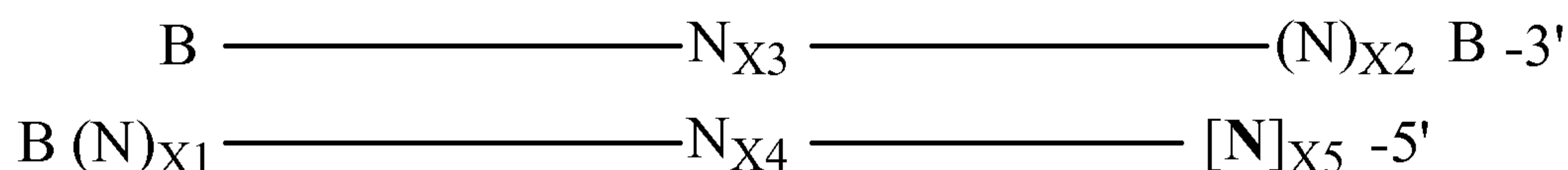


SXII

wherein the upper strand is the sense strand and the lower strand is the antisense strand of the double stranded nucleic acid molecule; said antisense strand comprises sequence complementary to a Proprotein Convertase Subtilisin Kexin 9 (PCSK9) RNA; each N is independently a nucleotide which can be unmodified or chemically modified; each B is a terminal cap moiety that can be present or absent; (N) represents non-base paired or overhanging nucleotides which can be unmodified or chemically modified; [N] represents nucleotide positions that are ribonucleotides; X1 and X2 are independently integers from about 0 to about 4; X3 is an integer from about 9 to about 30; X4 is an integer from about 11 to about 30, provided that the sum of X4 and X5 is about 17-36; X5 is an integer from about 1 to about 6; and

- (a) any pyrimidine nucleotides present in the antisense strand are 2'-deoxy-2'-fluoro nucleotides; any purine nucleotides present in the antisense strand other than the purines nucleotides in the [N] nucleotide positions, are 2'-O-methyl nucleotides;
- (b) any pyrimidine nucleotides present in the sense strand are 2'-deoxy-2'-fluoro nucleotides; any purine nucleotides present in the sense strand are deoxyribonucleotides; and
- (c) any (N) nucleotides are optionally 2'-O-methyl, 2'-deoxy-2'-fluoro, or deoxyribonucleotides.

5. A double stranded nucleic acid molecule having structure SXIII comprising a sense strand and an antisense strand:



SXIII

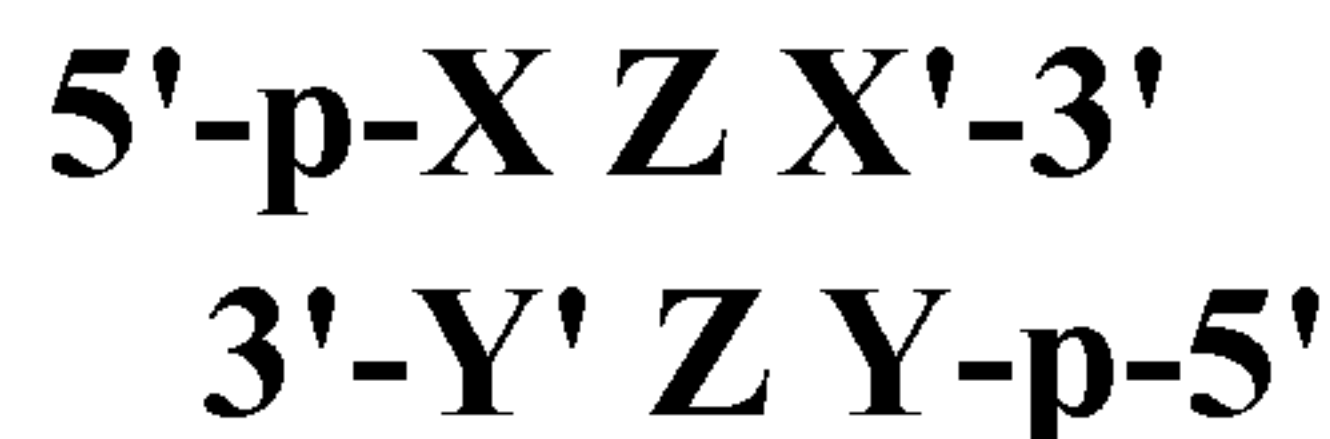
wherein the upper strand is the sense strand and the lower strand is the antisense strand of the double stranded nucleic acid molecule; said antisense strand comprises sequence complementary to a Proprotein Convertase Subtilisin Kexin 9 (PCSK9) RNA; each N is independently a nucleotide which can be unmodified or chemically modified; each B is a terminal cap moiety that can be present or absent; (N) represents non-base paired or overhanging nucleotides which can be unmodified or chemically modified; [N] represents nucleotide that are ribonucleotides; X1 and X2 are independently integers from about 0 to about 4; X3 is an integer from about 9 to about 30; X4 is an integer from about 11 to about 30, provided that the sum of X4 and X5 is about 17-36; X5 is an integer from about 1 to about 6; and

- (a) any pyrimidine nucleotides present in the antisense strand are nucleotides having a ribo-like, Northern or A-form helix configuration; any purine nucleotides present in the antisense strand other than the purines nucleotides in the [N] nucleotide positions, are 2'-O-methyl nucleotides;
- (b) any pyrimidine nucleotides present in the sense strand are nucleotides having a ribo-like, Northern or A-form helix configuration; any purine nucleotides present in the sense strand are 2'-O-methyl nucleotides; and

- (c) any (N) nucleotides are optionally 2'-O-methyl, 2'-deoxy-2'-fluoro, or deoxyribonucleotides.
6. The double stranded nucleic acid molecule of claim 1, wherein X5 = 1, 2, or 3; each X1 and X2 = 1 or 2; X3 = 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, or 30, and X4 = 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, or 30.
 7. The double stranded nucleic acid molecule of claim 2, wherein X5 = 1, 2, or 3; each X1 and X2 = 1 or 2; X3 = 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, or 30, and X4 = 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, or 30.
 8. The double stranded nucleic acid molecule of claim 3, wherein X5 = 1, 2, or 3; each X1 and X2 = 1 or 2; X3 = 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, or 30, and X4 = 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, or 30.
 9. The double stranded nucleic acid molecule of claim 4, wherein X5 = 1, 2, or 3; each X1 and X2 = 1 or 2; X3 = 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, or 30, and X4 = 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, or 30.
 10. The double stranded nucleic acid molecule of claim 5, wherein X5 = 1, 2, or 3; each X1 and X2 = 1 or 2; X3 = 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, or 30, and X4 = 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, or 30.
 11. The double stranded nucleic acid molecule of claim 1, wherein B is present at the 3' and 5' ends of the sense strand and at the 3'-end of the antisense strand.
 12. The double stranded nucleic acid molecule of claim 2, wherein B is present at the 3' and 5' ends of the sense strand and at the 3'-end of the antisense strand.
 13. The double stranded nucleic acid molecule of claim 3, wherein B is present at the 3' and 5' ends of the sense strand and at the 3'-end of the antisense strand.
 14. The double stranded nucleic acid molecule of claim 4, wherein B is present at the 3' and 5' ends of the sense strand and at the 3'-end of the antisense strand.
 15. The double stranded nucleic acid molecule of claim 5, wherein B is present at the 3' and 5' ends of the sense strand and at the 3'-end of the antisense strand.
 16. The double stranded nucleic acid molecule of claim 1, comprising one or more phosphorothioate internucleotide linkages at the first terminal (N) on the 3' end of the sense strand, antisense strand, or both sense strand and antisense strands of the siNA molecule.

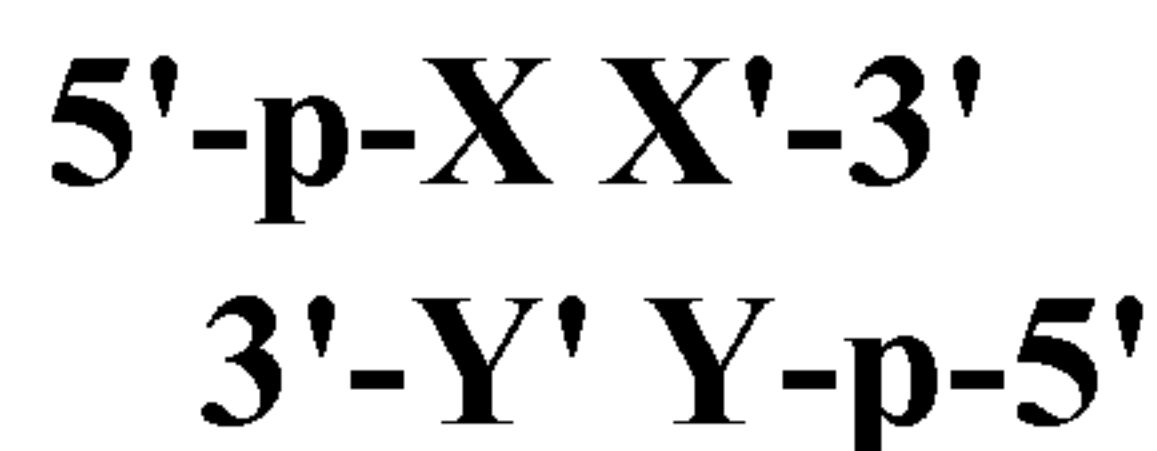
17. The double stranded nucleic acid molecule of claim 2, comprising one or more phosphorothioate internucleotide linkages at the first terminal (N) on the 3'end of the sense strand, antisense strand, or both sense strand and antisense strands of the siNA molecule.
18. The double stranded nucleic acid molecule of claim 3, comprising one or more phosphorothioate internucleotide linkages at the first terminal (N) on the 3'end of the sense strand, antisense strand, or both sense strand and antisense strands of the siNA molecule.
19. The double stranded nucleic acid molecule of claim 4, comprising one or more phosphorothioate internucleotide linkages at the first terminal (N) on the 3'end of the sense strand, antisense strand, or both sense strand and antisense strands of the siNA molecule.
20. The double stranded nucleic acid molecule of claim 5, comprising one or more phosphorothioate internucleotide linkages at the first terminal (N) on the 3'end of the sense strand, antisense strand, or both sense strand and antisense strands of the siNA molecule.
21. A composition comprising the double stranded nucleic acid molecule of claim 1 in a pharmaceutically acceptable carrier or diluent.
22. A composition comprising the double stranded nucleic acid molecule of claim 2 in a pharmaceutically acceptable carrier or diluent.
23. A composition comprising the double stranded nucleic acid molecule of claim 3 in a pharmaceutically acceptable carrier or diluent.
24. A composition comprising the double stranded nucleic acid molecule of claim 4 in a pharmaceutically acceptable carrier or diluent.
25. A composition comprising the double stranded nucleic acid molecule of claim 5 in a pharmaceutically acceptable carrier or diluent.
26. A composition comprising two or more double stranded nucleic acid molecule of claim 1 in a pharmaceutically acceptable carrier or diluent.
27. A composition comprising two or more double stranded nucleic acid molecules of claim 2 in a pharmaceutically acceptable carrier or diluent.

28. A composition comprising two or more double stranded nucleic acid molecules of claim 3 in a pharmaceutically acceptable carrier or diluent.
29. A composition comprising two or more double stranded nucleic acid molecules of claim 4 in a pharmaceutically acceptable carrier or diluent.
30. A composition comprising two or more double stranded nucleic acid molecules of claim 5 in a pharmaceutically acceptable carrier or diluent.
31. The composition of claim 1, wherein one or more double stranded nucleic acid molecules of said composition comprises sequence having 15 to 30 nucleotides that are complementary to a first PCSK9 target, and wherein one or more one or more double stranded nucleic acid molecules of said composition comprises sequence having 15 to 30 nucleotides that are complementary to a second PCSK9 target.
32. The composition of claim 2, wherein one or more double stranded nucleic acid molecules of said composition comprises sequence having 15 to 30 nucleotides that are complementary to a first PCSK9 target, and wherein one or more one or more double stranded nucleic acid molecules of said composition comprises sequence having 15 to 30 nucleotides that are complementary to a second PCSK9 target.
33. The composition of claim 3, wherein one or more double stranded nucleic acid molecules of said composition comprises sequence having 15 to 30 nucleotides that are complementary to a first PCSK9 target, and wherein one or more one or more double stranded nucleic acid molecules of said composition comprises sequence having 15 to 30 nucleotides that are complementary to a second PCSK9 target.
34. The composition of claim 4, wherein one or more double stranded nucleic acid molecules of said composition comprises sequence having 15 to 30 nucleotides that are complementary to a first PCSK9 target, and wherein one or more one or more double stranded nucleic acid molecules of said composition comprises sequence having 15 to 30 nucleotides that are complementary to a second PCSK9 target.
35. The composition of claim 5, wherein one or more double stranded nucleic acid molecules of said composition comprises sequence having 15 to 30 nucleotides that are complementary to a first PCSK9 target, and wherein one or more one or more double stranded nucleic acid molecules of said composition comprises sequence having 15 to 30 nucleotides that are complementary to a second PCSK9 target.
36. A multifunctional double stranded nucleic acid molecule of Formula I:



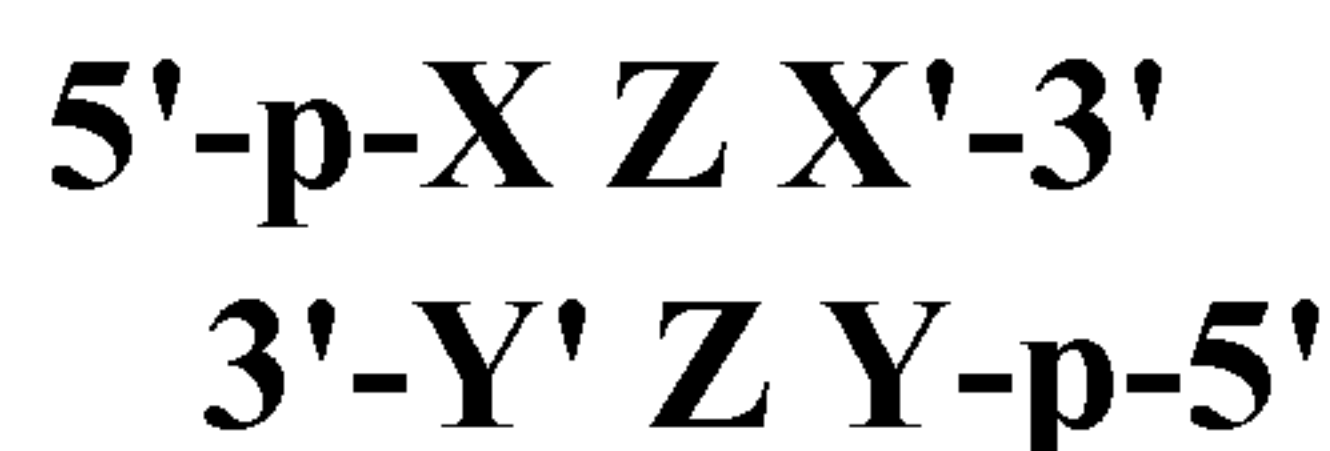
wherein each 5'-p-XZX'-3' and 5'-p-YZY'-3' independently comprise an oligonucleotide of length between about 24 and about 38 nucleotides, XZ comprises a nucleic acid sequence that is complementary to a first PCSK9 target nucleic acid sequence, YZ comprises an oligonucleotide comprising nucleic acid sequence that is complementary to a second PCSK9 target nucleic acid sequence, Z comprises nucleotide sequence of length about 1 to about 24 nucleotides that is complementary between regions XZ and YZ, X comprises nucleotide sequence of length about 1 to about 21 nucleotides that is complementary to nucleotide sequence present in region Y', Y comprises nucleotide sequence of length about 1 to about 21 nucleotides that is complementary to nucleotide sequence present in region X', p comprises a terminal phosphate group that can independently be present or absent, and wherein each said XZ and said YZ are independently of length sufficient to stably interact with said first and said second target nucleic acid sequence, respectively, or a portion thereof.

37. A multifunctional double stranded nucleic acid molecule of Formula II:



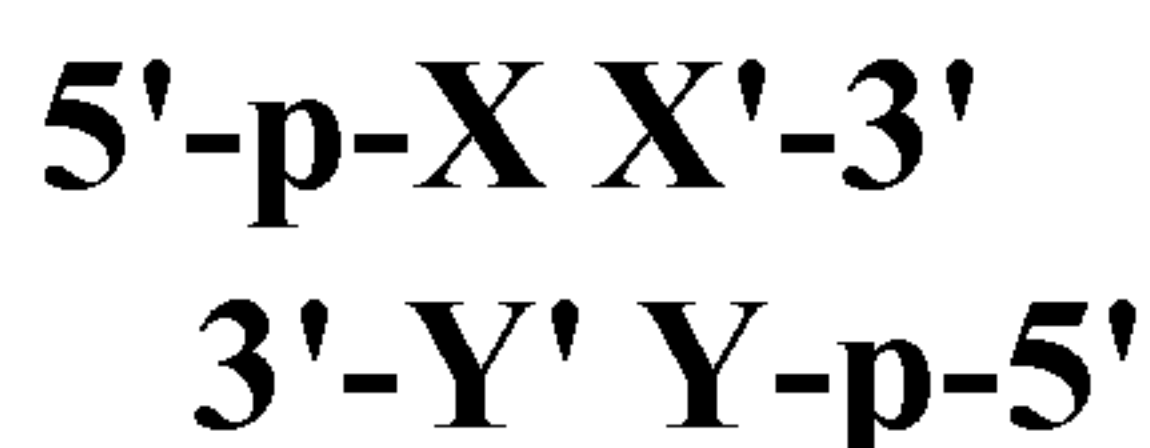
wherein each 5'-p-XX'-3' and 5'-p-YY'-3' independently comprise an oligonucleotide of length between about 24 and about 38 nucleotides, X comprises a nucleic acid sequence that is complementary to a first PCSK9 target nucleic acid sequence, Y comprises an oligonucleotide comprising nucleic acid sequence that is complementary to a second PCSK9 target nucleic acid sequence, said X further comprises nucleotide sequence of length about 1 to about 21 nucleotides that is complementary to nucleotide sequence present in region Y', said Y further comprises nucleotide sequence of length about 1 to about 21 nucleotides that is complementary to nucleotide sequence present in region X', p comprises a terminal phosphate group that can independently be present or absent, and wherein each said X and said Y are independently of length sufficient to stably interact with said first and said second target nucleic acid sequence, respectively, or a portion thereof.

38. A multifunctional double stranded nucleic acid molecule of Formula I:



wherein each 5'-p-XZX'-3' and 5'-p-YZY'-3' independently comprise an oligonucleotide of length between about 24 and about 38 nucleotides, XZ comprises a nucleic acid sequence that is complementary to a first PCSK9 target nucleic acid sequence, YZ comprises an oligonucleotide comprising nucleic acid sequence that is complementary to a second nucleic acid sequence of a PCSK9 pathway target nucleic acid sequence, Z comprises nucleotide sequence of length about 1 to about 24 nucleotides that is complementary between regions XZ and YZ, X comprises nucleotide sequence of length about 1 to about 21 nucleotides that is complementary to nucleotide sequence present in region Y', Y comprises nucleotide sequence of length about 1 to about 21 nucleotides that is complementary to nucleotide sequence present in region X', p comprises a terminal phosphate group that can independently be present or absent, and wherein each said XZ and said YZ are independently of length sufficient to stably interact with said first and said second target nucleic acid sequence, respectively, or a portion thereof.

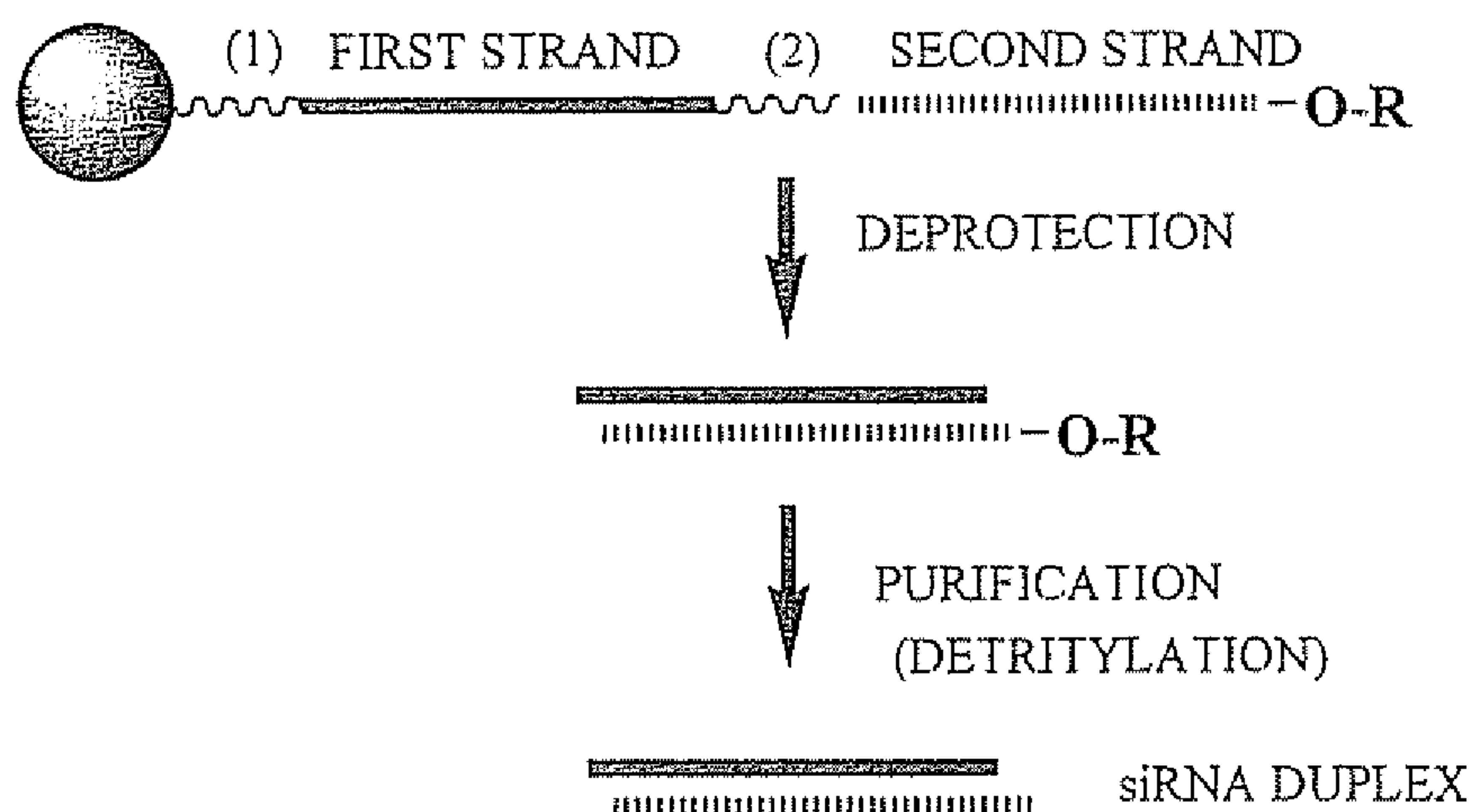
39. A multifunctional double stranded nucleic acid molecule of Formula II:



wherein each 5'-p-XX'-3' and 5'-p-YY'-3' independently comprise an oligonucleotide of length between about 24 and about 38 nucleotides, X comprises a nucleic acid sequence that is complementary to a first PCSK9 target nucleic acid sequence, Y comprises an oligonucleotide comprising nucleic acid sequence that is complementary to a second nucleic acid sequence of a PCSK9 pathway target nucleic acid sequence, said X further comprises nucleotide sequence of length about 1 to about 21 nucleotides that is complementary to nucleotide sequence present in region Y', said Y further comprises nucleotide sequence of length about 1 to about 21 nucleotides that is complementary to nucleotide sequence present in region X', p comprises a terminal phosphate group that can independently be present or absent, and wherein each said X and said Y are independently of length sufficient to stably interact with said first and said second target nucleic acid sequence, respectively, or a portion thereof.

