(12) INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(19) World Intellectual Property Organization
International Bureau

(43) International Publication Date
30 April 2009 (30.04.2009)

PCT

(10) International Publication Number
WO 2009/055445 A2

(51) International Patent Classification:
C12N 15/11 (2006.01) *C07H 21/00* (2006.01)
A61K 31/713 (2006.01) *A61P 31/00* (2006.01)

(21) International Application Number:
PCT/US2008/080755

(22) International Filing Date: 22 October 2008 (22.10.2008)

(25) Filing Language: English

(26) Publication Language: English

(30) Priority Data:

60/981,702	22 October 2007 (22.10.2007)	US
60/981,704	22 October 2007 (22.10.2007)	US
60/981,709	22 October 2007 (22.10.2007)	US
60/981,697	22 October 2007 (22.10.2007)	US

(71) Applicant *(for all designated States except US)*: **SIRNA THERAPEUTICS, INC.**; 1700 Owens Street, 4th Floor, San Francisco, CA 941158 (US).

(72) Inventors; and

(75) Inventors/Applicants *(for US only)*: **VARGEESE, Chandra** [US/US]; 111 Amberly Drive, Blue Bell, PA 19422 (US). **VAISH, Narendra** [IN/US]; 1313 Williams Street, Apt.503, Denver, CO 80218-2669 (US). **MCSWIGGEN, James** [US/US]; 4866 Franklin Drive, Boulder, CO 80301-6201 (US).

(74) Agent: **SINGER, Christopher, P.**; McDonnell Boehnen Hulbert & Berghoff LLP, 300 South Wacker Drive, Suite 3200, Chicago, IL 60606 (US).

(81) Designated States *(unless otherwise indicated, for every kind of national protection available)*: AE, AG, AL, AM, AO, AT, AU, AZ, BA, BB, BG, BH, BR, BW, BY, BZ, CA, CH, CN, CO, CR, CU, CZ, DE, DK, DM, DO, DZ, EC, EE, EG, ES, FI, GB, GD, GE, GH, GM, GT, HN, HR, HU, ID, IL, IN, IS, JP, KE, KG, KM, KN, KP, KR, KZ, LA, LC, LK, LR, LS, LT, LU, LY, MA, MD, ME, MG, MK, MN, MW, MX, MY, MZ, NA, NG, NI, NO, NZ, OM, PG, PH, PL, PT, RO, RS, RU, SC, SD, SE, SG, SK, SL, SM, ST, SV, SY, TJ, TM, TN, TR, TT, TZ, UA, UG, US, UZ, VC, VN, ZA, ZM, ZW.

(84) Designated States *(unless otherwise indicated, for every kind of regional protection available)*: ARIPO (BW, GH, GM, KE, LS, MW, MZ, NA, SD, SL, SZ, TZ, UG, ZM, ZW), Eurasian (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European (AT, BE, BG, CH, CY, CZ, DE, DK, EE, ES, FI, FR, GB, GR, HR, HU, IE, IS, IT, LT, LU, LV, MC, MT, NL, NO, PL, PT, RO, SE, SI, SK, TR), OAPI (BF, BJ, CF, CG, CI, CM, GA, GN, GQ, GW, ML, MR, NE, SN, TD, TG).

Published:
— *without international search report and to be republished upon receipt of that report*

WO 2009/055445 A2

(54) **Title:** RNA INTERFERENCE MEDIATED INHIBITION OF RESPIRATORY SYNCYTIAL VIRUS (RSV) EXPRESSION USING SHORT INTERFERING NUCLEIC ACID (siNA)

(57) **Abstract:** Compounds, compositions, and methods for mediating RNA interference (RNAi) against respiratory syncytial virus (RSV) gene expression are provided, useful for preventing, inhibiting, or reducing RSV infection, liver failure, hepatocellular carcinoma, cirrhosis, and/or other disease states associated with RSV infection in a subject or organism.

RNA INTERFERENCE MEDIATED INHIBITION OF RESPIRATORY SYNCYTIAL VIRUS (RSV) EXPRESSION USING SHORT INTERFERING NUCLEIC ACID (siNA)

[0001] This application claims priority to U.S. Provisional Patent Application serial number 60/981,697, filed October 22, 2007; U.S. Provisional Patent Application serial number 60/981,702, filed October 22, 2007; U.S. Provisional Patent Application serial number 60/981,704, filed October 22, 2007; and U.S. Provisional Patent Application serial number 60//981,709, filed October 22, 2007 each titled, "RNA Interference Mediated Inhibition of Respiratory Syncytial Virus (RSV) Expression Using Short Interfering Nucleic Acid (siNA), and of which is incorporated herein by reference in the entirety.

FIELD OF THE INVENTION

[0002] Compounds, compositions, and methods, more particularly, double stranded nucleic acid (siNA) molecules including small nucleic acid molecules, such as short interfering nucleic acid (siRNA), short interfering RNA (siRNA), double-stranded RNA (dsRNA), micro-RNA (miRNA), and short hairpin RNA (shRNA) molecules capable of mediating RNA interference (RNAi) against respiratory syncytial virus (RSV) gene expression are provided. Such small nucleic acid molecules are useful, for example, in providing compositions to prevent, inhibit, or reduce RSV infection, liver failure, hepatocellular carcinoma, cirrhosis, and/or other disease states associated with RSV infection 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; Berstein *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.

[00010] 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-alpha 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.

[0010] McSwiggen et al., WO 03/070918 describe double stranded nucleic acid molecules, including short interfering nucleic acids, targeting RSV and conserved sequences within the RSV genome.

[0011] Bushman et al., US 2003/0203868 describe the inhibition of certain pathogens, including RSV, using certain RNA interference mediating ribonucleic acid molecules.

[0012] Vaillant et al., US 2004/0229828 describe certain antiviral single-stranded oligonucleotides targeting RSV.

[0013] Mohapatra et al., WO 05/056021 describe certain siRNA molecules targeting RSV.

[0014] Bitco *et al.,* 2005, *Nature Medicine*, 11, 50-55, describes the use of certain nasally administered vector expressed siRNA constructs targeting RSV.

[0015] Zhang *et al.,* 2005, *Nature Medicine*, 11, 56-62, describes the use of certain nasally administered vector expressed siRNA constructs targeting the N1 gene of RSV.

SUMMARY OF THE INVENTION

[0016] In various aspects are provided chemically-modified synthetic short interfering nucleic acid (siNA) molecules capable of modulating target gene expression or activity in cells by RNA interference (RNAi). The use of chemically-modified siNA improves various properties of native siNA molecules through increased resistance to nuclease degradation in vivo and/or through improved cellular uptake. Further, contrary to earlier published studies, siNA having multiple chemical modifications, including fully modified siNA, retains its RNAi activity. 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.

[0017] In some embodiments are provided one or more siNA molecules and methods that independently or in combination modulate the expression of gene(s) encoding RSV and/or cellular proteins associated with the maintenance or development of RSV infection, respiratory distress, bronchiolitis, and pneumonia.

[0018] Chemical modifications described herein can be applied to any siNA construct of the invention. In a non-limiting example, the introduction of chemically-modified

nucleotides into nucleic acid 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 RSV 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.

[0019] In a first aspect, the invention comprises a double stranded nucleic acid (siNA) molecule comprising a first strand and a second strand, wherein said first strand is a sense strand and said second strand is an antisense strand, wherein said sense strand comprises the following structure:

5' { B- uu*AA*u*AGG*u*A*u*G*uu*A*u*A*u*G*[N]n-B } 3' (SEQ ID NO:145);

5' { B- cu*AGAA*uc*AA*u*AAAGGG*c*A*[N]n-B } 3' (SEQ ID NO:146);

5' { B- uu*GGAG*u*G*cu*AGAGAG*uu*A*[N]n-B } 3' (SEQ ID NO:147); or

5' { B- *G*ccu*AAAAAAG*u*GGA*ucuu[N]n-B } 3' (SEQ ID NO:148);

wherein *A* is deoxyadenosine and *G* is deoxyguanosine; u is 2'-deoxy-2'-fluorouridine; c is 2'-deoxy-2'-fluorocytidine; each N is independently an unmodified or a chemically modified nucleotide; n is 0, 1, or 2; and each B is independently a terminal cap abasic moiety, a terminal cap inverted abasic moiety, a terminal cap inverted nucleotide, or other terminal cap moiety. Further, each B is independently present or absent.

[0020] The second (antisense) strand of this aspect of the invention is any nucleotide sequence that, together with the sense strand, can induce RNA interference (RNAi) activity. The antisense strand comprises a length that is similar to the length of the sense strand, such as, for example, from 15-30 nucleotides in length (e.g., 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, or 30 nucleotides). The antisense strand comprises nucleotides that are complementary to portions of the sense strand, such as, for example, from about 30-100% of the nucleotides of the antisense strand are complementary to the sense strand (e.g., 30, 40, 50, 55, 60, 65, 70, 75, 80, 85, 90, 95, 96, 97, 98, 99, or 100% of the nucleotides of the antisense strand are complementary to nucleotides of the sense strand). Embodiments of the antisense strand also comprise one or more chemically modified nucleotides, such as, for example, one

or more chemically modified purine nucleotide (e.g., 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, or more chemically modified purine nucleotides) and/or one or more chemically modified pyrimidine nucleotide (e.g., 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, or more chemically modified pyrimidine nucleotides). In such embodiments, the chemical modifications independently comprise any chemical modification known in the art, including the exemplary modifications disclosed herein for the sense strand.

[0021] In some embodiments of this aspect, the sense strand comprises SEQ ID NO: 145. In some embodiments of this aspect, the sense strand comprises SEQ ID NO: 146. In other embodiments of this aspect, the sense strand comprises SEQ ID NO: 147. In even other embodiments of this aspect, the sense strand comprises SEQ ID NO: 148. In any of these embodiments wherein the sense strand comprises the various SEQ ID NOs, each N can be independently selected from 2'-O-methyl, 2'-deoxy (e.g., deoxythymidine), 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, LNA nucleotide, and 5-C-methyl nucleotide. In some embodiments of the first aspect of the invention, each N is independently selected from 2'-O-methyl Uridine (U) or deoxythymidine (T). In some embodiments of the first aspect, the double stranded nucleic acid molecule comprises a sequence wherein each N is T (deoxythymidine).

[0022] In some embodiments of the first aspect of the invention, B is absent from both ends. In one embodiment, B is present on the 3' terminal end only; in another, B is present on the 5' terminal end only. In one embodiment, B is present and the same at both ends. In one embodiment, B is present and different at both ends.

[0023] In one embodiment, n is 0. In another embodiment, n is 1. In another embodiment, n is 2.

[0024] In one embodiment, there are overhangs at both terminal ends. In another embodiment, there is at least one blunt end. In another embodiment there are two blunt ends.

[0025] In some embodiments of the first aspect, n is 2 and each N is T:

5' { B-uu*AA*u*AGG*u*A*u*G*uu*A*u*A*u*G*TT-B } 3' (SEQ ID NO:55);

5' { B-cu*AGAA*uc*AA*u*AAAGGGcA* TT-B } 3' (SEQ ID NO:75);

5' { B-uu*GGAG*u*G*cu*AGAGAG*uu*A*TT-B } 3' (SEQ ID NO:99); or

5' { B-*G*ccu*AAAAAAG*u*GGA*ucuuTT-B } 3' (SEQ ID NO:123).

[0026] In a second aspect, the invention comprises a double stranded nucleic acid molecule comprising a first strand and a second strand, wherein said first strand is an

antisense strand and said second strand is a sense strand, wherein said antisense strand comprises the following structure:

5' { CAUAuAAcAuAccuAuuAA[N]p } 3' (SEQ ID NO:149);

5' { UGCccuuuAuuGAuucuAG [N]p } 3' (SEQ ID NO:150);

5' { UAAcucucuAGcAcuccAA [N]p } 3' (SEQ ID NO:151); or

5' { AAGAuccAcuuuuuuAGGc[N]p } 3' (SEQ ID NO:152);

wherein C, A, G, and U are cytidine, adenosine, guanosine, and uridine, respectively; A is 2'-O-methyl adenosine; G is 2'-O-methyl guanosine; c and u are 2'-deoxy-2'-fluorocytidine and 2'-deoxy-2'-fluorouridine, respectively; each N is independently an unmodified or a chemically modified nucleotide; and p = 0, 1, or 2. In some embodiments, the double stranded nucleic acid molecule comprises a sequence wherein each N is independently selected from a 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, LNA nucleotide, and 5-C-methyl nucleotide. In some embodiments, the double stranded nucleic acid molecule comprises a sequence wherein each N is independently selected from 2'-O-methyl Uridine (U) or deoxythymidine (T). In some embodiments, the double stranded nucleic acid molecule comprises a sequence wherein each N is U (2'-O-methyl uridine).

[0027] In this second aspect of the invention the sense strand is any nucleotide sequence that, together with the antisense strand, is adequate to induce RNA interference (RNAi) activity. The sense strand comprises length that is similar to the length of the antisense strand, such as, for example, from 15-30 nucleotides in length (e.g., 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, or 30 nucleotides). The sense strand comprises nucleotides that are complementary to portions of the antisense strand, such as, for example, from about 30-100% of the nucleotides of the sense strand are complementary to the antisense strand (e.g., 30, 40, 50, 55, 60, 65, 70, 75, 80, 85, 90, 95, 96, 97, 98, 99, or 100% of the nucleotides of the sense strand are complementary to nucleotides of the antisense strand). Embodiments of the sense strand also comprise one or more chemically modified nucleotides, such as, for example one or more chemically modified purine nucleotide (e.g., 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, or more chemically modified purine nucleotides) and/or one or more chemically modified pyrimidine nucleotide (e.g., 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, or more chemically modified pyrimidine nucleotides). In such embodiments, the chemical modification independently comprise any chemical modification known in the art, including the exemplary modifications disclosed for the antisense strand in this second aspect of the invention.

[0028] In some embodiments, p is 0. In others, p is 1. In still others, p is 2.

[0029] In an embodiment, p is 2 and each N is U (2'-O-methyl uridine):

5' { CAUAuAAcAuAccuAuuAAUU } 3' (SEQ ID NO:56);

5' { UGCccuuuAuuGAuucuAGUU } 3' (SEQ ID NO:76);

5' { UAAcucucuAGcAcuccAAUU } 3' (SEQ ID NO:100); or

5' { AAGAuccAcuuuuuuAGGcUU } 3' (SEQ ID NO:124).

[0030] In one embodiment, there are overhangs at both terminal ends. In another embodiment, there is at least one blunt end. In another embodiment there are two blunt ends.

[0031] In any of the preceding aspects and embodiments a first nucleotide strand is provided and a second nucleotide strand allows for RNAi activity where either (i) said second strand has at least partial complimentarity to said first strand or (ii) said second strand is perfectly complimentary at all corresponding residues with said first strand. In one embodiment, the siNA molecules contain 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 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 molecules with blunt ends, where both ends are blunt, or alternatively, where one of the ends is blunt.

[0032] In an embodiment of the first and second aspects, the invention comprises a siNA molecule in which the sense strand is as described for the first aspect of the invention and the antisense strand is as described for the second aspect.

[0033] In one embodiment, a double stranded nucleic acid (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 nucleic acid molecule (see for example **Figure 4**). In one embodiment, a double stranded nucleic acid 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 molecule. For example, a double stranded nucleic acid 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 nucleic acid molecule. In another embodiment, the nucleotide overhang portion of a

double stranded nucleic acid (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 (siNA) molecule of the invention comprises glyceryl, abasic, or inverted deoxy abasic non-nucleotides or other terminal cap.

[0034] In one embodiment, the second strand of a double stranded siNA molecule of the invention comprises a different pattern of chemical modifications, such as the modification patterns described herein or any combination thereof (see **Table 15**). Other exemplary patterns of chemical modifications are described in Published U.S. Patent Application No. 2006/0287267, incorporated herein by reference. Any combination of the chemical modifications disclosed in US 2006/0287267 can be included in the invention, some of which are summarized in **Table 15**.

[0035] In one embodiment, a double stranded nucleic acid molecule comprises a sense strand and an antisense strand, wherein said sense strand comprises the following structure [RSV-I]:

[RSV-I] 5' { B- uu*AA*u*AGG*u*A*u*G*uu*A*u*A*u*G*[N]n-B } 3' (SEQ ID NO:145); and

wherein said antisense strand comprises the following structure:

5' { CAUAuAAcAuAccuAuuAA[N]p } 3' (SEQ ID NO:149); or

wherein said sense strand comprises the following structure [RSV-II]:

[RSV-II] 5' { B- cu*AGAA*uc*AA*u*AAAGGGcA*[N]n-B } 3' (SEQ ID NO:146); and

wherein said antisense strand comprises the following structure:

5' { UGCccuuuAuuGAuucuAG[N]p } 3' (SEQ ID NO:150); or

wherein said sense strand comprises the following structure [RSV-III]:

[RSV-III] 5' { B- uu*GGAG*u*G*cu*AGAGAG*uu*A*[N]n-B } 3' (SEQ ID NO:147); and

wherein said antisense strand comprises the following structure:

5' { UAAcucucuAGcAcuccAA[N]p } 3' (SEQ ID NO:151); or

wherein said sense strand comprises the following structure [RSV-IV]:

[RSV-IV] 5' { B- *G*ccu*AAAAAAG*u*GGA*ucuu[N]n-B } 3' (SEQ ID NO:148); and

wherein said antisense strand comprises the following structure:

5' { AAGAuccAcuuuuuuAGGc[N]p } 3' (SEQ ID NO:152);

wherein *A* is deoxyadenosine and *G* is deoxyguanosine; wherein C, A, G, and U are cytidine, adenosine, guanosine, and uridine, respectively; A is 2'-O-methyl adenosine; G is 2'-O-methyl guanosine; c and u are 2'-deoxy-2'-fluorocytidine and 2'-deoxy-2'-fluorouridine, respectively; N is an unmodified or a chemically modified nucleotide; n is independently 0, 1, or 2; p is independently 0, 1, or 2; and B is a terminal cap abasic moiety, a terminal cap inverted abasic moiety, a terminal cap inverted nucleotide, or other terminal cap moiety; wherein B is further independently present or absent. B, if present on both terminal ends, is the same or different at both ends. In an embodiment, each N is independently selected from a 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, LNA nucleotide, and 5-C-methyl nucleotide. In an embodiment, each N deoxynucleotide is independently selected from deoxythymidine, deoxyguanosine, deoxycytidine, or deoxyadenosine. In an embodiment, each N is independently selected from 2'-O-methyl Uridine (U) and deoxythymidine (T).

[0036] In some embodiments, a double stranded nucleic acid molecule comprising a sense strand and an antisense strand, comprising:

```
[RSV-Ia]  5' B-uuAAuAGGuAuGuuAuAuGTT-B 3' (SEQ ID NO:55)
             ||||||||||||||||||
          3' UUAAuuAuccAuAcAAuAUAC     5' (SEQ ID NO:56); or


[RSV-IIa] 5' B-cuAGAAucAAuAAAGGGcATT-B 3' (SEQ ID NO:75)
             ||||||||||||||||||
          3' UUGAucuuAGuuAuuuccCGU     5' (SEQ ID NO:76); or

[RSV-IIIa] 5' B-uuGGAGuGcuAGAGAGuuATT-B 3' (SEQ ID NO:99)
              ||||||||||||||||||
           3' UUAAccucAcGAucucucAAU     5' (SEQ ID NO:100); or
```

```
[RSV-IVa] 5' B-GccuAAAAAAGuGGAucuuTT-B 3' (SEQ ID NO:123)
                ||||||||||||||||||
          3' UUcGGAuuuuuucAccuAGAA     5' (SEQ ID NO:124)
```

wherein *A* is deoxyadenosine and *G* is deoxyguanosine; wherein C, A, G, and U are cytidine, adenosine, guanosine, and uridine, respectively; A and U are 2'-O-methyl adenosine and 2'-O-methyl uridine, respectively; c and u are 2'-deoxy-2'-fluoro cytidine and 2'-deoxy-2'-fluoro uridine, respectively; and B is a terminal cap abasic moiety, a terminal cap inverted abasic moiety, a terminal cap inverted nucleotide, or other terminal cap moiety; wherein B is further independently present or absent. B, if present on both terminal ends, is the same or different at both ends.

[0037] In one embodiment, B is an inverted abasic cap moiety which is linked to the 5'-end or 3'-end of the double stranded nucleic acid (siNA) molecule. Non-limiting examples of such moieties are illustrated in **FIG. 5** and **FIG. 25**. In an embodiment B is an inverted abasic moiety as shown in **FIG. 25**.

[0038] In one embodiment, the siNA molecule is assembled from two separate oligonucleotide fragments wherein at least a first fragment of one separate oligonucleotide comprises the sense region and at least a second fragment of the other separate oligonucleotide 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, 23, 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.

[0039] By "blunt ends" is meant symmetric termini or termini of a double stranded siNA molecule having no overhanging nucleotides. The two strands of a double stranded siNA molecule align with each other without over-hanging 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.

[0040] In one embodiment, the invention features a medicament comprising a siNA molecule of the invention. In one embodiment, the invention features an active ingredient comprising a siNA molecule of the invention. In one embodiment are provided compositions

comprising the double stranded nucleic acid molecule described herein in a pharmaceutically acceptable carrier or diluent.

[0041] Methods are also provided for treating, preventing, or reducing RSV infection in a subject comprising contacting the subject with the compositions described herein under conditions suitable to modulate the expression of RSV in the subject whereby the treatment, prevention, or reduction of RSV infection can be achieved. In one embodiment, the methods comprise administration of ribavirin.

[0042] Other features and advantages of the invention will be apparent from the following description of the embodiments thereof, and from the claims.

BRIEF DESCRIPTION OF THE DRAWINGS

[0043] **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.

[0044] **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.

[0045] **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.

[0046] **Figure 4A-C** shows non-limiting examples of different siNA constructs of the invention.

[0047] The examples shown in **Figure 4A** (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*.

[0048] The examples shown in **Figure 4B** 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.

[0049] The example shown in **Figure 4C** 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.

[0050] **Figure 5** 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.

[0051] **Figure 6** shows a non-limiting example of a strategy used to identify chemically modified siNA constructs of the invention that are nuclease resistance 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 in 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). The 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.

[0052] **Figure 7** shows non-limiting examples of phosphorylated siNA molecules of the invention, including linear and duplex constructs and asymmetric derivatives thereof.

[0053] **Figure 8** shows non-limiting examples of chemically modified terminal phosphate groups of the invention.

[0054] **Figure 9A** 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 9B** shows a non-limiting representative example of a duplex forming oligonucleotide sequence. **Figure 9C** shows a non-limiting example of the self assembly schematic of a representative duplex forming oligonucleotide sequence. **Figure 9D** 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.

[0055] **Figure 10** 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.

[0056] **Figure 11** 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 11A** 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 11B** 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.

[0057] **Figure 12** 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. **Figure 12A** 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. **Figure 12B** 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 11**.

[0058] **Figure 13** 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 bifuctional siNA constructs that can mediate RNA interference against differing target nucleic acid sequences. **Figure 13A** 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. **Figure 13B** 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.

[0059] **Figure 14** 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 bifuctional siNA constructs that can mediate RNA interference against differing target nucleic acid sequences. **Figure 14A** 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. **Figure 14B** 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 13**.

[0060] **Figure 15** 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, 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.

[0061] **Figure 16** 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.

[0062] **Figure 17(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.

[0063] **Figure 18** shows a non-limiting example of various dendrimer based multifunctional siNA designs.

[0064] **Figure 19** shows a non-limiting example of various supramolecular multifunctional siNA designs.

[0065] **Figure 20** 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.

[0066] **Figure 21** 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 multiifunctional 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.

[0067] **Figure 22** 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.

[0068] **Figure 23** 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.

[0069] **Figure 24** 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.

[0070] **Figure 25** depicts an embodiment of 5' and 3' inverted abasic cap moieties linked to a nucleic acid strand.

DETAILED DESCRIPTION OF THE INVENTION

Mechanism of Action of Nucleic Acid Molecules of the Invention

[0071] 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 siNA molecule can be decreased (i.e., less than ten-fold), but the overall activity of the siNA molecule is enhanced *in vitro* and/or *in vivo*.

[0072] 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 though 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.

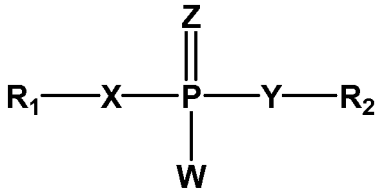
[0073] The presence of long dsRNAs in cells stimulates the activity of a ribonuclease III enzyme referred to as Dicer. Dicer is involved in the processing of the dsRNA into short pieces of dsRNA known as short interfering RNAs (siRNAs) (Berstein *et al.,* 2001, *Nature,* 409, 363). Short interfering 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 though 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, siNA 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.

[0074] 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*.

[0075] In one embodiment, the invention features a chemically-modified short interfering nucleic acid (siNA) molecule capable of mediating RNA interference (RNAi) against RSV

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:

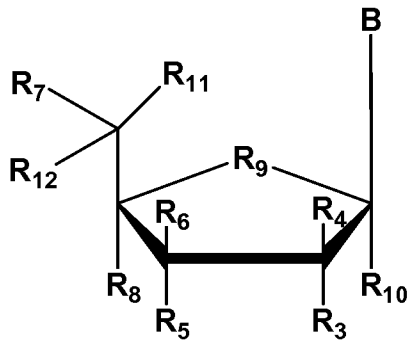


wherein each R1 and R2 is independently any nucleotide, non-nucleotide, or polynucleotide which can be naturally-occurring or chemically-modified and which can be included in the structure of the siNA molecule or serve as a point of attachment to the siNA molecule, each X and Y is independently O, S, 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).

[0076] 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 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, a 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.

[0077] In one embodiment, the invention features a chemically-modified short interfering nucleic acid (siNA) molecule capable of mediating RNA interference (RNAi) against RSV 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:

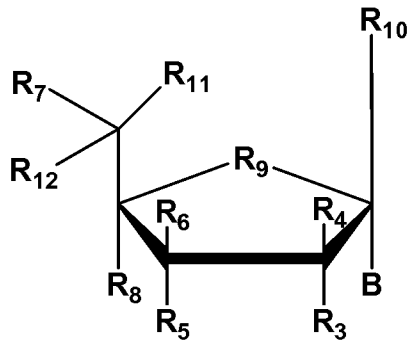


wherein each R3, R4, R5, R6, R7, R8, R10, R11 and R12 is independently H, OH, alkyl, substituted alkyl, alkaryl or aralkyl, F, Cl, Br, CN, CF3, OCF3, OCN, O-alkyl, S-alkyl, N-alkyl, O-alkenyl, S-alkenyl, N-alkenyl, SO-alkyl, alkyl-OSH, alkyl-OH, O-alkyl-OH, O-alkyl-SH, S-alkyl-OH, S-alkyl-SH, alkyl-S-alkyl, alkyl-O-alkyl, ONO2, NO2, N3, NH2, aminoalkyl, aminoacid, aminoacyl, ONH2, O-aminoalkyl, O-aminoacid, O-aminoacyl, heterocycloalkyl, heterocycloalkaryl, aminoalkylamino, polyalklylamino, 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.

[0078] 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 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 anther 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.

[0079] In one embodiment, the invention features a chemically-modified short interfering nucleic acid (siNA) molecule capable of mediating RNA interference (RNAi) against RSV 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:



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, 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,

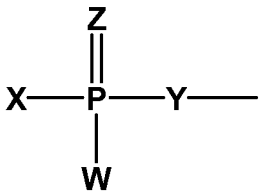
heterocycloalkyl, heterocycloalkaryl, aminoalkylamino, polyalklylamino, substituted silyl, or a group having any of Formula I, II, III, IV, V, VI and/or VII, any of which can be included in the structure of the siNA molecule or serve as a point of attachment to the siNA molecule; R9 is O, S, CH2, S=O, 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.

[0080] 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 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 anther 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.

[0081] In another embodiment, a 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.

[0082] In one embodiment, the invention features a chemically-modified short interfering nucleic acid (siNA) molecule capable of mediating RNA interference (RNAi) against RSV 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.

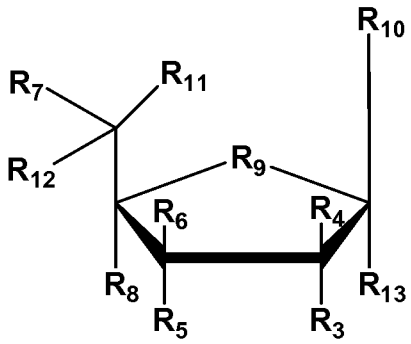
[0083] In one embodiment, the invention features a siNA molecule having a 5'-terminal phosphate group having Formula IV on the RSV target-complementary strand, for example, a strand complementary to a RSV target RNA, wherein the siNA molecule comprises an all RNA siNA molecule. In another embodiment, the invention features a siNA molecule having a 5'-terminal phosphate group having Formula IV on the RSV 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 RSV target-complementary strand of a siNA molecule of the invention, for example a siNA molecule having chemical modifications having any of Formulae I-VII.

[0084] In one embodiment, the invention features a chemically-modified short interfering nucleic acid (siNA) molecule capable of mediating RNA interference (RNAi) against RSV 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 (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 (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.

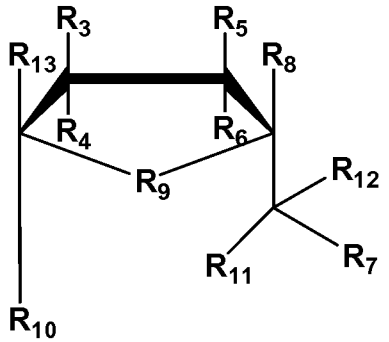
[0085] In another embodiment, the invention features a siNA molecule comprising 2'-5' internucleotide linkages. The 2'-5' internucleotide linkage(s) can be at the 3'-end, the 5'-end, or both of the 3'- and 5'-ends of one or both siNA sequence strands. In addition, the 2'-5' internucleotide linkage(s) 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.

[0086] In one embodiment, a 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:



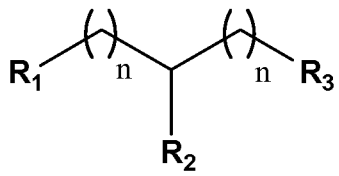
wherein each R3, R4, R5, R6, R7, R8, R10, R11, R12, and R13 is independently H, OH, alkyl, substituted alkyl, alkaryl or aralkyl, F, Cl, Br, CN, CF3, OCF3, OCN, O-alkyl, S-alkyl, N-alkyl, O-alkenyl, S-alkenyl, N-alkenyl, SO-alkyl, alkyl-OSH, alkyl-OH, O-alkyl-OH, O-alkyl-SH, S-alkyl-OH, S-alkyl-SH, alkyl-S-alkyl, alkyl-O-alkyl, ONO2, NO2, N3, NH2, aminoalkyl, aminoacid, aminoacyl, ONH2, O-aminoalkyl, O-aminoacid, O-aminoacyl, heterocycloalkyl, heterocycloalkaryl, aminoalkylamino, polyalklylamino, 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.

[0087] In one embodiment, a 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, 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, polyalklylamino, 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.

[0088] In another embodiment, a 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, 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, polyalklylamino, 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.

[0089] 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).

[0090] Each nucleotide within the double stranded siNA molecule can independently have a chemical modification comprising the structure of any of Formulae I-VIII. 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.

[0091] In one embodiment, one or more nucleotide positions 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. 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.

[0092] 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 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 5**).

[0093] 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 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.

[0094] In another embodiment, a 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.

[0095] In one embodiment, a 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.

[0096] In one embodiment, a 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.

[0097] In another embodiment, a 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.

[0098] In one embodiment, a chemically-modified short interfering nucleic acid (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, or abasic chemical modifications or any combination thereof.

[0099] In one embodiment, a chemically-modified short interfering nucleic acid (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, or abasic chemical modifications or any combination thereof.

[00100] In one embodiment, a chemically-modified short interfering nucleic acid (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, or abasic chemical modifications or any combination thereof.

[00101] In one embodiment, the sense strand of a double stranded siNA molecule of the invention comprises a terminal cap moiety, (see for example **Figure 5**) such as an inverted deoxyabaisc moiety, at the 3'-end, 5'-end, or both 3' and 5'-ends of the sense strand.

[00102] In one embodiment, the invention features a chemically-modified short interfering nucleic acid molecule (siNA) capable of mediating RNA interference (RNAi) against RSV 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 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.