40. A composition comprising the multifunctional double stranded nucleic acid molecule of claim 36 in a pharmaceutically acceptable carrier or diluent.

41. A composition comprising the multifunctional double stranded nucleic acid molecule of claim 37 in a pharmaceutically acceptable carrier or diluent.
42. A composition comprising the multifunctional double stranded nucleic acid molecule of claim 38 in a pharmaceutically acceptable carrier or diluent.
43. A composition comprising the multifunctional double stranded nucleic acid molecule of claim 39 in a pharmaceutically acceptable carrier or diluent.
44. A composition comprising the double stranded nucleic acid molecule of claim 1 formulated as any of LNP-051, 052, 053, 054, 060, 061, 069, 073, 077, 080, 082, 083, 086, 097, 098, 099, 100, 101, 102, 103, 104, 105, 106, 107, 108, 109, 110, 111, 112, 113, 114, 115, 116, or 117.
45. A composition comprising the double stranded nucleic acid molecule of claim 2 formulated as a lipid nanoparticle (LNP).
46. A composition comprising the double stranded nucleic acid molecule of claim 3 formulated as a lipid nanoparticle (LNP).
47. A composition comprising the double stranded nucleic acid molecule of claim 4 formulated as a lipid nanoparticle (LNP).
48. A composition comprising the double stranded nucleic acid molecule of claim 5 formulated as a lipid nanoparticle (LNP).

Figure 1

= SOLID SUPPORT

R = TERMINAL PROTECTING GROUP

FOR EXAMPLE:

DIMETHOXYTRITYL (DMT)



= CLEAVABLE LINKER

(FOR EXAMPLE: NUCLEOTIDE SUCCINATE OR
INVERTED DEOXYABASIC SUCCINATE)



= CLEAVABLE LINKER

(FOR EXAMPLE: NUCLEOTIDE SUCCINATE OR
INVERTED DEOXYABASIC SUCCINATE)

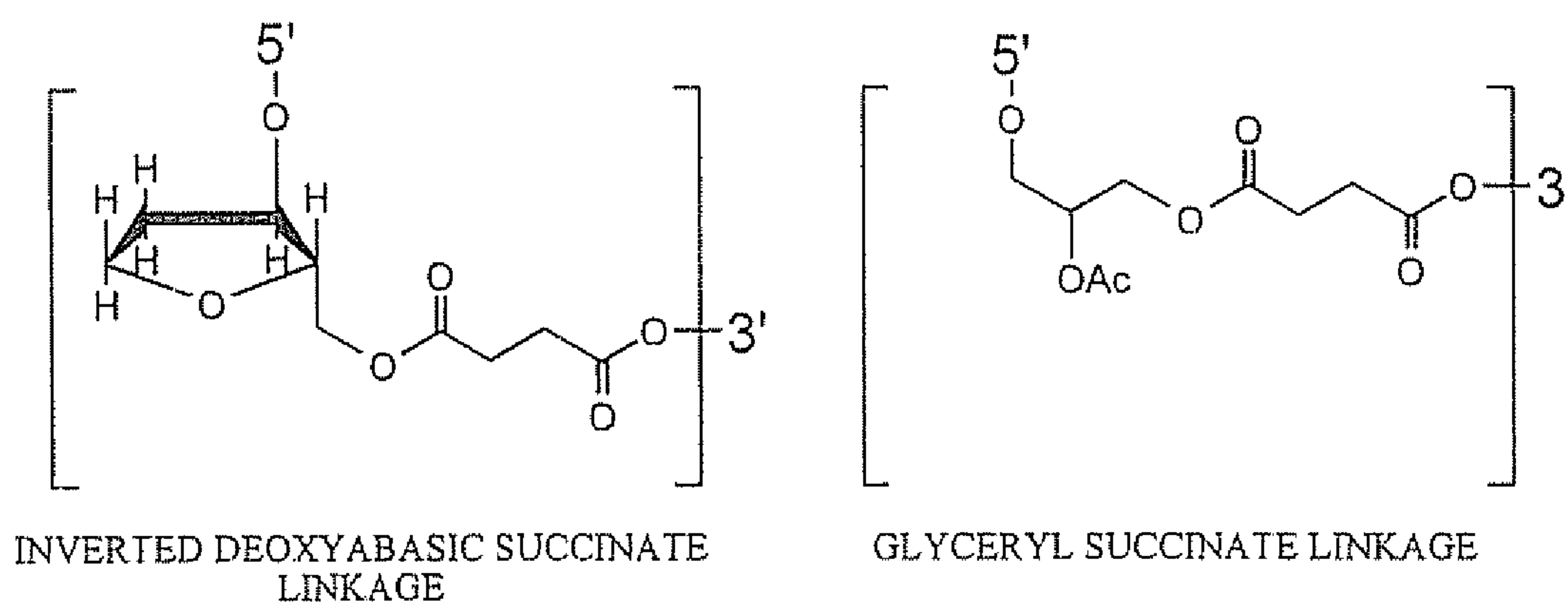


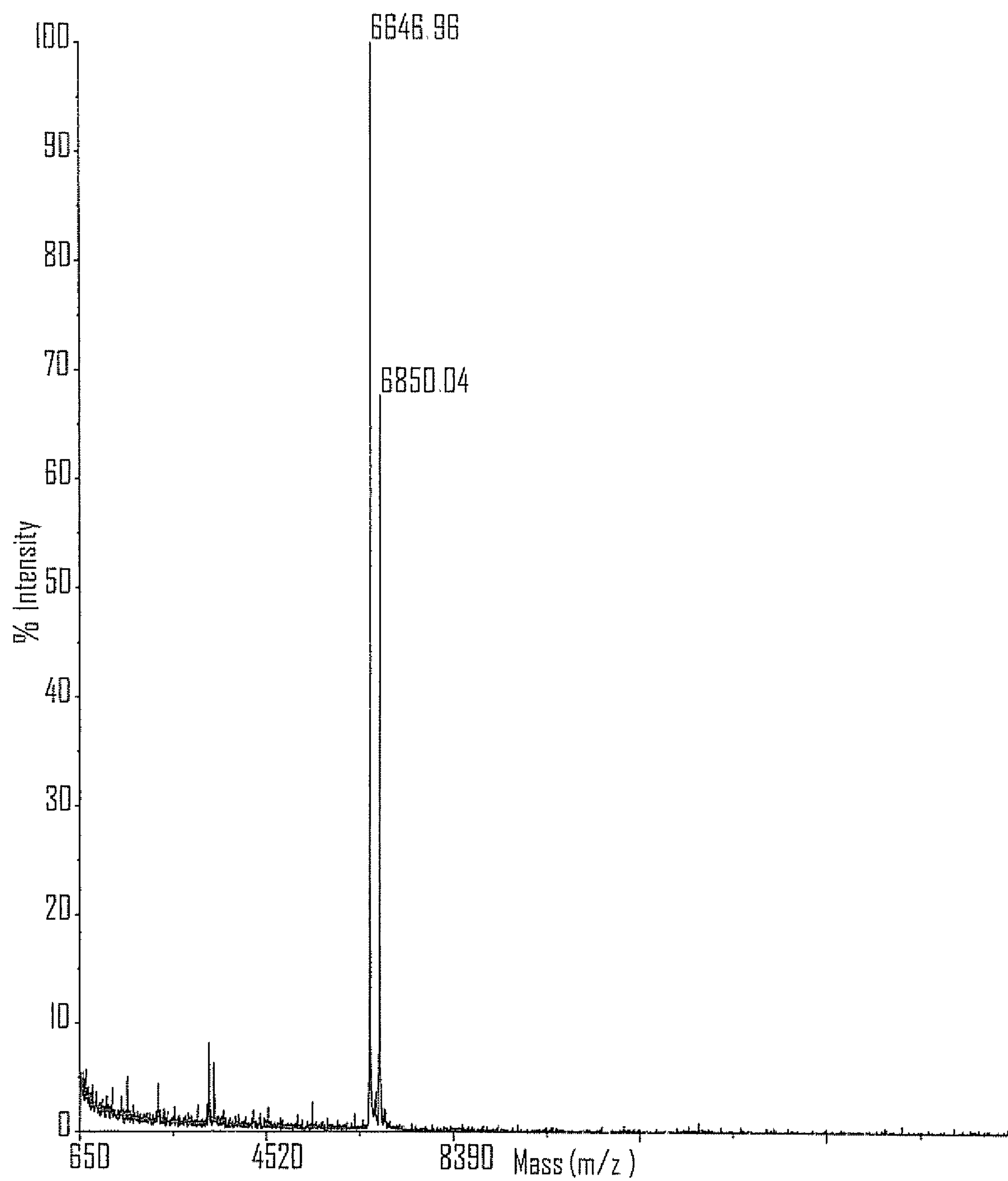
Figure 2

Figure 3

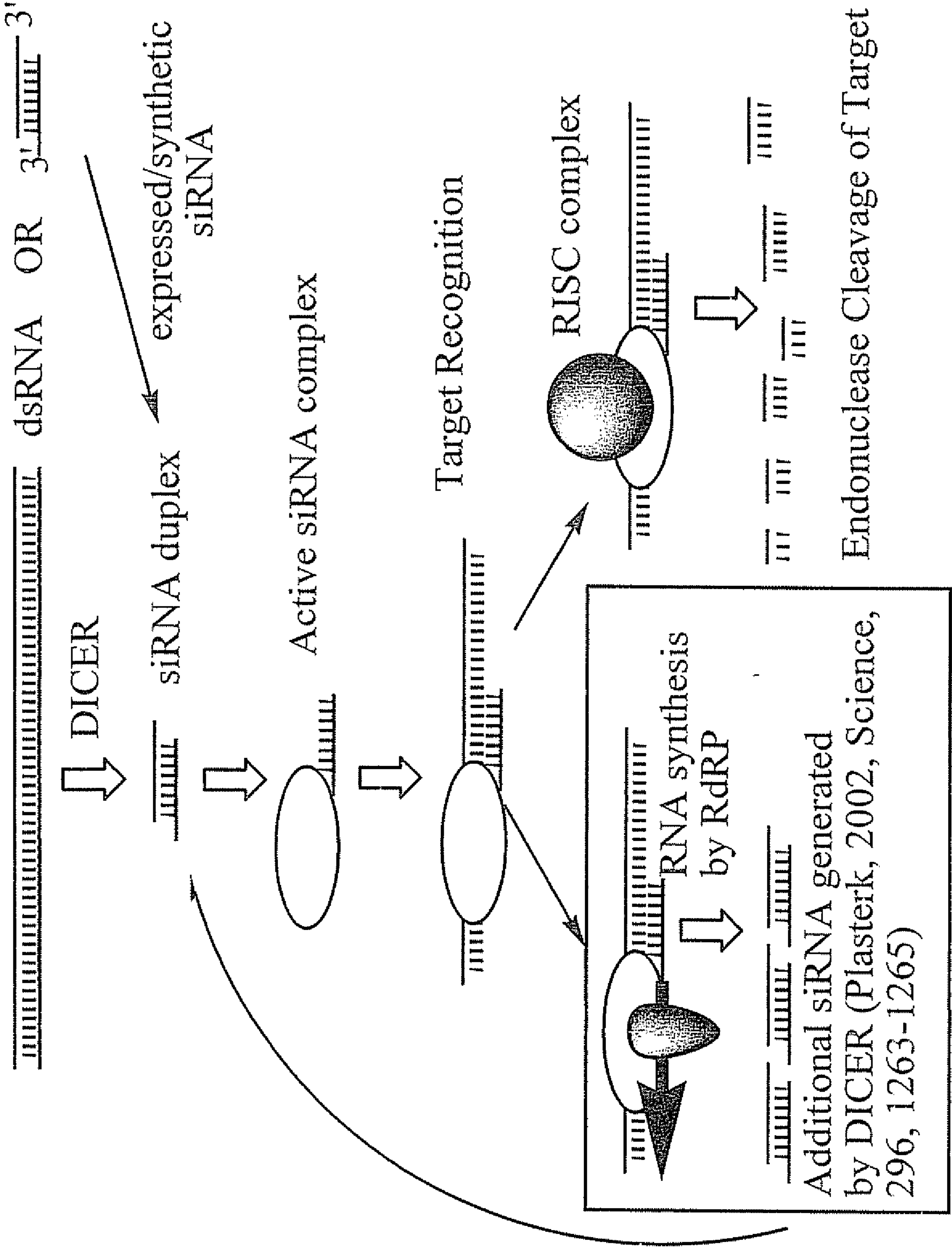
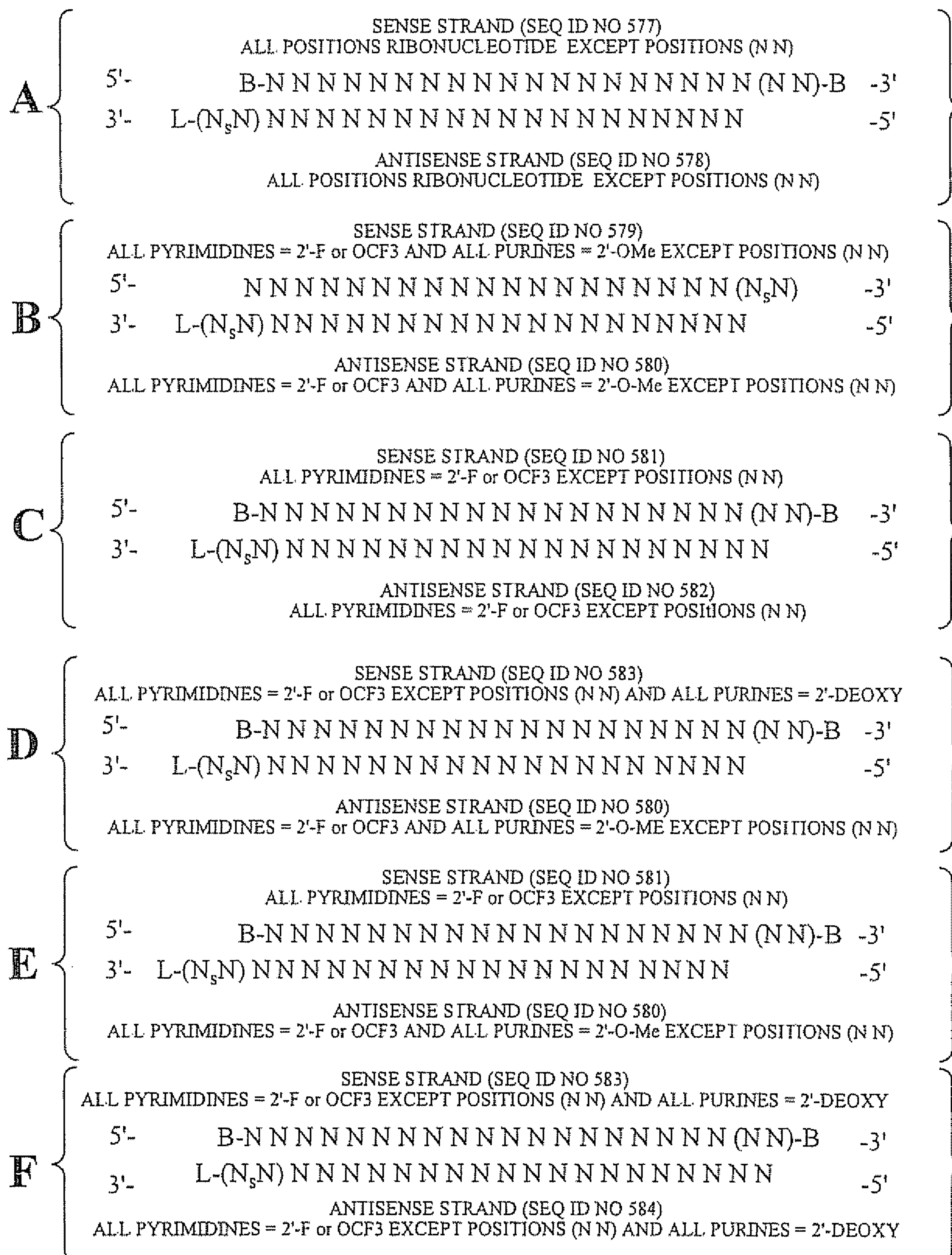
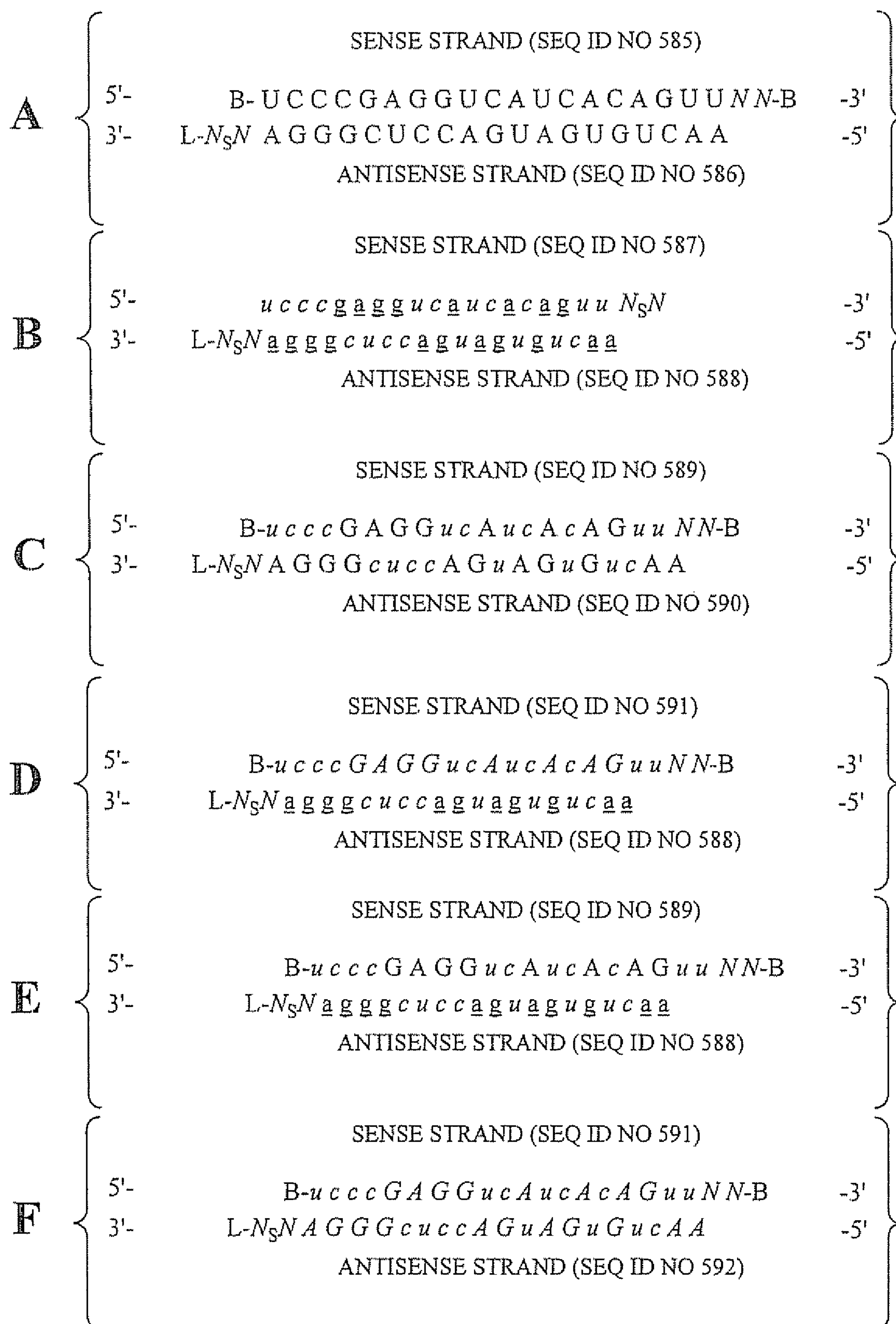


Figure 4

POSITIONS (NN) CAN COMPRISE ANY NUCLEOTIDE, SUCH AS DEOXYNUCLEOTIDES (eg. THYMIDINE), 2'-O-METHYL, 2'-DEOXY-2'-FLUORO, OR UNIVERSAL BASES
B = ABASIC, INVERTED ABASIC, INVERTED NUCLEOTIDE OR OTHER TERMINAL CAP THAT IS OPTIONALLY PRESENT
L = GLYCERYL or B THAT IS OPTIONALLY PRESENT
S = PHOSPHOROTHIOATE OR PHOSPHORODITHIOATE that is optionally absent

Figure 5*italic lower case* = 2'-deoxy-2'-fluoro or 2'-OCF₃underline = 2'-O-methyl*ITALIC UPPER CASE* = DEOXY*N* = Deoxy, 2'-OMe, 2'-deoxy-2'-fluoro, LNA etc.B = ABASIC, INVERTED ABASIC, INVERTED
NUCLEOTIDE OR OTHER TERMINAL CAP THAT
IS OPTIONALLY PRESENT

L = GLYCERYL MOIETY or B OPTIONALLY PRESENT

S = PHOSPHOROTHIOATE OR
PHOSPHORODITHIOATE OPTIONALLY PRESENT

Figure 6A

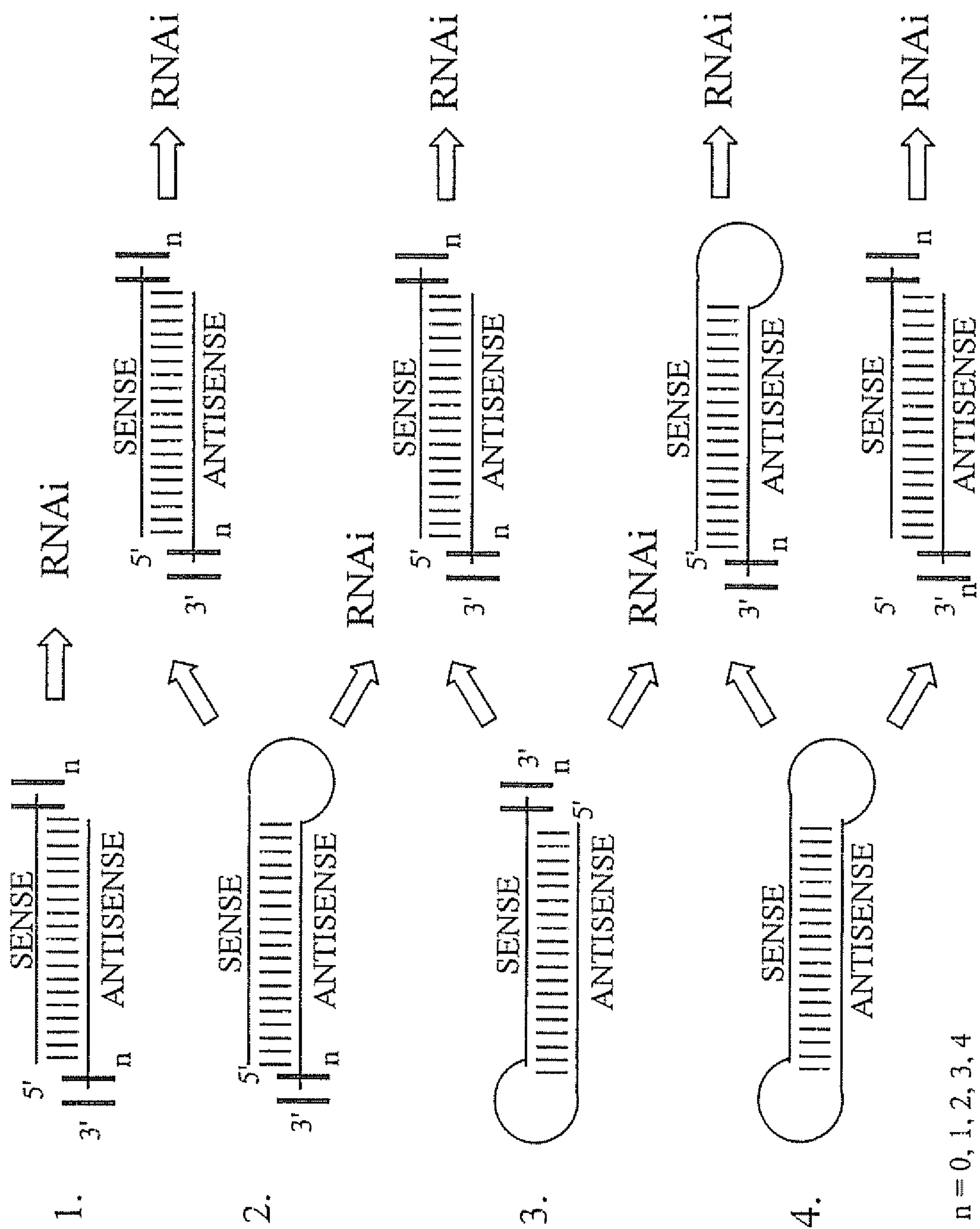
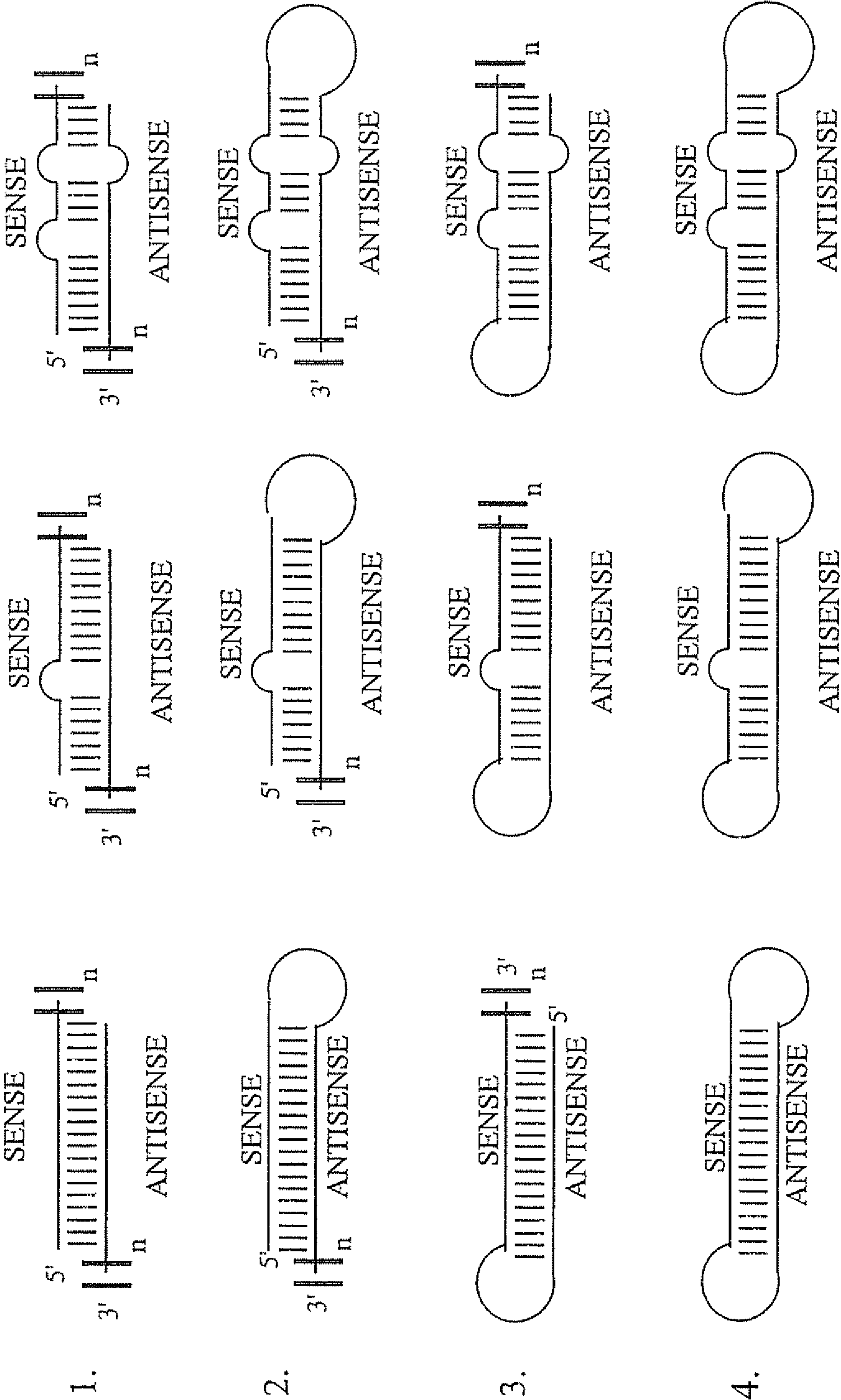
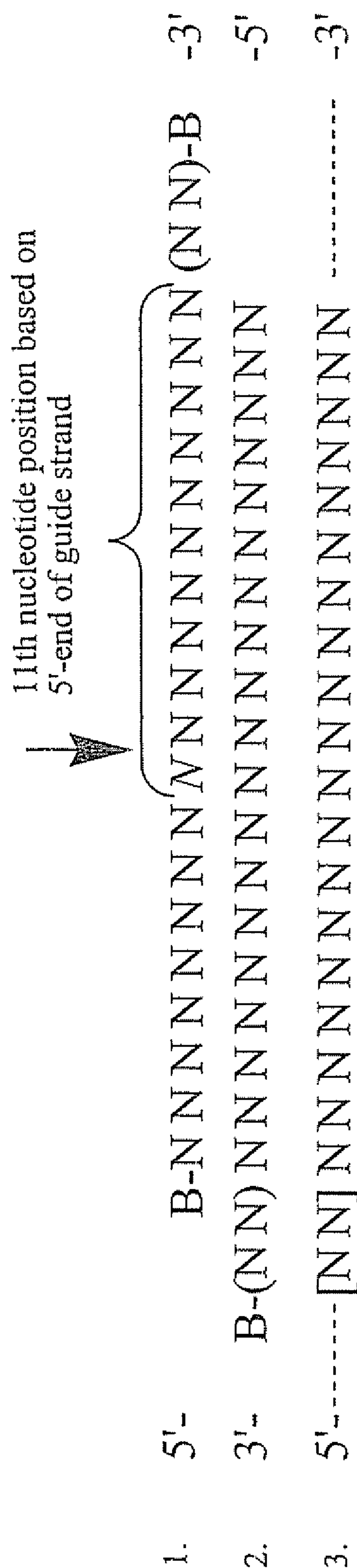


Figure 6B



n = 0, 1, 2, 3, 4

Figure 6C



1. = sense strand (passenger strand)
2. = antisense strand (guide strand)
3. = target polynucleotide sequence

The guide strand is complementary to the target sequence and the passenger strand is complementary to the guide strand. Overhang nucleotides (NN) in the guide strand can be complementary to nucleotides [NN] in target sequence. Overhang nucleotides (NN) in the passenger strand can comprise nucleotides [NN] in target sequence. Position *N* of the passenger strand can comprise a ribonucleotide. For the representative 19 base pair 21 mer duplex shown, position *N* is 9 nucleotides in from the 3' end of the passenger strand. However, in duplexes of differing length, the position *N* is determined based on the 5'-end of the guide strand by counting 11 nucleotide positions in from the 5'-terminus of the guide strand and picking the corresponding base paired nucleotide in the passenger strand. Cleavage by Ago2 takes place between positions 10 and 11 as indicated by the arrow.

Representative 2 nucleotide overhangs are shown, but can vary for example from 0 to about 4 nucleotides.

$B =$ terminal cap which can be present or absent

This generalized motif can be applied to all Stab 00-34 chemistries herein.

Figure 7

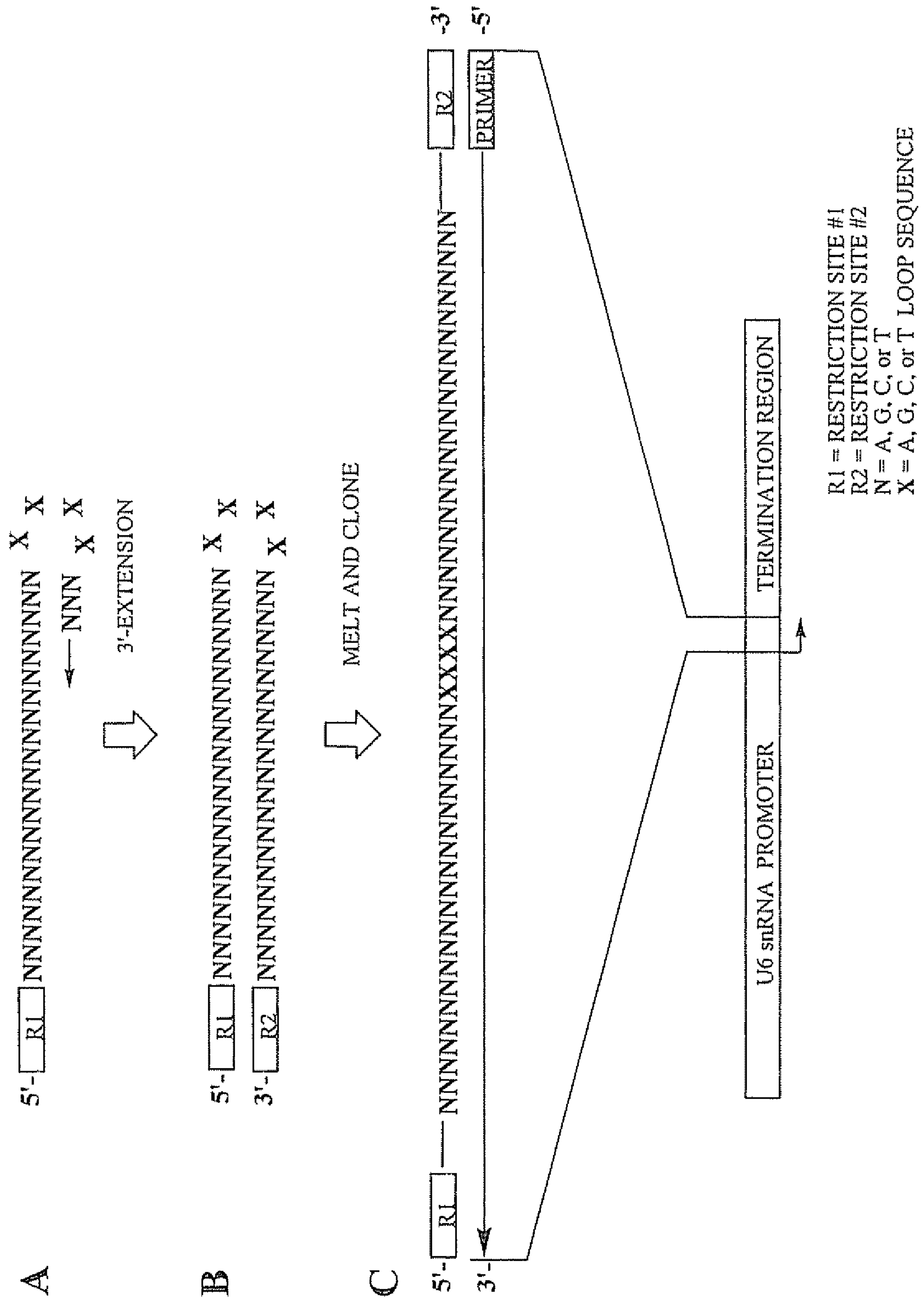


Figure 9: Target site Selection using siRNA

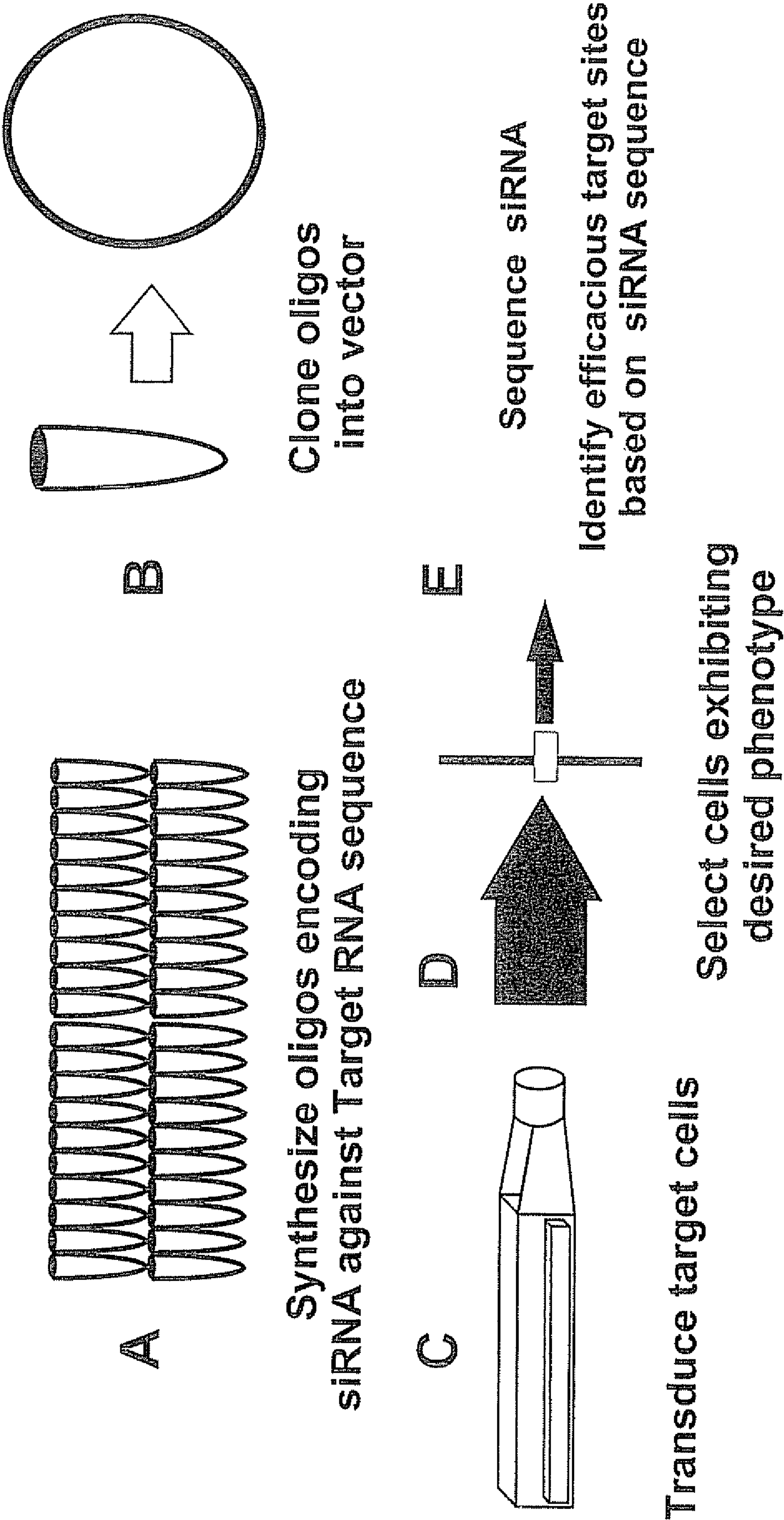
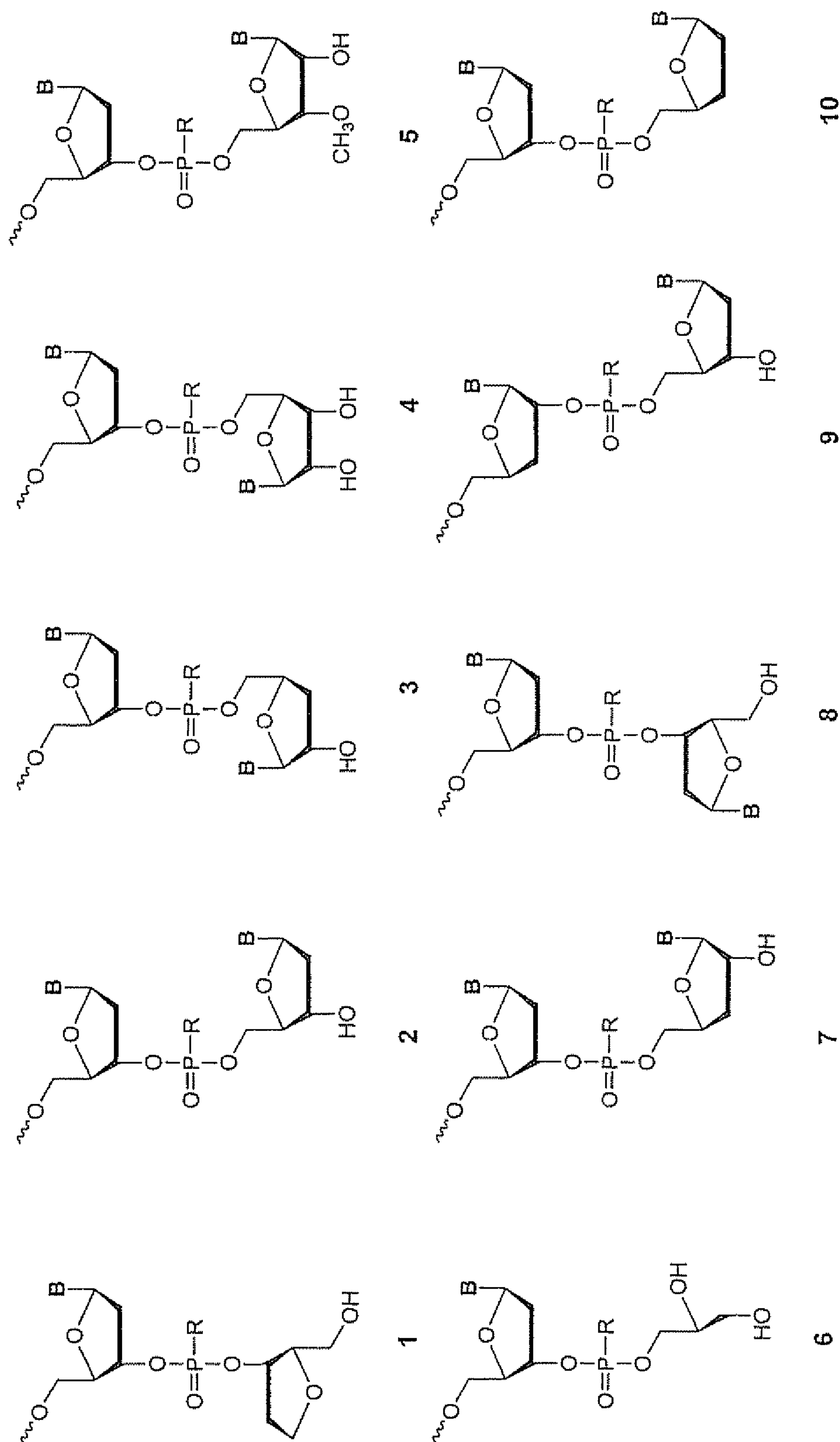


Figure 10

R = O, S, N, alkyl, substituted alkyl, O-alkyl, S-alkyl, alkaryl, or aralkyl
 B = Independently any nucleotide base, either naturally occurring or chemically modified, or optionally H (abasic).