[00103] In one embodiment, the invention features a short interfering nucleic acid (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 RSV target molecule wherein the nucleic acid molecule has sequence that comprises a

sequence recognized by the RSV target molecule in its natural setting. Alternately, an aptamer can be a nucleic acid molecule that binds to a RSV target molecule where the RSV target molecule does not naturally bind to a nucleic acid. The RSV 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.)

[00104] 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; Jschke *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 remaining bases to exhibit their enzymatic 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.

[00105] In one embodiment, the invention features a short interfering nucleic acid (siNA) molecule capable of mediating RNA interference (RNAi) against RSV 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. For example, a siNA molecule can be assembled from a single oligonculeotide 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 oligonculeotide 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.

Duplex Forming Oligonucleotides (DFO) of the Invention

[00106] 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.

[00107] Applicant demonstrates herein that certain oligonucleotides, refered 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 RSV 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 RSV target nucleic acid molecule.

[00108] 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 distict nucleotide sequence.

[00109] 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 9 and 10**. 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.

[00110] 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 RSV 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.

[00111] 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 RSV target nucleic acid molecule (*e.g.*, RSV target RNA).

[00112] 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 moleucles 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 RSV 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 RSV target nucleic acid sequence.

[00113] 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 RSV target nucleic acid molecule or a portion thereof (*e.g.*, RSV 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.

[00114] In one embodiment, a DFO molecule of the invention comprises a structure having Formula DFO-I:

5'-p-X Z X'-3'

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 RSV target nucleic acid sequence or a portion thereof and is of length sufficient to interact (*e.g.*, base pair) with the RSV target nucleic acid sequence or a portion thereof (*e.g.*, RSV 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 RSV 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 RSV target RNA or a portion thereof (*e.g.*, RSV 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 RSV 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 RSV target RNA or a portion thereof (*e.g.*, RSV 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.

[00115] 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 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.

[00116] In one embodiment, the invention features a double stranded oligonucleotide construct having Formula DFO-I(a):

5'-p-X Z X'-3'
3'-X' Z X-p-5'

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 RSV target nucleic acid sequence or a portion thereof (*e.g.*, RSV RNA target) and is of length sufficient to interact with the RSV target nucleic acid sequence of a portion thereof (*e.g.*, RSV 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 RSV target RNA or a portion thereof (*e.g.*, RSV 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 RSV target RNA 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 RSV 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 RSV target RNA or a portion thereof (*e.g.*, RSV 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 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 RSV target gene expression.

[00117] In one embodiment, a DFO molecule of the invention comprises structure having Formula DFO-II:

5'-p-X X'-3'

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 RSV target nucleic acid sequence (*e.g.*, RSV target RNA) or a portion thereof and is of length sufficient to interact (*e.g.*, base pair) with the RSV 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 sufficint to form a relatively stable double stranded oligonucleotide.

[00118] In one embodiment, the invention features a double stranded oligonucleotide construct having Formula DFO-II(a):

5'-p-X X'-3'
3'-X' X-p-5'

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 RSV target nucleic acid sequence or a portion thereof (*e.g.*, RSV RNA target) and is of length sufficient to interact (*e.g.*, base pair) with the RSV target nucleic acid sequence (*e.g.*, RSV target 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 sufficint to form a relatively stable double stranded oligonucleotide. In one embodiment, the double stranded oligonucleotide construct of Formula 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 RSV target gene expression.

[00119] In one embodiment, the invention features a DFO molecule having Formula DFO-I(b):

5'-p-Z-3'

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 RSV target nucleic acid (*e.g.*, RSV target 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.

[00120] 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.

[00121] In one embodiment, the palidrome 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 RSV target nucleic acid sequence (*e.g.*, modified base analogs that can form Watson Crick base pairs or non-Watson Crick base pairs).

[00122] 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

[00123] 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 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 RSV or cellular/host target nucleic acid sequence or can target sequences of more than one distinct target nucleic acid molecules (*e.g.*, RSV RNA or cellular/host RNA 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.

[00124] Applicant demonstrates herein that certain oligonucleotides, refered 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 nucleic acid molecules encoding a virus or viral proteins (e.g. nucleopretein (N), large (L) and phosphoproteins (P), matrix (M), fusion (F), glycoprotein (G), NS1 and 2 non-structural proteins, including small hydrophobic (SH) and M2 protein targets) and corresponding cellular proteins required for viral infection and/or replication, or differing strains or subtypes of a particular virus (*e.g.*, RSV subtype A and subtype B and different strains 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 or pathogen 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.

[00125] Use of multifunctional siNA molecules that target more then one region of a target nucleic acid molecule (*e.g.*, messenger RNA or RSV 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.*, RSV RNA), thereby allowing down regulation or inhibition of different strain variants or a virus, or splice variants encoded by a single host gene, or allowing for targeting of both coding and non-coding regions of the host target nucleic acid molecule.

[00126] 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 seqeunce 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.

[00127] 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.

[00128] 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 11-23**.

[00129] 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 seqeunces 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 11 and 12** 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 13 and 14**).

[00130] In one embodiment, the invention features a multifunctional short interfering nucleic acid (multifunctional siNA) molecule, wherein each strand of the 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.

[00131] 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.

[00132] 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.*, RSV or host 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 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.

[00133] 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.*, RSV or host 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 distinct from the 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.

[00134] 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 seqeunce 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.

[00135] 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 seqeunce 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.

[00136] 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.

[00137] 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.

[00138] 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 differeing genes or gene transcripts.

[00139] 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 viral genome (*e.g.*, RSV) 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 proteins (*e.g.*, two differing host proteins involved in the RSV life-cycle) can be used to down regulate, inhibit, or shut down a particular biologic pathway by targeting, for example, a viral RNA (*e.g.*, RSV RNA) and one or more host RNAs that are involved in viral infection or the viral life-cycle (*e.g.*, Rho-A, ICAM-1, or interferon regulatory factors).

[00140] In another non-limiting example, a multifunctional siNA molecule comprising a region in one strand having a nucleotide sequence complementarity to a first target nucleic acid sequence derived from a target nucleic acid molecule encoding a virus or a viral protein (*e.g.*, RSV) and the second strand comprising a region having a nucleotide sequence complementarity to a second target nucleic acid sequence present in target nucleic acid molecule encoding a cellular protein (*e.g.*, a host cell receptor for the virus) can be used to down regulate, inhibit, or shut down the viral replication and infection by targeting the virus and cellular proteins necessary for viral infection or replication.

[00141] In another nonlimiting example, a multifunctional siNA molecule comprising a region in one strand having a nucleotide sequence complementarity to a first target nucleic acid sequence (*e.g.*, conserved sequence) present in a target nucleic acid molecule such as a viral genome (*e.g.*, RSV RNA) and the second strand comprising a region having a nucleotide sequence complementarity to a second target nucleic acid sequence (*e.g.*, conserved sequence) present in target nucleic acid molecule derived from a gene encoding a viral protein (*e.g.*, RSV proteins) to down regulate, inhibit, or shut down the viral replication and infection by targeting the viral genome and viral encoded proteins necessary for viral infection or replication.

[00142] In one embodiment the invention takes advantage of conserved nucleotide sequences present in different strains, isotypes or forms of a virus and genes encoded by these different strains, isotypes and forms of the virus (*e.g.*, RSV). By designing multifunctional siNAs in a manner where one strand includes a sequence that is complementary to target nucleic acid sequence conserved among various strains, isotypes or forms of a virus and the other strand includes sequence that is complementary to target nucleic acid sequence conserved in a protein encoded by the virus, it is possible to selectively and effectively inhibit viral replication or infection using a single multifunctional siNA.

[00143] 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 RSV viral RNA of a first viral strain and the second region comprises nucleotide sequence complementary to a RSV viral RNA of a second viral strain. In one embodiment, the first and second regions can comprise nucleotide sequence complementary to shared or conserved RNA sequences of differing viral strains or classes or viral strains.

[00144] 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 a nucleotide sequence complementary to a RSV viral RNA encoding one or more RSV viruses (*e.g.*, one or more strains of RSV) and the second region comprises a nucleotide sequence complementary to a viral RNA encoding one or more interferon agonist proteins. In one embodiment, the first region can comprise a nucleotide sequence complementary to shared or conserved RNA sequences of differing RSV viral strains or classes of RSV viral strains. Non-limiting example of interferon agonist proteins include any protein that is capable of inhibition or suppressing RNA silencing (*e.g.*, RNA binding proteins such as E3L or NS1 or equivalents thereof.

[00145] 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 RSV viral RNA and the second region comprises nucleotide sequence complementary to a cellular RNA that is involved in RSV viral infection and/or replication. Non-limiting examples of cellular RNAs involved in viral infection and/or replication include cellular receptors (see for example Ghildyal et al., 2005, J Gen Virol., 86: 1879-94), cell surface molecules, cellular enzymes,

cellular transcription factors, and/or cytokines, second messengers, and cellular accessory molecules including, but not limited to, La antigen, FAS, interferon agonsit protein, interferon regulatory factors (IRFs); cellular PKR protein kinase (PKR); human eukaryotic initiation factors 2B (elF2B gamma and/or elF2gamma); human DEAD Box protein (DDX3); and cellular proteins that bind to the poly(U) tract of the RSV 3'-UTR, such as polypyrimidine tract-binding protein.

[00146] In one embodiment, a double stranded multifunctional siNA molecule of the invention comprises a structure having Formula MF-I:

5'-p-X Z X'-3'
3'-Y' Z Y-p-5'

wherein each 5'-p-XZX'-3' and 5'-p-YZY'-3' are independently an oligonucleotide of length 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; XZ comprises a nucleic acid sequence that is complementary to a first RSV target nucleic acid sequence; YZ is an oligonucleotide comprising nucleic acid sequence that is complementary to a second RSV 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 complementary; 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 RSV 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 RSV 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 RSV target nucleic acid sequence and the second RSV target nucleic acid sequence are present in the same target nucleic acid molecule (*e.g.*, RSV RNA or host RNA). In another embodiment, the first RSV target nucleic acid sequence and the second RSV target nucleic acid sequence are present in different target nucleic acid molecules (*e.g.*, RSV RNA and host 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.

[00147] In one embodiment, a multifunctional siNA molecule of the invention comprises a structure having Formula MF-II:

5'-p-X X'-3'
3'-Y' Y-p-5'

wherein each 5'-p-XX'-3' and 5'-p-YY'-3' are independently an oligonucleotide of length 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 RSV target RNAs or a portion thereof. In one embodiment, the first RSV target nucleic acid sequence and the second RSV target nucleic acid sequence are present in the same target nucleic acid molecule (*e.g.*, RSV RNA or host RNA). In another embodiment, the first RSV target nucleic acid sequence and the second RSV target nucleic acid sequence are present in different target nucleic acid molecules (*e.g.*, RSV RNA and host 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.

[00148] In one embodiment, a multifunctional siNA molecule of the invention comprises a structure having Formula MF-III:

X X'
Y'-W-Y

wherein each X, X', Y, and Y' is independently an oligonucleotide of length 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 RSV 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 RSV target sequence via RNA interference. In one embodiment, the first RSV target nucleic acid sequence and the second RSV target nucleic acid sequence are present in the same target nucleic acid molecule (*e.g.*, RSV RNA or host RNA). In another embodiment, the first RSV target nucleic acid sequence and the second RSV target nucleic acid sequence are present in different target nucleic acid molecules (*e.g.*, RSV RNA and host 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, lable, aptamer, ligand, lipid, or polymer.

[00149] In one embodiment, a multifunctional siNA molecule of the invention comprises a structure having Formula MF-IV:

X X'
Y'-W-Y

wherein each X, X', Y, and Y' is independently an oligonucleotide of length 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 RSV 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 RSV target sequence via RNA interference. In one embodiment, the first RSV target nucleic acid sequence and the second RSV target nucleic acid sequence are present in the same target nucleic acid molecule (*e.g.*, RSV RNA or host

RNA). In another embodiment, the first RSV target nucleic acid sequence and the second RSV target nucleic acid sequence are present in different target nucleic acid molecules (*e.g.*, RSV RNA and host 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, lable, aptamer, ligand, lipid, or polymer.

[00150] In one embodiment, a multifunctional siNA molecule of the invention comprises a structure having Formula MF-V:

X X'
Y'-W-Y

wherein each X, X', Y, and Y' is independently an oligonucleotide of length 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 RSV 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 RSV target nucleic acid sequence are all present in the same target nucleic acid molecule (*e.g.*, RSV RNA or host RNA). In another embodiment, the first, second, third and fourth RSV target nucleic acid sequence are independently present in different target nucleic acid molecules (*e.g.*, RSV RNA and host 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, lable, aptamer, ligand, lipid, or polymer.

[00151] 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.

[00152] 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 **Tables 13 and 15**, or any other combination of modified nucleotides and non-nucleotides as described in the various embodiments herein.

[00153] In one embodiment, the palidrome 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).

[00154] 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.

[00155] 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 17**). The tethers or linkers can be nucleotide-based linkers or non-nucleotide based linkers as generally known in the art and as described herein.

[00156] 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 17 (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.

[00157] 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 17 (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.

[00158] 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 17 (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.

[00159] 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 17 (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 interefence-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.

[00160] 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 17 (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 interefence-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.

[00161] 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 17 (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 interefence-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.

[00162] In any of the above embodiments, a first target nucleic acid sequence or second target nucleic acid sequence can independently comprise RSV RNA or a portion thereof or a polynucleotide coding or non-coding sequence of cellular or host target that is invoved in RSV infection or replication, or disease processes associated with RSV infection such as such as cellular receptors, cell surface molecules, cellular enzymes, cellular transcription factors, and/or cytokines, second messengers, and cellular accessory molecules including, but not limited to, ICAM-1, RhoA (see for example Budge *et al.,* 2004, Journal of Antimicrobial Chemotherapy, 54(2):299-302, e.g., Genbank Accession No. NM_044472); FAS (*e.g.*, Genbank Accession No. NM_000043) or FAS ligand (*e.g.*, Genbank Accession No. NM_000639); interferon regulatory factors (IRFs; *e.g.*, Genbank Accession No. AF082503.1); cellular PKR protein kinase (*e.g.*, Genbank Accession No. XM_002661.7); human eukaryotic initiation factors 2B (elF2Bgamma; *e.g.*, Genbank Accession No. AF256223, and/or elF2gamma; *e.g.*, Genbank Accession No. NM_006874.1); human DEAD Box protein (DDX3; *e.g.*, Genbank Accession No. XM_018021.2); and cellular proteins that

bind to the poly(U) tract of the RSV 3'-UTR, such as polypyrimidine tract-binding protein (*e.g.*, Genbank Accession Nos. NM_031991.1 and XM_042972.3). In one embodiment, the first RSV target nucleic acid sequence is a RSV RNA or a portion thereof and the second RSV target nucleic acid sequence is a RSV RNA of a portion thereof. In one embodiment, the first RSV target nucleic acid sequence is a RSV RNA or a portion thereof and the second RSV target nucleic acid sequence is a host RNA or a portion thereof. In one embodiment, the first RSV target nucleic acid sequence is a host RNA or a portion thereof and the second RSV target nucleic acid sequence is a host RNA or a portion thereof. In one embodiment, the first RSV target nucleic acid sequence is a host RNA or a portion thereof and the second RSV target nucleic acid sequence is a RSV RNA or a portion thereof.

Synthesis of Nucleic Acid Molecules

[00163] 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. Some molecules of the instant invention are chemically synthesized, and others can similarly be synthesized.

[00164] 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 μmol 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 16** 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 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 μL of 0.11 M = 6.6 μmol) of 2'-O-methyl phosphoramidite and a 105-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 22-fold excess (40 μL of 0.11 M = 4.4 μmol) of deoxy phosphoramidite and a 70-fold excess of S-ethyl tetrazole (40 μL of 0.25 M = 10 μmol) 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 I_2, 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.

[00165] 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.

[00166] 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 16** 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 I_2, 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-dioxide0.05 M in acetonitrile) is used.

[00167] 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 μL of a solution of 1.5 mL N-

methylpyrrolidinone, 750 µL 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 NH_4HCO_3. 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.

[00168] 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 NH_4HCO_3.

[00169] For purification of the trityl-on oligomers, the quenched NH_4HCO_3 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.

[00170] 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.

[00171] 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.

[00172] 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.

[00173] 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.

[00174] 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.

[00175] 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.

[00176] In one embodiment, the synthesis of a 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.

[00177] In one embodiment, the invention features a method for synthesizing a 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 than 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.

[00178] In a further embodiment, the method for 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.

[00179] In another embodiment, the invention features a method for synthesizing a 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.

[00180] In another embodiment, the invention features a method for making a double-stranded 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.

[00181] In another embodiment, the method of synthesis of 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.

[00182] In one embodiment, the invention features siNA constructs that mediate RNAi against a target polynucleotide (*e.g.*, RNA or DNA target), 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.

[00183] In another embodiment, the invention features a method for generating 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.

[00184] In another embodiment, the invention features a method for generating 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 15**) 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.

[00185] In another embodiment, the invention features a method for generating 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 formualtion of step (a) under conditions suitable for isolating siNA formulations having improved toxicologic profiles.

[00186] In another embodiment, the invention features a method for generating 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 15**) 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.

[00187] In another embodiment, the invention features a method for generating 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 formualtion 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.

[00188] In another embodiment, the invention features a method for generating 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 15**) 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).

[00189] In another embodiment, the invention features a method for generating 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 formualtion 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).

[00190] In another embodiment, the invention features a method for generating 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 15**) 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.

[00191] In another embodiment, the invention features a method for generating 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 formualtion 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.

[00192] In one embodiment, the invention features a chemically synthesized double stranded short interfering nucleic acid (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.

[00193] By "improved toxicologic profile", is meant that the chemically modified or formulated 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. In a non-limiting example, siNA molecules and formulations with improved toxicologic profiles are associated with reduced immunostimulatory properties, such as a reduced, decreased or attenuated 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. Such an improved toxicologic profile is 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, *e.g.*, Published U.S. Patent Application No: US 2006/0287267 (Dec. 21, 2006) **Table IV**). Herein, numeric Stab chemistries include both 2'-fluoro and 2'-OCF3 versions of the chemistries shown in **Table IV** of US 2006/0287267. 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.

[00194] In one embodiment, the level of immunostimulatory response associated with a given 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% of the nucleotide positions in the siNA molecule modified can be selected to have a corresponding degree of immunostimulatory properties as described herein.

[00195] 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 siNA molecules that posess minimal immunostimulatory properties to prevent non-specific toxicity or off target effects (*e.g.*, about 90% to about 100% reduction in immunostimulation).

[00196] In other embodiments, the siNA molecule comprisisng 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.

[00197] In one embodiment, the invention features 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.

[00198] In another embodiment, the invention features a method for generating 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.

[00199] In one embodiment, the invention features 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.

[00200] In one embodiment, the invention features 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.

[00201] In another embodiment, the invention features a method for generating 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.

[00202] In another embodiment, the invention features a method for generating 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.

[00203] In one embodiment, the invention features 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.

[00204] In another embodiment, the invention features a method for generating 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.

[00205] In one embodiment, the invention features chemically-modified 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.

[00206] In another embodiment, the invention features a method for generating 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.

[00207] In another embodiment, the invention features a method for generating 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.

[00208] In yet another embodiment, the invention features a method for generating 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.

[00209] In yet another embodiment, the invention features a method for generating 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.

[00210] In one embodiment, the invention features 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.

[00211] In another embodiment, the invention features a method for generating 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.

[00212] In one embodiment, the invention features 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.

[00213] In one embodiment, the invention features a method for generating 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.

[00214] In one embodiment, the invention features a double stranded short interfering nucleic acid (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).

[00215] In one embodiment, the invention features a double stranded short interfering nucleic acid (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.

[00216] In one embodiment, the invention features a double stranded short interfering nucleic acid (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).

[00217] In one embodiment, the invention features a double stranded short interfering nucleic acid (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.

[00218] In one embodiment, the invention features a double stranded short interfering nucleic acid (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**

5, 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.

[00219] In one embodiment, the invention features a double stranded short interfering nucleic acid (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 5**, 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.

[00220] In one embodiment, the invention features a method for generating 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 5** (*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. In a non-limiting example, a 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, *e.g.*, Published U.S. Patent Application No: US 2006/0287267 (Dec. 21, 2006) **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** of US 2006/0287267. For example, "Stab 7/8" refers to both Stab 7/8 and Stab 7F/8F etc.

[00221] In one embodiment, the invention features a method for generating 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, *e.g.*, Published U.S. Patent Application No: US 2006/0287267 (Dec. 21, 2006) **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** of US 2006/0287267, or in **Table 15** herein. For example, "Stab 7/35" refers to both Stab 7/35 and Stab 7F/35F etc.

[00222] 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 receptor. Interaction of the

ligand with the receptor can result in a biochemical reaction, or can simply be a physical interaction or association.

[00223] In another embodiment, the invention features a method for generating 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.

[00224] In another embodiment, the invention features a method for generating 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.

[00225] In another embodiment, polyethylene glycol (PEG) can be covalently attached to siNA compounds of the present invention. The attached PEG can be any molecular weight, preferably from about 100 to about 50,000 daltons (Da).

[00226] 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 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.

[00227] 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" 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. For example 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 linkers 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 nucleotide sequence that is complementary to nucleotide sequence of a target gene. In another embodiment, the siNA molecule of the invention interacts with 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 disclosed herein (see, *e.g.*, Example 2). 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.

[00228] 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).

[00229] In one embodiment, a siNA molecule of the invention is a duplex forming oligonucleotide "DFO", (see for example **Figures 9-10** and Vaish *et al.*, USSN 10/727,780

filed December 3, 2003 and International PCT Application No. US04/16390, filed May 24, 2004).

[00230] In one embodiment, a siNA molecule of the invention is a multifunctional siNA, (see for example **Figures 11-23** 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 RSV RNA. In one embodiment, the multifunctional siNA of the invention can comprise sequence targeting RSV RNA and one or more cellular targets involved in the RSV lifecyle, such as cellular receptors, cell surface molecules, cellular enzymes, cellular transcription factors, and/or cytokines, second messengers, and cellular accessory molecules including, but not limited to, La antigen (see for example Costa-Mattioli *et al.,* 2004, *Mol Cell Biol.,* 24, 6861-70, *e.g.*, Genbank Accession No. NM_003142) (*e.g.*, interferon regulatory factors (IRFs; *e.g.*, Genbank Accession No. AF082503.1); cellular PKR protein kinase (*e.g.*, Genbank Accession No. XM_002661.7); human eukaryotic initiation factors 2B (elF2Bgamma; *e.g.*, Genbank Accession No. AF256223, and/or elF2gamma; *e.g.*, Genbank Accession No. NM_006874.1); human DEAD Box protein (DDX3; *e.g.*, Genbank Accession No. XM_018021.2); and cellular proteins that bind to the poly(U) tract of the RSV 3'-UTR, such as polypyrimidine tract-binding protein (*e.g.*, Genbank Accession Nos. NM_031991.1 and XM_042972.3).

[00231] 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.

[00232] By "asymmetric duplex" as used herein is meant a 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.

[00233] 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.

[00234] 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 molecules (*e.g.*, siNA) 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.

[00235] 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.

[00236] 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 (snRNA), ribosomal RNA (rRNA), transfer RNA (tRNA) and precursor RNAs thereof. Such non-coding RNAs can serve as target nucleic acid molecules for siNA mediated RNA interference in modulating the activity of fRNA or ncRNA involved in functional or

regulatory cellular processes. Abberant fRNA or ncRNA activity leading to disease can therefore be modulated by siNA molecules of the invention. siNA 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.

[00237] 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.

[00238] By "RSV" as used herein is meant, any respiratory syncytial virus or RSV protein, peptide, or polypeptide having RSV activity, such as encoded by RSV Genbank Accession Nos. shown in **Table 12**. The term RSV also refers to nucleic acid sequences encoding any RSV protein, peptide, or polypeptide having RSV activity (e.g., any of protein such as nucleopretein (N), large (L) and phosphoproteins (P), matrix (M), fusion (F), glycoprotein (G), NS1 and 2 non-structural proteins, including small hydrophobic (SH) and M2 proteins). The term "RSV" is also meant to include other RSV encoding sequence, such as other RSV isoforms, mutant RSV genes, splice variants of RSV genes, and RSV gene polymorphisms. In one embodiment, the term RSV as used herein refers to cellular or host proteins or polynucleotides encoding such proteins or that are otherwise involved in RSV infection and/or replication.

[00239] By "target" as used herein is meant, any target protein, peptide, or polypeptide, such as encoded by Genbank Accession Nos. herein and in USSN 10/923,536 and USSN 10/923536, 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 or in USSN 10/923,536 and USSN 10/923536. 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, sRNA) 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.

[00240] 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.).

[00241] 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.

[00242] By "sense region" is meant a nucleotide sequence of a 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.

[00243] By "antisense region" is meant a nucleotide sequence of a 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.

[00244] By "target nucleic acid" or "target polynucleotide" is meant any nucleic acid 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.

[00245] 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 stand 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 based 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.

[00246] In one embodiment, a double stranded nucleic acid molecule of the invention, such as siNA molecule, 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.

[00247] In one embodiment, double stranded nucleic acid molecule of the invention, such as 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.

[00248] In one embodiment, double stranded nucleic acid molecule of the invention is a microRNA (miRNA). By "microRNA" or "miRNA" is meant, 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; Ying *et al.,* 2004, Gene, 342, 25-28; and Sethupathy *et al.,* 2006, RNA, 12:192-197). 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.

[00249] In one embodiment, the invention features nucleic acids that inhibit, down-regulate, or disrupt miRNAs that are involved in RSV infection and/or the RSV life-cycle. For example, a double stranded nucleic acid molecule of the invention (e.g., siNA) can be used to inhibit the function of a miRNA. In certain embodiments herein, the micro RNA is the target RNA in any of the embodiments herein. Double stranded nucleic acid molecules of the invention (e.g., siNA) having a antisense strand or antisense region that is complementary to a target miRNA sequence and a sense strand complementary to the antisense strand can be used to inhibit the activity of miRNAs involved in the RSV life-cycle or in RSV infection to prevent RSV activity or treat RSV infection in a cell or organism. Similarly, an single stranded nucleic acid molecule having complementary to a target miRNA sequence can be used to inhibit the activity of miRNAs involved in the RSV life-cycle or in RSV infection to

prevent RSV activity or treat RSV infection in a cell or organism (see for example Zamore *et al.,* US 2005/0227256 and Tuschl *et al.,* US 2005/0182005 both incorporated by reference herein in their entirety; Zamore *et al.,* 2005, Science, 309: 1519-24; Czech, 2006, NEJM, 354:1194-5; Krutzfeldt *et al,* 2005, Nature, 438:685-9).

[00250] In one embodiment, siNA molecules of the invention that down regulate or reduce target gene expression are used for treating, preventing or reducing RSV infection, respiratory distress, bronchiolitis and/or pneumonia in a subject or organism as described herein or otherwise known in the art.

[00251] 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.

[00252] The 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 of SEQ ID NOs 1-148. Examples of such nucleic acid molecules consist essentially of sequences defined in these tables and figures. Furthermore, the chemically modified constructs described in **Tables 13 & 15** can be applied to any siNA sequence of the invention.

[00253] In another aspect, the invention provides mammalian cells containing one or more siNA molecules of this invention. The one or more siNA molecules can independently be targeted to the same or different sites.

[00254] 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.

[00255] 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).

[00256] 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 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, 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.

[00257] 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).

[00258] 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.

[00259] 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 diseases, disorders, conditions, and traits described herein or otherwise known in the art, in a subject or organism.

[00260] In one embodiment, the 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.

[00261] In a further embodiment, the siNA molecules can be used in combination with other known treatments to prevent or treat RSV infection, respiratory distress, bronchiolitis and/or pneumonia 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.

Optimizing Activity of the nucleic acid molecule of the invention.

[00262] 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.

[00263] 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 is cells is not significantly inhibited.

[00264] 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.

[00265] 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.

[00266] 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.

[00267] 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; see also US Patent 7,060,809 and International Patent Publication WO 00/56746, all incorporated by reference herein).

[00268] 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.

[00269] 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.

[00270] The term "biodegradable" as used herein, refers to degradation in a biological system, for example, enzymatic degradation or chemical degradation.

[00271] 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.

[00272] 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.

[00273] 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.

[00274] 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.

[00275] 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.

[00276] 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.

[00277] 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-erythrofuranosyl) nucleotide, 4'-thio nucleotide; carbocyclic nucleotide; 1,5-anhydrohexitol nucleotide; L-nucleotides; alpha-nucleotides; modified base nucleotide; phosphorodithioate linkage; *threo*-pentofuranosyl 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 5.**

[00278] 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-erythrofuranosyl) 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*-pentofuranosyl 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).

[00279] 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.

[00280] 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, NO_2 or $N(CH_3)_2$, 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, NO_2, halogen, $N(CH_3)_2$, 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, NO_2 or $N(CH_3)_2$, amino or SH.

[00281] 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.

[00282] 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.

[00283] 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.

[00284] 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. .

[00285] 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.

[00286] 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.

[00287] In connection with 2'-modified nucleotides as described for the present invention, by "amino" is meant 2'-NH_2 or 2'-*O*- NH_2, 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.

[00288] 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

[00289] A siNA molecule of the invention can be adapted for use to treat, prevent, inhibit, or reduce RSV infection, respiratory distress, bronchiolitis and pneumonia and/or any other trait, disease or condition that is related to or will respond to the levels of RSV 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 the lung as is described herein and as is generally known in the art.

[00290] In one embodiment, a 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.

[00291] In one embodiment, a 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 formuations 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 IV (see USSN 11/353,630 *supra*).

[00292] In one embodiment, the 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.

[00293] In one embodiment, a 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.

[00294] In one embodiment, a 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.

[00295] In one embodiment, the nucleic acid 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.

[00296] 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.

[00297] 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.

[00298] In one non-limiting example, the term "metered dose inhaler" or "MDI" means a unit comprising a can, a crimped cap covering the mouth of the can, and a drug metering valve situated in the cap, while the term "MDI system" also includes a suitable channeling device. The term "MDI can" means the container without the cap and valve. The term "drug metering valve" or "MDI valve" refers to a valve and its associated mechanisms which delivers a predetermined amount of drug formulation of the double stranded nucleic acid molecules disclosed herein from an MDI upon each activation. By "drug" is intended the compositions comprising the nucleic acid molecules described elsewhere herein. The channeling device may comprise, for example, an actuating device for the valve and a cylindrical or cone-like passage through which medicament may be delivered from the filled MDI can via the MDI valve to the nose or mouth of a patient, e.g. a mouthpiece actuator. The relation of the parts of a typical MDI is illustrated in U.S. Pat. No. 5,261,538 incorporated herein by reference. An exemplary MDI is disclosed in WO 96/26755, the entire contents of

which is hereby incorporated by reference. Other exemplary pressurized containers for use in MDIs are disclosed in WO 96/32151, WO 96/32345, WO 96/32150 and WO 96/32099.

[00299] The pressurized container may be a vial made from aluminum. However, other materials may be used, including, but are not limited to, ferrous alloys, non-ferrous alloys, such as stainless steel, ceramic materials, polymers, composite materials, and mixtures thereof. Suitable containers which contain a polymeric coating on the inside thereof are disclosed in WO 96/32151.

[00300] Most often the MDI can and cap are made of aluminum or an alloy of aluminum, although other metals not affected by the drug formulation, such as stainless steel, an alloy of copper or tin plate, may be used. An MDI can may also be fabricated from glass or plastic. Preferably, however, the MDI cans employed in the present invention are made of aluminum or an alloy thereof. Advantageously, strengthened aluminum or aluminum alloy MDI cans may be employed. Such strengthened MDI cans are capable of withstanding particularly stressful coating and curing conditions, e.g., particularly high temperatures, which may be required for certain fluorocarbon polymers. Strengthened MDI cans which have a reduced tendency to malform under high temperatures include MDI cans comprising side walls and a base of increased thickness and MDI cans comprising a substantially ellipsoidal base (which increases the angle between the side walls and the base of the can), rather than the hemispherical base of standard MDI cans. MDI cans having an ellipsoidal base offer the further advantage of facilitating the coating process.

[00301] The MDI cans include MDI cans supplied by Presspart of Cary, N.C., USA or the United Kingdom, or by Neotechnic of the United Kingdom. The MDI cans typically have a neck diameter of 20 millimeters, although any suitable neck diameter may be used and can vary in height from 30 millimeters to 60 millimeters.