Figure 11: Modification Strategy

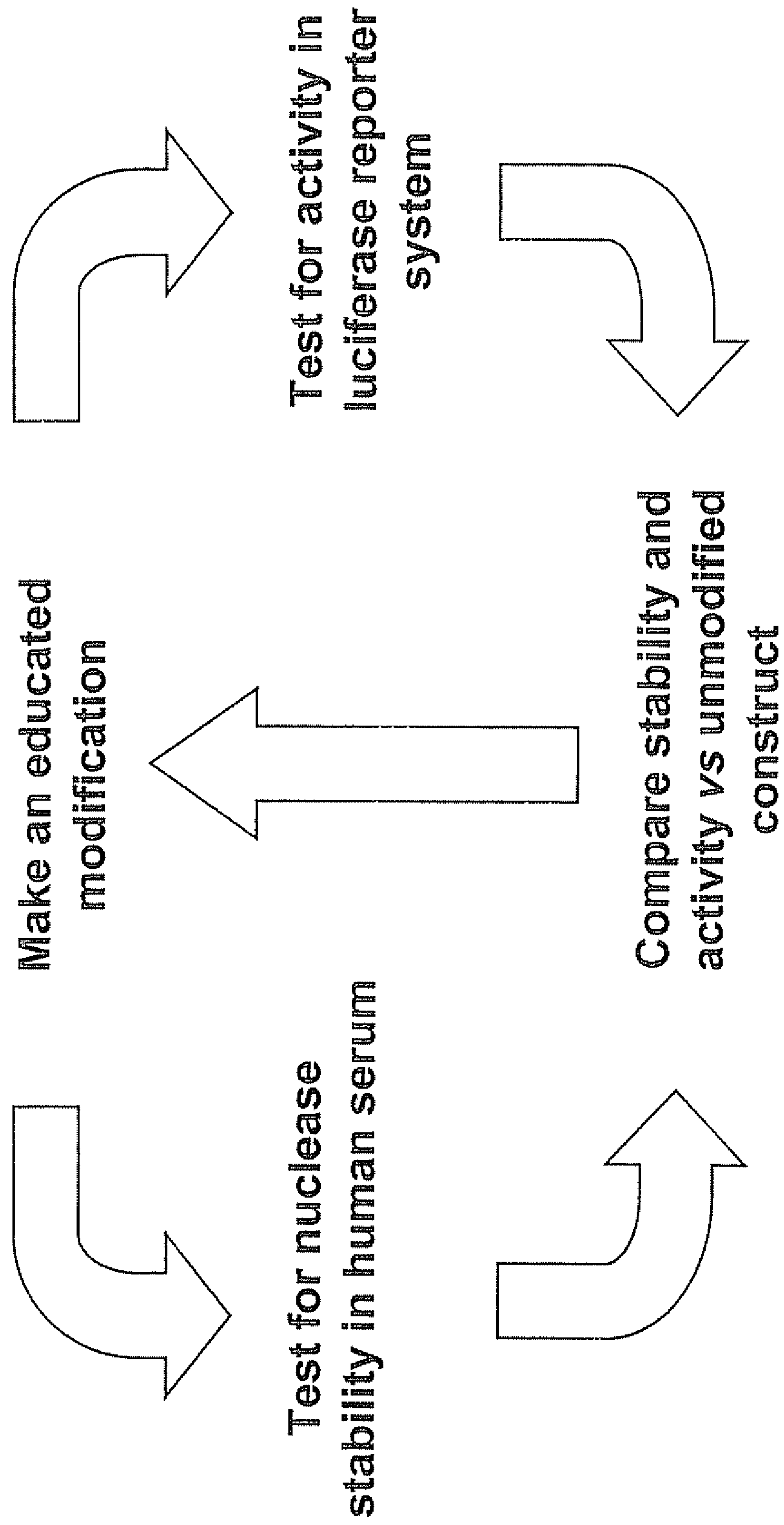


Figure 12: Phosphorylated siNA constructs

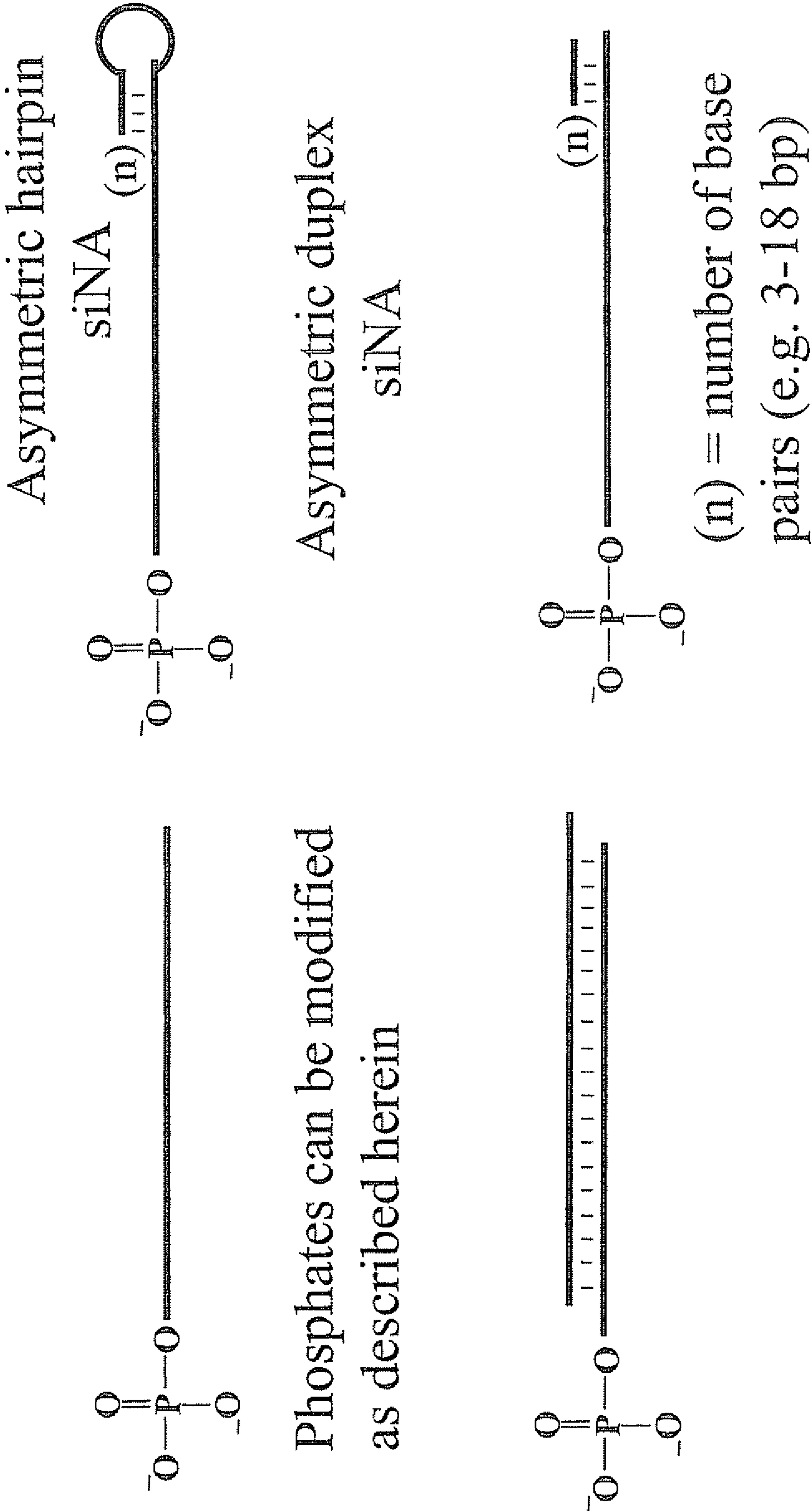


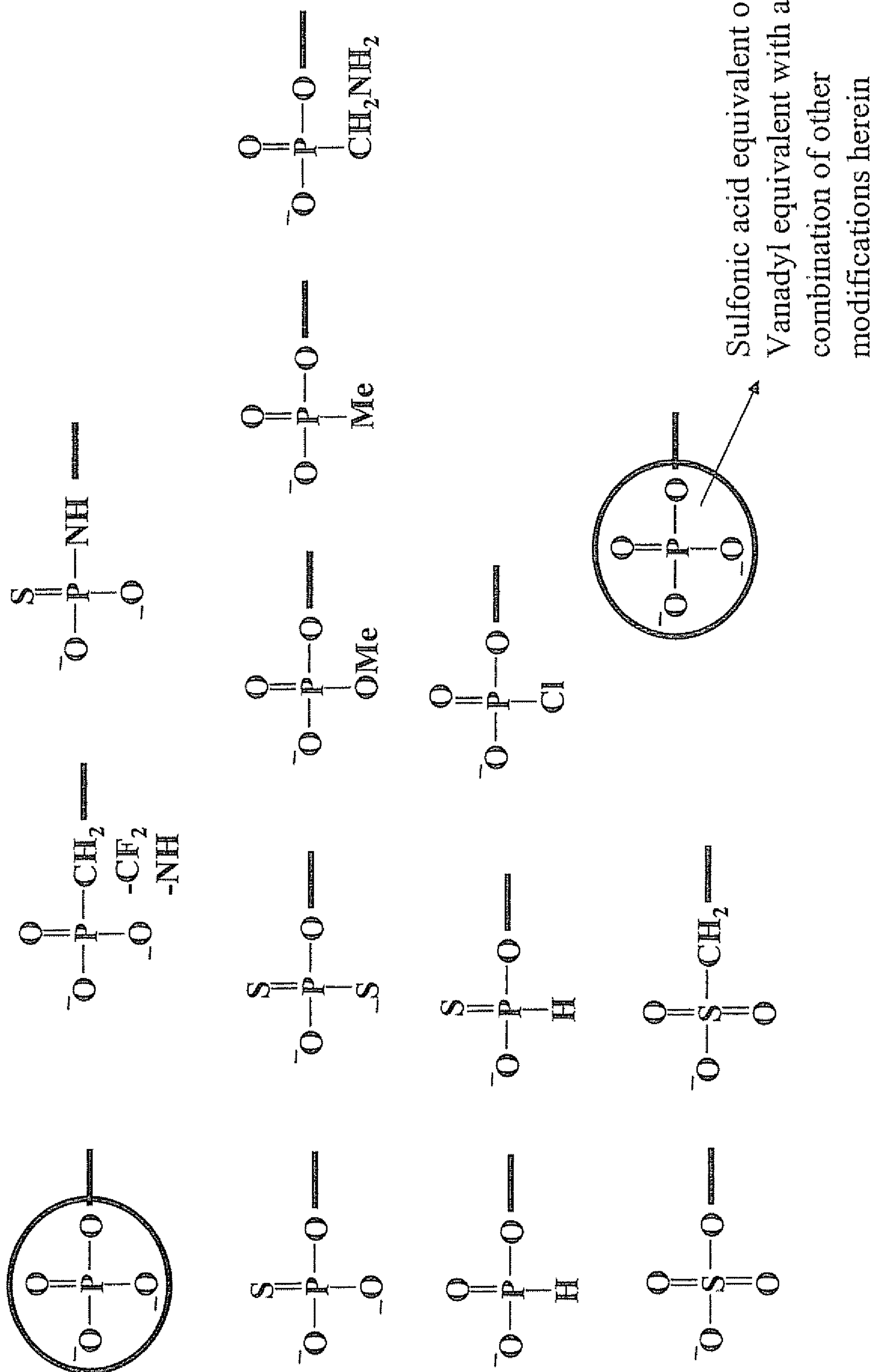
Figure 13: 5'-phosphate modifications

Figure 14A: Duplex forming oligonucleotide constructs that utilize Palindrome or repeat sequences

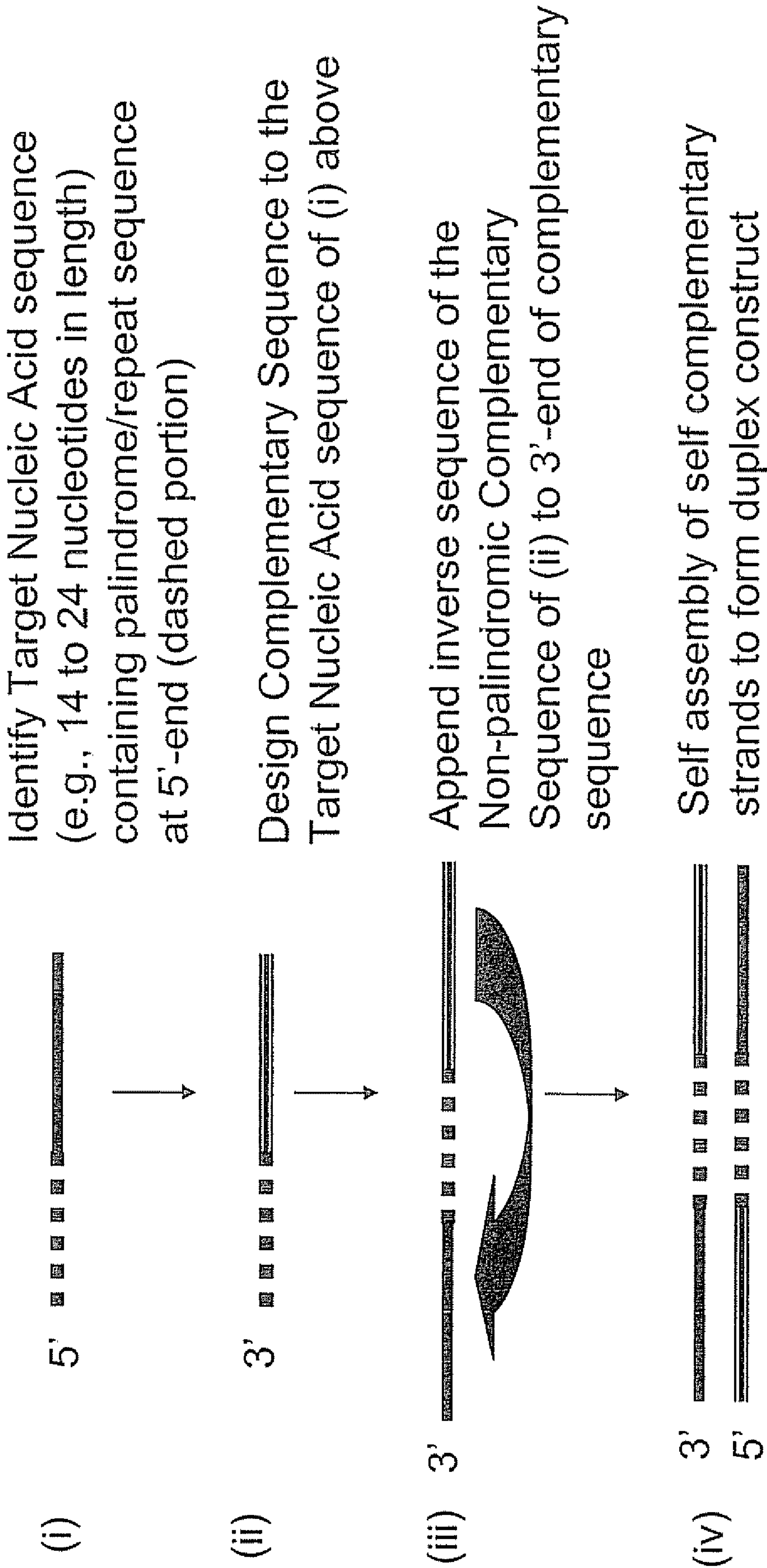


Figure 14B: Example of a duplex forming oligonucleotide sequence that utilizes a palindrome or repeat sequence

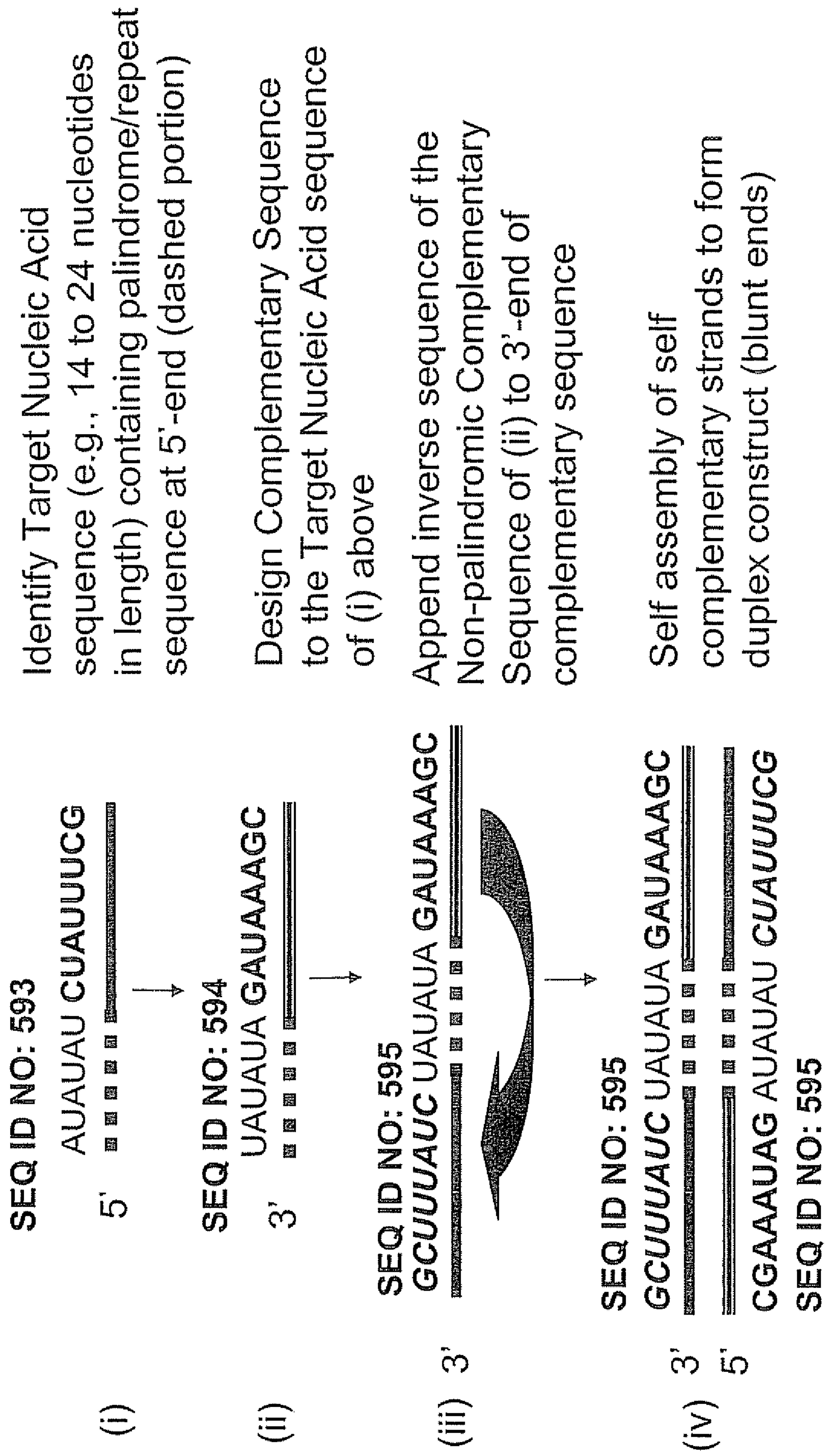


Figure 14C: Example of a duplex forming oligonucleotide sequence that utilizes a palindrome or repeat sequence, self assembly

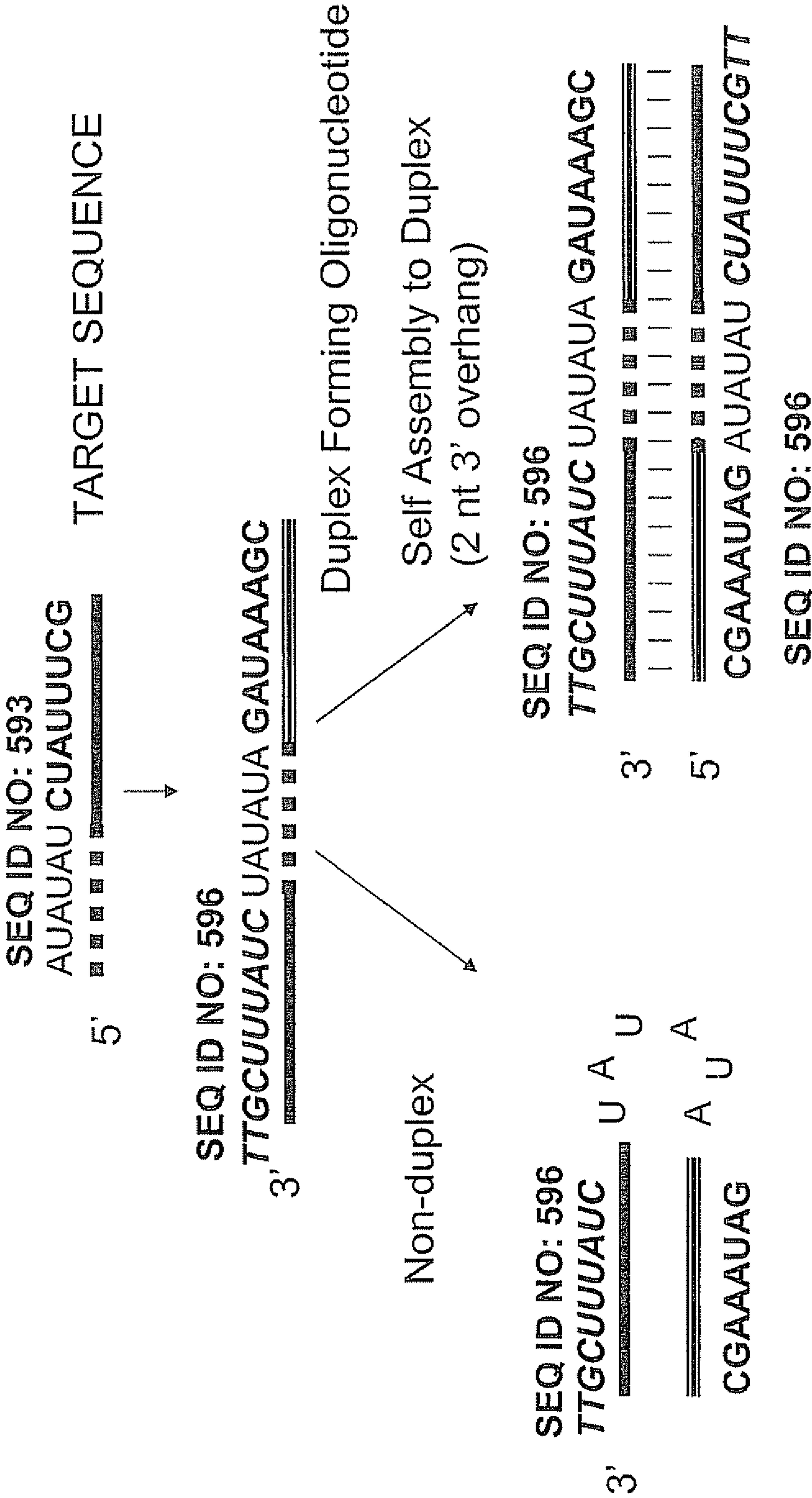


Figure 14D: Example of a duplex forming oligonucleotide sequence that utilizes a palindrome or repeat sequence, self assembly and inhibition of Target Sequence Expression

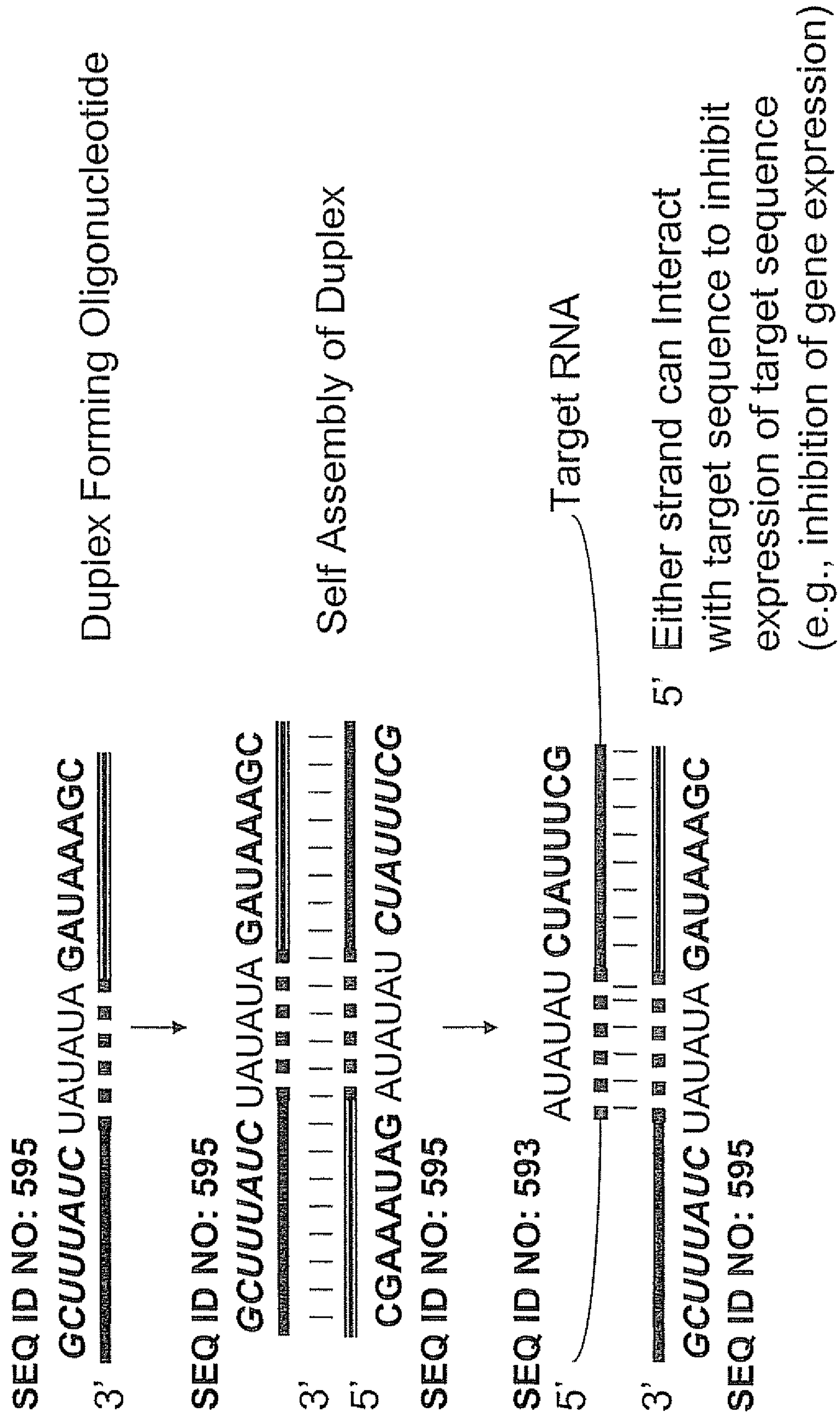


Figure 15: Duplex forming oligonucleotide constructs that utilize artificial palindrome or repeat sequences

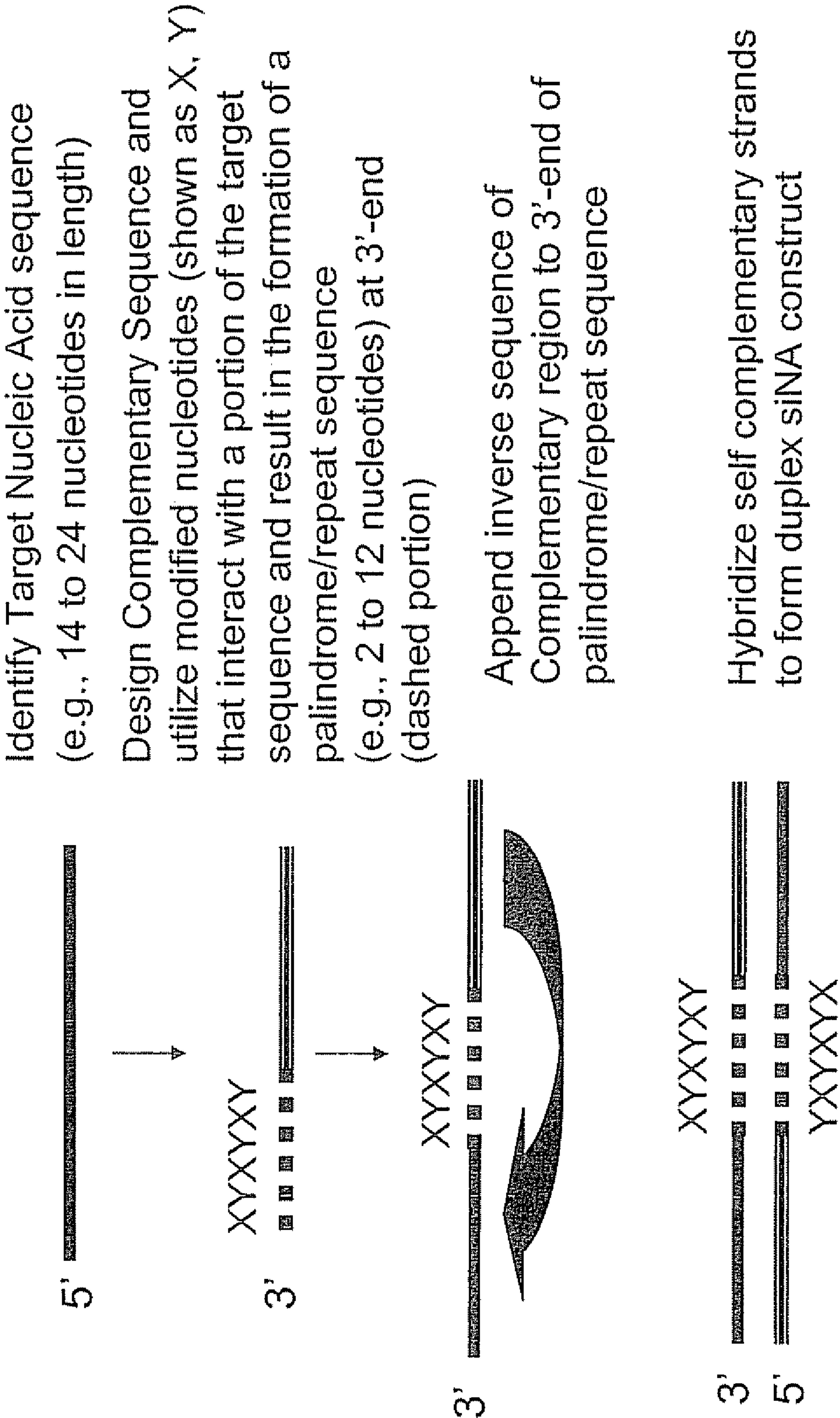


Figure 16: Examples of double stranded multifunctional siNA constructs with distinct complementary regions

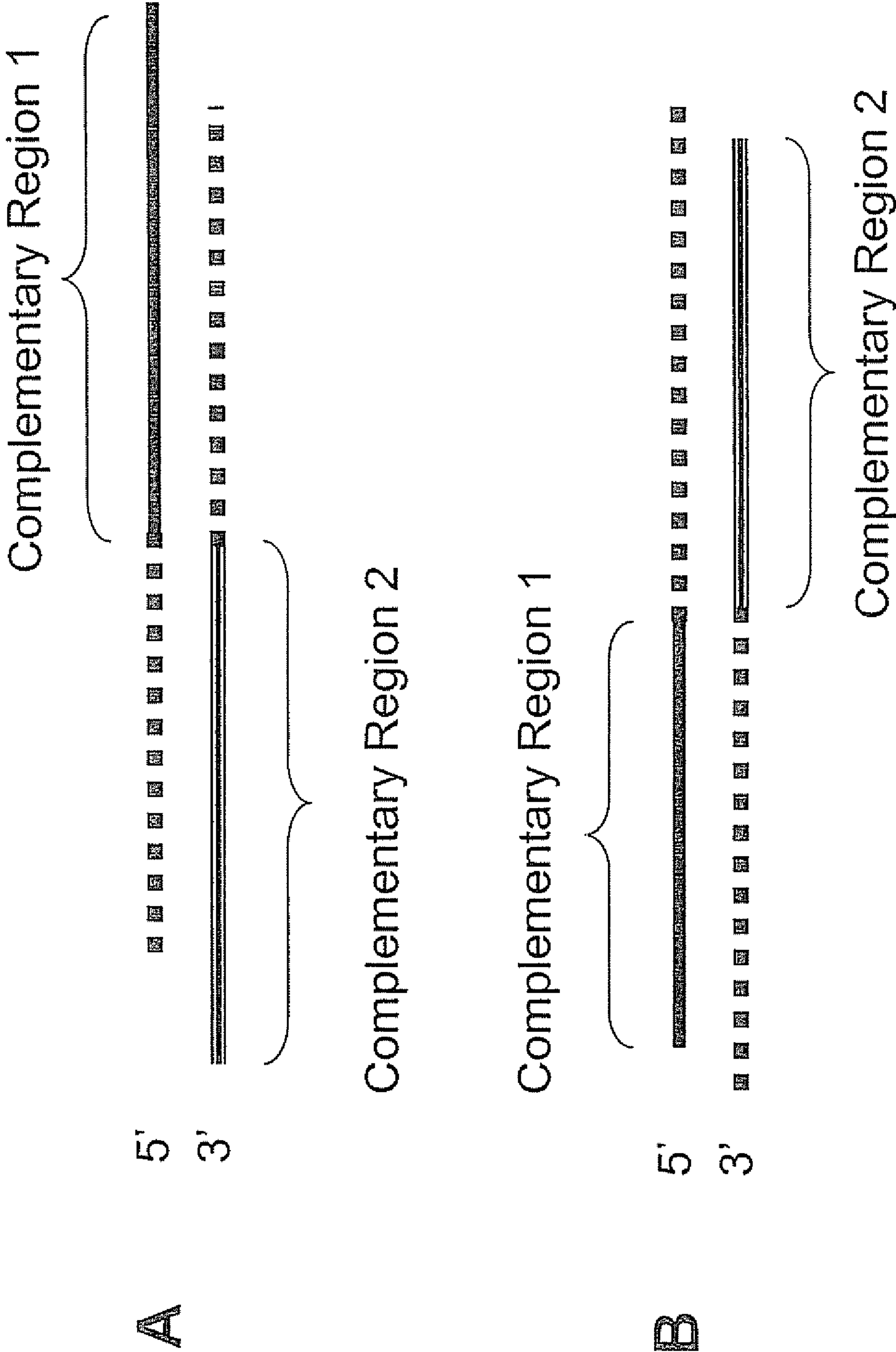


Figure 17: Examples of hairpin multifunctional siNA constructs with distinct complementary regions

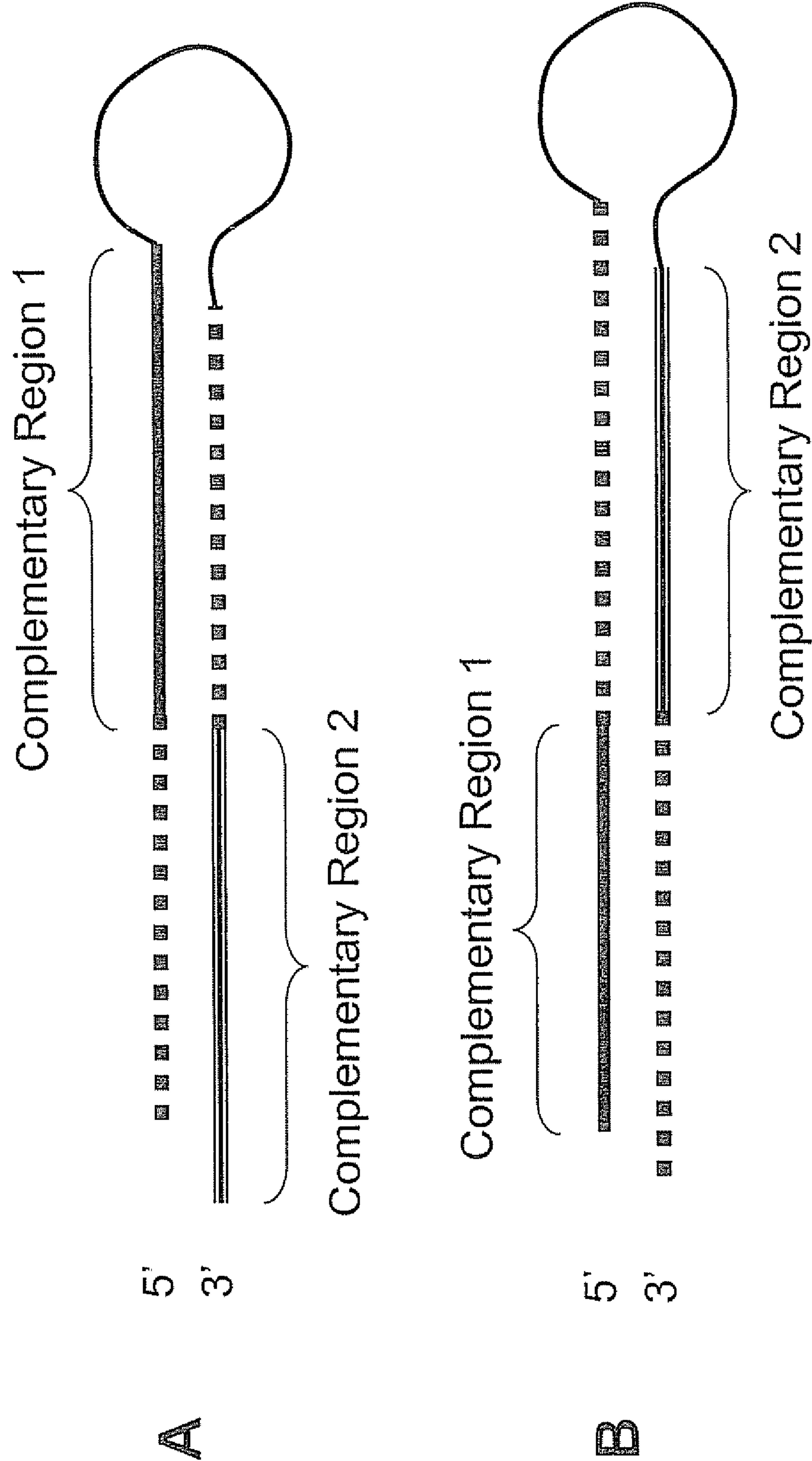


Figure 18: Examples of double stranded multifunctional siNA constructs with distinct complementary regions and a self complementary/palindrome region

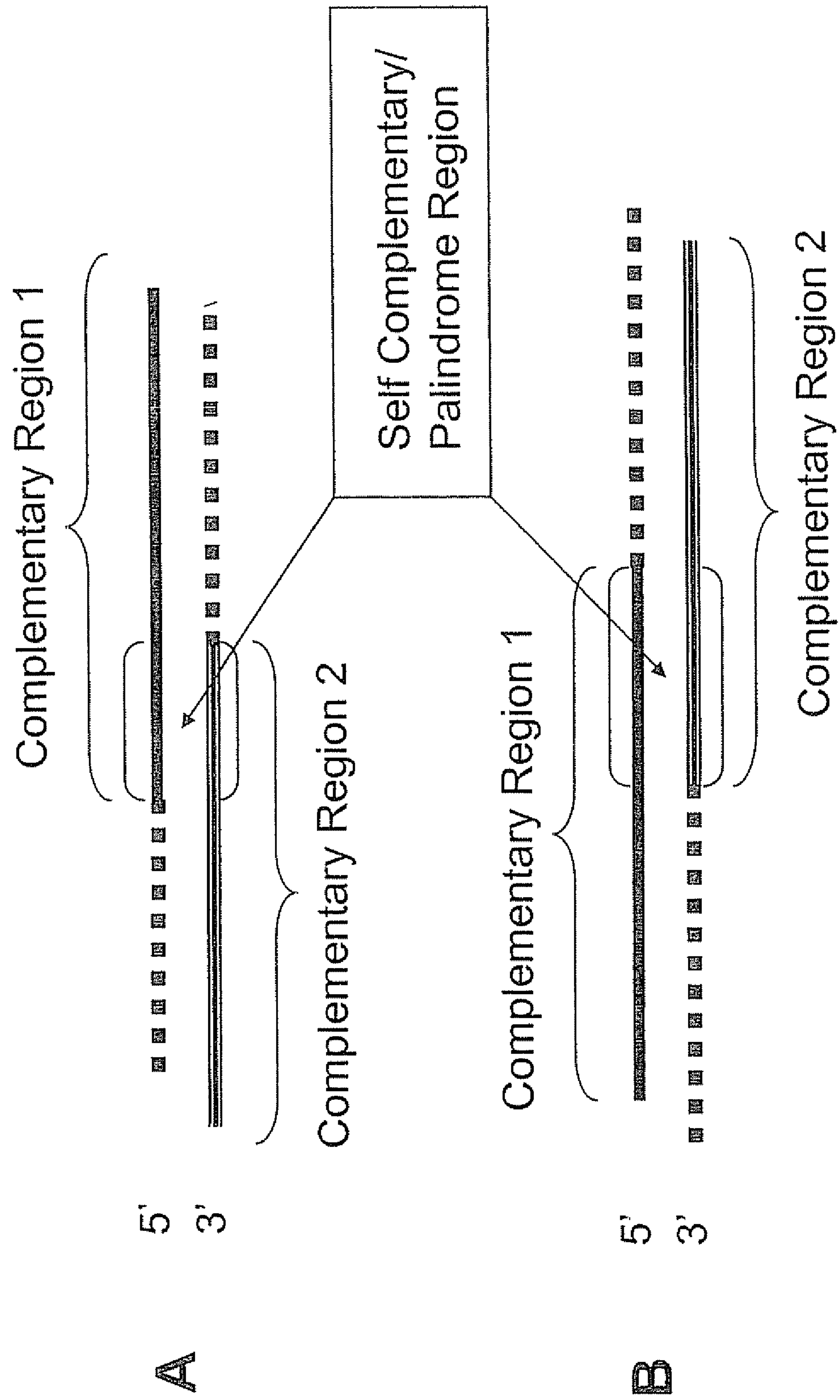


Figure 19: Examples of hairpin multifunctional siNA constructs with distinct complementary regions and a self complementary/palindrome region

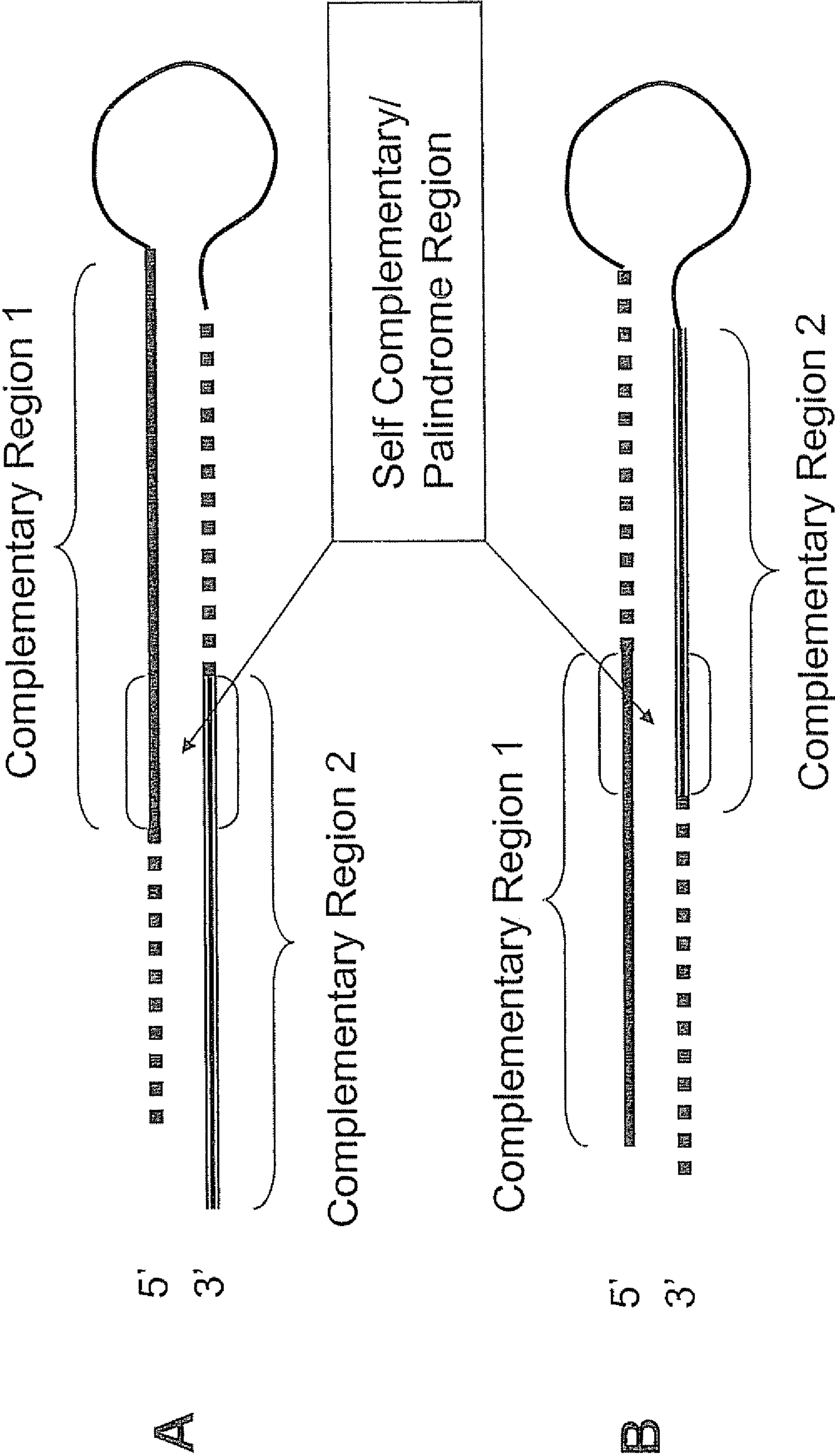


Figure 20: Example of multifunctional siRNA targeting two
Separate Target nucleic acid sequences

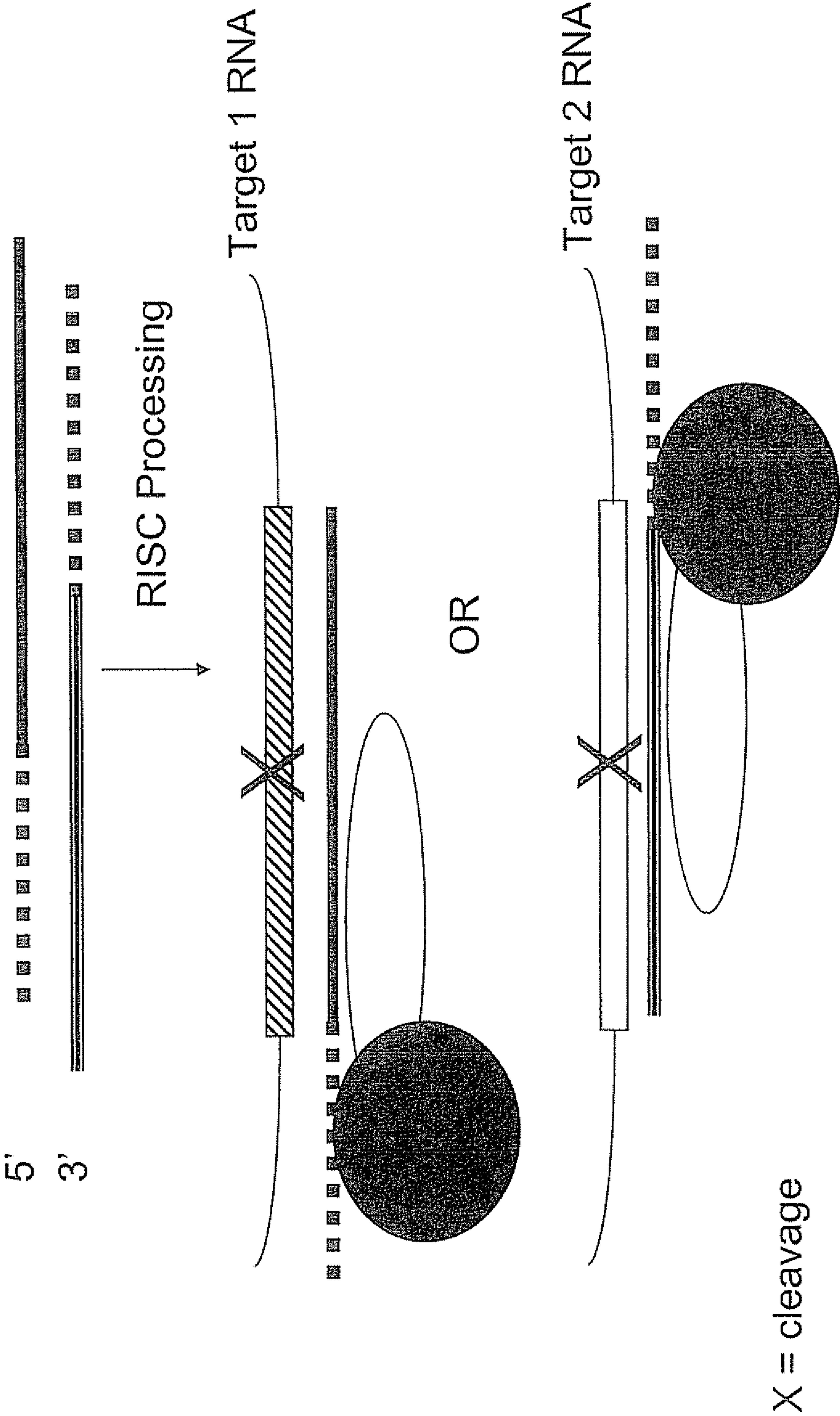


Figure 21: Example of multifunctional siNA targeting two regions within the same target nucleic acid sequence