[00302] The drug metering valve consists of parts usually made of stainless steel, a pharmacologically inert and propellant resistant polymer, such as acetal (polyoxymethylene), polyamide (e.g., Nylon.RTM.), polycarbonate, polyester, fluorocarbon polymer (e.g., Teflon.RTM.) or a combination of these materials. Additionally, seals and "O" rings of various materials (e.g., nitrile rubbers, polyurethane, acetyl resin, fluorocarbon polymers), or other elastomeric materials are employed in and around the valve.

[00303] The preferred MDI valves have typical metering chamber volumes of 25 to 63 microliters. The valves preferably have a ferrule skirt to suit a 20 mm neck diameter can. Typical suppliers of MDI valves include Valois Pharm, France; Bespak of Europe or the United Kingdom; or Neotechnic, United Kingdom.

[00304] "Propellants" used herein mean pharmacologically inert liquids with boiling points from about room temperature (25 degrees C to about -25 degrees C) which singly or in combination exert a high vapor pressure at room temperature, including CFCs such as Freon and hydrofluorocarbons. Upon activation of the MDI system, the high vapor pressure of the propellant in the MDI forces a metered amount of drug formulation out through the metering valve then the propellant very rapidly vaporizes dispersing the drug particles. The propellants used in the present invention are low boiling fluorocarbons; in particular, hydrofluorocarbons or hydrofluoroalkanes such as HFA-134a and HFA-227. This is particularly useful with propellants (including propellant mixtures) which are more hygroscopic than P11, P114 and/or P12 such as HFA-134a and HFA-227.

[00305] The MDIs described herein are particularly useful for containing and dispensing inhaled drug formulations with hydrofluoroalkane propellants such as 134a with little, or essentially no, excipient and which tend to deposit or cling to the interior walls and parts of the MDI system. In certain cases, it is advantageous to dispense an inhalation drug with essentially no excipient, e.g., where the subject may be allergic to an excipient or the drug reacts with an excipient.

[00306] Drug formulations for use in the invention may be free or substantially free of formulation excipients, e.g., surfactants and cosolvents, etc. Such drug formulations are advantageous since they may be substantially taste and odor free, less irritant and less toxic than excipient-containing formulations. Thus, a preferred drug formulation consists essentially of a drug, or a physiologically acceptable salt or solvate thereof, optionally in combination with one or more other pharmacologically active agent, and a fluorocarbon propellant.

[00307] Further drug formulations for use in the invention may be free or substantially free of surfactant. Thus, a further preferred drug formulation comprises or consists essentially of a drug (or a physiologically acceptable salt or solvate thereof), optionally in combination with one or more other pharmacologically active agents, a fluorocarbon propellant and 0.01 to 5%

w/w based on the propellant of a polar cosolvent, which formulation is substantially free of surfactant. Preferred propellants are 1,1,1,2-tetrafluoroethane, 1,1,1,2,3,3,3-heptafluoro-n-propane or mixtures thereof, and especially 1,1,1,2-tetrafluoroethane or 1,1,1,2,3,3,3-heptafluoro-n-propane. However, the drug formulation may contain any additional excipients which are necessary or desirable to prepare a suitable drug formulation.

[00308] The term "excipients" as used herein means chemical agents having little or no pharmacological activity (for the quantities used) but which enhance the drug formulation or the performance of the MDI system. For example, excipients include but are not limited to surfactants, preservatives, flavorings, antioxidants, antiaggregating agents, and cosolvents, e.g., ethanol and diethyl ether.

[00309] Suitable surfactants are generally known in the art, for example, those surfactants disclosed in European Patent Application No. 0327777. The amount of surfactant employed is desirably in the range of 0.0001% to 50% w/w ratio relative to the drug, in particular 0.05 to 5% w/w ratio.

[00310] A polar cosolvent such as C_{2-6} aliphatic alcohols and polyols, e.g., glycerol, ethanol, isopropanol and propylene glycol, preferably ethanol, may be included in the drug formulation in the desired amount, either as the only excipient or in addition to other excipients, such as surfactants. Suitably, the drug formulation may contain 0.01 to 5% w/w based on the propellant of a polar cosolvent, e.g., ethanol, preferably 0.1 to 5% w/w, e.g., about 0.1 to 1% w/w.

[00311] In one embodiment, the 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), dipalmitoylphosphatidylchol- ine, phosphatidylcholine, phosphatidylglycerol, phosphatidylinositol, phosphatidylethanolamine, phosphatidylserine; phosphatidic acid, ubiquinones, lysophosphatidylethanolamine, lysophosphatidylcholine, palmitoyl-lysophosphatidylcholine, dehydroepiandrosterone, dolichols, sulfatidic acid, glycerol-3-phosphate, dihydroxyacetone phosphate, glycerol, glycero-3-phosphocholine,

dihydroxyacetone, palmitate, cytidine diphosphate (CDP) diacylglycerol, CDP choline, choline, choline phosphate; as well as natural and artificial lamelar 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 useed 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.

[00312] 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.

[00313] In one embodiment, the invention features the use of methods to deliver the nucleic acid molecules of the instant invention to the central nervous system and/or peripheral nervous system. 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. Pharmocol.,* 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.

[00314] 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.

[00315] In one embodiment, 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 comounds and/or therapeis 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 comounds and/or therapies described herein and/or otherwise known in the art.

[00316] In one embodiment, a siNA molecule of the invention is administered iontophoretically, for example to a particular organ or compartment (*e.g.*, lung, liver, 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.

[00317] 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. Phamacol. Exp. Ther.,* 285(2), 920-928; Kronenwett *et al.,* 1998, *Blood,* 91(3), 852-862; Filion 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 oligonucleitide, 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. In certain embodiment, the nucleic acid molecules of the invention are delivered to hematopoetic cells as is 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, and USSN 11/353,630, filed February 14, 2006 (Vargeese *et al.*), all of which are incorporated by reference herein in their entirety.

[00318] 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 polyvinylpyrolidone). 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,NI,NII,NIII-tetramethyl-N,NI,NII,NIII-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 Manheim); and (4) Lipofectamine, 3:1 (M/M) liposome formulation of the polycationic lipid DOSPA and the neutral lipid DOPE (GIBCO BRL).

[00319] 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).

[00320] In one embodiment, 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, Phramaceutical 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.

[00321] In one embodiment, a 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.

[00322] 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.

[00323] 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.

[00324] A pharmacological composition or formulation 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.

[00325] In one embodiment, siNA molecules of the invention are administered to a subject by systemic administration in a pharmaceutically acceptable composition or formulation. 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, 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.

[00326] By "pharmaceutically acceptable formulation" or "pharmaceutically acceptable composition" is meant, a composition or formulation that allows for the effective distribution of the nucleic acid molecules of the instant invention in the physical location most suitable for their desired activity. Non-limiting examples of agents suitable for formulation with the nucleic acid molecules of the instant invention include: P-glycoprotein inhibitors (such as Pluronic P85),; biodegradable polymers, such as poly (DL-lactide-coglycolide) 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.

[00327] 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 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, 42, 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.

[00328] In one embodiment, a liposomal formulation of the invention comprises a double stranded nucleic acid molecule of the invention (e.g, siNA) 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.

[00329] In one embodiment, the invention features a composition comprising a siNA molecule or double stranded nucleic acid molecule formulated as any of formulation 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**).

[00330] In one embodiment, the invention features a composition comprising a first double stranded nucleic 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 RSV target sequence and the second strand of the second double stranded nucleic acid molecule comprises sequence complementary to a second RSV target sequence. 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.

[00331] In one embodiment, 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 RSV target cells from a patient are extracted. These extracted cells are contacted with siNAs RSV 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.

[00332] In one embodiment, the invention features a method of modulating the expression of a RSV target gene in a subject or organism comprising contacting the subject or organism with a siNA molecule of the invention under conditions suitable to modulate (*e.g.*, inhibit) the expression of the RSV target gene in the subject or organism.

[00333] 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 RSV target gene 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.

[00334] In one embodiment, the invention features a method for treating or preventing RSV infection 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 RSV target gene in the subject or organism whereby the treatment or prevention of RSV infection 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 liver cells and tissues. 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 involved in the maintenance or development of RSV infection 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 tisssues 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 RSV infection in a subject or organism.

[00335] In one embodiment, the invention features a method for treating or preventing respiratory distress 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 RSV target gene in the subject or organism whereby the treatment or prevention of respiratory failure 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 lung cells and tissues involved in respiratory failure. 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 involved in the maintenance or development of the respiratory failure 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 tisssues 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 respiratory failure in a subject or organism.

[00336] In one embodiment, the invention features a method for treating or preventing bronchiolitis 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 RSV target gene in the subject or organism whereby the treatment or prevention of bronchiolitis 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 liver cells and tissues involved in bronchiolitis. 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 involved in the maintenance or development of bronchiolitis 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 tisssues 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 bronchiolitis in a subject or organism.

[00337] In one embodiment, the invention features a method for treating or preventing pneumonia 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 RSV target gene in the subject or organism whereby the treatment or prevention of pneumonia 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 cells and tissues involved in pneumonia. 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 involved in the maintenance or development of pneumonia 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 tisssues 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 pneumonia in a subject or organism.

[00338] In one embodiment, the invention features a method for treating or preventing RSV infection in a subject or organism comprising contacting the subject or organism with a siNA

molecule of the invention under conditions suitable to modulate (*e.g.*, inhibit) the expression of an inhibitor of RSV gene expression in the subject or organism.

[00339] In one embodiment, the invention features a method for treating or preventing respiratory failure in a subject or organism comprising contacting the subject or organism with a siNA molecule of the invention under conditions suitable to modulate (*e.g.*, inhibit) the expression of an inhibitor of RSV gene expression in the subject or organism.

[00340] In one embodiment, the invention features a method for treating or preventing bronchiolitis in a subject or organism comprising contacting the subject or organism with a siNA molecule of the invention under conditions suitable to modulate (*e.g.*, inhibit) the expression of an inhibitor of RSV gene expression in the subject or organism.

[00341] In one embodiment, the invention features a method for treating or preventing pneumonia in a subject or organism comprising contacting the subject or organism with a siNA molecule of the invention under conditions suitable to modulate (*e.g.*, inhibit) the expression of an inhibitor of RSV gene expression in the subject or organism.

[00342] In one embodiment, the invention features a method for treating or preventing Respiratory syncytial virus (RSV) infection in a subject, comprising administering to the subject PEG Interferon in combination with a siNA molecule of the invention; wherein the PEG Interferon and the siNA molecule are administered under conditions suitable for reducing or inhibiting the level of Respiratory syncytial virus (RSV) in the subject compared to a subject not treated with the PEG Interferon and the siNA molecule. In one embodiment, a 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, and U.S. Provisional patent application No. 60/737,024, filed November 15, 2005 (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).

[00343] In one embodiment, the invention features a method for treating or preventing Respiratory syncytial virus (RSV) infection in a subject, comprising administering to the subject ribavirin in combination with a siNA molecule of the invention; wherein the ribavirin and the siNA are administered under conditions suitable for reducing or inhibiting the level of Respiratory syncytial virus (RSV) in the subject compared to a subject not treated with the

ribavirin and the siNA molecule. 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.*).

[00344] In one embodiment, the invention features a method for treating or preventing Respiratory syncytial virus (RSV) infection in a subject, comprising administering to the subject PEG Interferon and ribavirin in combination with a siNA molecule of the invention; wherein the PEG Interferon and ribavirin and the siNA molecule are administered under conditions suitable for reducing or inhibiting the level of Respiratory syncytial virus (RSV) in the subject compared to a subject not treated with the PEG Interferon and ribavirin and the siNA molecule. 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.*).

[00345] In one embodiment, in addition to the methods described herein or in combination with the methods described herein, a subject is further treated with palivizumab, RespiGam, A-60444, or other antiviral compounds and fusion inhibitors that may be used to treat RSV infection, alone, or in combination with other therapeutic modalities.

[00346] In any of the above method for treating or preventing respiratory syncytial virus (RSV) infection in a subject, the treatment is combined with administration of a corticosteroid composition as is generally recognized in the art, including Triamcinolone acetonide, methylprednisolone, and dexamethasone.

[00347] In any of the above method for treating or preventing respiratory syncytial virus (RSV) infection in a subject, the treatment is combined with administration of a beta-2 agonist composition as is generally recognized in the art, including for example, albuterol or albuterol sulfate.

[00348] 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 phamaceutically acceptable carrier or diluent. In another embodiment, the

invention features a composition comprising PEG Interferon, ribavirin, Vertex VX-950, Actilon (CPG 10101), and/or Isatoribine (TLR-7 agonist) and one or more double stranded nucleic acid molecules or siNA molecules of the invention in a phamaceutically acceptable carrier or diluent.

[00349] 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 Interferon (e.g., Interferon-alpha, or PEG interferon such as PEG-Intron, Rebetol, Rebetron, or Pegasys), ribavirin, Vertex VX-950, Actilon (CPG 10101), or Isatoribine (TLR-7 agonist). In another embodiment, such combination therapies can be utilized in any of the embodiments herein.

[00350] In any of the methods of treatment of the invention, the 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).

[00351] In one embodiment, a course of treatment 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).

[00352] In any of the methods of treatment of the invention, the 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 gastrointestinal administration as is generally known in the art.

[00353] In one embodiment, in any of the methods of treatment or prevention of the invention, the 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.

[00354] In another embodiment, the invention features a method of modulating the expression of more than one RSV target gene in a subject or organism comprising contacting the subject or organism with one or more siNA molecules of the invention under conditions suitable to modulate (*e.g.*, inhibit) the expression of the RSV target genes in the subject or organism.

[00355] 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.

[00356] 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.

[00357] 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 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 hearing loss, deafness, tinnitus, and/or motion and balance 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.

[00358] 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.

[00359] By "phenotypic change" is meant any detectable change to a cell that occurs in response to contact or treatment with a nucleic acid 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.

[00360] In one embodiment, the invention features a kit containing a 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.

[00361] In one embodiment, the invention features a cell containing one or more siNA molecules of the invention, which can be chemically-modified. In another embodiment, the cell containing a 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.

[00362] The present invention also includes compositions prepared for storage or administration that include a pharmaceutically 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.

[00363] 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.

[00364] The nucleic acid 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.

[00365] 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 monosterate or glyceryl distearate can be employed.

[00366] 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.

[00367] 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.

[00368] 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

[00369] 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.

[00370] 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.

[00371] 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.

[00372] 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.

[00373] 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.

[00374] 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.

[00375] 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.

[00376] 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.

[00377] 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.

[00378] In one embodiment, the invention comprises compositions suitable for administering nucleic acid 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, triatennary structures are bound with greater affinity than

biatenarry or monoatennary 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.

[00379] Alternatively, certain 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.

[00380] 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. 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).

[00381] In one aspect the invention features an expression vector comprising a nucleic acid sequence encoding at least one 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).

[00382] 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).

[00383] 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; Lisziewicz *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).*

[00384] In another aspect the invention features an expression vector comprising a nucleic acid sequence encoding at least one of the 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.

[00385] 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 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.

[00386] 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 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.

RSV biology and biochemistry

[00387] Respiratory syncytial virus (RSV) is the most common cause of bronchiolitis and pneumonia among infants and children under 1 year of age. Illness begins most frequently with fever, runny nose, cough, and sometimes wheezing. During their first RSV infection, between 25% and 40% of infants and young children have signs or symptoms of bronchiolitis or pneumonia, and 0.5% to 2% require hospitalization. Most children recover from illness in 8 to 15 days. The majority of children hospitalized for RSV infection are under 6 months of age. RSV also causes repeated infections throughout life, usually associated with moderate-to-severe cold-like symptoms; however, severe lower respiratory tract disease may occur at any age, especially among the elderly or among those with compromised cardiac, pulmonary, or immune systems.

[00388] Human respiratory syncytial virus (hRSV or RSV) is the type species of the genus Pneumovirus. Along with other members of the family Paramyxoviridae, hRSV is an enveloped virus with a negative sense, single-stranded RNA genome. These viruses are 150-200nm in diameter with a helical nucleocapsid. Other members of the family include human

parainfluenzavirus 1 (HPIV-1; genus Respirovirus), mumps virus (MuV), human parainfluenzavirus 2 and 4 (HPIV-2 and HPIV-4; genus Rubulavirus), measles virus (MeV; genus Morbillivirus), Hendravirus and Nipahvirus (genus Henipavirus) and human metapneumovirus (hMPV; genus Metapneumovirus).

[00389] The hRSV virion enters its target cell by fusion of the hRSV envelope with the cell membrane and release of the viral genome into the cell's cytoplasm where translation will occur. The nucleopretein (N), large (L) and phosphoproteins (P) together with the RNA genome form the nucleoprotein core. These together with the matrix (M), fusion (F), and glycoprotein (G) are classified as structural proteins. The nonstructural proteins include NS1 and 2, small hydrophobic (SH) and M2 (formerly 22-kDa). The respiratory syncytial virus genome is transcribed from the 3' end into monocistronic (each species only encodes a single protein) mRNA molecules. New viruses are released form the infected cell buy budding. In the presence of newly synthesized hRSV fusion (F) protein, neighbouring infected cells may form a clump whose membranes have fused to form a "giant cell" called a syncytium. New virions can then spread more effectively from cell-to-cell.

[00390] RSV is spread from respiratory secretions through close contact with infected persons or contact with contaminated surfaces or objects. Infection can occur when infectious material contacts mucous membranes of the eyes, mouth, or nose, and possibly through the inhalation of droplets generated by a sneeze or cough. In temperate climates, RSV infections usually occur during annual community outbreaks, often lasting 4 to 6 months, during the late fall, winter, or early spring months. The timing and severity of outbreaks in a community vary from year to year. RSV spreads efficiently among children during the annual outbreaks, and most children will have serologic evidence of RSV infection by 2 years of age.

[00391] Diagnosis of RSV infection can be made by virus isolation, detection of viral antigens, detection of viral RNA, demonstration of a rise in serum antibodies, or a combination of these approaches. Most clinical laboratories use antigen detection assays to diagnose infection.

[00392] For children with mild disease, no specific treatment is necessary other than the treatment of symptoms (e.g., acetaminophen to reduce fever). Children with severe disease may require oxygen therapy and sometimes mechanical ventilation. Ribavirin aerosol may be used in the treatment of some patients with severe disease. Some investigators have used a

combination of immune globulin intravenous (IGIV) with high titers of neutralizing RSV antibody (RSV-IGIV) and ribavirin to treat patients with compromised immune systems.

[00393] The use of nucleic molecules of the invention targeting RSV genes and cellular/host gene targets associated with the RSV life cycle therefore provides a class of novel therapeutic agents that can be used in the treatment and diagnosis of RSV infection, respiratory distress, stridor, bronchiolitis and pneumonia, or any other disease or condition that responds to modulation (*e.g.*, inhibition) of RSV genes in a subject or organism.

Examples:

[00394] 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

[00395] The 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.

[00396] 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.

[00397] 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 Bromotripyrrolidinophosphoniumhexaflurorophosphate (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 $NH_4H_2CO_3$.

[00398] 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 H20 followed by 1 CV 1M NaCl and additional H2O. The siNA duplex product is then eluted, for example, using 1 CV 20% aqueous CAN.

[00399] Isolated and purified siRNAs can be characterized using any one or all of a variety of techniques. Matrix assisted laser desorption ionization (MALDI-TOF) mass spectrometry analysis of a purified siNA construct allows for identification and confirmation of 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 can be performed using a luciferase reporter assay that can assess RNAi activity of siNA constructs generated as double strands or from separately synthesized oligonucleotide sequence strands.

Example 2: Design, Synthesis, and Identification of siRNAs Active Against RSV

RSV siRNA synthesis

[00400] The RSV used in the following experiments was sucrose density gradient purified RSV Strain A-2 (Cat # 10-247-000) from Advanced Biotechnologies Incorporated (Columbia, MD). All transfections were carried out using Lipofectamine 2000 (LF2K; Invitrogen Cat# 11668-027) as per the manufacturer's instructions. Detailed synthetic and transfection conditions are described below.

[00401] A series of 48 total siRNAs (12 for each RSV gene critical cell replication (*i.e.*, N, P, M2, and L)) were designed, synthesized and evaluated for efficacy against RSV as well as for general cytotoxicity profile. The primary criteria for design of RSV siNAs were (i) conservation across selected strains of RSV and (ii) high efficacy scores as determined by a proprietary algorithm. Screening assays were conducted such that the cells were infected with RSV and transfected with siRNA on the same day. The primary endpoints for the screen were the syncytia/plaque forming assay at low and high multiplicity of infection (MOI), and a cell viability assay. The effects of the siRNAs on RSV protein production, RSV mRNA levels and cell cytotoxicity were also examined. The sequences of the siRNAs that were designed, synthesized, and evaluated for efficacy against RSV and for general cytotoxicity profiles are described in Table 1a (duplexes) and Table 1b (target sequence and modified sequence).

Table 1a: RSV siRNAs Duplexes Synthesized.

Duplex #	Duplexing ID	Target Name	Target Position	S Cmpnd #	AS Cmpnd #	Target Sequence	SEQ ID NO:
15277-DC	35131	RSVN	1140	50419	50420	AUGGCUCUUAGCAAAGUCA	1
15279-DC	35133	RSVN	1143	50423	50424	GCUCUUAGCAAAGUCAAGU	2
15278-DC	35132	RSVN	1144	50421	50422	CUCUUAGCAAAGUCAAGUU	3
15287-DC	35141	RSVN	1329	50439	50440	UUAAUAGGUAUGUUAUAUG	4
15286-DC	35140	RSVN	1518	50437	50438	AUUGAGAUAGAAUCUAGAA	5
15284-DC	35138	RSVN	1519	50433	50434	UUGAGAUAGAAUCUAGAAA	6
15280-DC	35134	RSVN	1539	50425	50426	UCCUACAAAAAAAUGCUAA	7
15276-DC	35130	RSVN	1540	50417	50418	CCUACAAAAAAAUGCUAAA	8
15281-DC	35135	RSVN	1541	50427	50428	CUACAAAAAAAUGCUAAAA	9
15283-DC	35137	RSVN	1543	50431	50432	ACAAAAAAAUGCUAAAAGA	10
15285-DC	35139	RSVN	2037	50435	50436	UUCUACCAUAUAUUGAACA	11
15282-DC	35136	RSVN	2038	50429	50430	UCUACCAUAUAUUGAACAA	12
15299-DC	35153	RSVP	2401	50463	50464	AAUUCCUAGAAUCAAUAAA	13
15296-DC	35150	RSVP	2406	50457	50458	CUAGAAUCAAUAAAGGGCA	14
15288-DC	35142	RSVP	2477	50441	50442	CUCAAUAGAUAUAGAAGUA	15
15294-DC	35148	RSVP	2479	50453	50454	CAAUAGAUAUAGAAGUAAC	16
15291-DC	35145	RSVP	2668	50447	50448	CAUUUGAUAACAAUGAAGA	17
15295-DC	35149	RSVP	2671	50455	50456	UUGAUAACAAUGAAGAAGA	18
15292-DC	35146	RSVP	2672	50449	50450	UGAUAACAAUGAAGAAGAA	19
15297-DC	35151	RSVP	2673	50459	50460	GAUAACAAUGAAGAAGAAU	20
15293-DC	35147	RSVP	2759	50451	50452	UGAUGAAAAAUUAAGUGAA	21
15289-DC	35143	RSVP	2760	50443	50444	GAUGAAAAAUUAAGUGAAA	22
15290-DC	35144	RSVP	2895	50445	50446	GAAGCAUUAAUGACCAAUG	23
15298-DC	35152	RSVP	2896	50461	50462	AAGCAUUAAUGACCAAUGA	24

Duplex #	Duplexing ID	Target Name	Target Position	S Cmpnd #	AS Cmpnd #	Target Sequence	SEQ ID NO:
15300-DC	35154	RSVM2	7833	50465	50466	GUUGGAGUGCUAGAGAGUU	25
15301-DC	35155	RSVM2	7834	50467	50468	UUGGAGUGCUAGAGAGUUA	26
15302-DC	35156	RSVM2	7976	50469	50470	GAUAAGAGUGUACAAUACU	27
15303-DC	35157	RSVM2	7978	50471	50472	UAAGAGUGUACAAUACUGU	28
15304-DC	35158	RSVM2	8411	50473	50474	UAUAUAUAUUAGUGUCAUA	29
15305-DC	35159	RSVM2	8412	50475	50476	AUAUAUAUUAGUGUCAUAA	30
15306-DC	35160	RSVM2-L	8489	50477	50478	GGGACAAAAUGGAUCCCAU	31
15307-DC	35161	RSVM2-L	8490	50479	50480	GGACAAAAUGGAUCCCAUU	32
15308-DC	35162	RSVM2-L	8493	50481	50482	CAAAAUGGAUCCCAUUAUU	33
15309-DC	35163	RSVM2-L	8499	50483	50484	GGAUCCCAUUAUUAAUGGA	34
15310-DC	35164	RSVM2-L	8500	50485	50486	GAUCCCAUUAUUAAUGGAA	35
15311-DC	35165	RSVM2-L	8501	50487	50488	AUCCCAUUAUUAAUGGAAA	36
15312-DC	35166	RSVL	9091	50489	50490	CAUCCUCCAUCAUGGUUAA	37
15318-DC	35172	RSVL	10077	50501	50502	GCCUAAAAAAGUGGAUCUU	38
15319-DC	35173	RSVL	10365	50503	50504	CAGUGUAGGUAGAAUGUUU	39
15313-DC	35167	RSVL	12532	50491	50492	CCAUGUGAAUUCCCUGCAU	40
15314-DC	35168	RSVL	12544	50493	50494	CCUGCAUCAAUACCAGCUU	41
15315-DC	35169	RSVL	12545	50495	50496	CUGCAUCAAUACCAGCUUA	42
15323-DC	35177	RSVL	13364	50511	50512	GCCCUUGGGUUGUUAACAU	43
15320-DC	35174	RSVL	13365	50505	50506	CCCUUGGGUUGUUAACAUA	44
15321-DC	35175	RSVL	13369	50507	50508	UGGGUUGUUAACAUAGAUU	45
15316-DC	35170	RSVL	13370	50497	50498	GGGUUGUUAACAUAGAUUA	46
15317-DC	35171	RSVL	14252	50499	50500	GGUCUUAUUUACAUAUAAA	47
15322-DC	35176	RSVL	14676	50509	50510	CCCUAUAACAAAAAAAGGA	48

Table 1b: RSV siRNAs Strands Synthesized.

Compound #	Synthesis #	Target Name	Target Position	Target Sequence	Seq ID No	Modified sequence	Seq ID No
50419	65671	RSVNP	1140	AUGGCUCUUAGCAAAGUCA	1	B*A*u*GG*cucuu*AG*c*AAA*Guc*ATT*B	49
50420	65672	RSVNP	1140	AUGGCUCUUAGCAAAGUCA	1	UGAcuuuGcuAAGAGccAuUU	50
50423	65675	RSVNP	1143	GCUCUUAGCAAAGUCAAGU	2	B*G*cucuu*AG*c*AAA*Guc*AAG*u*TT*B	51
50424	65676	RSVNP	1143	GCUCUUAGCAAAGUCAAGU	2	ACUuGAcuuuGcuAAGAGcUU	52
50421	65673	RSVNP	1144	CUCUUAGCAAAGUCAAGUU	3	Bcucuu*AG*c*AAA*Guc*AAG*uu*TT*B	53
50422	65674	RSVNP	1144	CUCUUAGCAAAGUCAAGUU	3	AACuuGAcuuuGcuAAGAGUU	54
50439	65691	RSVNP	1329	UUAAUAGGUAUGUUAUAUG	4	Buu*AA*u*AGG*u*Au*Guu*Au*Au*GTT*B	55
50440	65887	RSVNP	1329	UUAAUAGGUAUGUUAUAUG	4	CAUAuAAcAuAccuAuuAAUU	56
50437	65689	RSVNP	1518	AUUGAGAUAGAAUCUAGAA	5	B*A*uu*GAG*u*AGAA*ucu*AGAATT*B	57
50438	65690	RSVNP	1518	AUUGAGAUAGAAUCUAGAA	5	UUCuAGAuucuAucucAAuUU	58
50433	65685	RSVNP	1519	UUGAGAUAGAAUCUAGAAA	6	Buu*GAG*u*AGAA*ucu*AGAAATT*B	59
50434	65845	RSVNP	1519	UUGAGAUAGAAUCUAGAAA	6	UUUcuAGAuucuAucucAAUU	60
50425	65677	RSVNP	1539	UCCUACAAAAAAAUGCUAA	7	Buccu*Ac*A*AAAAAA*u*Gcu*AATT*B	61
50426	65678	RSVNP	1539	UCCUACAAAAAAAUGCUAA	7	UUAGcAuuuuuuuGuAGGAUU	62
50417	65669	RSVNP	1540	CCUACAAAAAAAUGCUAAA	8	Bccu*Ac*A*AAAAAA*u*Gcu*AAATT*B	63
50418	65670	RSVNP	1540	CCUACAAAAAAAUGCUAAA	8	UUUAGcAuuuuuuuGuAGGUU	64
50427	65679	RSVNP	1541	CUACAAAAAAAUGCUAAAA	9	Bcu*Ac*A*AAAAAA*u*Gcu*AAAATT*B	65
50428	65680	RSVNP	1541	CUACAAAAAAAUGCUAAAA	9	UUUuAGcAuuuuuuuGuAGUU	66
50431	65683	RSVNP	1543	ACAAAAAAAUGCUAAAAGA	10	B*Ac*A*AAAAAA*u*Gcu*AAAAGATT*B	67
50432	65684	RSVNP	1543	ACAAAAAAAUGCUAAAAGA	10	UCUuuuAGcAuuuuuuuGuUU	68
50435	65687	RSVNP	2037	UUCUACCAUAUAUUGAACA	11	Buucu*Acc*A*u*A*u*Auu*GAAc*ATT*B	69
50436	65886	RSVNP	2037	UUCUACCAUAUAUUGAACA	11	UGUucAAuAuAuGGuAGAAUU	70
50429	65681	RSVNP	2038	UCUACCAUAUAUUGAACAA	12	Bucu*Acc*A*u*A*u*Auu*GAAc*A*ATT*B	71

Compound #	Synthesis #	Target Name	Target Position	Target Sequence	Seq ID No	Modified sequence	Seq ID No
50430	65682	RSVNP	2038	UCUACCAUAUAUUGAACAA	12	UUGuucAAuAuAuGGuAGAUU	72
50463	65715	RSVP	2401	AAUUCCUAGAAUCAAUAAA	13	B*AA*uuccu*AGAA*uc*AA*u*AAATT*B	73
50464	65716	RSVP	2401	AAUUCCUAGAAUCAAUAAA	13	UUUAuuGAuucuAGGAAuuUU	74
50457	65709	RSVP	2406	CUAGAAUCAAUAAAGGGCA	14	Bcu*AGAA*uc*AA*u*AAAGGG*c*ATT*B	75
50458	65710	RSVP	2406	CUAGAAUCAAUAAAGGGCA	14	UGCccuuuAuuGAuucuAGUU	76
50441	65693	RSVP	2477	CUCAAUAGAUAUAGAAGUA	15	Bcuc*AA*u*AGA*u*Au**AGAAG*u*ATT*B	77
50442	65694	RSVP	2477	CUCAAUAGAUAUAGAAGUA	15	UACuucuAuAucuAuuGAGUU	78
50453	65705	RSVP	2479	CAAUAGAUAUAGAAGUAAC	16	Bc*AA*u*AGA*u*Au**AGAAG*u*AAc*TT*B	79
50454	65706	RSVP	2479	CAAUAGAUAUAGAAGUAAC	16	GUUAcuucuAuAucuAuuGUU	80
50447	65699	RSVP	2668	CAUUUGAUAACAAUGAAGA	17	Bc*A*uuu*GA*u*AA*c*AA*u*GAAGATT*B	81
50448	65700	RSVP	2668	CAUUUGAUAACAAUGAAGA	17	UCUucAuuGuuAucAAAuGUU	82
50455	65707	RSVP	2671	UUGAUAACAAUGAAGAAGA	18	Buu*GA*u*AA*c*AA*u*GAAGAAGATT*B	83
50456	65708	RSVP	2671	UUGAUAACAAUGAAGAAGA	18	UCUucuucAuuGuuAucAAUU	84
50449	65701	RSVP	2672	UGAUAACAAUGAAGAAGAA	19	Bu*GA*u*AA*c*AA*u*GAAGAAGAATT*B	85
50450	65702	RSVP	2672	UGAUAACAAUGAAGAAGAA	19	UUCuucuucAuuGuuAucAUU	86
50459	65711	RSVP	2673	GAUAACAAUGAAGAAGAAU	20	B*GA*u*AA*c*AA*u*GAAGAAGAA*u*TT*B	87
50460	65901	RSVP	2673	GAUAACAAUGAAGAAGAAU	20	AUUcuucuucAuuGuuAucUU	88
50451	65703	RSVP	2759	UGAUGAAAAAUUAAGUGAA	21	Bu*GA*u*GAAAAA*uu*AAG*u*GAATT*B	89
50452	65704	RSVP	2759	UGAUGAAAAAUUAAGUGAA	21	UUCAcuuAAuuuuucAucAUU	90
50443	65695	RSVP	2760	GAUGAAAAAUUAAGUGAAA	22	B*GA*u*GAAAAA*uu*AAG*u*GAAATT*B	91
50444	65696	RSVP	2760	GAUGAAAAAUUAAGUGAAA	22	UUUcAcuuAAuuuuucAucUU	92
50445	65697	RSVP	2895	GAAGCAUUAAUGACCAAUG	23	B*GAAG*c*AA*uu*AA*u*GA*cc*AA*u*GTT*B	93
50446	65698	RSVP	2895	GAAGCAUUAAUGACCAAUG	23	CAUuGGucAuuAAuGcuucUU	94
50461	65713	RSVP	2896	AAGCAUUAAUGACCAAUGA	24	B*AAG*c*AA*uu*AA*u*GA*cc*AA*u*GATT*B	95
50462	65892	RSVP	2896	AAGCAUUAAUGACCAAUGA	24	UCAuuGGucAuuAAuGcuuUU	96
50465	65717	RSVM2	7833	GUUGGAGUGCUAGAGAGUU	25	B*G*uu*GGAG*u*G*c*uAGAGAG*uu*TT*B	97
50466	65718	RSVM2	7833	GUUGGAGUGCUAGAGAGUU	25	AACucucuAGcAcuccAAcUU	98

Compound #	Synthesis #	Target Name	Target Position	Target Sequence	Seq ID No	Modified sequence	Seq ID No
50467	65719	RSVM2	7834	UUGGAGUGCUAGAGAGUUA	26	Buu*GGA*Gu*G*cu*AGAGAG*uu*ATT*B	99
50468	65720	RSVM2	7834	UUGGAGUGCUAGAGAGUUA	26	UAAcucucuAGcAcuccAAUU	100
50469	65721	RSVM2	7976	GAUAAGAGUGUACAAUACU	27	B*GA*u*AAGAG*u*G*u*Ac*A*Au*Acu*TT*B	101
50470	65722	RSVM2	7976	GAUAAGAGUGUACAAUACU	27	AGUAuuGuAcAcucuuAucUU	102
50471	65723	RSVM2	7978	UAAGAGUGUACAAUACUGU	28	Bu*AAGAG*u*G*u*Ac*A*Au*Acu*G*u*TT*B	103
50472	65724	RSVM2	7978	UAAGAGUGUACAAUACUGU	28	ACAGuAuuGuAcAcucuuAUU	104
50473	65725	RSVM2	8411	UAUAUAUAUUAGUGUCAUA	29	Bu*A*u*A*u*A*u*A*uu*AG*u*G*uc*A*u*ATT*B	105
50474	65726	RSVM2	8411	UAUAUAUAUUAGUGUCAUA	29	UAUGAcAcuAAuAuAuAuAUU	106
50475	65727	RSVM2	8412	AUAUAUAUUAGUGUCAUAA	30	B*A*u*A*u*A*u*A*uu*AG*u*G*uc*A*u*AATT*B	107
50476	65728	RSVM2	8412	AUAUAUAUUAGUGUCAUAA	30	UUAuGAcAcuAAuAuAuAuUU	108
50477	65846	RSVM2-L	8489	GGGACAAAAUGGAUCCCAU	31	B*GGGAcAAAA*u*GGA*ucccA*u*TT*B	109
50478	65730	RSVM2-L	8489	GGGACAAAAUGGAUCCCAU	31	AUGGGAuccAuuuuGucccUU	110
50479	65731	RSVM2-L	8490	GGACAAAAUGGAUCCCAUU	32	B*GGAcAAAA*u*GGA*ucccA*uu*TT*B	111
50480	65732	RSVM2-L	8490	GGACAAAAUGGAUCCCAUU	32	AAUGGGAuccAuuuuGuccUU	112
50481	65733	RSVM2-L	8493	CAAAAUGGAUCCCAUUAUU	33	Bc*AAAA*u*GGA*ucccA*uu*A*uu*TT*B	113
50482	65734	RSVM2-L	8493	CAAAAUGGAUCCCAUUAUU	33	AAUAAuGGGAuccAuuuuGUU	114
50483	65735	RSVM2-L	8499	GGAUCCCAUUAUUAAUGGA	34	B*GGA*ucccA*uu*A*uu*A*A*u*GGATT*B	115
50484	65736	RSVM2-L	8499	GGAUCCCAUUAUUAAUGGA	34	UCCAuuAAuAAuGGGAuccUU	116
50485	65737	RSVM2-L	8500	GAUCCCAUUAUUAAUGGAA	35	B*GA*ucccA*uu*A*uu*A*A*u*GGAATT*B	117
50486	65738	RSVM2-L	8500	GAUCCCAUUAUUAAUGGAA	35	UUCcAuuAAuAAuGGGAucUU	118
50487	65739	RSVM2-L	8501	AUCCCAUUAUUAAUGGAAA	36	B*A*ucccA*uu*A*uu*A*A*u*GGAAATT*B	119
50488	65740	RSVM2-L	8501	AUCCCAUUAUUAAUGGAAA	36	UUUccAuuAAuAAuGGGAuUU	120
50489	65741	RSVL	9091	CAUCCUCCAUCAUGGUUAA	37	Bc*A*uccuccA*uc*A*u*GGuu*AATT*B	121
50490	65742	RSVL	9091	CAUCCUCCAUCAUGGUUAA	37	UUAAccAuGAuGGAGGAuGUU	122
50501	65753	RSVL	10077	GCCUAAAAAAGUGGAUCUU	38	B*G*ccu*AAAAAAG*u*GGA*ucuu*TT*B	123
50502	65754	RSVL	10077	GCCUAAAAAAGUGGAUCUU	38	AAGAuccAcuuuuuuAGGcUU	124
50503	65755	RSVL	10365	CAGUGUAGGUAGAAUGUUU	39	Bc*AG*u*G*u*AGG*u*AGAA*u*G*uuu*TT*B	125

Compound #	Synthesis #	Target Name	Target Position	Target Sequence	Seq ID No	Modified sequence	Seq ID No
50504	65756	RSVL	10365	CAGUGUAGGUAGAAUGUUU	39	AAAcAuucuAccuAcAcuGUU	126
50491	65743	RSVL	12532	CCAUGUGAAUUCCCUGCAU	40	BccAuGuGAAuucccuGcAuTTB	127
50492	65744	RSVL	12532	CCAUGUGAAUUCCCUGCAU	40	AUGcAGGGAAuucAcAuGGUU	128
50493	65745	RSVL	12544	CCUGCAUCAAUACCAGCUU	41	BccuGcAucAAuAccAGcuuTTB	129
50494	65746	RSVL	12544	CCUGCAUCAAUACCAGCUU	41	AAGcuGGuAuuGAuGcAGGUU	130
50495	65747	RSVL	12545	CUGCAUCAAUACCAGCUUA	42	BcuGcAucAAuAccAGcuuATTB	131
50496	65748	RSVL	12545	CUGCAUCAAUACCAGCUUA	42	UAAGcuGGuAuuGAuGcAGUU	132
50511	65763	RSVL	13364	GCCCUUGGGUUGUUAACAU	43	BGcccuuGGGuuGuuAAcAuTTB	133
50512	65764	RSVL	13364	GCCCUUGGGUUGUUAACAU	43	AUGuuAAcAAcccAAGGGcUU	134
50505	65847	RSVL	13365	CCCUUGGGUUGUUAACAUA	44	BcccuuGGGuuGuuAAcAuATTB	135
50506	66052	RSVL	13365	CCCUUGGGUUGUUAACAUA	44	UAUGuuAAcAAcccAAGGGUU	136
50507	65759	RSVL	13369	UGGGUUGUUAACAUAGAUU	45	BuGGGuuGuuAAcAuAGAuuTTB	137
50508	65760	RSVL	13369	UGGGUUGUUAACAUAGAUU	45	AAUcuAuGuuAAcAAcccAUU	138
50497	65749	RSVL	13370	GGGUUGUUAACAUAGAUUA	46	BGGGuuGuuAAcAuAGAuuATTB	139
50498	65750	RSVL	13370	GGGUUGUUAACAUAGAUUA	46	UAAucuAuGuuAAcAAcccUU	140
50499	65751	RSVL	14252	GGUCUUAUUUACAUAUAAA	47	BGGucuuAuuuAcAuAuAAATTB	141
50500	65752	RSVL	14252	GGUCUUAUUUACAUAUAAA	47	UUUAuAuGuAAAuAAGAccUU	142
50509	65761	RSVL	14676	CCCUAUAACAAAAAAAGGA	48	BcccuAuAAcAAAAAAAGGATTB	143
50510	65762	RSVL	14676	CCCUAUAACAAAAAAAGGA	48	UCCuuuuuuuGuuAuAGGGUU	144

wherein:
A, C, G, and U = ribose A, C, G or U
c and u = 2'-fluoro C or U
A, U and G = 2'-O-methyl (2'-OMe) A U or G
A and *G* = deoxy A or G
B = inverted abasic

Small Scale Synthetic Procedure

[00402] For each oligonucleotide, the two individual, complementary strands of the siRNA are synthesized separately using solid phase synthesis, then purified separately by reversed phase solid phase extraction (SPE). The complementary strands are annealed to form the double strand (duplex) and delivered in the desired concentration and buffer of choice.

[00403] Briefly, the single strand oligonucleotides are synthesized using phosphoramidite chemistry on an automated solid-phase synthesizer. A small-scale 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 solution 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 final 5'-terminus protecting group (trityl or 5'-O-dimethoxytrityl).

[00404] Upon completion of the synthesis, the solid-support and associated oligonucleotide is dried under argon pressure. 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.

[00405] 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 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 allows for retention of the 5'-O-dimethoxytrityl group to remain on the 5' terminal position (trityl-on).

[00406] The following process is performed on single strands that contain ribonucleotides. After treating the solid support with the aqueous base, the mixture is filtered 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. Fluoride reagent such as triethylamine trihydrofluoride is added to the mixture, and the solution is heated. The reaction is quenched with suitable buffer to provide a solution of crude single strand with the 5'-O-dimethoxytrityl group on the final 5' terminal position.

[00407] The trityl–on solution of each crude single strand is purified using SPE RPC purification. The hydrophobic nature of the trityl group permits stronger retention of the desired full-length oligo than the non-tritylated truncated failure sequences. The failure sequences are selectively washed from the resin with a low percent acetonitrile rinse. Retained oligonucleotides are then detritylated on-column with trifluoroacetic acid to remove the acid-labile trityl group. Residual acid is washed from the column, a salt exchange is performed, and a final desalting of the material commences. The full-length oligo is recovered in a purified form with an aqueous-organic solvent. The final product is then analyzed for purity (HPLC), identity (Maldi-TOF MS), and yield (UV A_{260}). The oligos are dried via lyophilization or vacuum condensation.

[00408] *Annealing:* Based on the analysis of the product, the dried oligos are dissolved in appropriate buffers followed by mixing equal molar amounts (calculated using the theoretical extinction coefficient) of the sense and antisense oligonucleotide strands. The solution is then analyzed for purity of duplex by chromatographic methods and desired final concentration. 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 delivered and ready for use.

Large Scale siRNA Manufacture

[00409] For each oligonucleotide, the two individual, complementary strands of the siRNA are synthesized separately using phosphoramidite chemistry on an automated solid phase synthesis, then purified separately by ion exchange chromatography. Briefly, an adjustable/fixed 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.

[00410] 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.

[00411] For 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.

[00412] For 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.

[00413] *Anion Exchange Purification:* 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 containers, analyzed by HPLC, and the appropriate fractions are combined to provide a pool of product, which is analyzed for purity (HPLC), identity (MS), and concentration (UV A_{260}).

[00414] *Annealing:* 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.

[00415] *Ultrafiltration:* 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 Milli-Q water until the conductivity of the filtrate is that of water.

[00416] *Lyophilization:* The concentrated solution is transferred to a bottle, flash frozen and attached to a lyophilizer. The product is then freeze-dried to a powder. The bottle is removed from the lyophilizer and is now ready for use.

Initial Screening Protocol (96 well plate transfections)

[00417] Cells (Hep2) were plated in the center 60 wells of a tissue-culture treated, 96-well plate at a final count of 7500 cells/well in 100μL of the appropriate culture media. The outer wells of the plate were filled with 100μL of DPBS to prevent evaporation. The cells were cultured for 24 hours after plating at 37°C in the presence of 5% CO_2.

[00418] After 24 hours, complexes containing siRNA and LF2K were created as follows: A solution of LF2K in OPTI-MEM was prepared containing LF2K at a final concentration of 10 μg/mL. In parallel, solutions of the siRNAs to be tested were prepared to a final concentration of 150 nM in OPTI-MEM. After incubation of both solutions at 20 °C for 20 minutes, an equal volume of the siRNA solution and the LF2K solution were added together for each of the siRNAs.

[00419] This resulting siRNA/LF2K solution contained a final concentration of siRNA of 75 nM and a final concentration of LF2K of 5 μg/mL. This solution was incubated at 20°C for 20 minutes. After incubation, 50 uL of the solution was added to each of the relevant wells (each siRNA was screened in triplicate in each experiment). The final concentration of siRNA in each well was 25 nM and the final concentration of LF2K was 1.66 μg/mL.

Transfection and Infection of Hep2 cells

[00420] Using a standard transfection protocol (0.25 µl LF2K per well) Hep2 cells were transfected and subsequently incubated at 37˚C for 4 hours. The cells were infected with RSV, Strain A-2 by (1) diluting the RSV stock in Opti-MEM: 1:10,000 (MOI 0.005) or 1:1000 (MOI 0.05); (2) adding 10 µl of diluted virus to the wells containing transfected cells and controls; (3) incubating the cells at 37˚C for 48 hours.

6-Well Plate Transfection Protocol

[00421] One day before transfection, about 75,000 Hep2 cells are seeded in 6-well plate in 2 ml of growth medium. The cells are transfected with siRNA at 25 nM final concentration using Lipofectamine 2000 reagent (2.5 µl per well). The siRNA -Lipofectamine 2000 complex was prepared as follows: (a) dilute 1 µM siRNA stock solution in 250 µl of Opti-MEM Reduced Serum Medium (resulting concentration of siRNA is 250 nM); (b) prepare the working solution of Lipofectamine 2000 reagent by diluting the stock in Opti-MEM at 1:100 ratio. Mix and incubate for 5 min at room temperature; (c) combine 250 µl of diluted siRNA with an equal volume of the working solution of Lipofectamine 2000 (resulting concentration of siRNA is 125 nM); and (d) add 500 µl of the mixture to the well (resulting concentration of siRNA is 25 nM).

[00422] After 4 hours incubation at 37˚C infect the cells with RSV as follows: (a) dilute the RSV stock in Opt-MEM: 1:1000 (MOI 0.005) or 1:100 (MOI 0.05); (b) add 10 µl of diluted virus to the wells containing transfected cells and controls; (c) incubate cells at 37˚C for 48 hours.

Quantitative RT-PCR (Taqman)

[00423] A series of probes and primers were used to detect the various mRNA transcripts of the genes of RSV and GAPDH (as a control/normalization), and the assays performed according to the manufacturer's instructions.

RSV Western Blot

[00424] The protein source for the Western Blot experiments are from transfection and infection of Hep2 cells in 6 well plate. Protein samples were prepared using the "PARIS" Protein and RNA Isolation System (Ambion, Cat# 1921) according to manufacturer's

instructions. The amount of total protein in cell lysates are measured using a Quick Start Bradford Protein Assay Kit (Bio-Rad, Cat# 500-0205).

[00425] Western blot assays were performed by running an SDS protein gel (10% Tris-HCl) using about 2 ug of protein per lane. After the resolution by electrophoresis, the proteins are transferred to a PVDF membrane (1 hr at 100V). Using non-fat dry milk (3%) in PBST the membrane is blocked (30 min. at room temperature). The membrane is blotted with goat anti-RSV polyclonal antibody (Biodesign International, Cat # B65860G), diluted 1: 500 in 1% FBS in PBST, overnight at 4°C. Subsequently the membrane is washed three times (5 min. each) in PBST. The blot is incubated in rabbit anti-goat secondary antibody (Pierce, Cat # 31402) diluted 1 : 20,000 in 1% FBS in PBST for 30 minutes at room temperature on a rocker or plate shaker, and subsequently washed three times (5 min. each) in PBST. The blot is then incubated with ECL Plus Western Blotting Detection System (GE Healthcare, Cat # RPN2132), and is imaged on the Bio-Rad VersaDoc Imager.

Calculations

[00426] All IC_{50} values were calculated from the data using GraphPad Prism software, specifically a non-linear, sigmoidal, variable slope curve fitted to the data. Also, unless otherwise indicated, all calculations of the efficacy and potency of the siRNAs were done relative to a non-targeting control siRNA. For cell viability studies the specific calculation for % viable cells was based upon the luminescence (CPS) for cells which were not infected with RSV. The log reduction calculation used the following formula:

Log (# of syncytia in well+NTC siRNA) – Log (# of syncytia in well+active siRNA)

[00427] In all of the calculations of the % knock-down of mRNA, the calculation was made relative to the normalized level of expression of the gene of interest in the samples treated with the non-targeting control (NTC) unless otherwise indicated. The gene of interest expression level was divided by the level of expression of GAPDH in each sample. The three replicates for each condition in each experiment were averaged and the standard deviation of those samples was calculated. The following formula was then used to calculate the % of knock-down of the gene of interest:

$$(1) - \frac{\text{(Normalized active siRNA treated expression level)}}{\text{(Normalized NTC siRNA treated expression level)}} * 100\%.$$

Results

Low MOI Syncytia Assays

[00428] As determined experimentally, infecting the cells in a 96-well plate with RSV at an MOI of 0.005 generally produced 50-60 syncytia per well after 48 hours. This number of syncytia can be readily scored.

[00429] The cells were stained with a solution of Crystal Violet at 0.25% and syncytia were counted under a dissecting microscrope. The multiplicity of infection (MOI) in these experiments was 0.005. The data is summarized in **Table 1**:

Table 1: Syncytia assay results for various siRNAs directed against RSV at low MOI of 0.005.

Site	Average # of syncytia	SD	n
1140	2.3	1.2	3
1143	6.0	2.6	3
1144	4.7	2.1	3
1329	0.3	0.6	3
1518	2.3	0.6	3
1519	2.0	1.0	3
1539	9.3	2.1	3
1540	7.0	1.7	3
1541	1.7	2.1	3
1543	1.0	0.0	3
2037	6.7	2.5	3
2038	3.7	1.5	3
2401	24.7	5.8	3
2406	0.7	0.6	3
2477	7.7	4.2	3
2479	0.7	0.6	3
2668	3.3	2.1	3
2671	2.3	0.6	3
2672	0.7	0.6	3
2673	24.3	4.2	3
2759	1.7	1.2	3
2760	0.3	0.6	3
2895	1.0	1.0	3
2896	17.0	7.0	3
7833	7.2	4.2	6

Site	Average # of syncytia	SD	n
7834	4.2	3.1	6
7976	7.5	5.0	6
7978	5.0	3.6	6
8411	46.7	4.2	3
8412	45.3	2.9	3
8489	47.0	4.0	3
8490	53.0	3.6	3
8493	49.5	0.7	2
8499	47.0	0.0	2
8500	57.5	2.1	2
8501	47.5	6.4	2
9091	40.5	7.8	2
10077	1.3	2.3	3
10365	35.7	10.4	3
12532	45.0	0.0	2
12544	2.2	2.5	5
12545	44.0	7.1	2
13364	34.3	10.0	3
13365	13.7	0.6	3
13369	34.0	1.0	3
13370	1.7	0.6	3
14252	29.7	4.5	3
14676	24.3	8.0	3
Untreated	0	0	12
LF2K +RSV	43.8	9.4	11
NT siRNA + RSV	52.2	8.3	11
RSV alone	61.3	6.1	12

Cell Viability Assay

[00430] As determined experimentally, an MOI of 0.005 (the MOI used for to score for syncytia) was insufficient to cause high levels of cell death at 96 hours; and further, even relatively high MOIs (0.1 and 0.05) did not show a great amount of cell death at 72 hours. However these levels induced essentially complete cell death at 96 hours. A higher MOI was considered as being more stringent criteria for the potency of the siRNAs and the 96 hour timepoint was considered to be one at which RSV had been given optimal time to act. Cells infected with RSV undergo cell death approximately 72 to 96 hours after infection with the

virus. The ability of the siRNAs to prevent this RSV-related cell death is summarized in **Table 2**.

Table 2: Cell viability assay for 4 siRNAs directed against RSV showing two different multiplicities of infection and as a percentage of viable cells relative to cells which were neither infected nor transfected.

Site	CPS MOI 0.1	SD	CPS MOI 0.05	SD	% viable cells (MOI 0.1)	% viable cells (MOI 0.05)
1140	1020643	90193	1063142	31052	114%	119%
1143	969157	47023	960138	17924	108%	107%
1144	925591	45806	944115	43306	103%	105%
1329	902241	43403	921340	10478	101%	103%
1518	1009706	37860	1066580	5692	113%	119%
1519	932279	10523	959312	9214	104%	107%
1539	1052458	61024	1067502	49598	117%	119%
1540	1042524	53696	1009327	3427	116%	113%
1541	1085442	26321	1115046	38007	121%	124%
1543	920178	49534	916121	23161	103%	102%
2037	955554	20890	968936	11197	107%	108%
2038	1118500	5102	996934	19670	125%	111%
2401	382300	47459	441489	27404	43%	49%
2406	1097973	80955	1038183	64525	122%	116%
2477	867243	35742	935887	31243	97%	104%
2479	831099	35408	809768	33874	93%	90%
2668	886193	18492	914222	22395	99%	102%
2671	1093397	93876	1019056	77079	122%	114%
2672	983283	89961	933986	12043	110%	104%
2673	964215	71240	916324	55440	108%	102%

2759	892701	26274	866389	29229	100%	97%
2760	895781	24354	935257	18525	100%	104%
2895	875846	25335	911996	61150	98%	102%
2896	419529	68742	589389	58933	47%	66%
7833	1044871	3503	925401	24414	117%	103%
7834	1037547	54759	894013	62821	116%	100%
7976	999365	48365	937804	51933	111%	105%
7978	1119458	10668	1002705	99036	125%	112%
8411	992065	39693	892834	59898	111%	100%
8412	403751	38785	416796	40800	45%	46%
8489	284922	23226	344237	121841	32%	38%
8490	633767	67782	487615	60288	71%	54%
8493	561083	73098	467003	42015	63%	52%
8499	978268	17692	861204	17057	109%	96%
8500	725005	125693	539731	44029	81%	60%
8501	619076	81490	599366	119623	69%	67%
9091	498752	70428	510806	122310	56%	57%
10077	1089716	44547	1148171	151234	122%	128%
10365	539029	52724	624639	92009	60%	70%
12532	226852	39248	272417	28332	25%	30%
12544	1057352	14814	990284	50878	118%	110%
12545	467354	68008	624557	77542	52%	70%
13364	286651	84823	267108	53022	32%	30%
13365	867692	18059	889723	31024	97%	99%
13369	653533	38430	617085	70981	73%	69%
13370	872430	16221	899879	4988	97%	100%

14252	442111	44839	444781	50711	49%	50%
14676	445330	102809	319504	8649	50%	36%
LF2K +RSV	244859	86497	276427	42257	27%	31%
NT siRNA + RSV	273059	56025	369015	132157	30%	41%
RSV alone	367911	69330	284765	88414	41%	32%
Untreated	896855	76591	896855	76591	100%	100%

Quantitative RT-PCR of RSV mRNA transcripts

[00431] A series of specific primers and probes were designed against the transcripts of the four genes of interest from RSV. These sets of primers and probes were then tested to determine the level of expression of each of the genes when cells were transfected with siRNAs directed against that gene and infected with RSV. The data of the expression levels of the RSV genes was normalized to GAPDH in order to control for the quality and efficiency of the RNA extraction from the cells in all cases. The different levels of expression of the mRNAs from the four genes tested is consistent with published data on the polarity of RSV transcription (Barik, S. J. Virol, Nov. 1992 pp 6813-6818). Genes transcribed from the 3' end of the negative strand genome (i.e. N and P genes) are transcribed at much higher level than those at the 5' end of the genome (i.e. M2 and L). **Tables 3-6** summarize the RT-PCR data for the siRNAs targeted against the various RSV genes.

Table 3. RT-PCR (Taqman) data of cells treated with RSV N specific siRNA. The data show the level of expression of the RSV N mRNA transcript (with accompanying standard deviation), and as the % knockdown of the RSV N mRNA transcript (with accompanying standard deviation) relative to cells transfected with a non-targeting control (NTC) before being infected with RSV.

siRNA	RSVN/GAPDH	SD	%knockdown	SD
UNT	0.001	0.001	100.0%	0.0%
LF2K-RSV	8.822	0.987	67.5%	3.6%
NT-siRNA	27.138	1.393	0.0%	5.1%
RSV-no siRNA	46.491	26.113	-71.3%	96.2%
Site 1140	0.026	0.011	99.9%	0.0%
Site 1143	0.014	0.003	100.0%	0.0%
Site 1144	0.030	0.019	99.9%	0.1%

siRNA	RSVN/GAPDH	SD	%knockdown	SD
Site 1329	0.002	0.000	100.0%	0.0%
Site 1518	0.037	0.010	99.9%	0.0%
Site 1519	0.008	0.002	100.0%	0.0%
Site 1539	0.258	0.063	99.0%	0.2%
Site 1540	0.013	0.005	100.0%	0.0%
Site 1541	0.070	0.030	99.7%	0.1%
Site 1543	0.024	0.011	99.9%	0.0%
Site 2037	0.089	0.028	99.7%	0.1%
Site 2038	0.026	0.019	99.9%	0.1%

Table 4. RT-PCR (Taqman) data of cells treated with RSV P specific siRNAs. The data show the level of expression of the RSV P mRNA transcript (with accompanying standard deviation) and as the % knockdown of the RSV P mRNA transcript (with accompanying standard deviation) relative cells transfected with a non-targeting control (NTC) before being infected with RSV.

siRNA	RSVP/GAPDH	SD	%knockdown	SD
UNT	0.000	0.000	100.0%	0.0%
LF2K-RSV	0.180	0.020	26.4%	8.2%
NTC-RSV	0.245	0.053	0.0%	21.5%
RSV-no siRNA	0.252	0.108	-2.7%	43.9%
Site 2401	0.058	0.023	76.4%	9.4%
Site 2406	0.000	0.000	99.9%	0.1%
Site 2477	0.009	0.001	96.3%	0.5%
Site 2479	0.000	0.000	99.9%	0.1%
Site 2668	0.004	0.001	98.3%	0.3%
Site 2671	0.000	0.000	99.9%	0.1%
Site 2672	0.001	0.000	99.6%	0.1%
Site 2673	0.017	0.007	93.1%	2.9%
Site 2759	0.001	0.001	99.5%	0.4%
Site 2760	0.000	0.000	99.9%	0.1%
Site 2895	0.000	0.000	99.9%	0.1%
Site 2896	0.040	0.001	83.7%	0.4%

Table 5. RT-PCR (Taqman) data of cells treated with RSV M2 specific siRNAs. The data show the level of expression of the RSV M2 mRNA transcript (with accompanying standard deviation) and as the % knockdown of the RSV M2 mRNA transcript (with accompanying

standard deviation) relative to cells transfected with a non-targeting control (NTC) before being infected with RSV.

siRNA	RSVM2/GAPDH	SD	%knockdown	SD
UNT	0.000	0.000	100.0%	0.0%
LF2K-RSV	0.219	0.037	-30.2%	21.7%
NTC-RSV	0.168	0.013	0.0%	7.6%
RSV-no siRNA	0.289	0.071	-72.0%	42.4%
Site 7833	0.005	0.001	96.9%	0.6%
Site 7834	0.004	0.001	97.8%	0.5%
Site 7976	0.007	0.002	95.8%	1.5%
Site 7978	0.003	0.001	98.1%	0.6%
Site 8411	0.016	0.005	90.4%	2.7%
Site 8412	0.090	0.018	46.6%	10.4%
Site 8489	0.118	0.021	29.8%	12.3%
Site 8490	0.081	0.009	52.0%	5.5%
Site 8493	0.067	0.012	60.2%	7.4%
Site 8499	0.027	0.002	83.7%	1.4%
Site 8500	0.049	0.001	71.1%	0.6%
Site 8501	0.050	0.004	70.3%	2.5%

Table 6. RT-PCR (Taqman) data of cells treated with RSV L specific siRNAs. The data show the level of expression of the RSV L mRNA transcript (with accompanying standard deviation) and as the % knockdown of the RSVL mRNA transcript (with accompanying standard deviation) relative to cells transfected with a non-targeting control (NTC) before being infected with RSV.

siRNA	RSVL/GAPDH	sd	%knockdown	SD
UNT	0.000	0.000	100.0%	0.0%
LF2K-RSV	0.073	0.014	-18.4%	22.4%
NTC-RSV	0.062	0.006	0.0%	9.1%
RSV-no siRNA	0.108	0.052	-74.3%	83.7%
Site 9091	0.048	0.002	22.3%	3.7%
Site 10077	0.001	0.000	97.7%	0.4%
Site 10365	0.028	0.005	53.9%	8.0%
Site 12532	0.111	0.012	-79.9%	19.5%
Site 12544	0.002	0.000	96.2%	0.4%
Site 12545	0.047	0.005	24.1%	7.4%
Site 13364	0.056	0.007	9.2%	10.7%

siRNA	RSVL/GAPDH	sd	%knockdown	SD
Site 13365	0.011	0.002	82.7%	2.6%
Site 13369	0.027	0.007	55.5%	10.7%
Site 13370	0.001	0.000	98.3%	0.1%
Site 14252	0.041	0.004	34.1%	6.1%
Site 14676	0.043	0.010	31.1%	15.8%

High MOI Syncytia Assays

[00432] The siRNAs against the four genes of RSV were screened a second time using a 10-fold higher multiplicity of infection (MOI) in an attempt to determine siRNA potency. The MOI in these experiments was 0.05. In scoring these samples, the MOI was so high that in wells where there was no suppression of syncytia formation the number of synctia formed was so high that the practical limit of counting individual syncytia was 150 per well. Consequently this conservative number was used in those cases where it was impossible to separate individual syncytia. The following Tables (**7-10**) summarize the results.