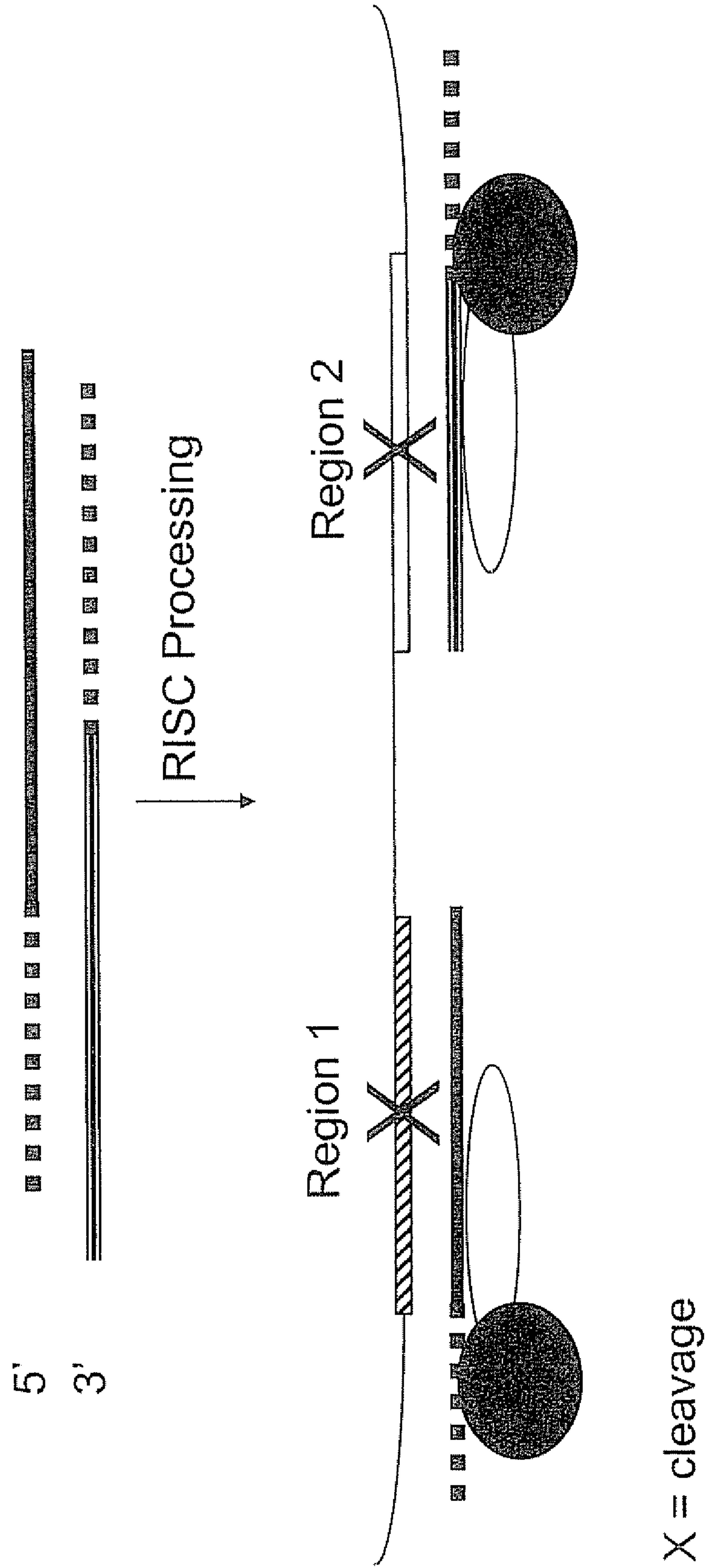
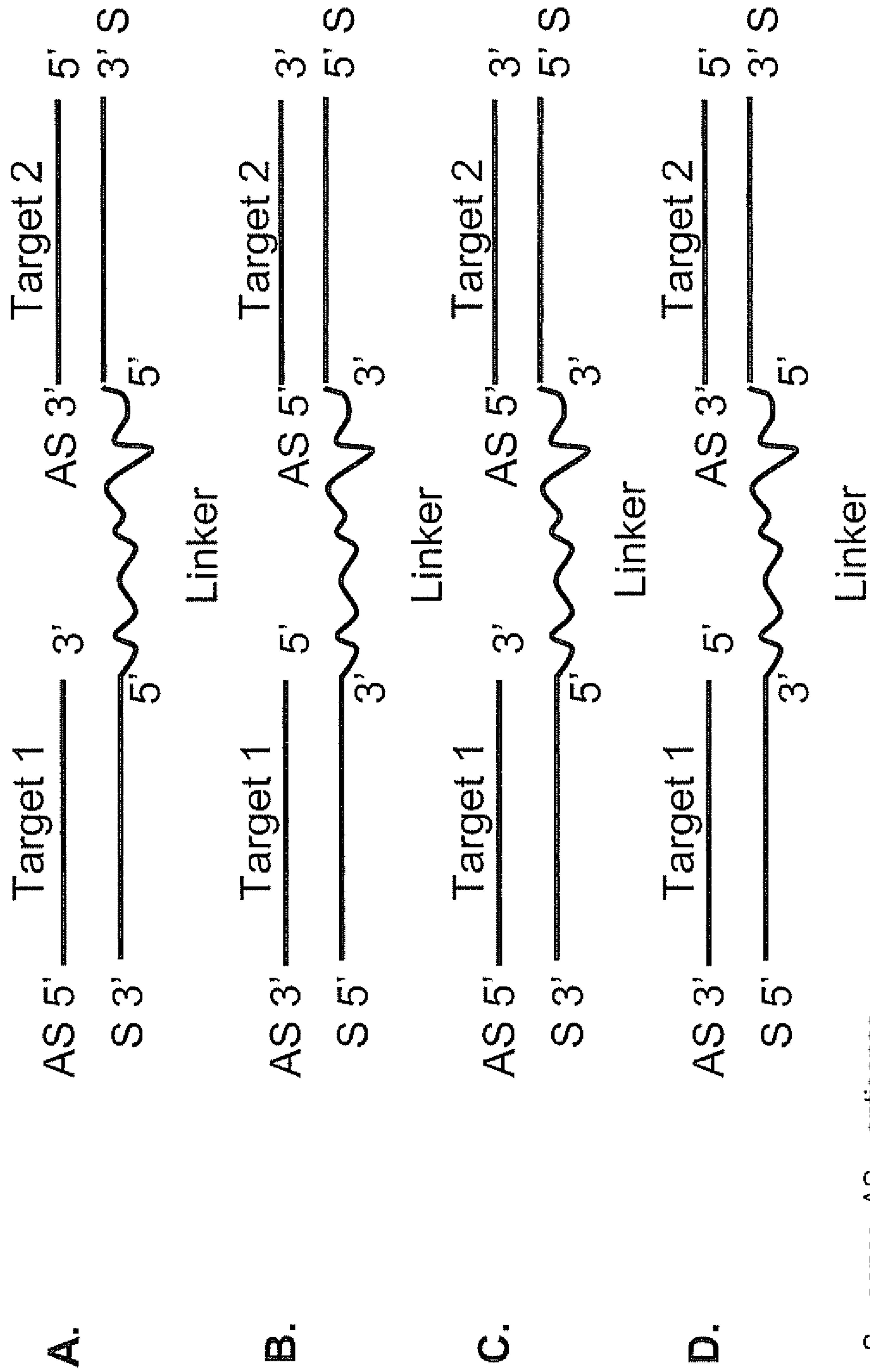
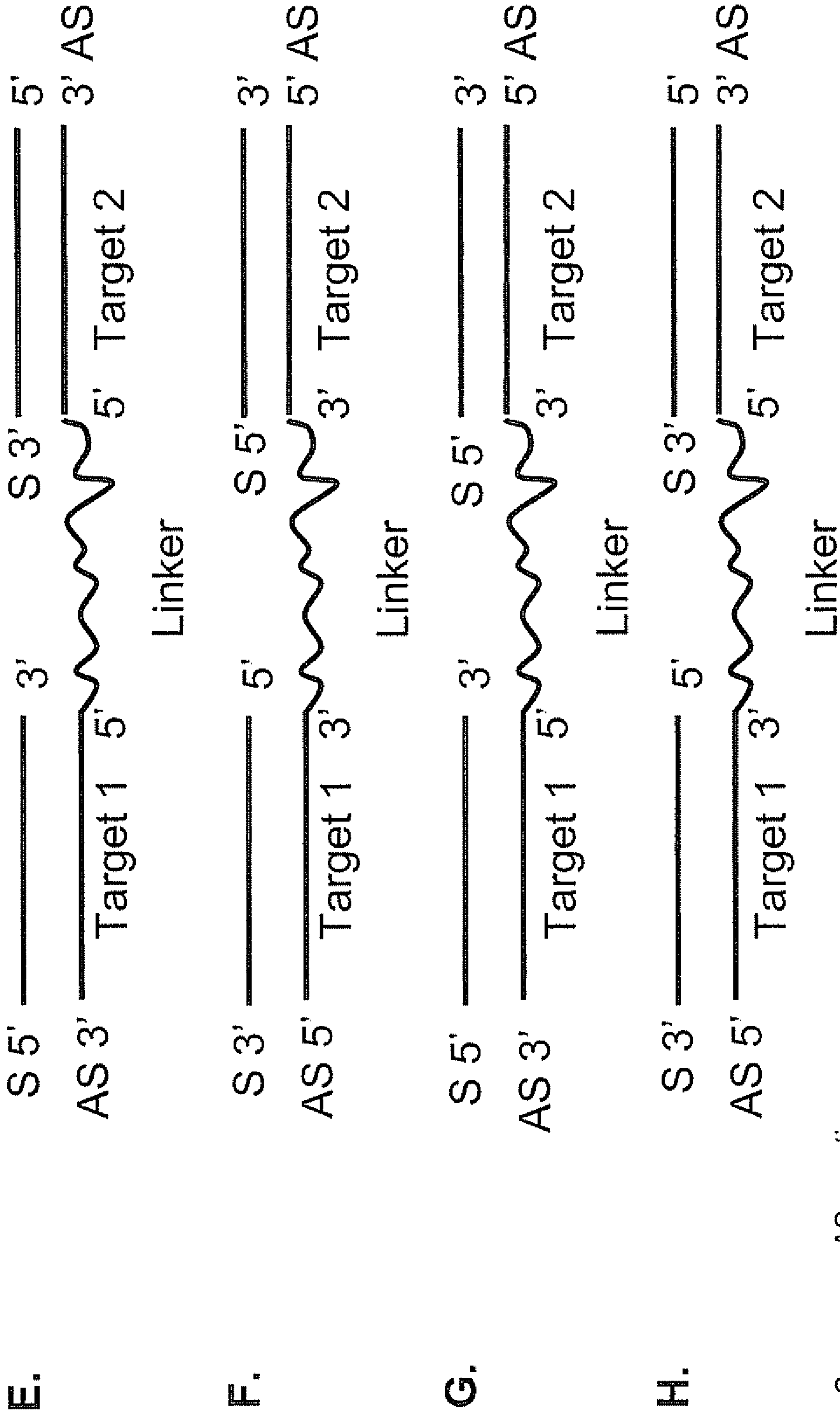


Figure 22: Tethered Multifunctional siNA design

S = sense, AS = antisense

Linker region can be nucleotide or non-nucleotide linker, and can optionally be decorated, for example with conjugates polymers or aptamers, such as for delivery purposes.

Figure 22: Tethered Multifunctional siNA design



S = sense, AS = antisense
Linker region can be nucleotide or non-nucleotide linker, and can optionally be decorated, for example with conjugates polymers or aptamers, such as for delivery purposes.

Figure 23: Dendrimer Multifunctional siNA designs

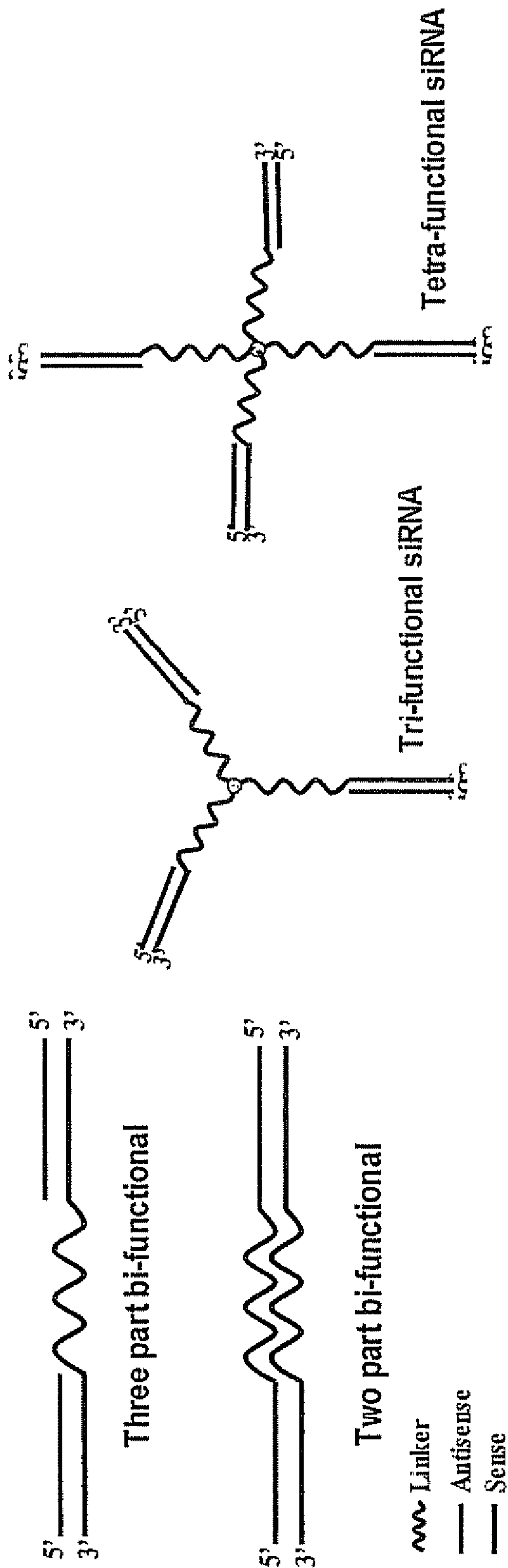
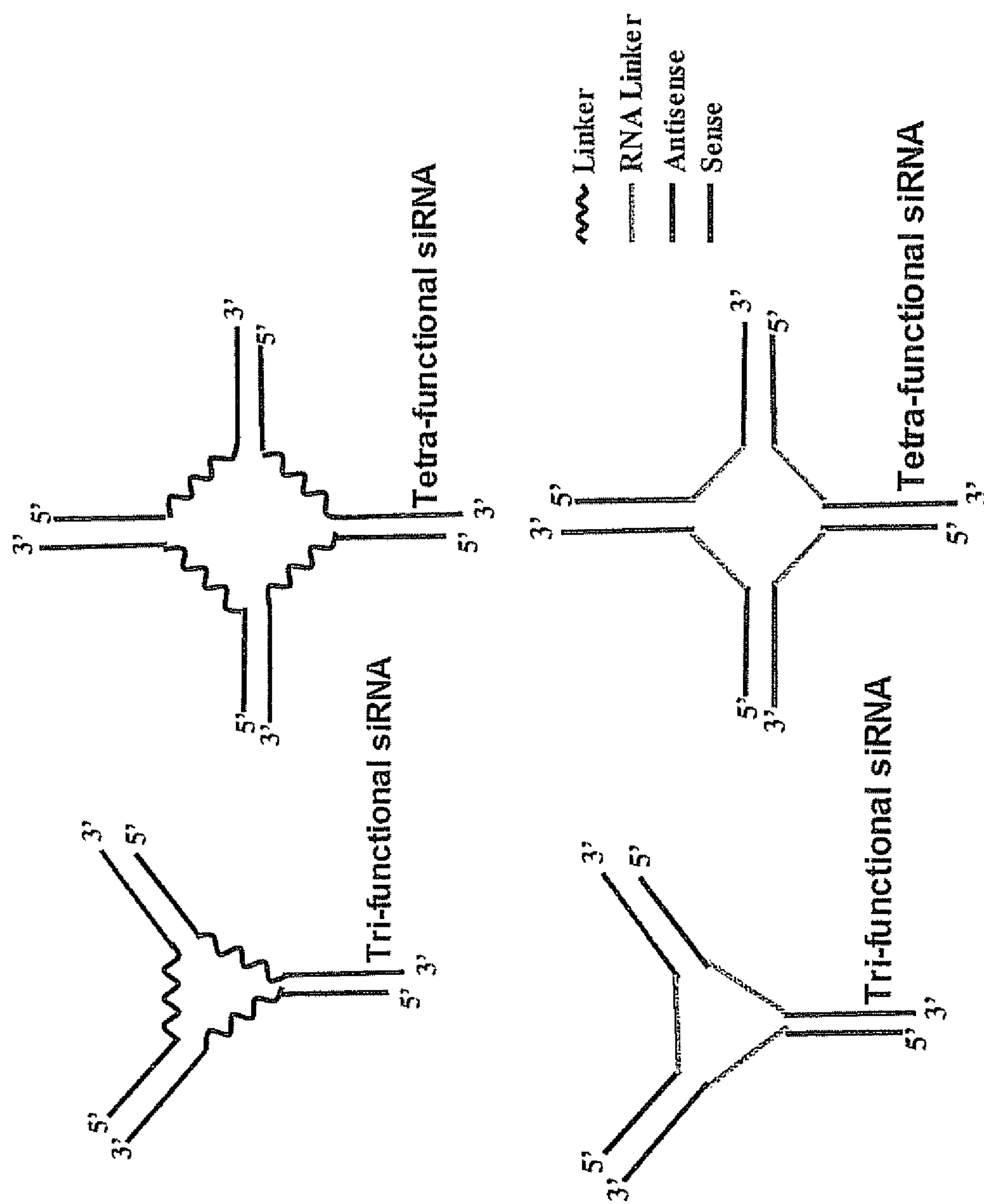