Table 7. Syncytia formed in wells where the cells were treated with RSV N specific siRNA (MOI = 0.05). The data are expressed as the average number of syncytia per well (n = 3) with accompanying standard deviations.

siRNA	# of syncytia	SD
untreated	0.67	1.15
LF2K + RSV	139.00	19.05
NTC + RSV	150.00	0.00
RSV	150.00	0.00
1140	0.67	1.15
1143	5.00	1.00
1144	6.00	3.46
1329	3.67	3.21
1518	6.33	1.53
1519	2.00	1.00
1539	14.33	2.08
1540	2.67	2.08
1541	5.67	2.52

siRNA	# of syncytia	SD
1543	2.00	2.00
2037	15.67	2.89
2038	4.33	0.58

Table 8. Syncytia formed in wells where the cells were treated with RSV P specific siRNAs (MOI = 0.05). The data are expressed as the average number of syncytia per well (n = 3) with accompanying standard deviations.

siRNA	# of syncytia	SD
untreated	0.33	0.58
LF2K + RSV	150.00	0.00
NTC + RSV	150.00	0.00
RSV	150.00	0.00
2401	25.00	4.36
2406	0.67	0.58
2477	17.67	2.08
2479	1.33	1.53
2668	5.33	1.53
2671	1.67	1.53
2672	4.00	0.00
2673	9.67	6.43
2759	4.33	2.31
2760	2.67	3.79
2895	0.67	1.15
2896	6.00	1.73

Table 9. Syncytia formed in wells where the cells were treated with RSV M2 specific siRNAs (MOI = 0.05). The data are expressed as the average number of syncytia per well (n = 3) with accompanying standard deviations.

siRNA	# of syncytia	SD
untreated	0.00	0.00
LF2K + RSV	150.00	0.00
NTC + RSV	150.00	0.00
RSV	150.00	0.00

siRNA	# of syncytia	SD
7833	7.00	3.61
7834	1.33	1.15
7976	10.00	4.36
7978	14.00	2.00
8411	63.33	22.68
8412	150.00	0.00
8489	100.00	0.00
8490	150.00	0.00
8493	150.00	0.00
8499	51.00	8.72
8500	150.00	0.00
8501	150.00	0.00

Table 10. Syncytia formed in wells where the cells were treated with RSV L specific siRNAs (MOI = 0.05). The data are expressed as the average number of syncytia per well (n = 3) with accompanying standard deviations.

siRNA	# of syncytia	SD
untreated	0.00	0.00
LF2K + RSV	150.00	0.00
NTC + RSV	150.00	0.00
RSV	150.00	0.00
9091	150.00	0.00
10077	2.00	3.46
10365	150.00	0.00
12532	150.00	0.00
12544	14.00	2.00
12545	150.00	0.00
13364	150.00	0.00
13365	150.00	0.00
13369	150.00	0.00
13370	2.67	1.53
14252	150.00	0.00
14676	150.00	0.00

[00433] Potency of these siRNAs was assessed using a syncytia assay where many more cells were transfected than is typical of the above experiments, allowing for scoring an extremely large number of syncytia in untreated wells and comparison of those numbers to the number of syncytia formed in treated wells. The results from this potency experiment for a subset of the siRNAs is summarized in the tables that follow.

Table 11. Summary of the efficacy experiments for a subset of the siRNAs directed against RSV. Data are expressed as the number of syncytia formed in wells after a particular treatment and as a comparison with the RSV specific siRNAs relative to a non-targeting control (NTC). The comparison data is expressed as log(NTC syncytia) – log(siRNA syncytia)

siRNA (25 nM)	# of Syncytia	Log Reduction (relative to NTC)
1140	8	2.46
1143	6	2.59
1144	13	2.25
1329	3	2.89
1518	5	2.67
2406	3	2.89
2479	4	2.76
2668	25	1.97
2671	7	2.52
7833	28	1.92
7834	5	2.67
10077	3	2.89
NTC	2328	

Table 12. Summary of the efficacy experiments for a subset of the siRNAs directed against RSV. Data are expressed as the number of syncytia formed in wells after a particular treatment and as a comparison with the RSV specific siRNAs relative to a non-targeting control (NTC). The comparison data is expressed as log(NTC syncytia) – log(siRNA syncytia)

siRNA (25 nM)	# of Syncytia	Log Reduction (relative to NTC)
1519	14	2.11

siRNA (25 nM)	# of Syncytia	Log Reduction (relative to NTC)
1540	10	2.26
1541	35	1.72
1543	6	2.48
2038	7	2.41
2672	9	2.31
2759	8	2.36
2760	9	2.31
2895	13	2.15
NTC	1820	

[00434] The in vitro results for the siRNAs against each of the four genes are summarized in **Table 13**. The Western blot data are presented as degree of inhibition of RSV protein production using the following designations: F= Full, P=Partial, M=Minimal, N=None. The IC_{50} determination was done with the syncytia assay, and the log reduction of syncytia formation and percent inhibition of RSV mRNA were both performed at a siRNA concentration of 25 nM. The potency of a commercially available monoclonal antibody was IC_{50} 4 nM (data not shown).

Table 13. Summary of Experimental Data for siRNAs.

Site	Gene	Sense/ Antisense	Modified Sequence (SEQ ID NO)	% Syncytia		% viable cells	% mRNA red.	Protein Red.(Full, Near Full or Partial)	Log red. Syncytia	IC_{50} nM
				Low MOI	High MOI					
1140	N	S	B*A*u*GG*cucuu*A Gc AAAG*uc*ATT*B (49)							
		AS	UGAcuuuGcu AAGAGccAuUU (50)	95.5%	99.5%	114%	99.9%	F	2.46	0.58
1143	N	S	B*G*cucuu*AGcA AAG*uc*AAG*u*TT*B (51)							
		AS	ACUuGAcuuu GcuAAGAGcUU (52)	88.5%	96.6%	108%	100.0%	N	2.59	1.20
1144	N	S	Bcucuu*AGcAA AG*uc*AAG*uu*TT*B (53)							
		AS	AACuuGAcuu uGcuAAGAGUU (54)	90.9%	96.0%	103%	99.9%	N	2.25	1.40
1329	N	S	Buu*AA*u*AGGuA* u*G*uu*A*u*A*u*GTT*B (55)							
		AS	CAUAuAAcAu AccuAuuAAUU (56)	99.4%	97.5%	101%	100.0%	F	2.89	0.30
1518	N	S	B*A*uu*GAGA*u*AG AA*ucu*AGAATT*B (57)							
		AS	UUCuAGAuuc uAucucAAuUU (58)	95.5%	95.8%	113%	99.9%	P	2.67	1.70
2406	P	S	Bcu*AGAA*uc*AA* u*AAAGGGcATT*B (75)							
		AS	UGCccuuuAu uGAuucuAGUU (76)	98.6%	99.5%	122%	99.9%	F	2.89	0.39
2479	P	S	Bc*AA*u*AGA*u*A*u *AGAAG*u*AAcTT*B (79)							
		AS	GUUAcuucuA uAucuAuuGUU (80)	98.6%	99.1%	93%	99.9%	F	2.76	0.42
2668	P	S	Bc*A*uuu*GA*u*AA* c*AA*u*GAAGATT*B (81)							
		AS	UCUucAuuGu uAucAAAuGUU (82)	93.6%	96.4%	99%	98.3%	N	1.97	11.7
2671	P	S	Buu*GA*u*AA*c*AA* u*GAAGAAGATT*B (83)							
		AS	UCUucuucAu uGuuAucAAUU (84)	95.5%	98.8%	122%	99.9%	F	2.52	0.38
7833	M2	S	B*G*uu*GGAG*u*G*c u*AGAGAG*uu*TT*B (97)							
		AS	AACucucuAG cAcuccAAcUU (98)	86.2%	95.3%	117%	96.9%	N	1.92	6.80
7834	M2	S	Buu*GGAG*u*G*cu *AGAGAG*uu*ATT*B (99)							
		AS	UAAcucucuA GcAcuccAAUU (100)	91.9%	99.1%	116%	97.8%	N	2.67	1.30
10077	L	S	B*G*ccu*AAAAAA G*u*GGA*ucuu*TT*B (123)							
		AS	AAGAuccAcu uuuuuAGGcUU (124)	97.5%	98.7%	122%	97.7%	P	2.89	1.40

[00435] It is to be understood that the double stranded siNA molecules of Table 13 have been generated according to the pattern of chemical modifications termed Stab 07 or Stab 35, set forth in **Table 15**. More specifically, it is to be understood that each particular sense strand embodiment set forth in **Table 13** is a representative member of a genus of siNA molecules according to the Stab 07 pattern of chemical modifications set forth in **Table 15** and the caption thereto. It is therefore understood that other members of that genus having a pattern of chemical modifications different from the particular sense strand in question that nonetheless follow the general pattern of Stab 07 may be synthesized and utilized as described in various embodiments herein. It is to be further understood that each antisense strand embodiment of the double stranded siNA molecules set forth in **Table 13** is a representative member of a genus of siNA molecules according to the Stab 35 pattern of chemical modifications set forth in **Table 15** and the caption thereto. It is therefore understood that other members of that genus having a pattern of chemical modifications different from the particular antisense strand in question that nonetheless follow the general pattern of Stab 35 may be synthesized and utilized as described in various embodiments herein.

Example 3: Models useful to evaluate the down-regulation of RSV gene expression

[00436] Evaluating the efficacy of anti-iRSV agents in vitro and in animal models is an important prerequisite to human clinical trials. Bitco *et al.,* 2005, *Nature Medicine*, 11, 50-55, describes the use of certain nasally administered vector expressed siRNA constructs targeting RSV. Zhang *et al.,* 2005, *Nature Medicine*, 11, 56-62, describes the use of certain nasally administered vector expressed siRNA constructs targeting the N1 gene of RSV. As such, these models can be used in evaluating the efficacy of siNA molecules of the invention in inhibiting RSV expression. Similarly, these models and others can be adapted to evaluate the safety and efficacy of siNA molecules of the invention in a pre-clinical setting. For example, the models used by Bitco *et al.* and Zhang *et al.* supra, can be adapted for use with synthetic siNA LNP formulations that are administered to the lungs of mice via intranasal inhalation or nebulization as is generally known in the art.

Example 4: Indications

[00437] The present body of knowledge in RSV research indicates the need for methods to assay RSV activity and for compounds that can regulate RSV expression for research, diagnostic, and therapeutic use. As described herein, the nucleic acid molecules of the present invention can be used in assays to diagnose disease state related of RSV levels. In addition, the nucleic acid molecules can be used to treat disease state related to RSV levels. Particular disease states that can be associated with RSV expression modulation include, but are not limited to, RSV infection, respiratory distress, stridor, bronchiolitis and pneumonia.

Example 5: Multifunctional siNA Inhibition of target RNA expression

Multifunctional siNA design

[00438] Once target sites have been identified for multifunctional siNA constructs, each strand of the siNA is designed with a complementary region of length, for example, of about 18 to about 28 nucleotides, that is complementary to a different target nucleic acid sequence. Each complementary region is designed with an adjacent flanking region of about 4 to about 22 nucleotides that is not complementary to the target sequence, but which comprises complementarity to the complementary region of the other sequence (see for example **Figure 11**). Hairpin constructs can likewise be designed (see for example **Figure 12**). Identification of complementary, palindrome or repeat sequences that are shared between the different target nucleic acid sequences can be used to shorten the overall length of the multifunctional siNA constructs (see for example **Figures 13 and 14**).

[00439] In a non-limiting example, three additional categories of additional multifunctional siNA designs are presented that allow a single siNA molecule to silence multiple targets. The first method utilizes linkers to join siNAs (or multiunctional siNAs) in a direct manner. This can allow the most potent siNAs to be joined without creating a long, continuous stretch of RNA that has potential to trigger an interferon response. The second method is a dendrimeric extension of the overlapping or the linked multifunctional design; or alternatively the organization of siNA in a supramolecular format. The third method uses helix lengths greater than 30 base pairs. Processing of these siNAs by Dicer will reveal new, active 5' antisense ends. Therefore, the long siNAs can target the sites defined by the original 5' ends and those defined by the new ends that are created by Dicer processing. When used in combination

with traditional multifunctional siNAs (where the sense and antisense strands each define a target) the approach can be used for example to target 4 or more sites.

I. Tethered Bifunctional siNAs

[00440] The basic idea is a novel approach to the design of multifunctional siNAs in which two antisense siNA strands are annealed to a single sense strand. The sense strand oligonucleotide contains a linker (*e.g.*, non-nulcoetide linker as described herein) and two segments that anneal to the antisense siNA strands (see **Figure 17**). The linkers can also optionally comprise nucleotide-based linkers. Several potential advantages and variations to this approach include, but are not limited to:

1. The two antisense siNAs are independent. Therefore, the choice of target sites is not constrained by a requirement for sequence conservation between two sites. Any two highly active siNAs can be combined to form a multifunctional siNA.

2. When used in combination with target sites having homology, siNAs that target a sequence present in two genes (*e.g.*, different isoforms), the design can be used to target more than two sites. A single multifunctional siNA can be for example, used to target RNA of two different target RNAs.

3. Multifunctional siNAs that use both the sense and antisense strands to target a gene can also be incorporated into a tethered multifuctional design. This leaves open the possibility of targeting 6 or more sites with a single complex.

4. It can be possible to anneal more than two antisense strand siNAs to a single tethered sense strand.

5. The design avoids long continuous stretches of dsRNA. Therefore, it is less likely to initiate an interferon response.

6. The linker (or modifications attached to it, such as conjugates described herein) can improve the pharmacokinetic properties of the complex or improve its incorporation into liposomes. Modifications introduced to the linker should not impact siNA activity to the same extent that they would if directly attached to the siNA.

7. The sense strand can extend beyond the annealed antisense strands to provide additional sites for the attachment of conjugates.

8. The polarity of the complex can be switched such that both of the antisense 3' ends are adjacent to the linker and the 5' ends are distal to the linker or combination thereof .

Dendrimer and supramolecular siNAs

[00441] In the dendrimer siNA approach, the synthesis of siNA is initiated by first synthesizing the dendrimer template followed by attaching various functional siNAs. Various constructs are depicted in **Figure 18**. The number of functional siNAs that can be attached is only limited by the dimensions of the dendrimer used.

Supramolecular approach to multifunctional siNA

[00442] The supramolecular format simplifies the challenges of dendrimer synthesis. In this format, the siNA strands are synthesized by standard RNA chemistry, followed by annealing of various complementary strands. The individual strand synthesis contains an antisense sense sequence of one siNA at the 5'-end followed by a nucleic acid or synthetic linker, such as hexaethyleneglyol, which in turn is followed by sense strand of another siNA in 5' to 3' direction. Thus, the synthesis of siNA strands can be carried out in a standard 3' to 5' direction. Representative examples of trifunctional and tetrafunctional siNAs are depicted in **Figure 19**. Based on a similar principle, higher functionality siNA constucts can be designed as long as efficient annealing of various strands is achieved.

Dicer enabled multifunctional siNA

[00443] Using bioinformatic analysis of multiple targets, stretches of identical sequences shared between differeing target sequences can be identified ranging from about two to about fourteen nucleotides in length. These identical regions can be designed into extended siNA helixes (*e.g.*, >30 base pairs) such that the processing by Dicer reveals a secondary functional 5'-antisense site (see for example **Figure 20**). For example, when the first 17 nucleotides of a siNA antisense strand (*e.g.*, 21 nucleotide strands in a duplex with 3'-TT overhangs) are complementary to a target RNA, robust silencing was observed at 25 nM. 80% silencing was observed with only 16 nucleotide complementarity in the same format.

[00444] Incorporation of this property into the designs of siNAs of about 30 to 40 or more base pairs results in additional multifunctional siNA constructs. The example in **Figure 20** illustrates how a 30 base-pair duplex can target three distinct sequences after processing by Dicer-RNaseIII; these sequences can be on the same mRNA or separate RNAs, such as viral and host factor messages, or multiple points along a given pathway (*e.g.*, inflammatory cascades). Furthermore, a 40 base-pair duplex can combine a bifunctional design in tandem, to provide a single duplex targeting four target sequences. An even more extensive approach can include use of homologous sequences to enable five or six targets silenced for one multifunctional duplex. The example in **Figure 20** demonstrates how this can be achieved. 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. Another non-limiting example is shown in **Figure 21**. 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 in four colors, blue, light-blue and red and orange. The required sequence identity overlapped is indicated by grey 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 multiifunctional 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.

Example 6: Diagnostic uses

[00445] The siNA molecules of the invention can be used in a variety of diagnostic applications, such as in the identification of molecular targets (*e.g.*, RNA) in a variety of applications, for example, in clinical, industrial, environmental, agricultural and/or research settings. Such diagnostic use of siNA molecules involves utilizing reconstituted RNAi

systems, for example, using cellular lysates or partially purified cellular lysates. siNA molecules of this invention can be used as diagnostic tools to examine genetic drift and mutations within diseased cells or to detect the presence of endogenous or exogenous, for example viral, RNA in a cell. The close relationship between siNA activity and the structure of the target RNA allows the detection of mutations in any region of the molecule, which alters the base-pairing and three-dimensional structure of the target RNA. By using multiple siNA molecules described in this invention, one can map nucleotide changes, which are important to RNA structure and function *in vitro*, as well as in cells and tissues. Cleavage of target RNAs with siNA molecules can be used to inhibit gene expression and define the role of specified gene products in the progression of disease or infection. In this manner, other genetic targets can be defined as important mediators of the disease. These experiments will lead to better treatment of the disease progression by affording the possibility of combination therapies (*e.g.*, multiple siNA molecules targeted to different genes, siNA molecules coupled with known small molecule inhibitors, or intermittent treatment with combinations siNA molecules and/or other chemical or biological molecules). Other *in vitro* uses of siNA molecules of this invention are well known in the art, and include detection of the presence of mRNAs associated with a disease, infection, or related condition. Such RNA is detected by determining the presence of a cleavage product after treatment with a siNA using standard methodologies, for example, fluorescence resonance emission transfer (FRET).

[00446] In a specific example, siNA molecules that cleave only wild-type or mutant forms of the target RNA are used for the assay. The first siNA molecules (*i.e.*, those that cleave only wild-type forms of target RNA) are used to identify wild-type RNA present in the sample and the second siNA molecules (*i.e.*, those that cleave only mutant forms of target RNA) are used to identify mutant RNA in the sample. As reaction controls, synthetic substrates of both wild-type and mutant RNA are cleaved by both siNA molecules to demonstrate the relative siNA efficiencies in the reactions and the absence of cleavage of the "non-targeted" RNA species. The cleavage products from the synthetic substrates also serve to generate size markers for the analysis of wild-type and mutant RNAs in the sample population. Thus, each analysis requires two siNA molecules, two substrates and one unknown sample, which is combined into six reactions. The presence of cleavage products is determined using an RNase protection assay so that full-length and cleavage fragments of each RNA can be analyzed in one lane of a polyacrylamide gel. It is not absolutely required to quantify the results to gain insight into the expression of mutant RNAs and putative risk of

the desired phenotypic changes in target cells. The expression of mRNA whose protein product is implicated in the development of the phenotype (*i.e.*, disease related or infection related) is adequate to establish risk. If probes of comparable specific activity are used for both transcripts, then a qualitative comparison of RNA levels is adequate and decreases the cost of the initial diagnosis. Higher mutant form to wild-type ratios are correlated with higher risk whether RNA levels are compared qualitatively or quantitatively.

[00447] All patents and publications mentioned in the specification are indicative of the levels of skill of those skilled in the art to which the invention pertains. All references cited in this disclosure are incorporated by reference to the same extent as if each reference had been incorporated by reference in its entirety individually.

[00448] One skilled in the art would readily appreciate that the present invention is well adapted to carry out the objects and obtain the ends and advantages mentioned, as well as those inherent therein. The methods and compositions described herein as presently representative of preferred embodiments are exemplary and are not intended as limitations on the scope of the invention. Changes therein and other uses will occur to those skilled in the art, which are encompassed within the spirit of the invention, are defined by the scope of the claims.

[00449] It will be readily apparent to one skilled in the art that varying substitutions and modifications can be made to the invention disclosed herein without departing from the scope and spirit of the invention. Thus, such additional embodiments are within the scope of the present invention and the following claims. The present invention teaches one skilled in the art to test various combinations and/or substitutions of chemical modifications described herein toward generating nucleic acid constructs with improved activity for mediating RNAi activity. Such improved activity can comprise improved stability, improved bioavailability, and/or improved activation of cellular responses mediating RNAi. Therefore, the specific embodiments described herein are not limiting and one skilled in the art can readily appreciate that specific combinations of the modifications described herein can be tested without undue experimentation toward identifying siNA molecules with improved RNAi activity.

[00450] The invention illustratively described herein suitably can be practiced in the absence of any element or elements, limitation or limitations that are not specifically disclosed herein. Thus, for example, in each instance herein any of the terms "comprising", "consisting essentially of", and "consisting of" may be replaced with either of the other two terms. The terms and expressions which have been employed are used as terms of description and not of limitation, and there is no intention that in the use of such terms and expressions of excluding any equivalents of the features shown and described or portions thereof, but it is recognized that various modifications are possible within the scope of the invention claimed. Thus, it should be understood that although the present invention has been specifically disclosed by preferred embodiments, optional features, modification and variation of the concepts herein disclosed may be resorted to by those skilled in the art, and that such modifications and variations are considered to be within the scope of this invention as defined by the description and the appended claims.

[00451] In addition, where features or aspects of the invention are described in terms of Markush groups or other grouping of alternatives, those skilled in the art will recognize that the invention is also thereby described in terms of any individual member or subgroup of members of the Markush group or other group.

Table 14: Accession Numbers of Sequences Used for RSV siRNA Design

1. AY911262
Human respiratory syncytial virus strain ATCC VR-26, complete genome
gi|60549163|gb|AY911262.1|[60549163]

2. AY353550
Human Respiratory syncytial virus 9320, complete genome
gi|38230482|gb|AY353550.1|[38230482]

3. M74568
Human respiratory syncytial virus nonstructural protein 1, nonstructural protein 2, nucleocapsid protein, phosphoprotein, matrix protein, small hydrophobic protein, glycoprotein, fusion glycoprotein, 22K/M2 protein and L protein mRNA, complete cds
gi|333959|gb|M74568.1|RSHSEQ[333959]

4. NC_001803
Respiratory syncytial virus, complete genome
gi|9629367|ref|NC_001803.1|[9629367]

5. NC_001781
Human respiratory syncytial virus, complete genome
gi|9629198|ref|NC_001781.1|[9629198]

Table 15

Non-limiting examples of Stabilization Chemistries for chemically modified siNA constructs

Chemistry	pyrimidine	Purine	cap	p=S	Strand
"Stab 7"	2'-fluoro	2'-deoxy	5' and 3'-ends	-	Usually S
"Stab 35"	2'-fluoro	2'-O-Methyl**			Usually AS
"Stab 35"	2'-OCF3	2'-O-Methyl**			Usually AS

CAP = any terminal cap, see for example **Figure 5**.
Each Stab chemistry can comprise 3'-terminal thymidine (TT) residues
Each Stab chemistry can comprise about 21 nucleotides, but can vary as described herein.
Each Stab chemistry can also include a single ribonucleotide in the sense or passenger strand at the 11th base paired position of the double stranded nucleic acid duplex as determined from the 5'-end of the antisense or guide strand.
S = sense strand
AS = antisense strand
p = phosphorothioate linkage
**Stab 35 has 2'-O-methyl U at 3'-overhangs and the first three 5' nucleotides may optionally be ribonucleotides

Table 16

A. 2.5 µmol Synthesis Cycle ABI 394 Instrument

Reagent	Equivalents	Amount	Wait Time* DNA	Wait Time* 2'-O-methyl	Wait Time*RNA
Phosphoramidites	6.5	163 µL	45 sec	2.5 min	7.5 min
S-Ethyl Tetrazole	23.8	238 µL	45 sec	2.5 min	7.5 min
Acetic Anhydride	100	233 µL	5 sec	5 sec	5 sec
N-Methyl Imidazole	186	233 µL	5 sec	5 sec	5 sec
TCA	176	2.3 mL	21 sec	21 sec	21 sec
Iodine	11.2	1.7 mL	45 sec	45 sec	45 sec
Beaucage	12.9	645 µL	100 sec	300 sec	300 sec
Acetonitrile	NA	6.67 mL	NA	NA	NA

B. 0.2 µmol Synthesis Cycle ABI 394 Instrument

Reagent	Equivalents	Amount	Wait Time* DNA	Wait Time* 2'-O-methyl	Wait Time*RNA
Phosphoramidites	15	31 µL	45 sec	233 sec	465 sec
S-Ethyl Tetrazole	38.7	31 µL	45 sec	233 min	465 sec
Acetic Anhydride	655	124 µL	5 sec	5 sec	5 sec
N-Methyl Imidazole	1245	124 µL	5 sec	5 sec	5 sec
TCA	700	732 µL	10 sec	10 sec	10 sec
Iodine	20.6	244 µL	15 sec	15 sec	15 sec
Beaucage	7.7	232 µL	100 sec	300 sec	300 sec
Acetonitrile	NA	2.64 mL	NA	NA	NA

C. 0.2 µmol Synthesis Cycle 96 well Instrument

Reagent	Equivalents:DNA/2'-O-methyl/Ribo	Amount: DNA/2'-O-methyl/Ribo	Wait Time* DNA	Wait Time* 2'-O-methyl	Wait Time* Ribo
Phosphoramidites	22/33/66	40/60/120 µL	60 sec	180 sec	360sec
S-Ethyl Tetrazole	70/105/210	40/60/120 µL	60 sec	180 min	360 sec
Acetic Anhydride	265/265/265	50/50/50 µL	10 sec	10 sec	10 sec
N-Methyl Imidazole	502/502/502	50/50/50 µL	10 sec	10 sec	10 sec
TCA	238/475/475	250/500/500 µL	15 sec	15 sec	15 sec
Iodine	6.8/6.8/6.8	80/80/80 µL	30 sec	30 sec	30 sec
Beaucage	34/51/51	80/120/120	100 sec	200 sec	200 sec
Acetonitrile	NA	1150/1150/1150 µL	NA	NA	NA

- Wait time does not include contact time during delivery.

- Tandem synthesis utilizes double coupling of linker molecule

Table 17

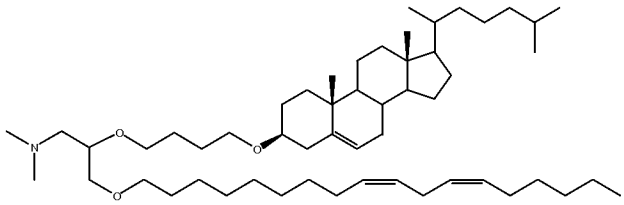
Lipid Nanoparticle (LNP) Formulations

Formulation #	Composition	Molar Ratio
L051	CLinDMA / DSPC / Chol / PEG-n-DMG	48 / 40 / 10 / 2
L053	DMOBA / DSPC / Chol / PEG-n-DMG	30 / 20 / 48 / 2
L054	DMOBA / DSPC / Chol / PEG-n-DMG	50 / 20 / 28 / 2
L069	CLinDMA / DSPC / Cholesterol / PEG-Cholesterol	48 / 40 / 10 / 2
L073	pCLinDMA or CLin DMA/ DMOBA / DSPC / Chol / PEG-n-DMG	25 / 25 / 20 / 28 / 2
L077	eCLinDMA / DSPC / Cholesterol / 2KPEG-Chol	48 / 40 / 10 / 2
L080	eCLinDMA / DSPC / Cholesterol / 2KPEG-DMG	48 / 40 / 10 / 2
L082	pCLinDMA / DSPC / Cholesterol / 2KPEG-DMG	48 / 40 / 10 / 2
L083	pCLinDMA / DSPC / Cholesterol / 2KPEG-Chol	48 / 40 / 10 / 2
L086	CLinDMA/DSPC/Cholesterol/2KPEG-DMG/Linoleyl alcohol	43 / 38 / 10 / 2 / 7
L061	DMLBA/Cholesterol/2KPEG-DMG	52 / 45 / 3
L060	DMOBA/Cholesterol/2KPEG-DMG N/P ratio of 5	52 / 45 / 3
L097	DMLBA/DSPC/Cholesterol/2KPEG-DMG	50 / 20 / 28
L098	DMOBA/Cholesterol/2KPEG-DMG, N/P ratio of 3	52 / 45 / 3
L099	DMOBA/Cholesterol/2KPEG-DMG, N/P ratio of 4	52 / 45 / 3
L100	DMOBA/DOBA/3% PEG-DMG, N/P ratio of 3	52 / 45 / 3
L101	DMOBA/Cholesterol/2KPEG-Cholesterol	52 / 45 / 3
L102	DMOBA/Cholesterol/2KPEG-Cholesterol, N/P ratio of 5	52 / 45 / 3
L103	DMLBA/Cholesterol/2KPEG-Cholesterol	52 / 45 / 3
L104	CLinDMA/DSPC/Cholesterol/2KPEG-cholesterol/Linoleyl alcohol	43 / 38 / 10 / 2 / 7
L105	DMOBA/Cholesterol/2KPEG-Chol, N/P ratio of 2	52 / 45 / 3
L106	DMOBA/Cholesterol/2KPEG-Chol, N/P ratio of 3	67 / 30 / 3

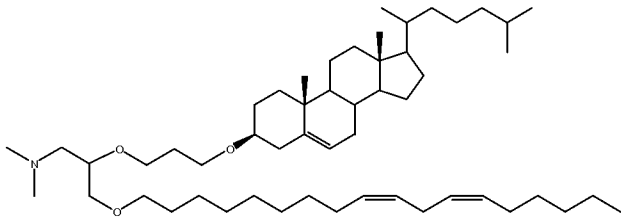
Formulation #	Composition	Molar Ratio
L107	DMOBA/Cholesterol/2KPEG-Chol, N/P ratio of 1.5	52 / 45 / 3
L108	DMOBA/Cholesterol/2KPEG-Chol, N/P ratio of 2	67 / 30 / 3
L109	DMOBA/DSPC/Cholesterol/2KPEG-Chol, N/P ratio of 2	50 / 20/28 / 2
L110	DMOBA/Cholesterol/2KPEG-DMG, N/P ratio of 1.5	52 / 45 / 3
L111	DMOBA/Cholesterol/2KPEG-DMG, N/P ratio of 1.5	67 / 30 / 3
L112	DMLBA/Cholesterol/2KPEG-DMG, N/P ratio of 1.5	52 / 45 / 3
L113	DMLBA/Cholesterol/2KPEG-DMG, N/P ratio of 1.5	67 /30 / 3
L114	DMOBA/Cholesterol/2KPEG-DMG, N/P ratio of 2	52 / 45 / 3
L115	DMOBA/Cholesterol/2KPEG-DMG, N/P ratio of 2	67 / 30 / 3
L116	DMLBA/Cholesterol/2KPEG-DMG, N/Pratio of 2	52 / 45 / 3
L117	DMLBA/Cholesterol/2KPEG-DMG, N/P ratio of 2	52 / 45 / 3

N/P ratio = Nitrogen:Phosphorous ratio between cationic lipid and nucleic acid

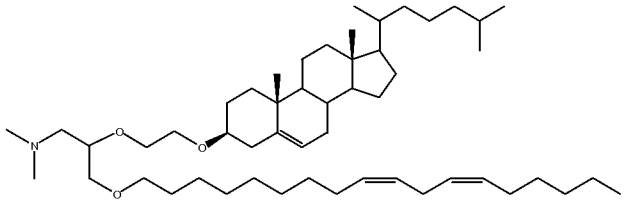
CLinDMA structure



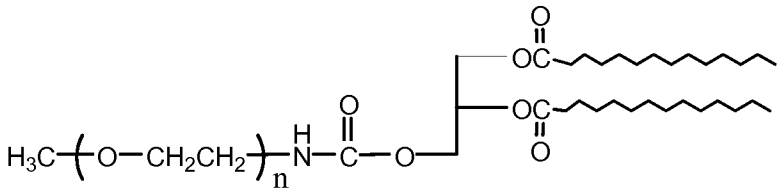
pCLinDMA structure



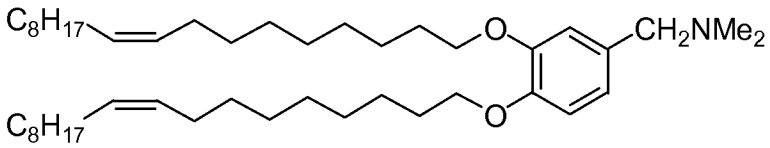
eCLinDMA structure



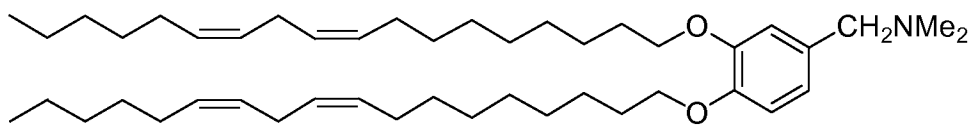
PEG-n-DMG structure



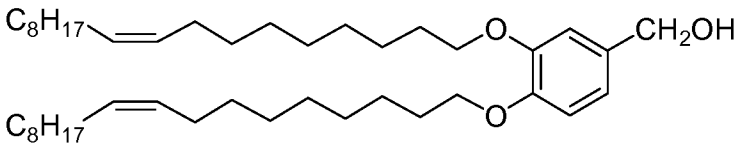
DMOBA structure



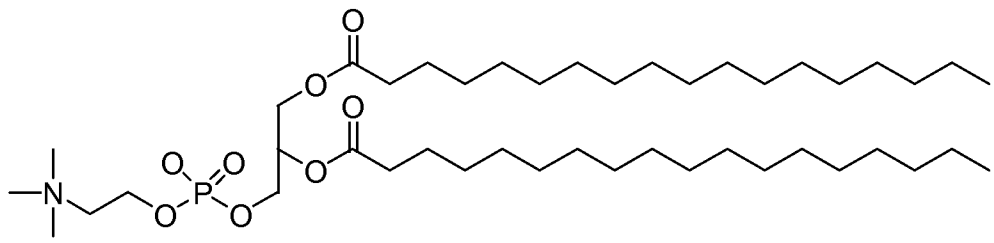
DMLBA structure



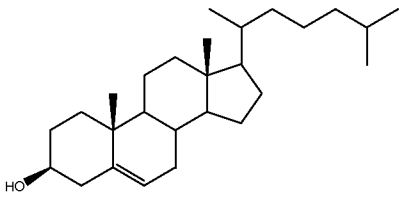
DOBA structure



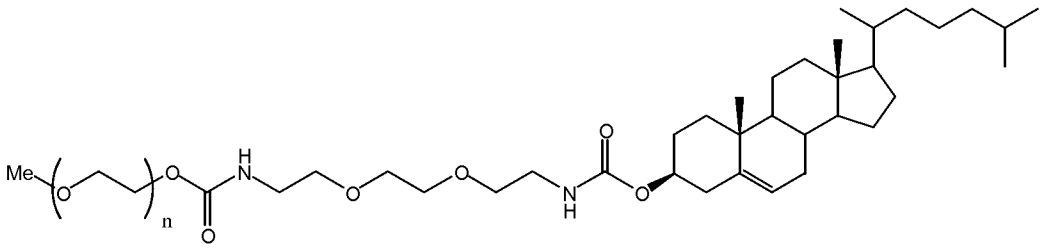
DSPC



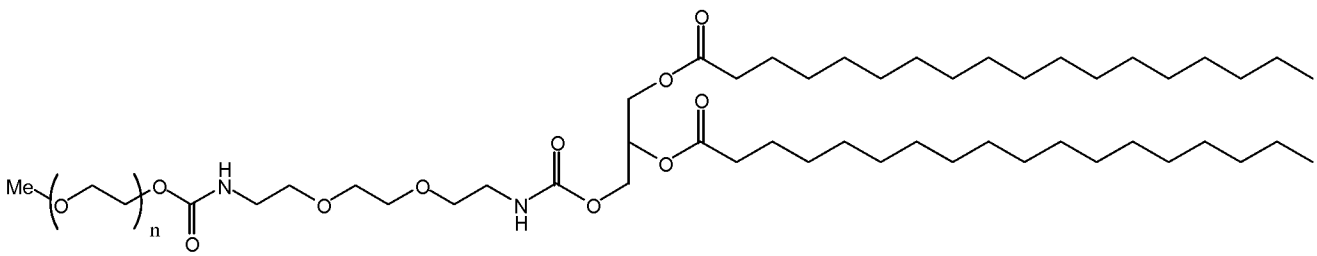
Cholesterol



2KPEG-***Cholesterol***



2KPEG-***DMG***



CLAIMS

What we claim is:

1. A double stranded nucleic acid (siNA) molecule comprising a first strand and a second strand that are complementary to each other, wherein said first strand is a sense strand and said second strand is an antisense strand, wherein said sense strand comprises:

5' { B- uu*AA*u*AGG*u*A*u*G*uu*A*u*A*u*G*[N]n-B } 3' (SEQ ID NO:145);

5' { B- cu*AGAA*uc*AA*u*AAAGGG*c*A*[N]n-B } 3' (SEQ ID NO:146);

5' { B- uu*GGAG*u*G*cu*AGAGAG*uu*A*[N]n-B } 3' (SEQ ID NO:147); or

5' { B- *G*ccu*AAAAAAG*u*GGA*ucuu[N]n-B } 3' (SEQ ID NO:148);

and wherein

A is deoxyadenosine

G is deoxyguanosine;

u is 2'-deoxy-2'-fluorouridine;

c is 2'-deoxy-2'-fluorocytidine;

N is any chemically modified nucleotide, wherein each N is independently selected from a 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, LNA nucleotide, and 5-C-methyl nucleotide

n is 0, 1, or 2; and

B is a terminal cap abasic moiety, a terminal cap inverted abasic moiety, a terminal cap inverted nucleotide, or other terminal cap moiety; wherein B is further independently present or absent; wherein B, if present on both terminal ends, is the same or different at both ends.

2. The double stranded nucleic acid (siNA) molecule of claim 1 wherein B is (i) present on both terminal ends and (ii) the same or different at both ends.

3. The double stranded nucleic acid (siNA) molecule of claim 1 wherein n is 2.

4. A double stranded nucleic acid (siNA) molecule comprising a first strand and a second strand that are complementary to each other, wherein said first strand is a sense strand and said second strand is an antisense strand, wherein said antisense strand comprises:

5' { CAUAuAAcAuAccuAuuAA[N]p } 3' (SEQ ID NO:149);
5' { UGCccuuuAuuGAuucuAG[N]p } 3' (SEQ ID NO:150);
5' { UAAcucucuAGcAcuccAA[N]p } 3' (SEQ ID NO:151); or
5' { AAGAuccAcuuuuuuAGGc[N]p } 3' (SEQ ID NO:152);

wherein
C is cytidine;
A is adenosine;
G is guanosine;
U is uridine;
A is 2'-O-methyl adenosine;
G is 2'-O-methyl guanosine;
c is 2'-deoxy-2'-fluorocytidine;
u is 2'-deoxy-2'-fluoro uridine;
N is any chemically modified nucleotide, wherein each N is independently selected from a 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, LNA nucleotide, and 5-C-methyl nucleotide; and
p is 0, 1, or 2.

5. The double stranded nucleic acid (siNA) molecule of claim 4 wherein p is 2.

6. A double stranded nucleic acid (siNA) molecule comprising a first strand and a second strand that are complementary to each other, wherein said first strand is a sense strand and said second strand is an antisense strand, wherein said sense strand comprises the following structure:

5' { B- uu*AA*u*AG*G*u*A*u*G*uu*A*u*A*u*G*[N]n-B } 3' (SEQ ID NO:145);

5' { B- cu*AGAA*uc*AA* u*AAAGGGcA*[N]n-B } 3' (SEQ ID NO:146);

5' { B- uu*GGAG*u*G*cu *AGAGAG*uu*A*[N]n-B } 3' (SEQ ID NO:147); or

5' { B- *G*ccu*AAAAAA G*u*GGA*ucuu[N]n-B } 3' (SEQ ID NO:148);

wherein *A* is deoxyadenosine and *G* is deoxyguanosine; u is 2'-deoxy-2'-fluoro uridine; c is 2'-deoxy-2'-fluorocytidine; each N is independently selected from deoxythymidine (T) and 2'-O-methyl uridine (U); and B is a terminal cap abasic moiety, a terminal cap inverted abasic moiety, a terminal cap inverted nucleotide, or other terminal cap moiety; wherein B is further independently present or absent; and wherein B, if present on both terminal ends, is the same or different at both ends, and n is 0, 1, or 2.

7. The double stranded nucleic acid (siNA) molecule of claim 6 wherein B is (i) present on both terminal ends and (ii) the same or different at both ends.

8. The double stranded nucleic acid (siNA) molecule of claim 6 wherein n is 2.

9. A double stranded nucleic acid (siNA) molecule comprising a first strand and a second strand that are complementary to each other, wherein said first strand is an antisense strand and said second strand is a sense strand, wherein said antisense strand comprises the following structure:

5' { CAUAuAAcAuAccuAuuAA[N]p } 3' (SEQ ID NO:149);

5' { UGCccuuuAuuGAuucuAG[N]p } 3' (SEQ ID NO:150);

5' { UAAcucucuAGcAcuccAA[N]p } 3' (SEQ ID NO:151); or

5' { AAGAuccAcuuuuuuAGGc[N]p } 3' (SEQ ID NO:152);

wherein C, A, G, and U are cytidine, adenosine, guanosine, and uridine, respectively; A is 2'-O-methyl adenosine; G is 2'-O-methyl guanosine; c and u are 2'-deoxy-2'-fluoro cytidine and 2'-deoxy-2'-fluoro uridine, respectively; and each N is independently selected from 2'-O-methyl Uridine (U) or deoxythymidine (T).

10. The double stranded nucleic acid (siNA) molecule of claim 9 wherein p is 2.

11. A double stranded nucleic acid (siNA) molecule comprising a first strand and a second strand that are complementary to each other, wherein said first strand is a sense strand and said second strand is an antisense strand, wherein said sense strand comprises the following structure:

5' { B-uu*AA*u*AGG*u*A*u*G*uu*A*u*A*u*G*[T]n-B } 3' (SEQ ID NO:55);
5' { B-cu*AGAA*uc*AA*u*AAAGGG*c*A*[T]n-B } 3' (SEQ ID NO:75);
5' { B-uu*GGAG*u*G*cu*AGAGAG*uu*A*[T]n-B } 3' (SEQ ID NO:99);
5' { B-*G*ccu*AAAAAAG*u*GGA*ucuu[T]n-B } 3' (SEQ ID NO:123);

wherein *A* is deoxyadenosine and *G* is deoxyguanosine; u is 2'-deoxy-2'-fluorouridine; c is 2'-deoxy-2'-fluorocytidine; T is deoxythymidine; and B is a terminal cap abasic moiety, a terminal cap inverted abasic moiety, a terminal cap inverted nucleotide, or other terminal cap moiety; wherein B is further independently present or absent; and wherein B, if present on both terminal ends, is the same or different at both ends, and n is 0, 1, or 2.

12. The double stranded nucleic acid (siNA) molecule of claim 11 wherein B is (i) present on both terminal ends and (ii) the same or different at both ends.

13. The double stranded nucleic acid (siNA) molecule of claim 11 wherein n is 2.

14. A double stranded nucleic acid (siNA) molecule comprising a first strand and a second strand that are complementary to each other, wherein said first strand is an antisense strand and said second strand is a sense strand, wherein said antisense strand comprises the following structure:

5' { CAUAuAAcAuAccuAuuAA[U]p } 3' (SEQ ID NO:56);

5' { UGCccuuuAu uGAuucuAG[U]p } 3' (SEQ ID NO:76);

5' { UAAcucucuA GcAcuccAA[U]p } 3' (SEQ ID NO:100);

5' { AAGAuccAcu uuuuuAGGc[U]p } 3' (SEQ ID NO:124);

wherein C, A, G, and U are cytidine, adenosine, guanosine, and uridine, respectively; A is 2'-O-methyl adenosine; G is 2'-O-methyl guanosine; c and u are 2'-deoxy-2'-fluoro cytidine and 2'-deoxy-2'-fluoro uridine, respectively; U is 2'-O-methyl Uridine; and p is 0, 1, or 2.

15. The double stranded nucleic acid (siNA) molecule of claim 14 wherein p is 2.

16. Any one of claims 1-15, wherein either (i) said second strand has at least partial complimentarity to said first strand or (ii) said second strand is perfectly complimentary at all corresponding residues with said first strand.

17. A composition comprising the double stranded nucleic acid (siNA) molecule of claim 16 in a pharmaceutically acceptable carrier or diluent.

18. A method for treating, preventing, or reducing RSV infection in a subject comprising contacting the subject with the composition of claim 17 under conditions suitable to modulate the expression of RSV in the subject whereby the treatment, prevention, or reduction of RSV infection can be achieved.

19. The method of 18, further comprising administration of ribavirin.

20. A double stranded nucleic acid (siNA) molecule comprising the following structure:

[RSV-I] 5' B-uu*AA*u*AG*G*u*A*u*G*uu*A*u*A*u*G*[N]n-B 3' (SEQ ID NO:55)
||||||||||||||||||
3' [N]pAAuuAuccAuAcAAuAUAC 5' (SEQ ID NO:56); or

[RSV-II] 5' B-cu*AGAA*uc*AA*u*AAAGGG*c*A*[N]n-B 3' (SEQ ID NO:75)
||||||||||||||||||
3' [N]pGAucuuAGuuAuuuccCGU 5' (SEQ ID NO:76); or

[RSV-III] 5' B-uu*GGAG*u*G*cu*AGAGAG*uu*A*[N]n-B 3' (SEQ ID NO:99)
||||||||||||||||||
3' [N]pAAccucAcGAucucucAAU 5' (SEQ ID NO:100); or

[RSV-IV] 5' B-*G*ccu*AAAAAAG*u*GG*Aucuu[N]n-B 3' (SEQ ID NO:123)
||||||||||||||||||
3' [N]pcGGAuuuuuucAccuAGAA 5' (SEQ ID NO:124) ;

wherein

A is deoxyadenosine;

G is deoxyguanosine;

C is cytidine;

A is adenosine;

G is guanosine;

U is uridine;

A is 2'-O-methyl adenosine;

G is 2'-O-methyl guanosine;

c is 2'-deoxy-2'-fluorocytidine;

u is and 2'-deoxy-2'-fluorouridine;

N is a chemically modified nucleotide each independently selected from deoxythymidine, deoxyguanosine, deoxycytidine, or deoxyadenosine, 2'-O-methyl Uridine, 2'-O-methyl Cytidine, 2'-O-methyl Adenosine, or 2'-O-methyl Guanosine; a 2'-deoxy-2'-fluoro nucleotide, or a LNA nucleotide;

n is independently 0, 1, or 2;

p is independently 0, 1, or 2;

B is a terminal cap moiety each independently present or absent, wherein if present on both terminal ends is the same or different, and is selected from a terminal cap abasic moiety, a terminal cap inverted abasic moiety, a terminal cap inverted nucleotide, or other terminal cap moiety;

with the proviso that the sense strand nucleotide corresponding to the 11th position of the double stranded nucleic acid duplex as determined from the 5' terminal end of the antisense strand may optionally be a ribonucleotide.

21. The double stranded nucleic acid (siNA) molecule of claim 20 wherein B is (i) present on both terminal ends and (ii) the same or different at both ends.

22. The double stranded nucleic acid (siNA) molecule of claim 20 wherein n is 2.

23. The double stranded nucleic acid (siNA) molecule of claim 20 wherein p is 2.

24. The double stranded nucleic acid (siNA) molecule of claim 20 wherein the 11th position of the double stranded nucleic acid duplex as determined from the 5' terminal end of the antisense strand is a ribonucleotide.

25. The double stranded nucleic acid (siNA) molecule of claim 20 wherein the 11th position of the double stranded nucleic acid duplex as determined from the 5' terminal end of the antisense strand is a deoxyribonucleotide.

26. The double stranded nucleic acid (siNA) molecule of claim 20 wherein there are overhangs at both terminal ends.

27. A composition comprising the double stranded nucleic acid (siNA) molecule of any of claims 20-26 in a pharmaceutically acceptable carrier or diluent.

28. A method for treating, preventing, or reducing RSV infection in a subject comprising contacting the subject with the composition of claim 27 under conditions suitable to modulate the expression of RSV in the subject whereby the treatment, prevention, or reduction of RSV infection can be achieved.

29. The method of 28, further comprising administration of ribavirin.

30. A double stranded nucleic acid (siNA) molecule comprising the following structure:

[RSV-I] 5' B-uu*AA*u*AGG*u*Au*G*uu*Au*Au*G*[N]n-B 3' (SEQ ID NO:55)
||||||||||||||||||
3' [N]pAAuuAuccAuAcAAuAUAC 5' (SEQ ID NO:56); or

[RSV-II] 5' B-cu*AGAAuc*A*Au*A*AAGGGc*A[N]n-B 3' (SEQ ID NO:75)
||||||||||||||||||
3' [N]pGAucuuAGuuAuuuccCGU 5' (SEQ ID NO:76); or

[RSV-III] 5' B-uu*GGAGu*G*cu*A*GAGAG*uu*A*[N]n-B 3' (SEQ ID NO:99)
||||||||||||||||||
3' [N]pAAccucAcGAucucucAAU 5' (SEQ ID NO:100); or

[RSV-IV] 5' B-*G*ccu*AAAAAAG*u*GGA*ucuu[N]n-B 3' (SEQ ID NO:123)
||||||||||||||||||
3' [N]pcGGAuuuuuucAccuAGAA 5' (SEQ ID NO:124);

wherein

A is deoxyadenosine;

G is deoxyguanosine;

C is cytidine;

A is adenosine;

G is guanosine;

U is uridine;

A is 2'-O-methyl adenosine;

G is 2'-O-methyl guanosine;

c is 2'-deoxy-2'-fluorocytidine;

u is and 2'-deoxy-2'-fluorouridine;

N is a chemically modified nucleotide each independently selected from deoxythymidine, deoxyguanosine, deoxycytidine, or deoxyadenosine, 2'-O-methyl Uridine, 2'-O-methyl Cytidine, 2'-O-methyl Adenosine, or 2'-O-methyl Guanosine; a 2'-deoxy-2'-fluoro nucleotide, or a LNA nucleotide;

n is independently 0, 1, or 2;

p is independently 0, 1, or 2; and

B is a terminal cap moiety each independently present or absent, wherein if present on both terminal ends is the same or different, and is selected from a terminal cap abasic moiety, a

terminal cap inverted abasic moiety, a terminal cap inverted nucleotide, or other terminal cap moiety.

31. A composition comprising the double stranded nucleic acid (siNA) molecule of claim 30 in a pharmaceutically acceptable carrier or diluent.

32. A method for treating, preventing, or reducing RSV infection in a subject comprising contacting the subject with the composition of claim 31 under conditions suitable to modulate the expression of RSV in the subject whereby the treatment, prevention, or reduction of RSV infection can be achieved.

33. The method of 32, further comprising administration of ribavirin.

34. A double stranded nucleic acid (siNA) molecule comprising the following structure:

[RSV-Ia] 5' B-uu*AAuAGGuAuGuuAuAuGTT*-B 3' (SEQ ID NO:55)
||||||||||||||||||
3' UUAAuuAuccAuAcAAuAUAC 5' (SEQ ID NO:56); or

[RSV-IIa] 5' B-cu*AGAAucAAuAAAGGGcATT*-B 3' (SEQ ID NO:75)
||||||||||||||||||
3' UUGAucuuAGuuAuuuccCGU 5' (SEQ ID NO:76); or

[RSV-IIIa] 5' B-uu*GGAGuGcuAGAGAGuuATT*-B 3' (SEQ ID NO:99)
||||||||||||||||||
3' UUAAccucAcGAucucucAAU 5' (SEQ ID NO:100); or

[RSV-IVa] 5' B-*GccuAAAAAAGuGGAucuuTT*-B 3' (SEQ ID NO:123)
||||||||||||||||||
3' UUcGGAuuuuuucAccuAGAA 5' (SEQ ID NO:124);

wherein

A is deoxyadenosine;

G is deoxyguanosine;

C is cytidine;

A is adenosine;

G is guanosine;

U is uridine;

A is 2'-O-methyl adenosine;

U is 2'-O-methyl uridine;

G is 2'-O-methyl guanosine;

c is 2'-deoxy-2'-fluorocytidine;

u is and 2'-deoxy-2'-fluorouridine; and

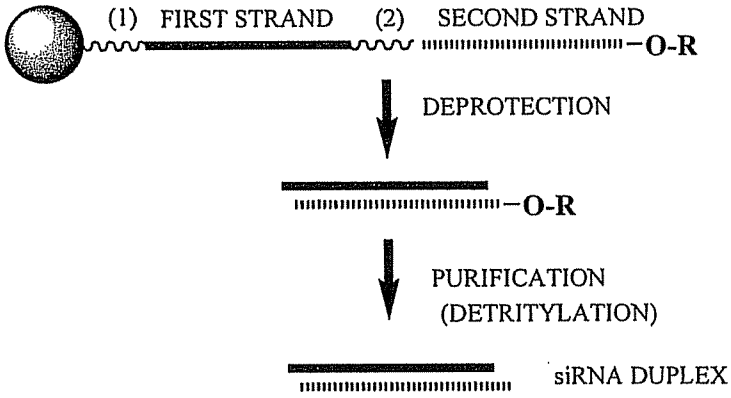
B is an inverted deoxyabasic moiety as described in FIG. 25.

35. A composition comprising the double stranded nucleic acid molecule of claim 34 in a pharmaceutically acceptable carrier or diluent.

36. A method for treating, preventing, or reducing RSV infection in a subject comprising contacting the subject with the composition of claim 35 under conditions suitable to modulate the expression of RSV in the subject whereby the treatment, prevention, or reduction of RSV infection can be achieved.

37. The method of Claim 36, further comprising administration of ribavirin.

Figure 1

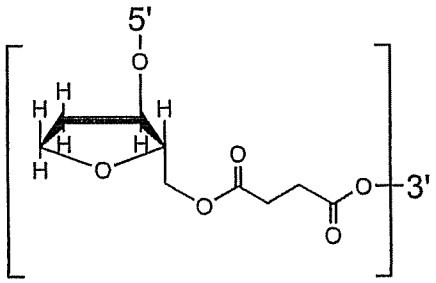


= SOLID SUPPORT

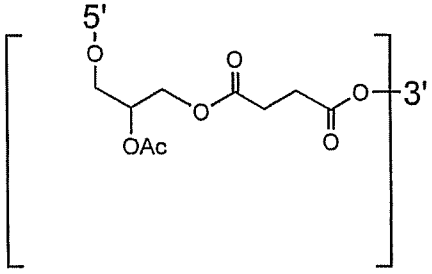
R = TERMINAL PROTECTING GROUP
FOR EXAMPLE:
DIMETHOXYTRITYL (DMT)

(1) = CLEAVABLE LINKER
(FOR EXAMPLE: NUCLEOTIDE SUCCINATE OR INVERTED DEOXYABASIC SUCCINATE)

(2) = CLEAVABLE LINKER
(FOR EXAMPLE: NUCLEOTIDE SUCCINATE OR INVERTED DEOXYABASIC SUCCINATE)



INVERTED DEOXYABASIC SUCCINATE LINKAGE



GLYCERYL SUCCINATE LINKAGE

Figure 2

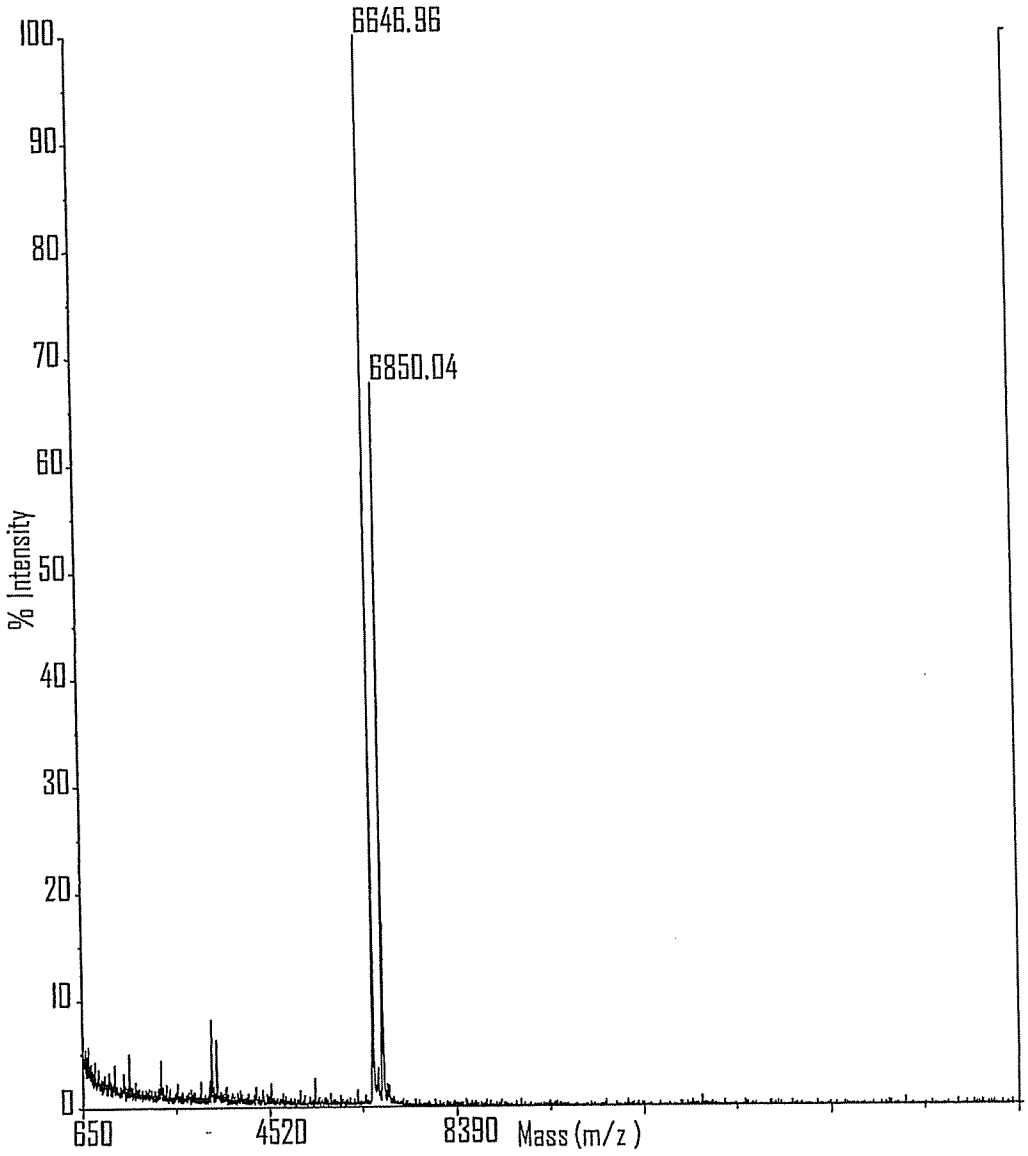

6646.96
6850.04
100
90
80
70
60
50
40
30
20
10
0
% Intensity
650
4520
8390
Mass (m/z)

Figure 3

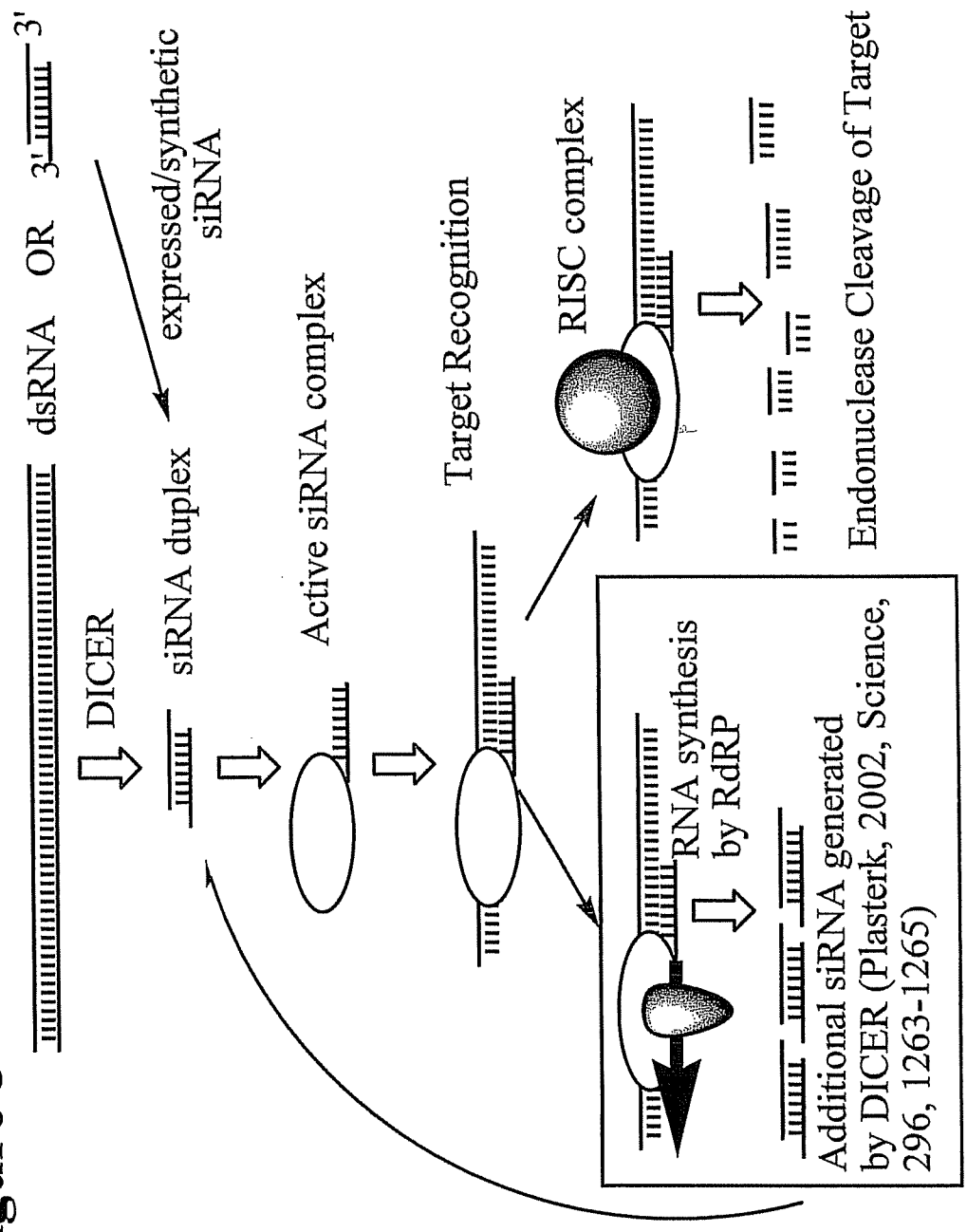

dsRNA OR 3' 3'
DICER
siRNA duplex
expressed/synthetic siRNA
Active siRNA complex
Target Recognition
RISC complex
RNA synthesis by RdRP
Additional siRNA generated by DICER (Plasterk, 2002, Science, 296, 1263-1265)
Endonuclease Cleavage of Target