Figure 24: Supramolecular Multifunctional siRNA designs



30 base pair precursor

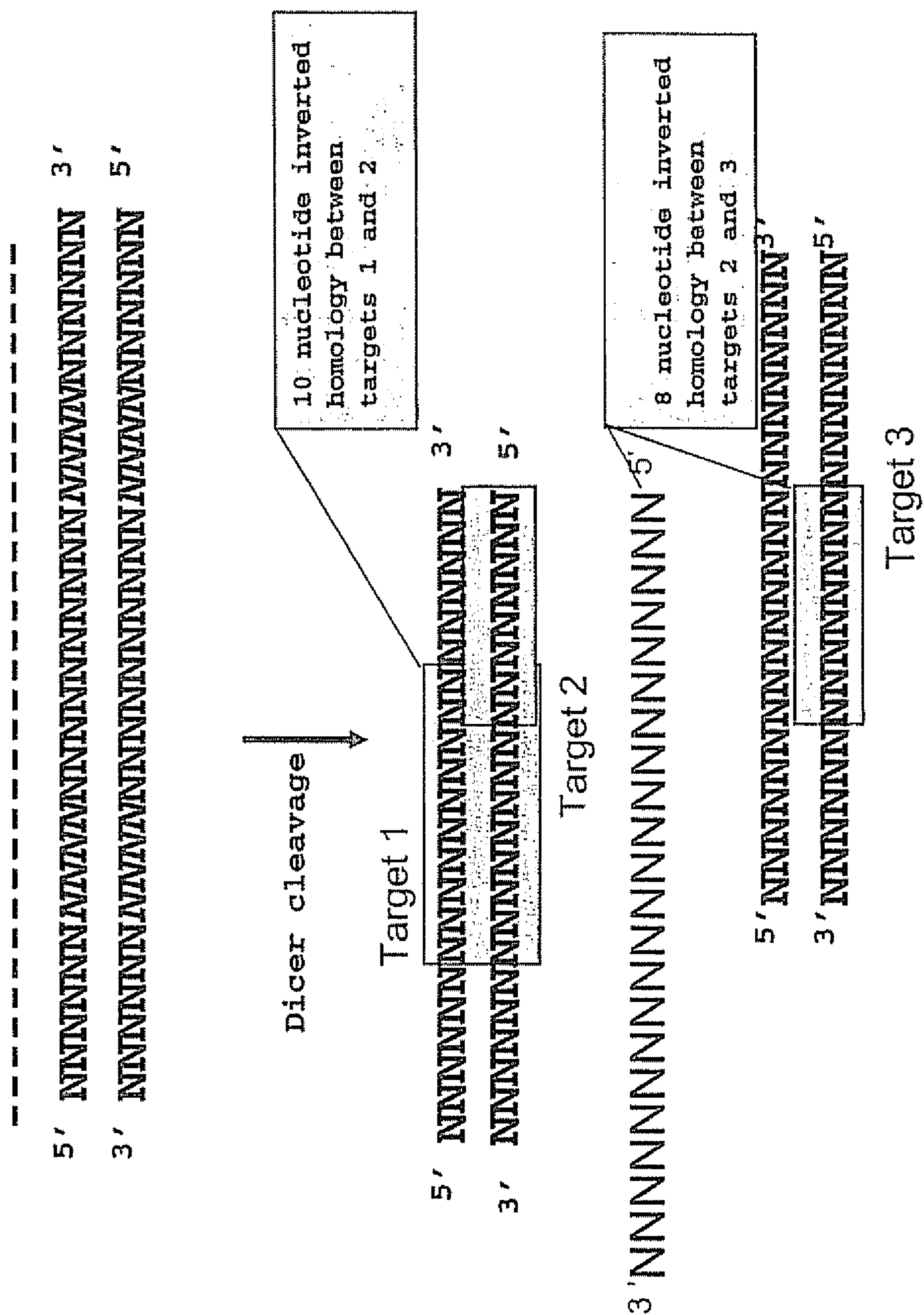


Figure 26: Dicer enabled multifunctional siNA design

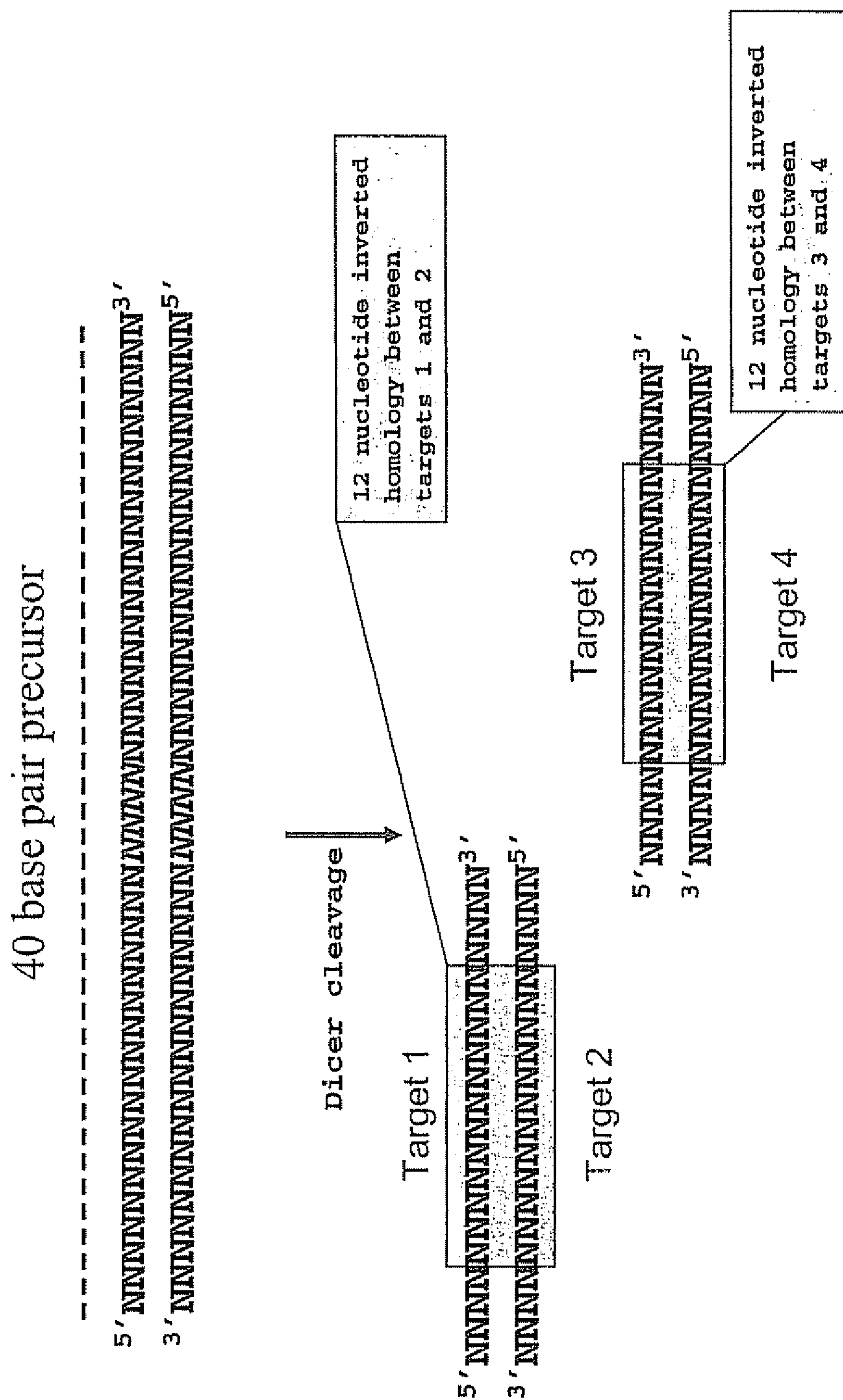
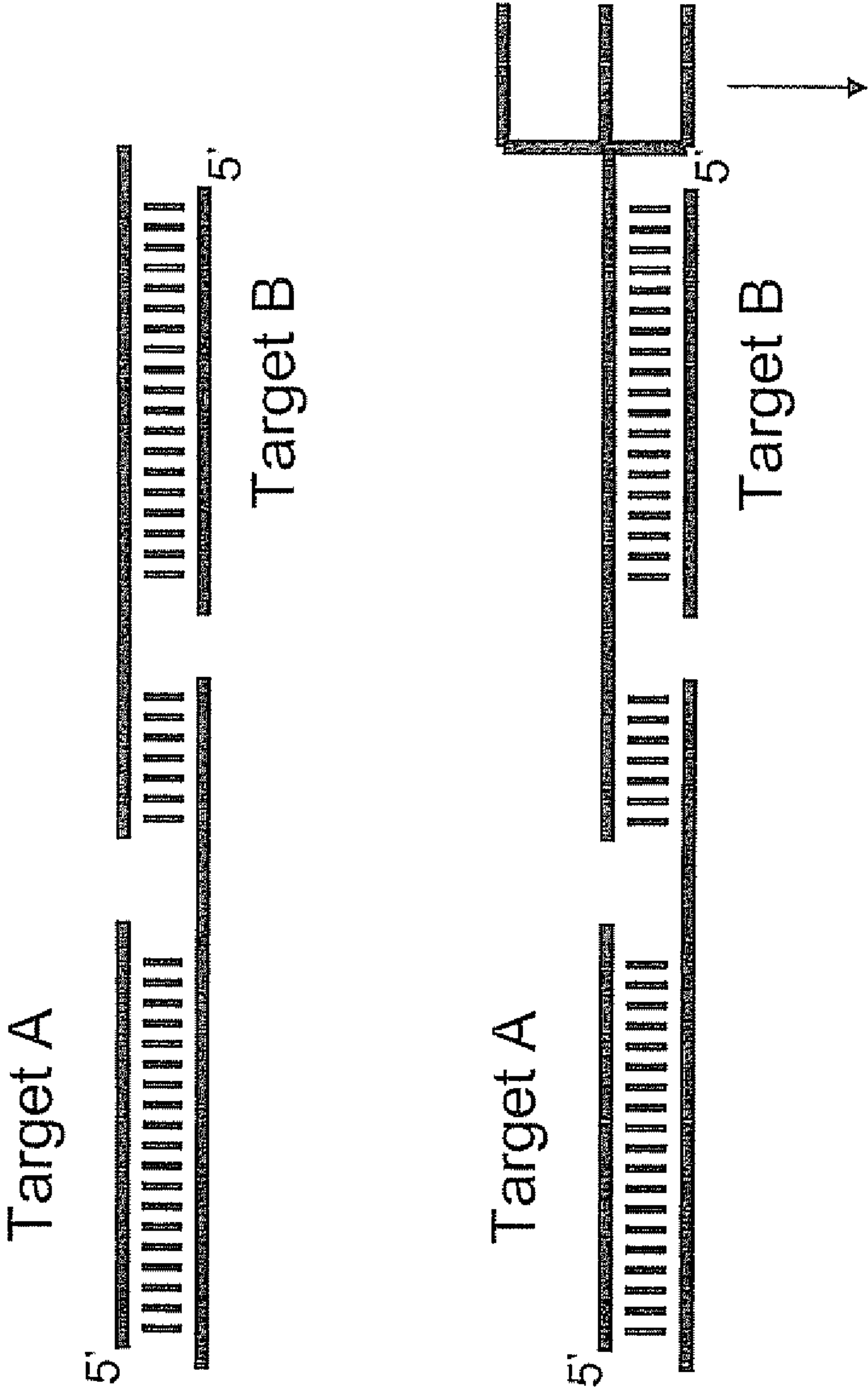


Figure 27: Additional Multifunctional siNA designs



Targeting Ligand/branched Ligand
e.g. Cholesterol, N-acetyl Galactosamine,
Lipid, Peptide, RGD etc.