Figure 4A

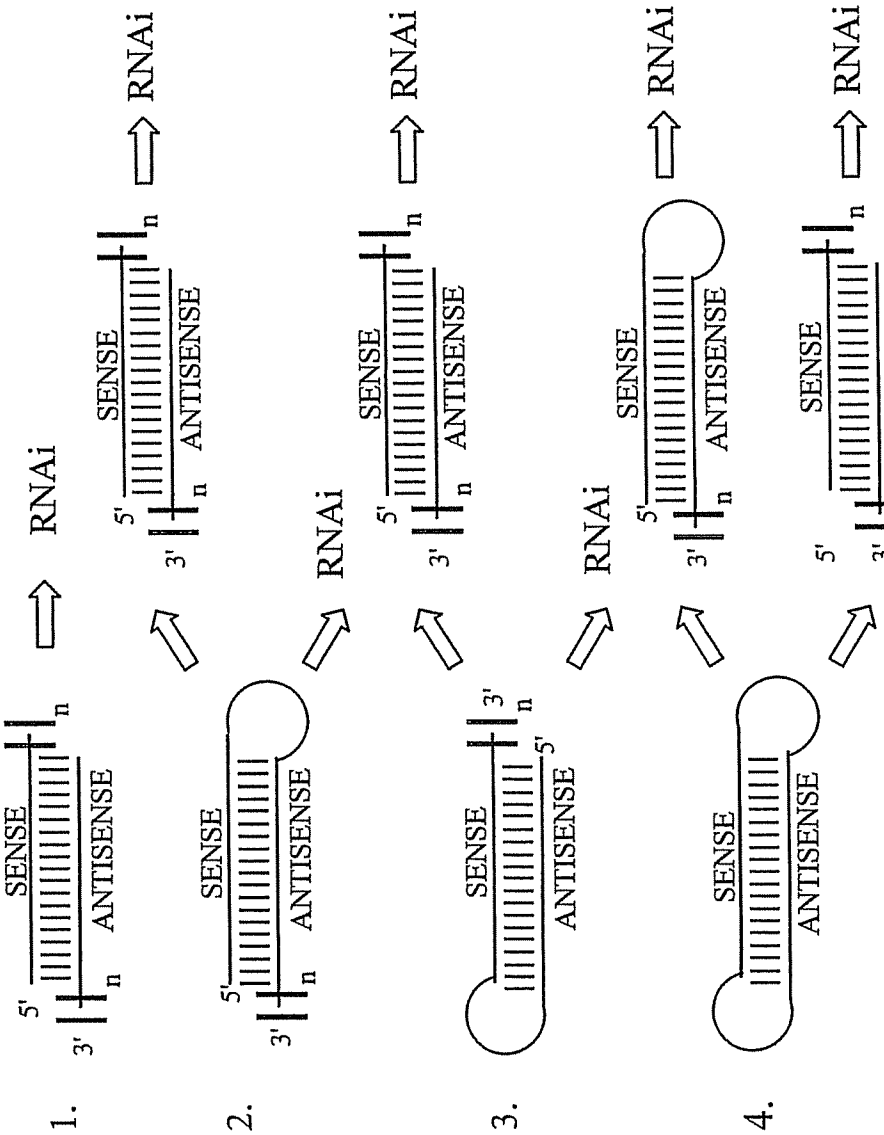
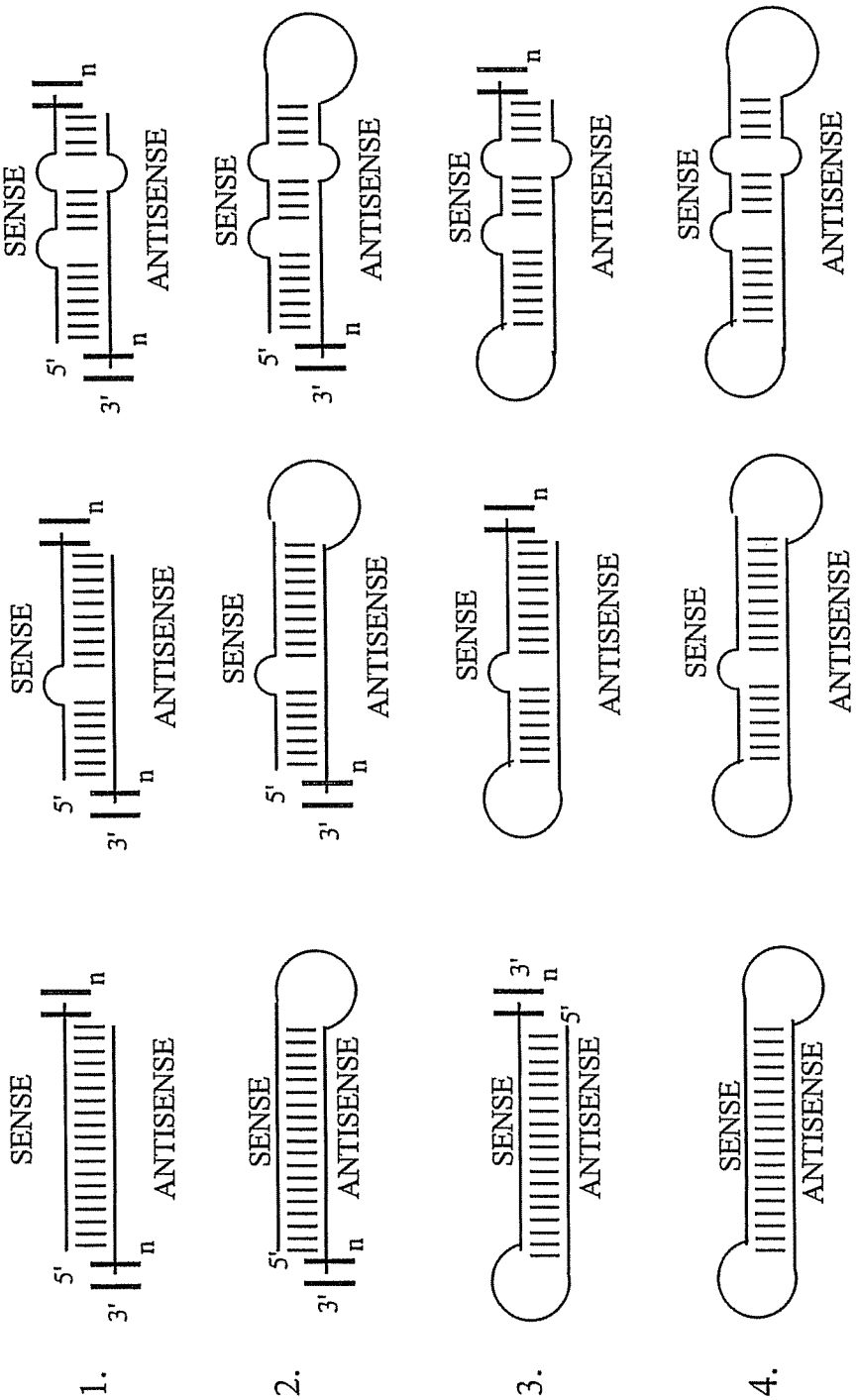
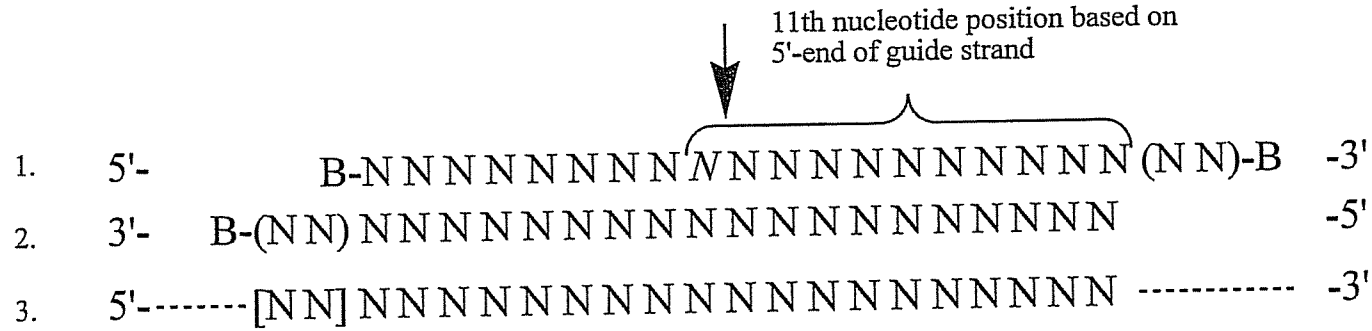


Figure 4B



n = 0, 1, 2, 3, 4

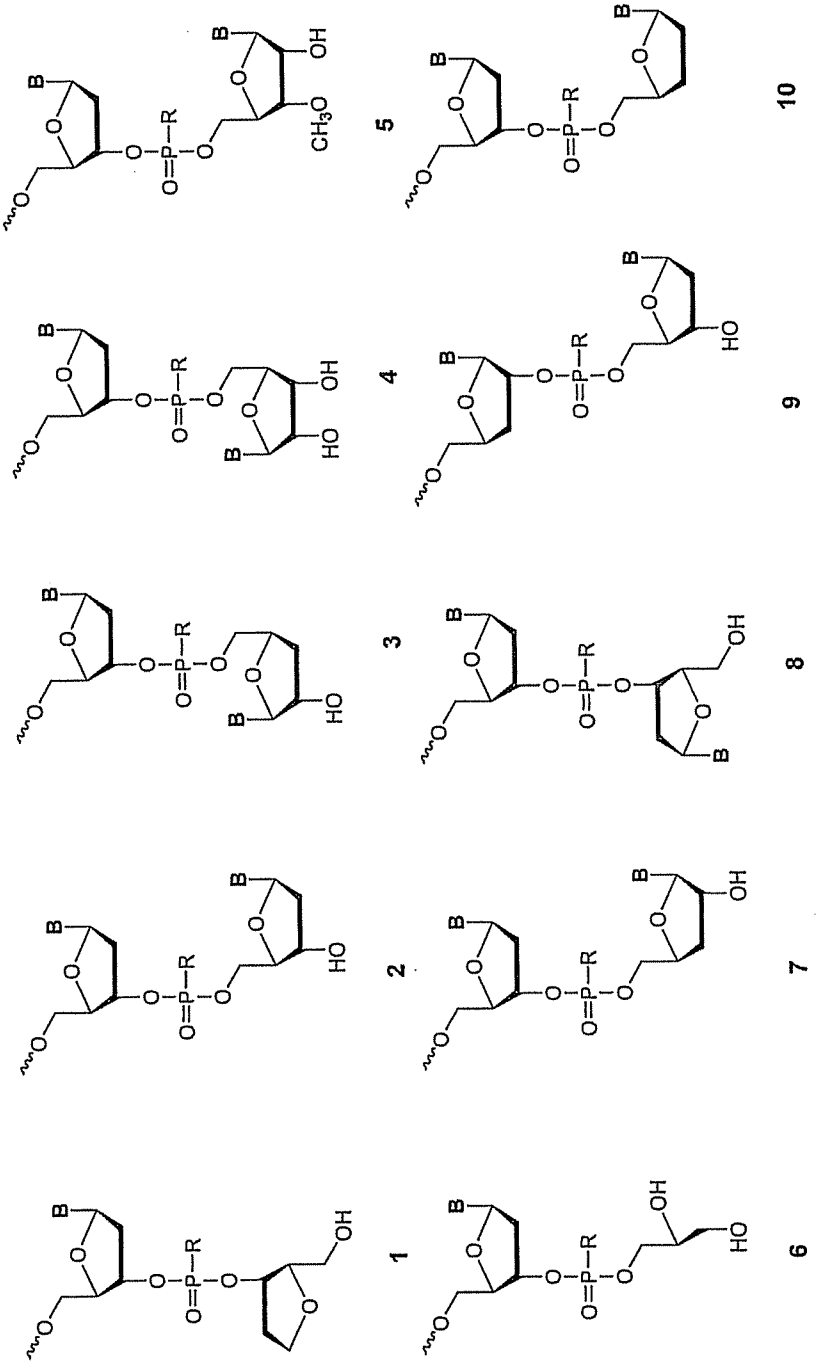
Figure 4C



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 5



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 6: Modification Strategy

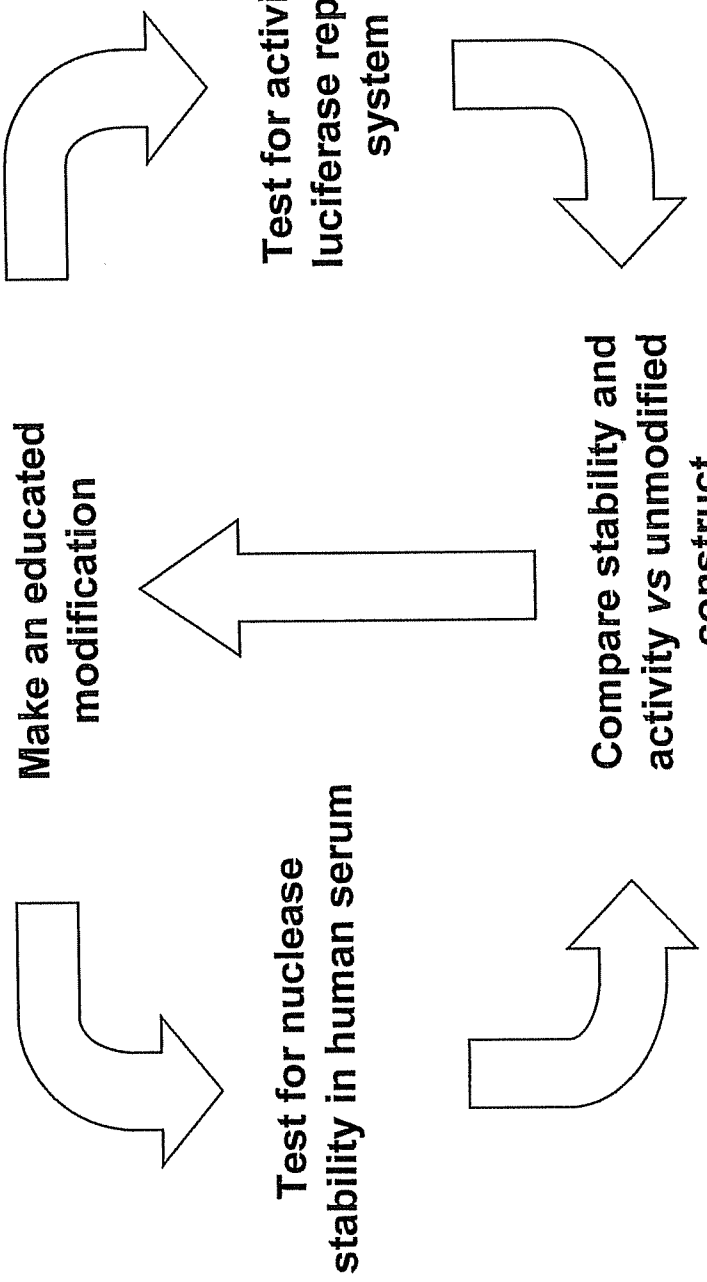


Figure 7: Phosphorylated siNA constructs

Figure 8: 5'-phosphate modifications

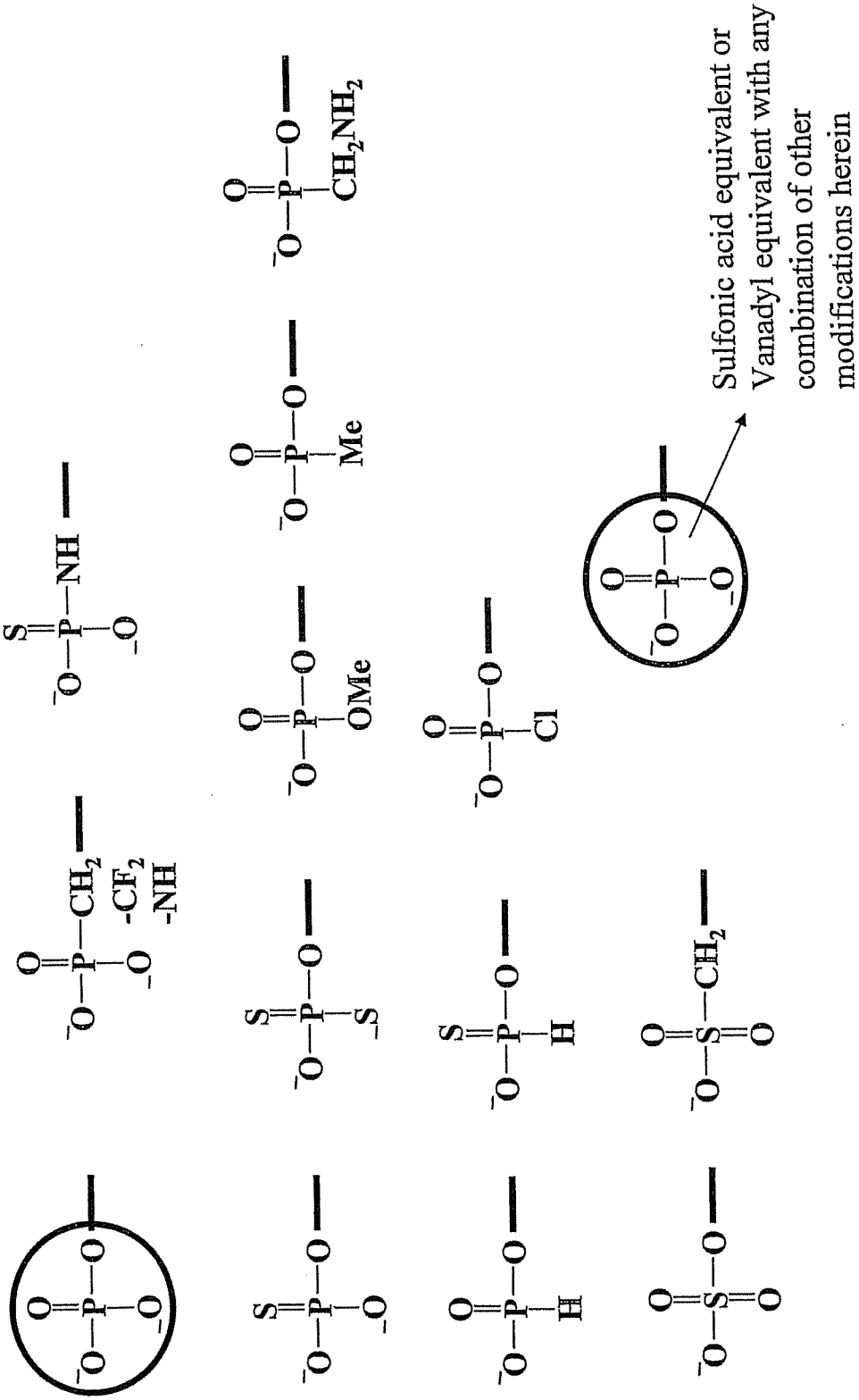
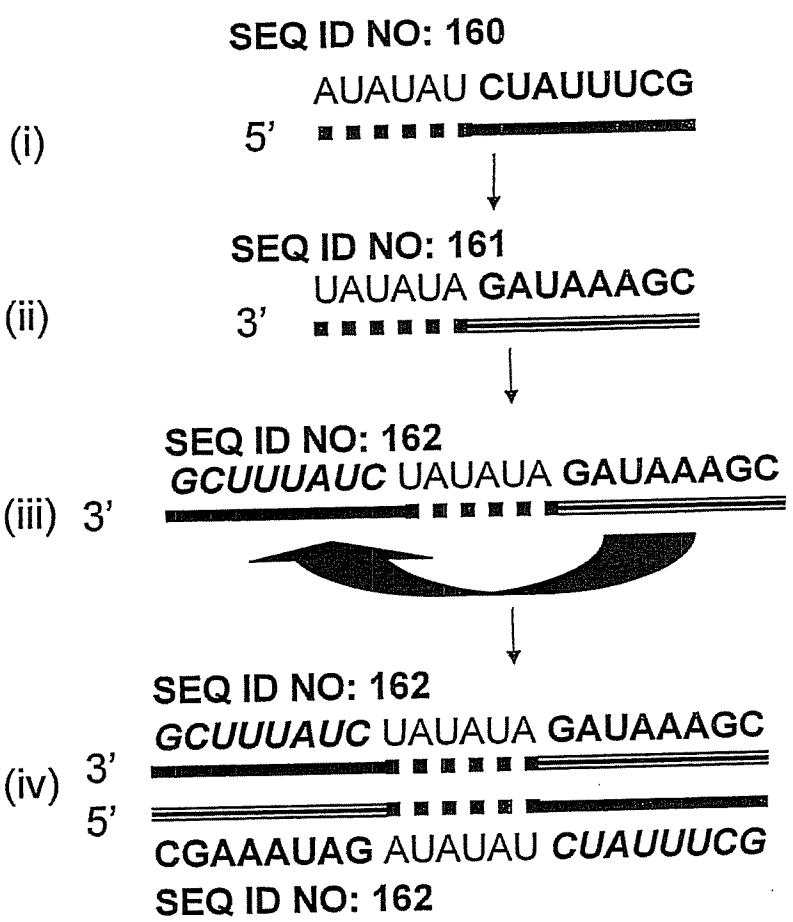


Figure 9A: Duplex forming oligonucleotide constructs that utilize Palindrome or repeat sequences

Figure 9B: Example of a duplex forming oligonucleotide sequence that utilizes a palindrome or repeat sequence



Identify Target Nucleic Acid sequence (e.g., 14 to 24 nucleotides in length) containing palindrome/repeat sequence at 5'-end (dashed portion)

Design Complementary Sequence to the Target Nucleic Acid sequence of (i) above

Append inverse sequence of the Non-palindromic Complementary Sequence of (ii) to 3'-end of complementary sequence

Self assembly of self complementary strands to form duplex construct (blunt ends)

Figure 9C: Example of a duplex forming oligonucleotide sequence that utilizes a palindrome or repeat sequence, self assembly

Figure 9D: Example of a duplex forming oligonucleotide sequence that utilizes a palindrome or repeat sequence, self assembly and inhibition of Target Sequence Expression

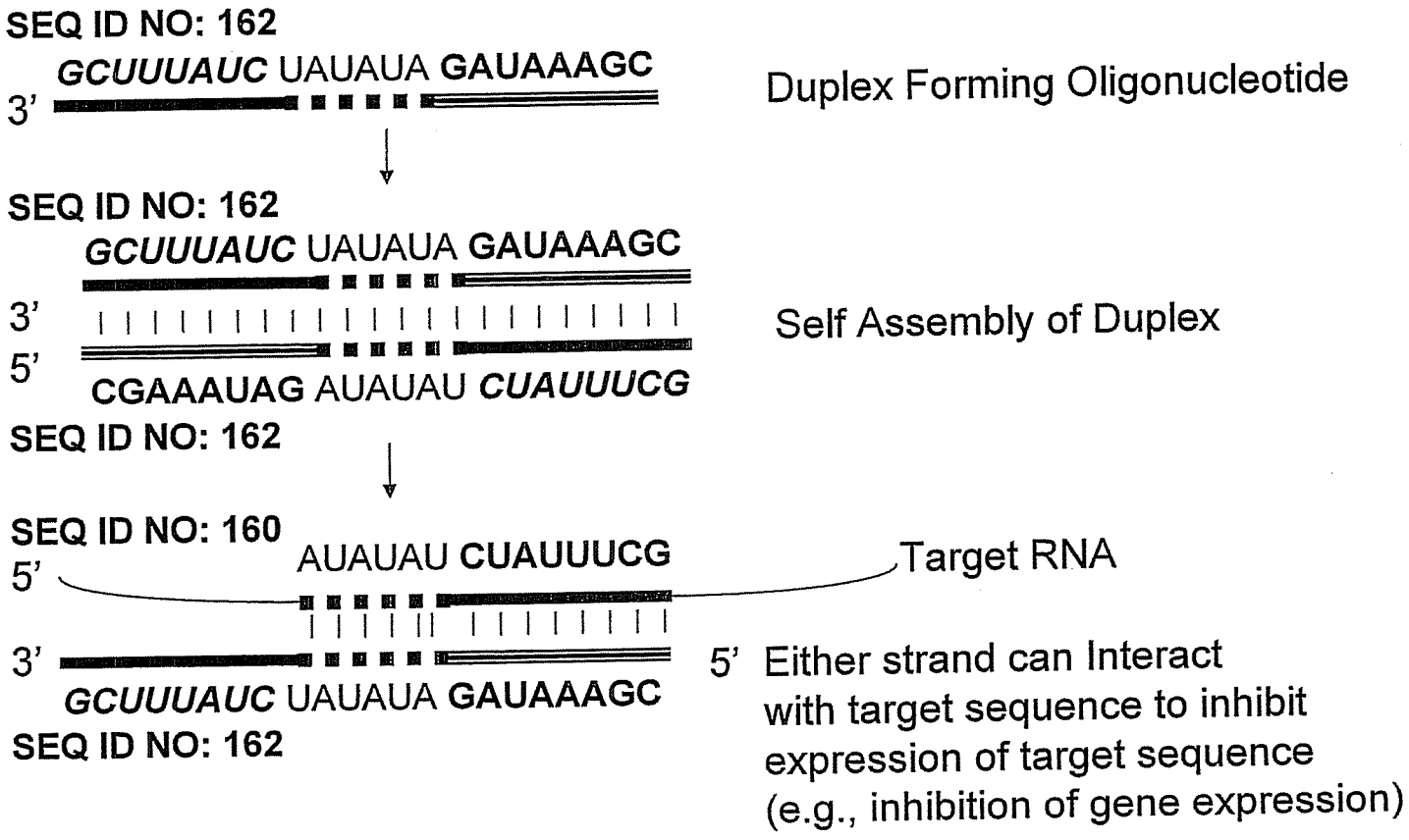


Figure 10: Duplex forming oligonucleotide constructs that utilize artificial palindrome or repeat sequences

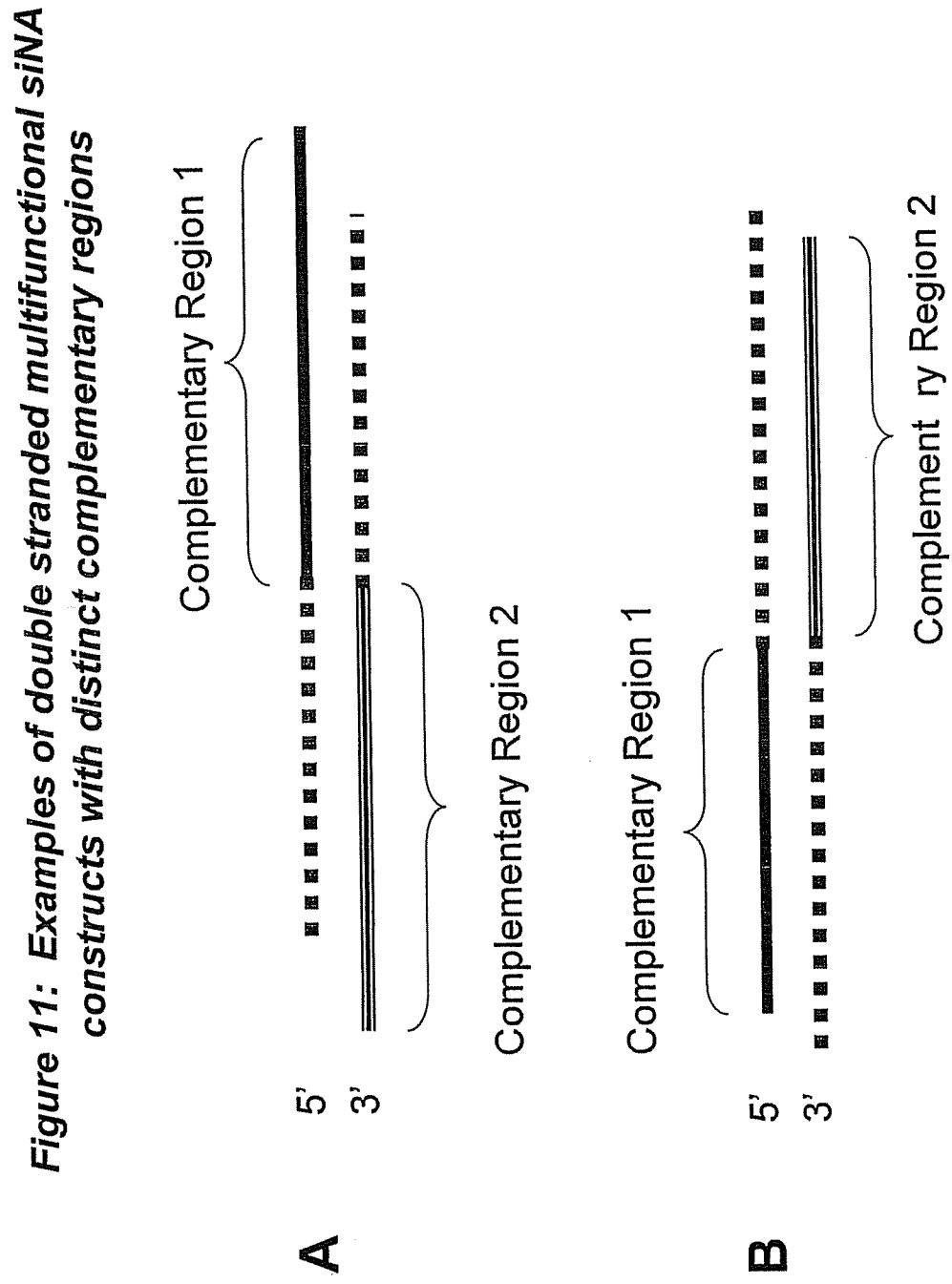


Figure 11: Examples of double stranded multifunctional siNA constructs with distinct complementary regions

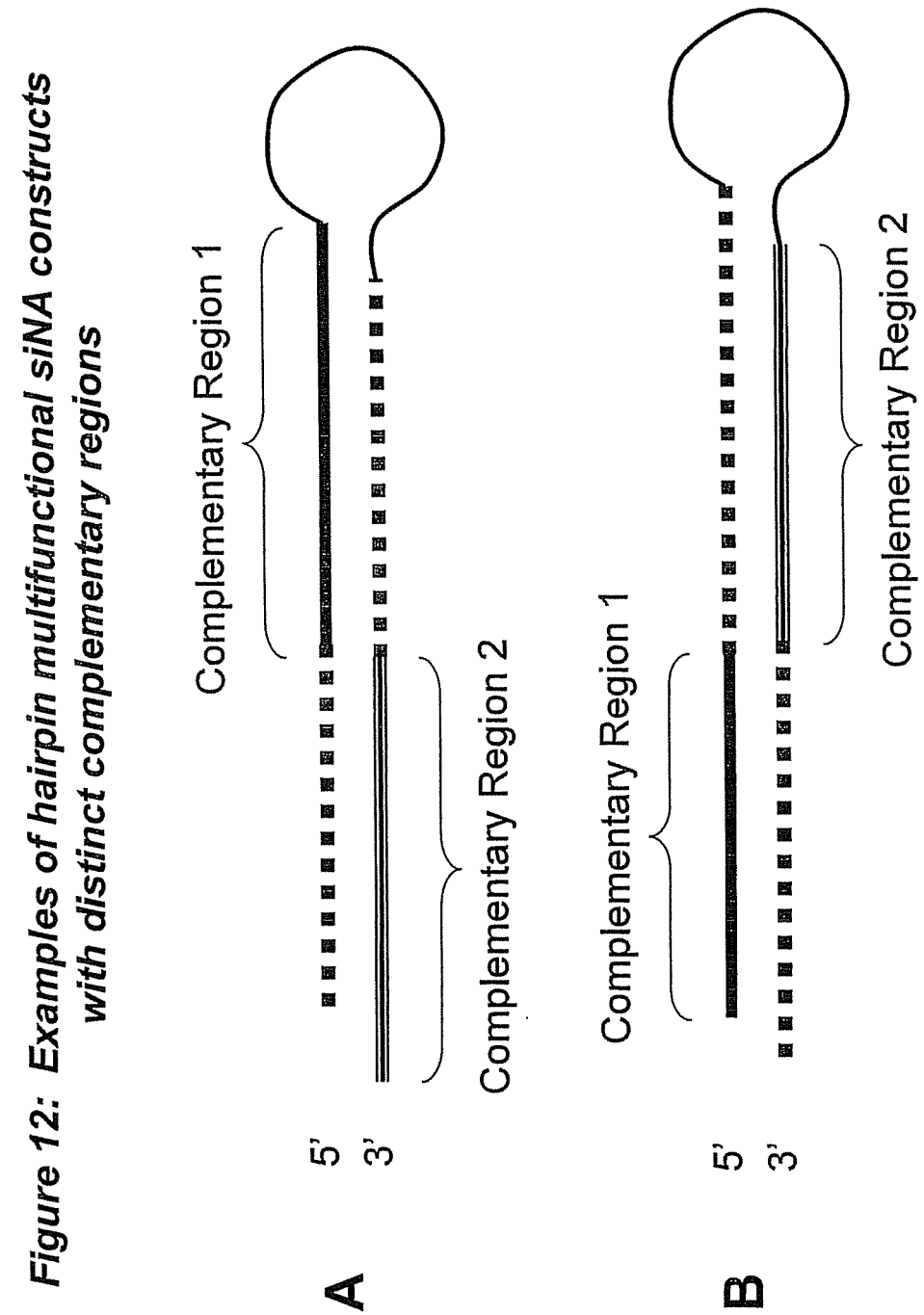


Figure 12: Examples of hairpin multifunctional siNA constructs with distinct complementary regions