Figure 28: Additional Multifunctional siNA designs

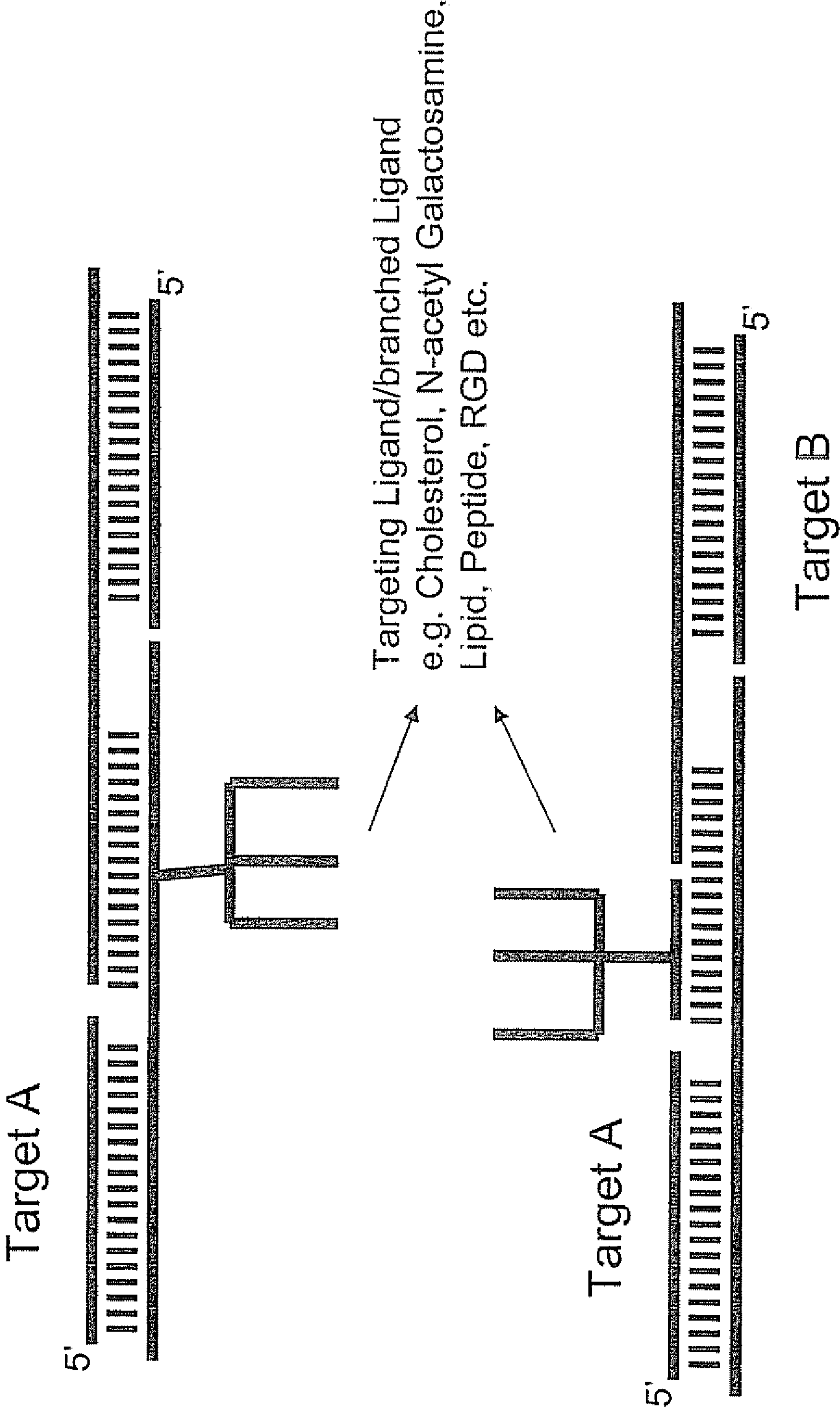


Figure 29: Cholesterol Conjugate Approach

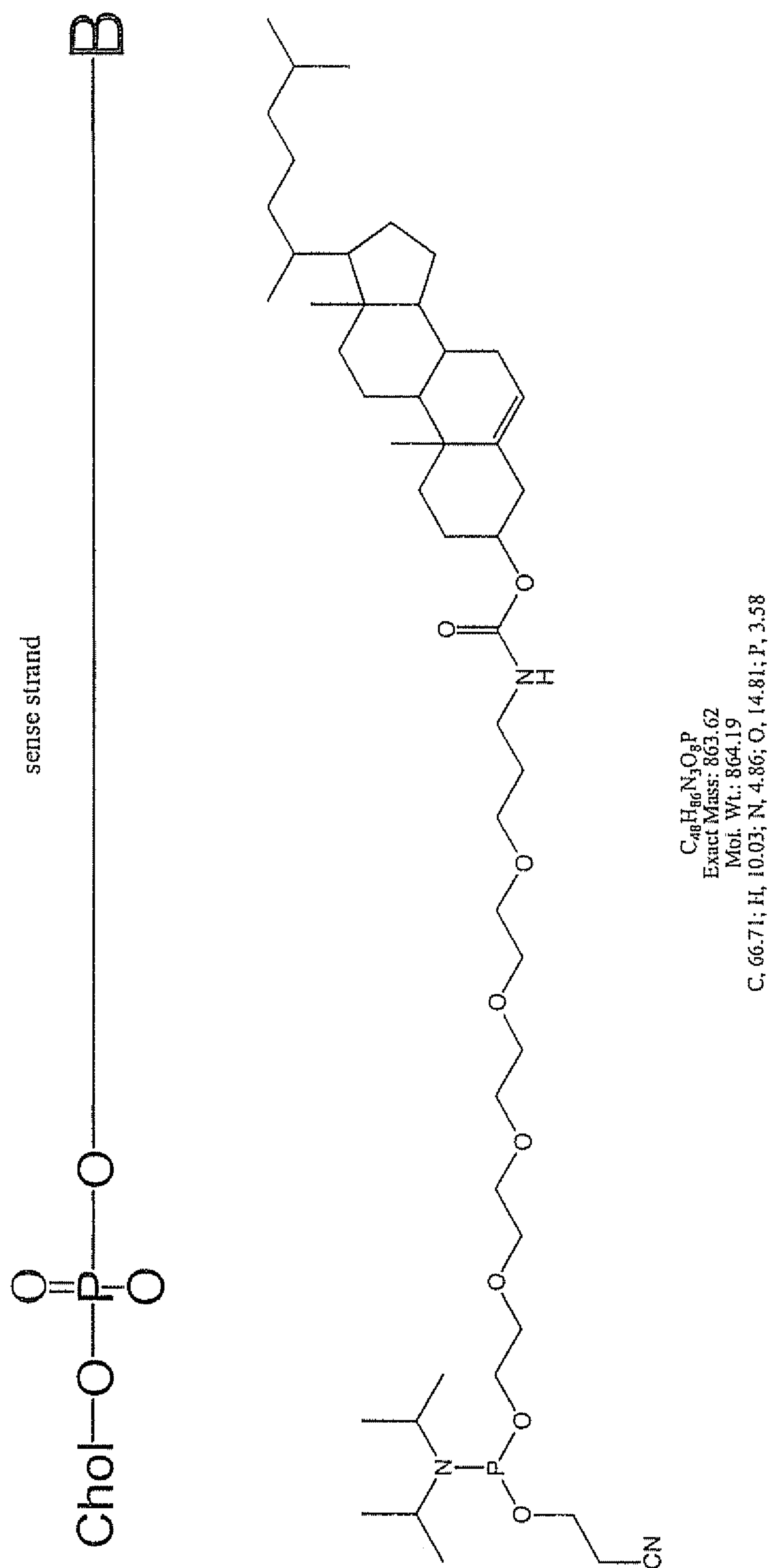


Figure 30 A-B: PCSK9 and LDLR Western Blot

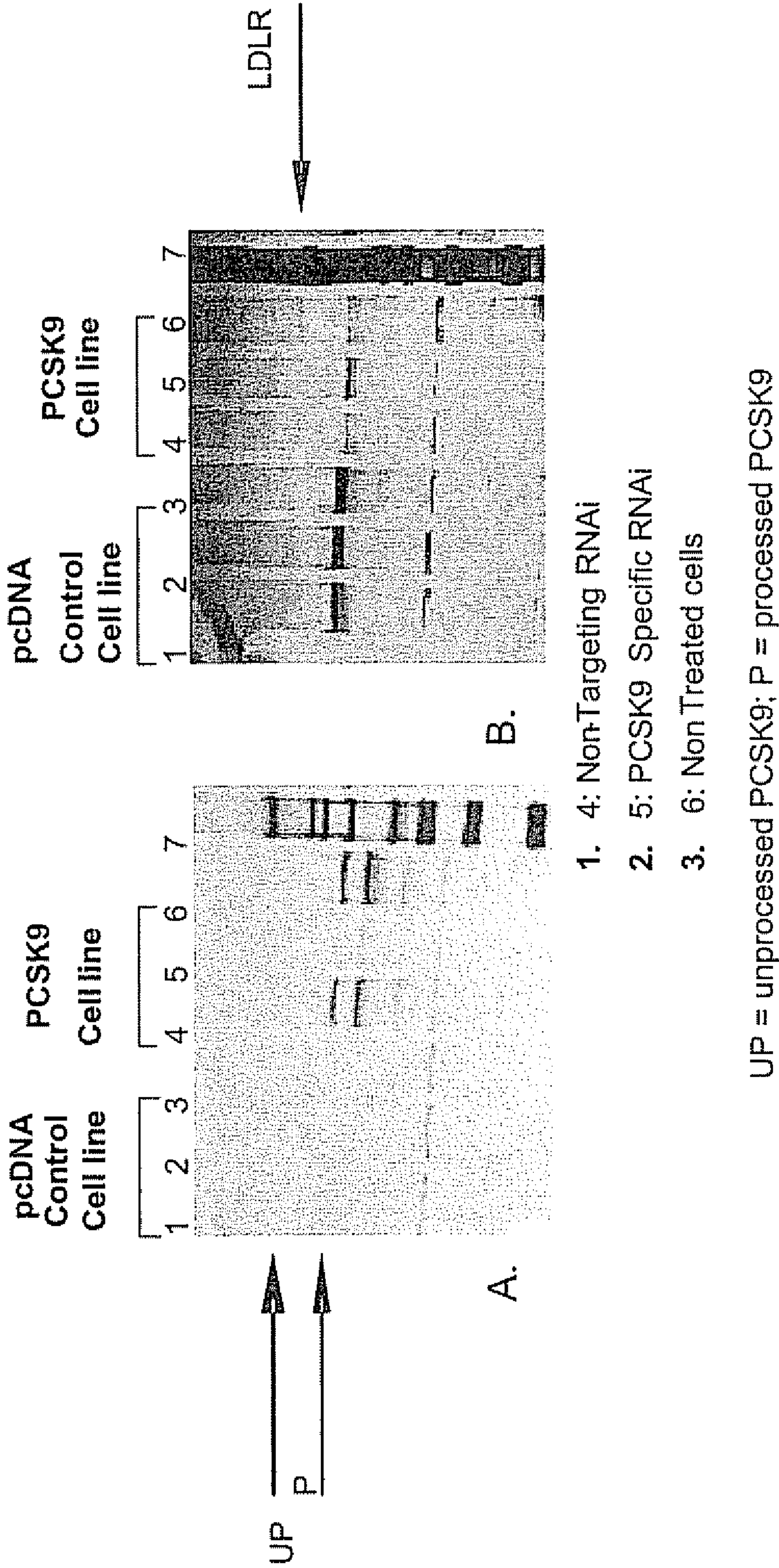


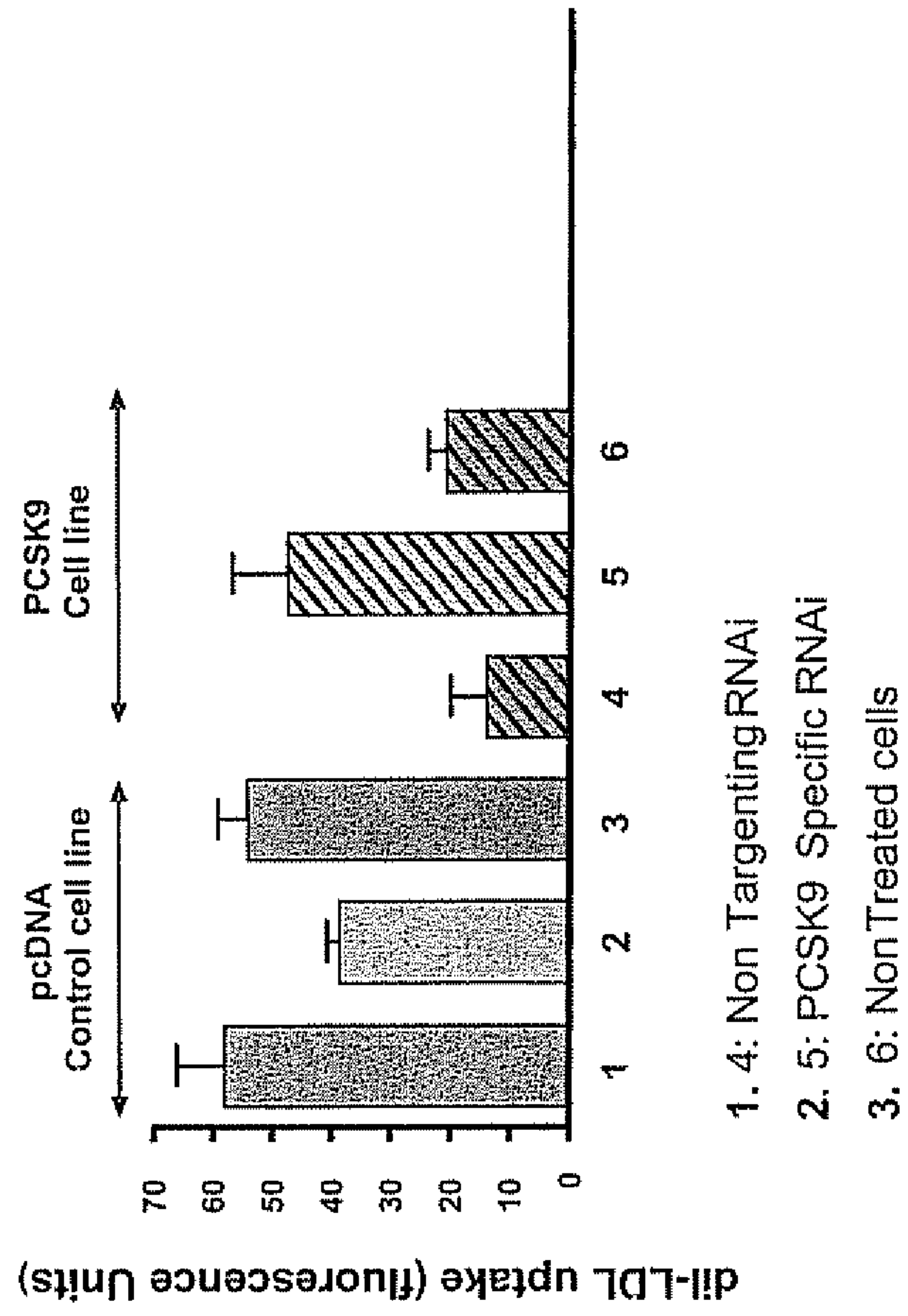
Figure 31: dil - LDL Uptake Assay

Figure 32. In vitro Screening of PCSK9 ShRNA Plasmids in Hepa1-6 Cells

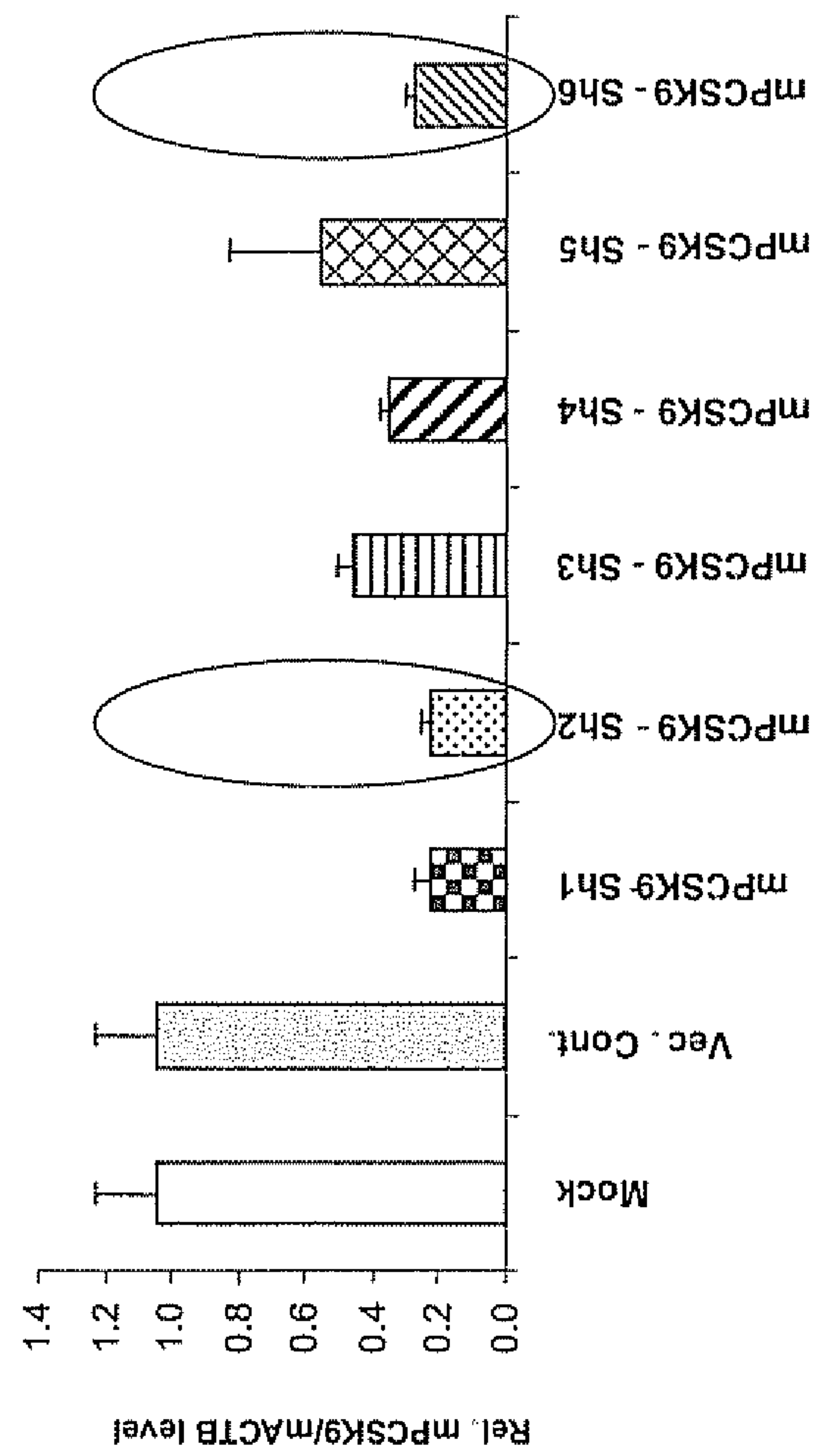


Figure 33. Confirmation of Ad-PCSK9 ShRNA Mediated Knockdown of Mouse PCSK9 in Hepa1-6 Cells

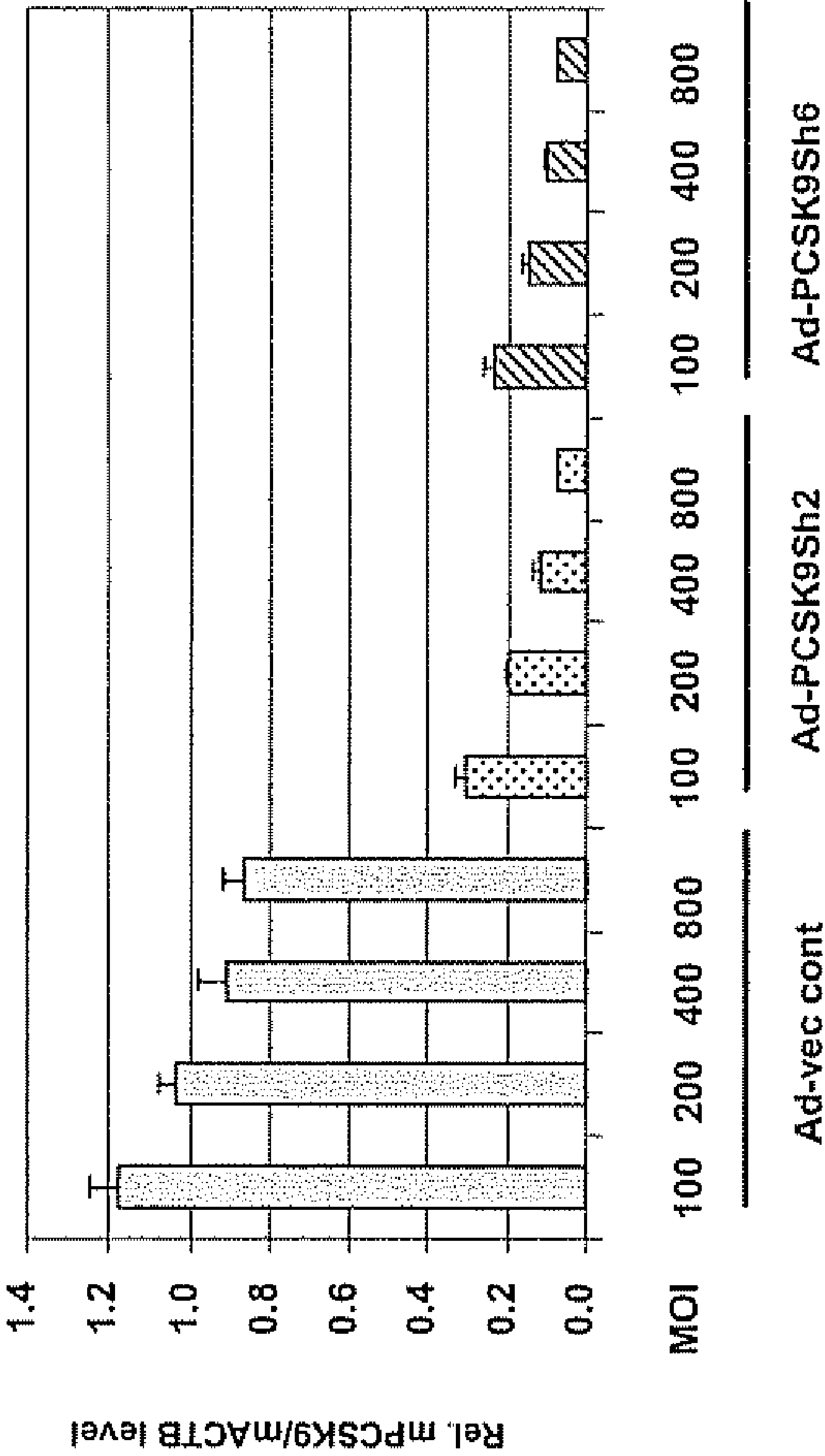


Figure 34A: Adenovirus - Mediated Inhibition of PCSK9 in Liver of C57BL6 Mice

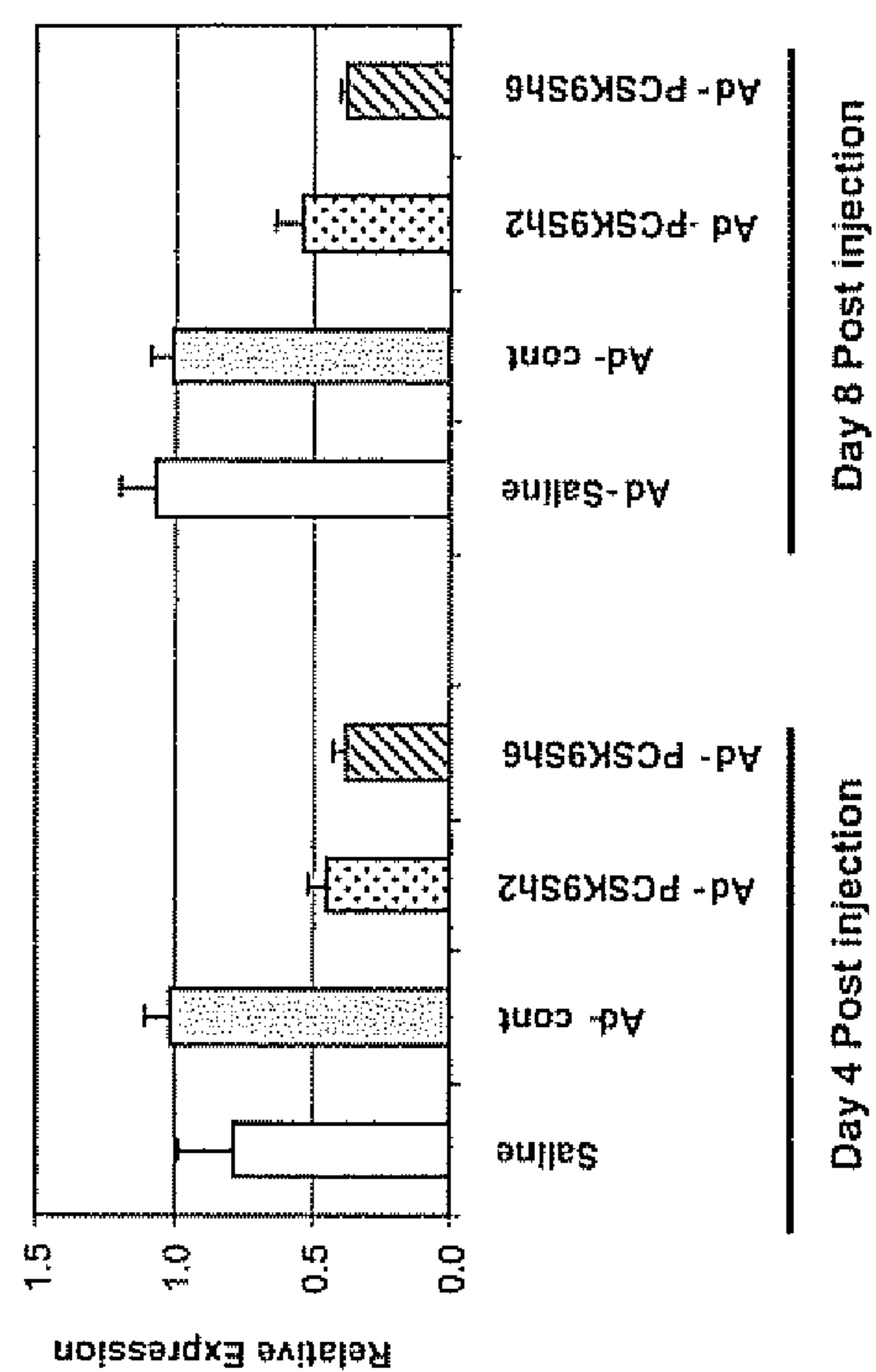


**Figure 34B. Effect of Hepatic Knockdown
on Plasma LDL Level**

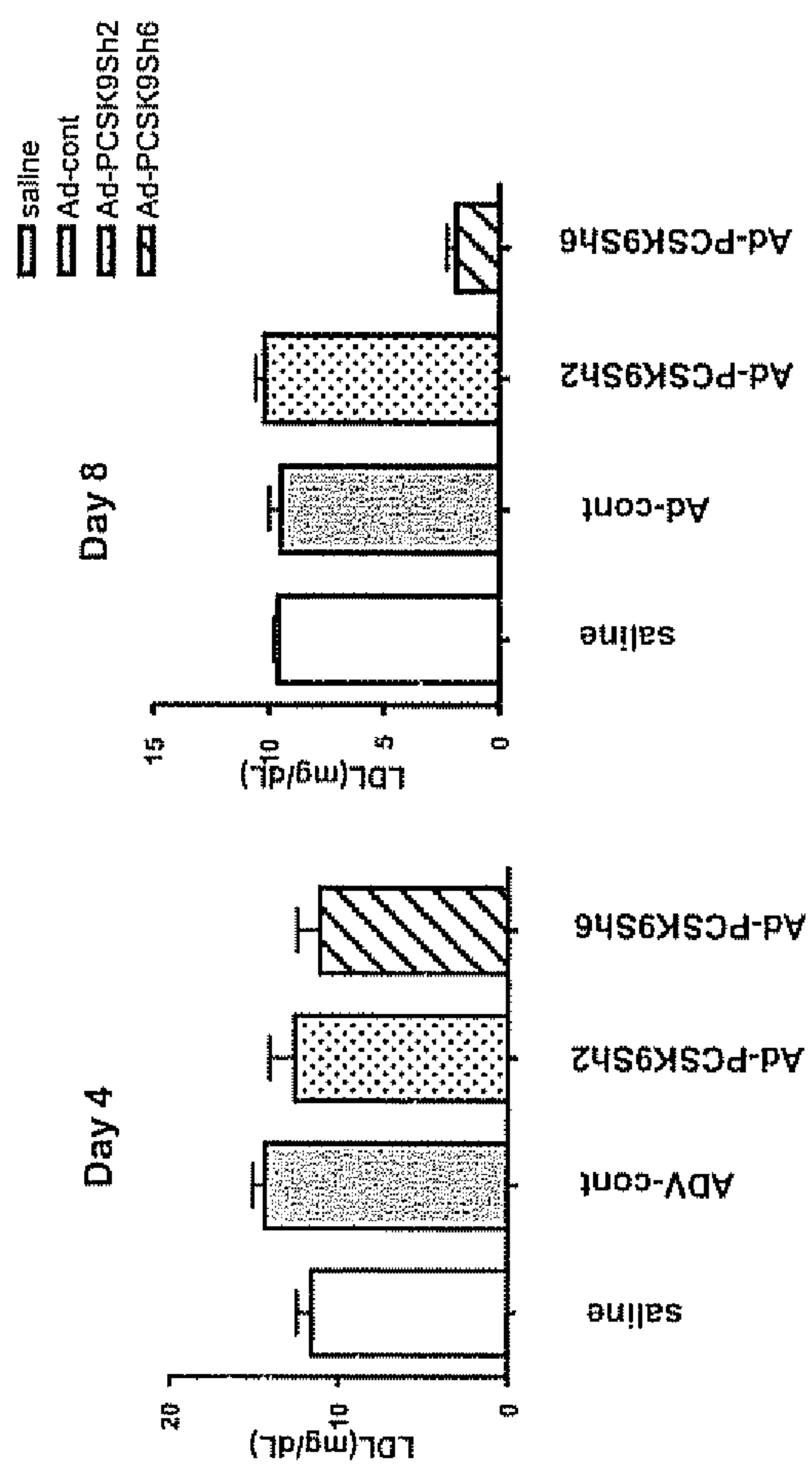
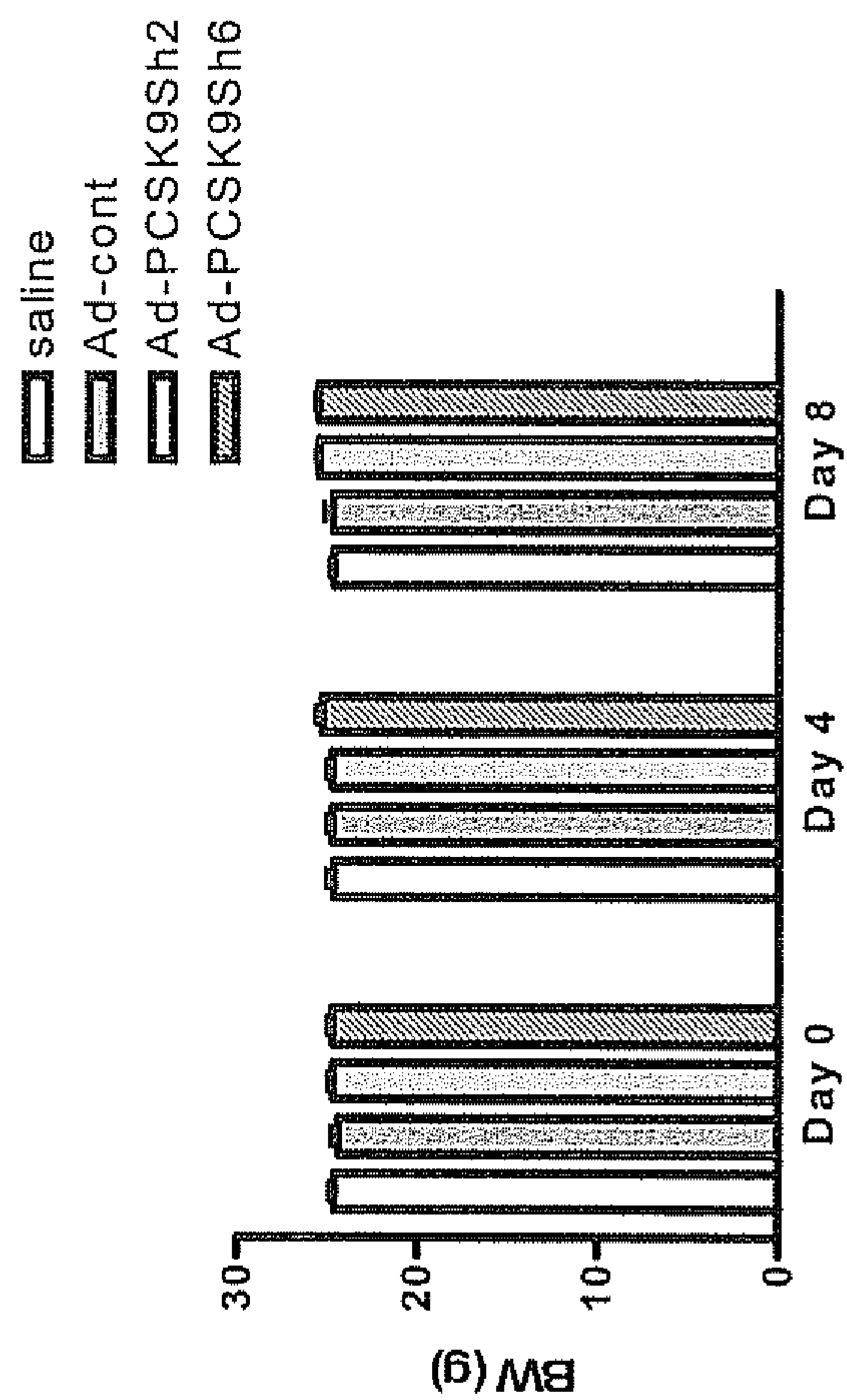
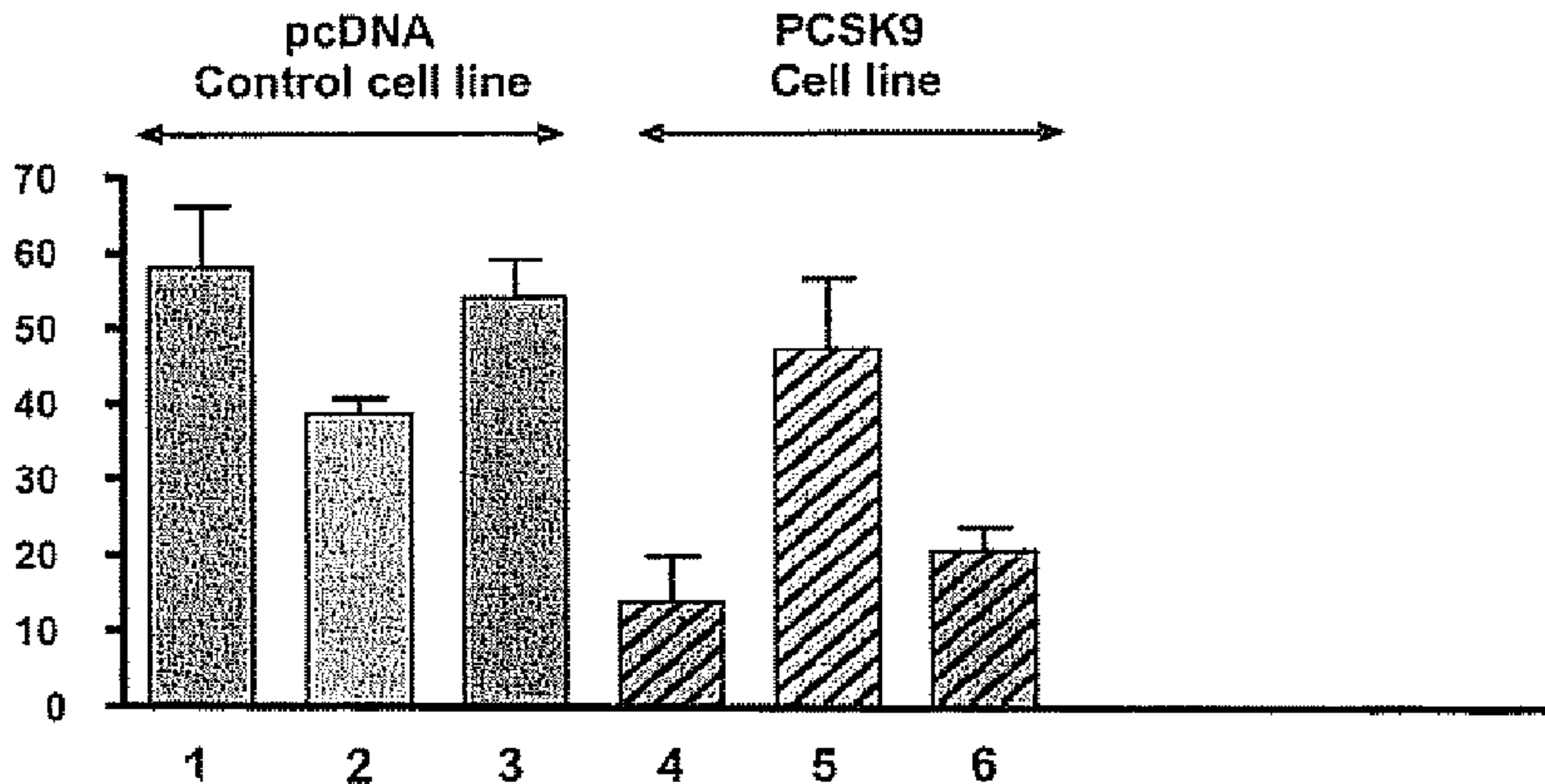


Figure 35. Effect of Hepatic Knockdown of PCSK9 on Body Weight



dil - LDL Uptake Assay

dil-LDL uptake (fluorescence Units)



- 1. 4: Non Targeting RNAi
- 2. 5: PCSK9 Specific RNAi
- 3. 6: Non Treated cells