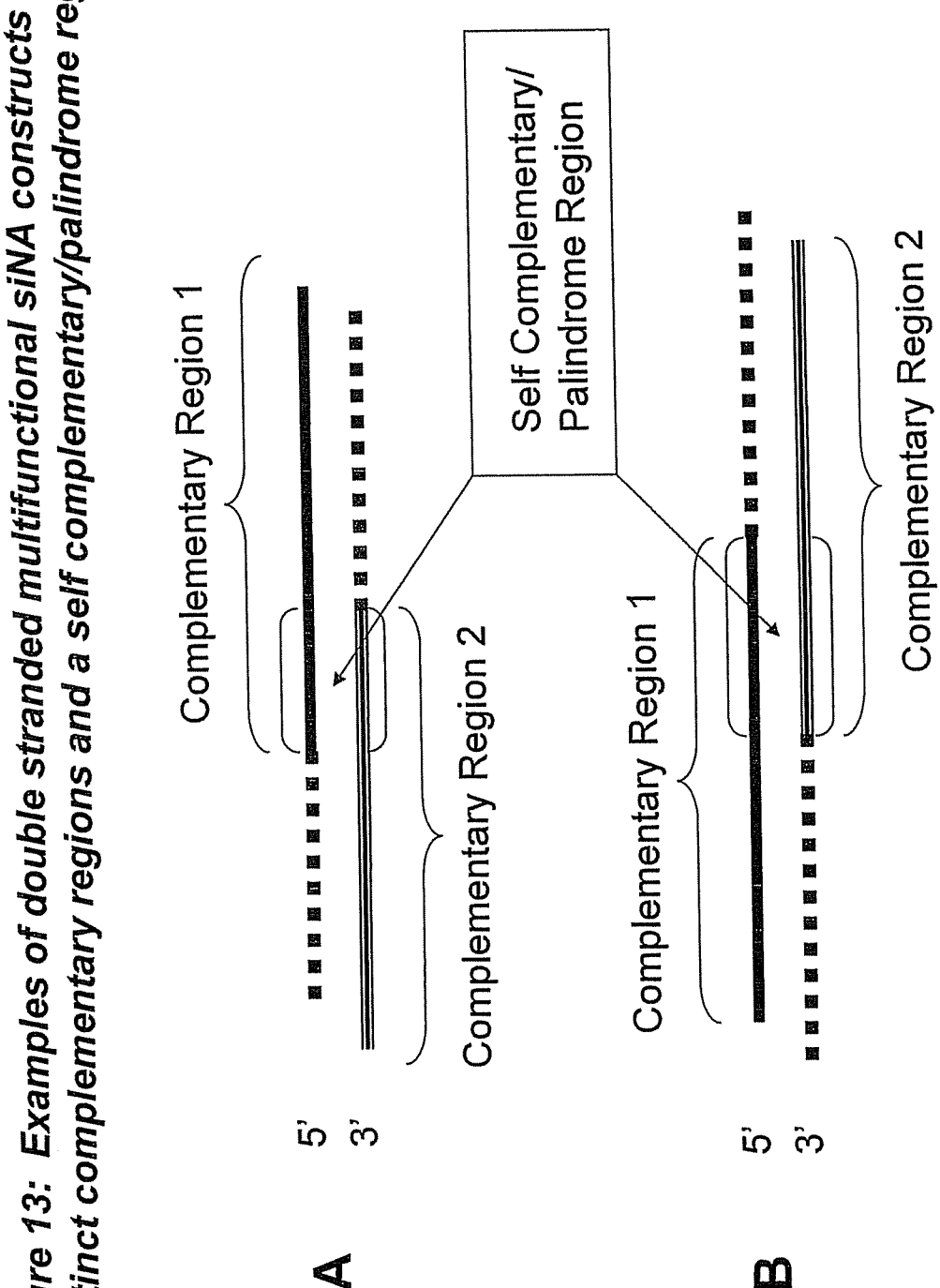


Figure 13: Examples of double stranded multifunctional siNA constructs with distinct complementary regions and a self complementary/palindrome region

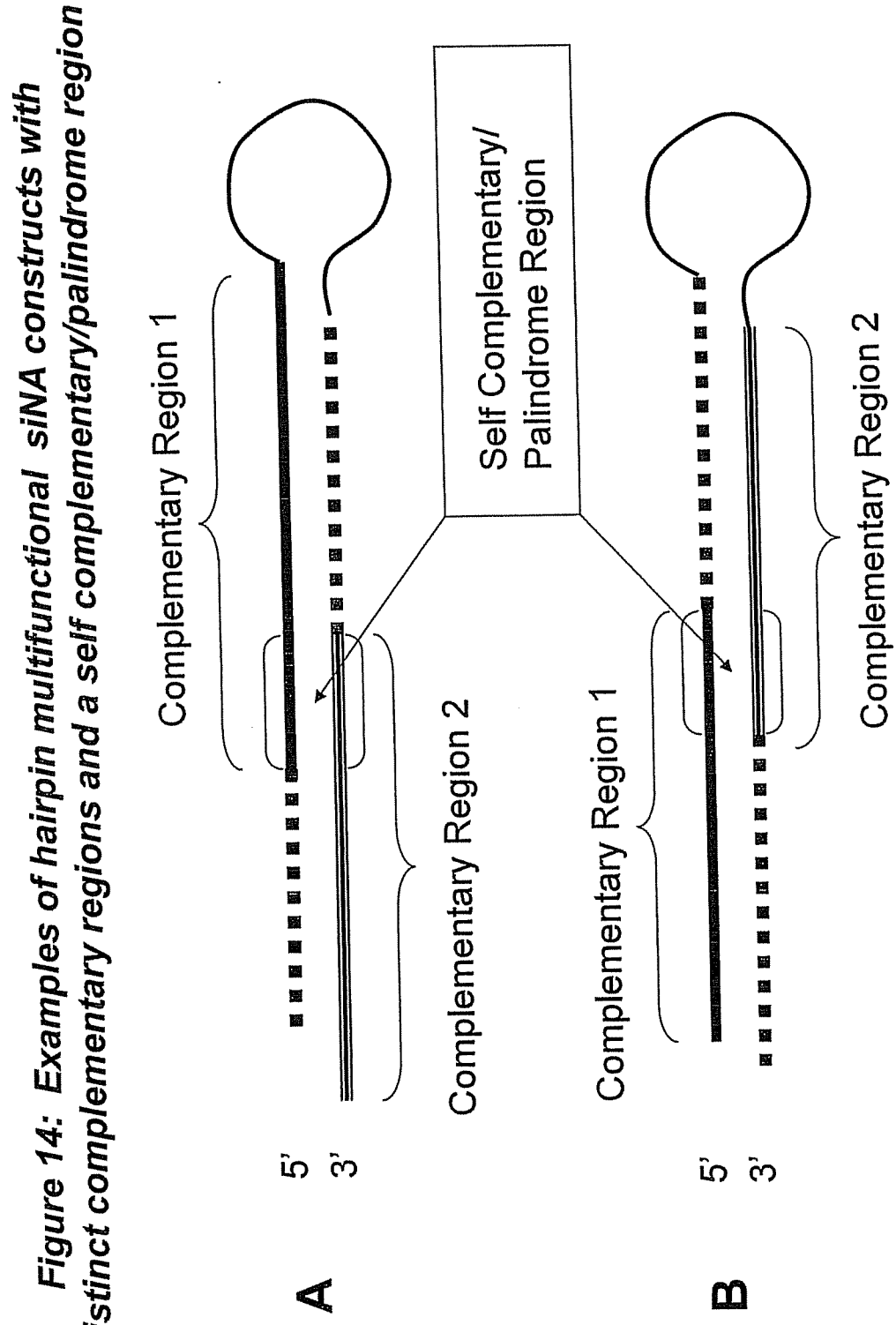


Figure 14: Examples of hairpin multifunctional siNA constructs with distinct complementary regions and a self complementary/palindrome region

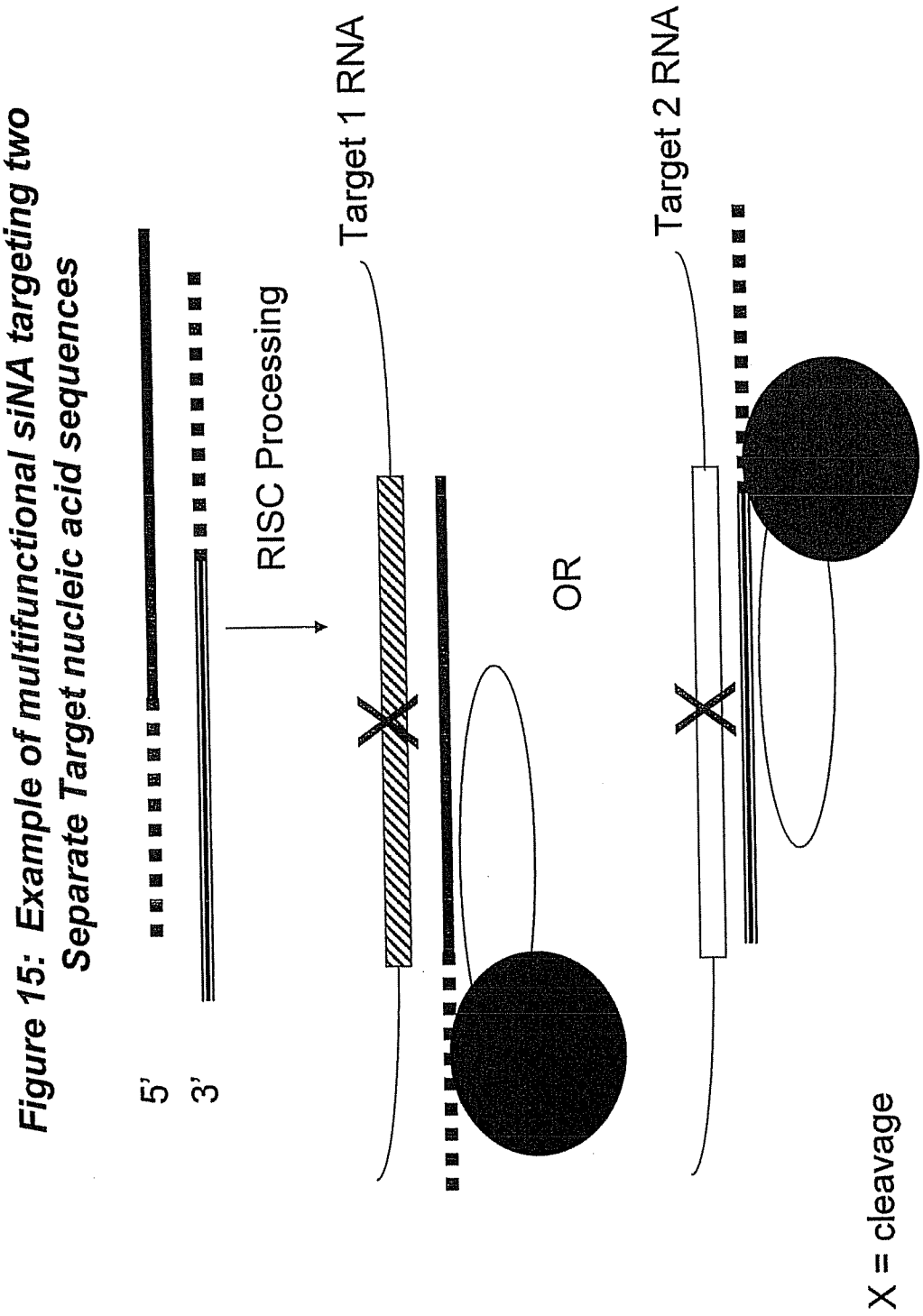


Figure 15: Example of multifunctional siNA targeting two Separate Target nucleic acid sequences

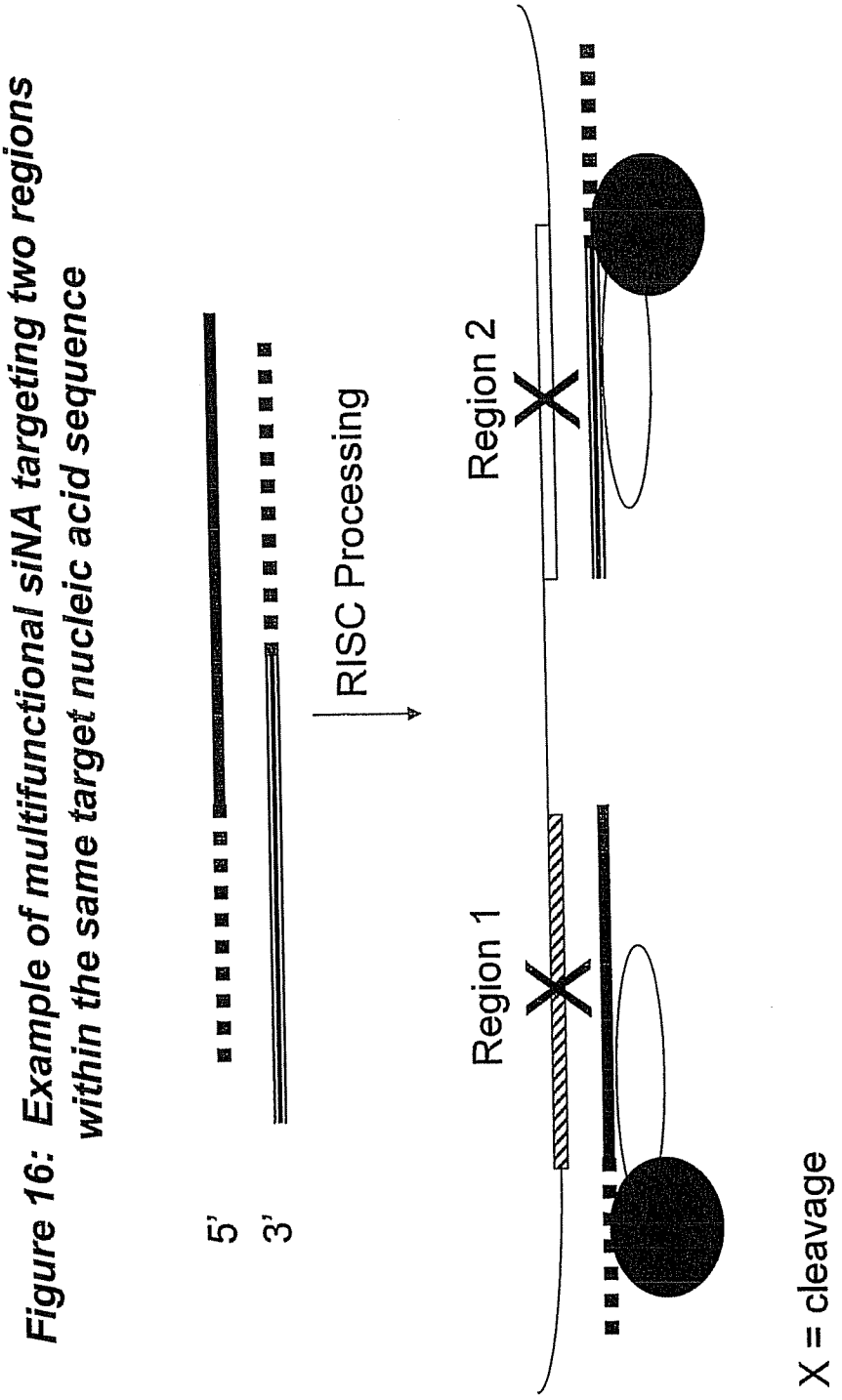
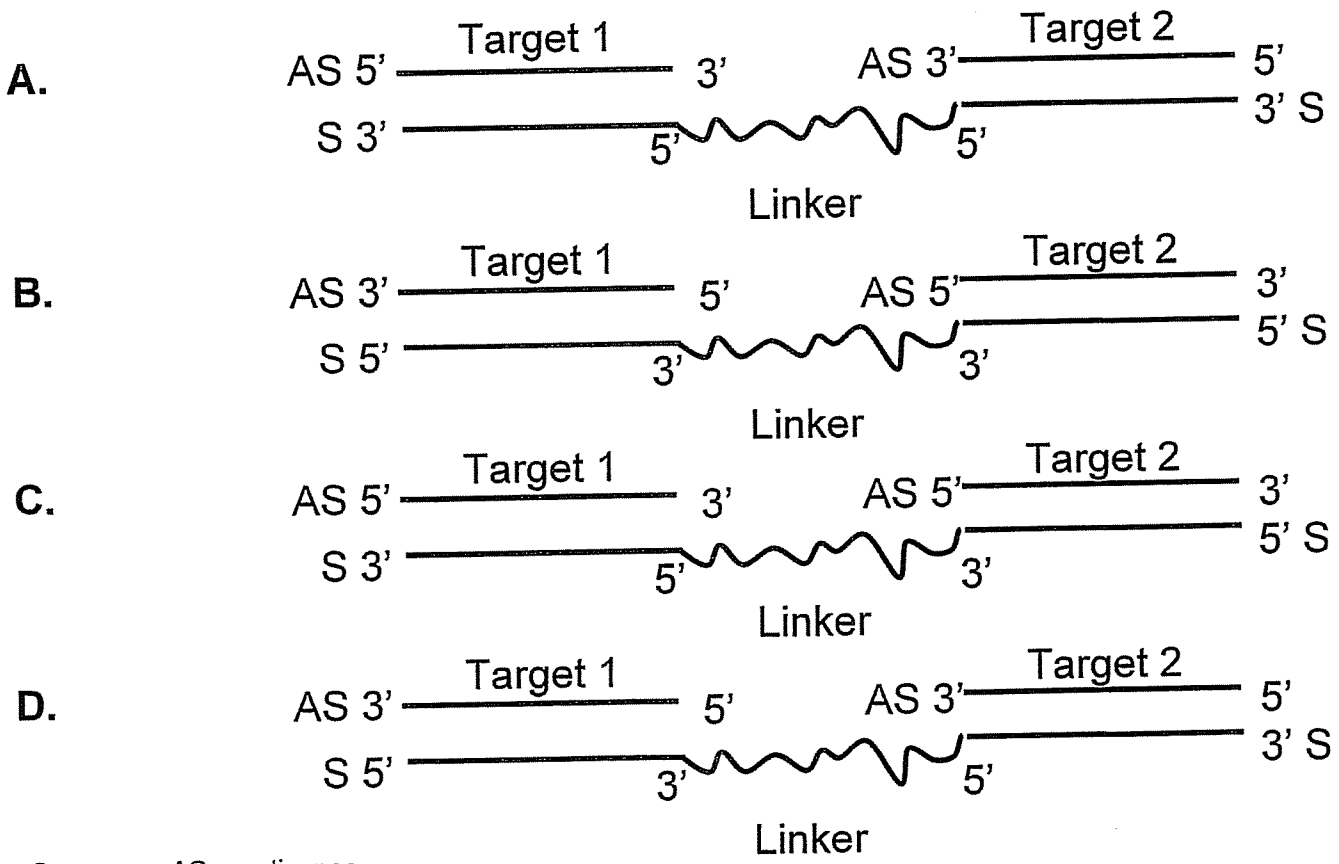


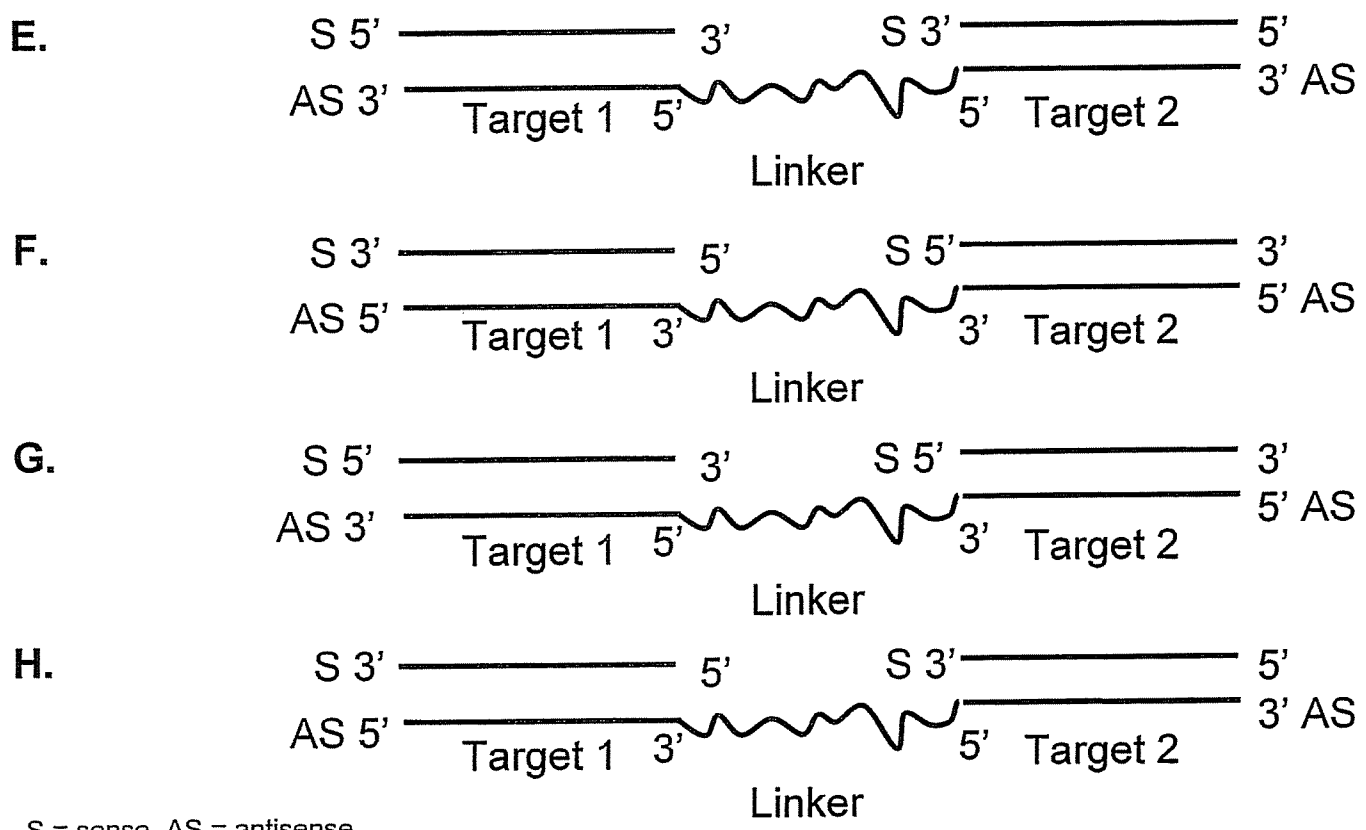
Figure 16: Example of multifunctional siNA targeting two regions within the same target nucleic acid sequence

Figure 17: 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 17: 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 18: Dendrimer Multifunctional siNA designs

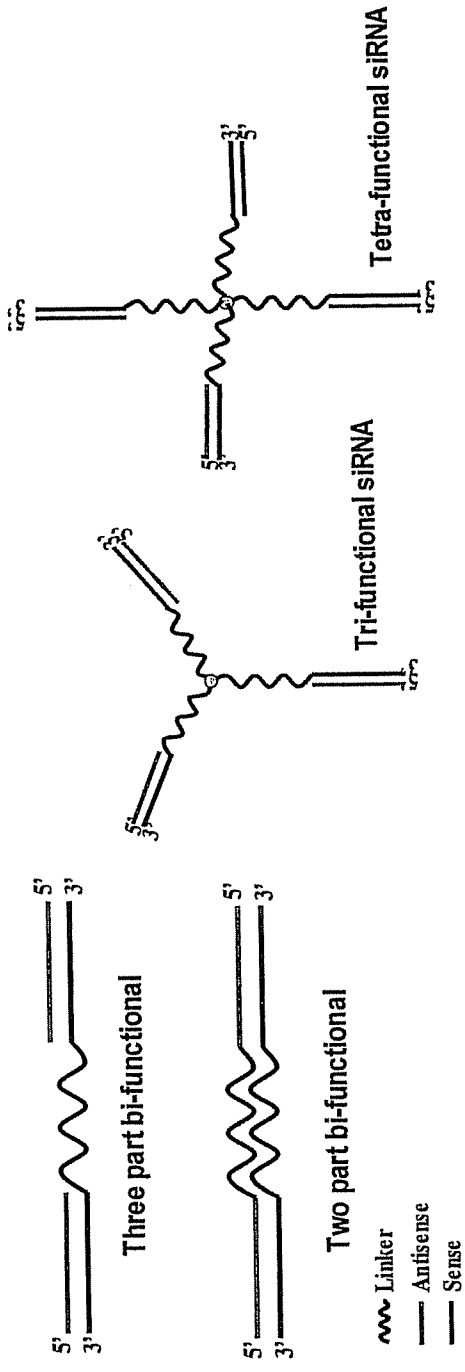


Figure 19: Supramolecular Multifunctional siNA designs

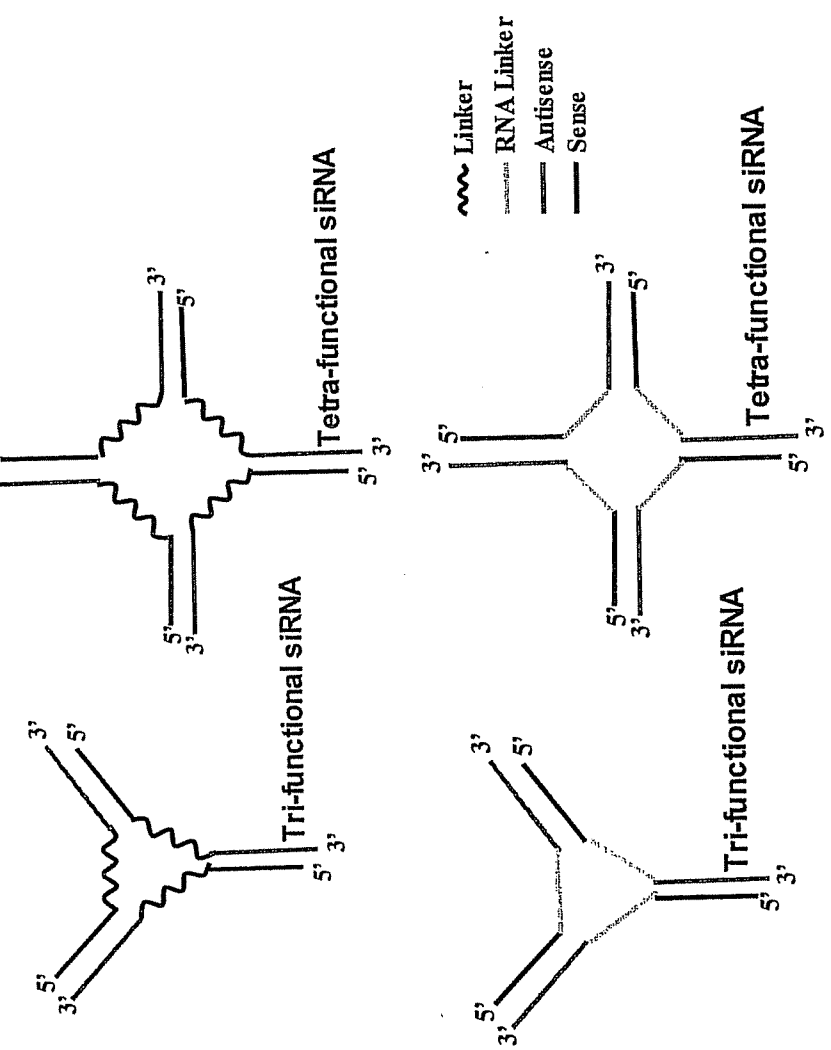


Figure 20: Dicer enabled multifunctional siNA design

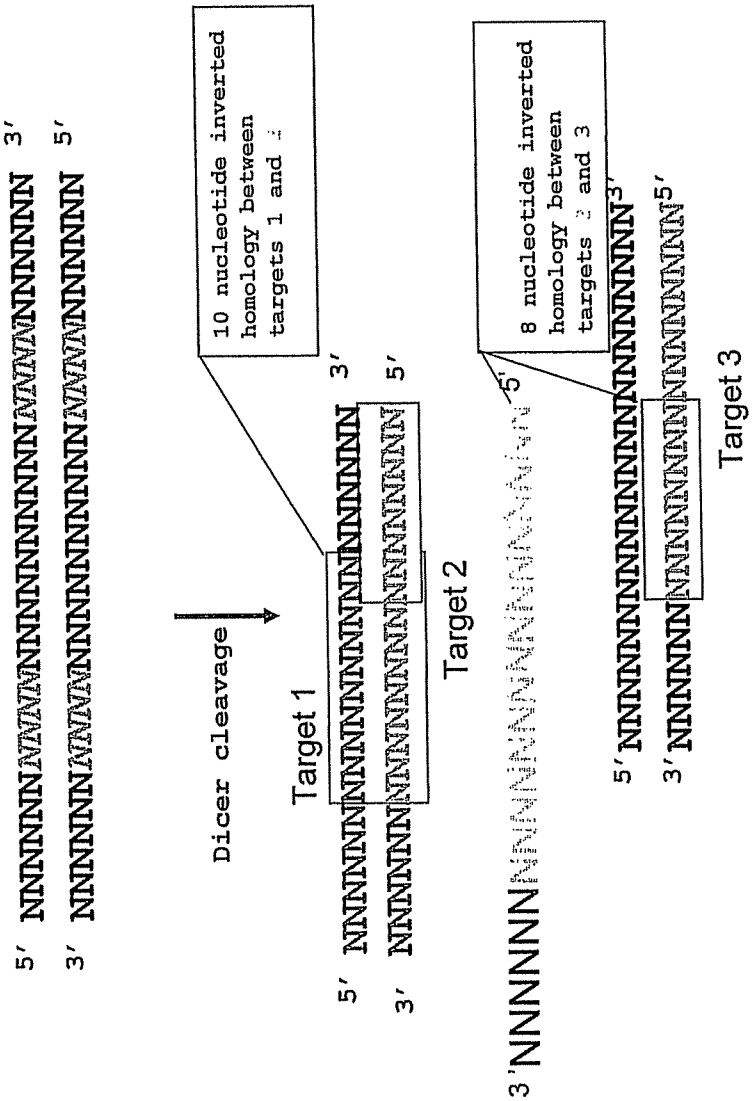


Figure 21: Dicer enabled multifunctional siNA design

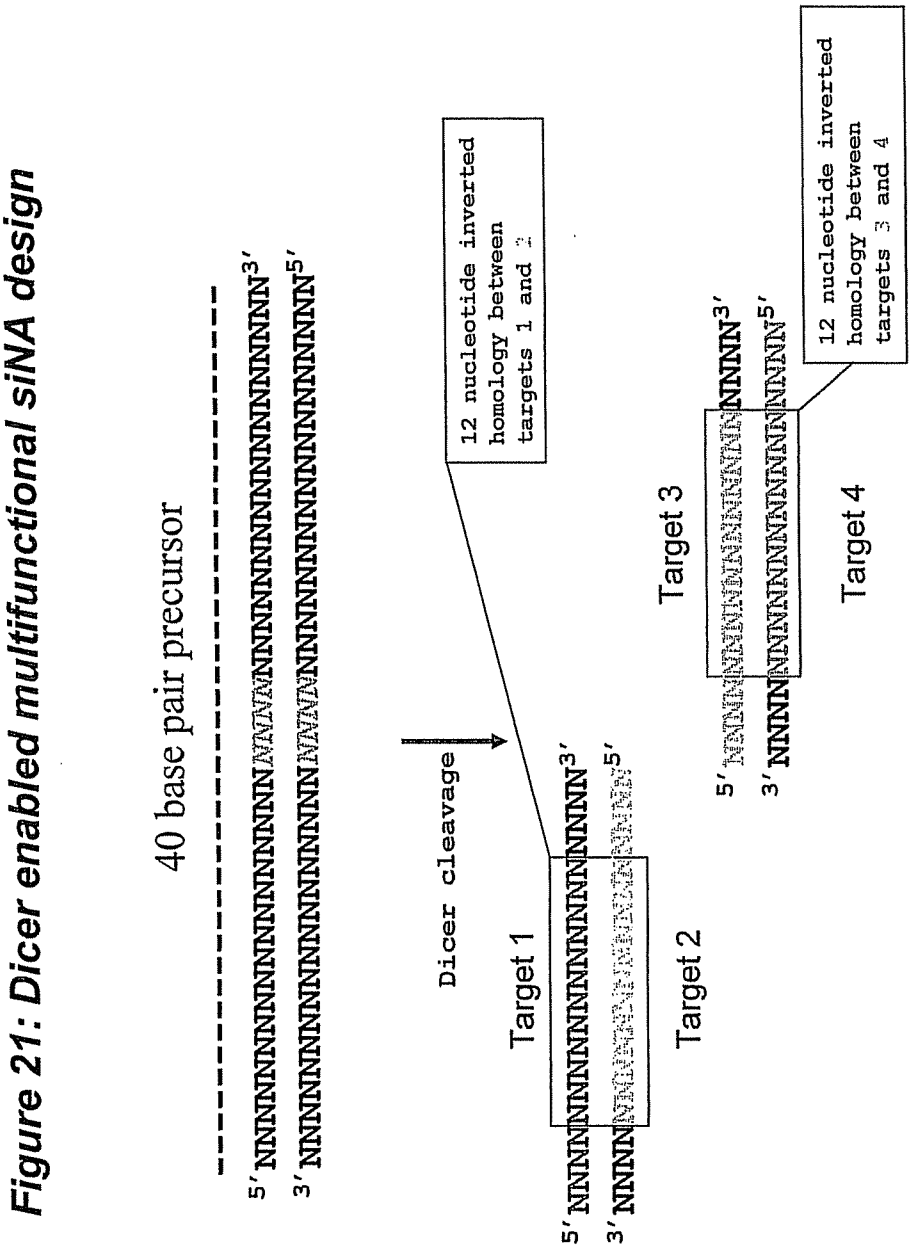


Figure 22: Additional Multifunctional siNA designs

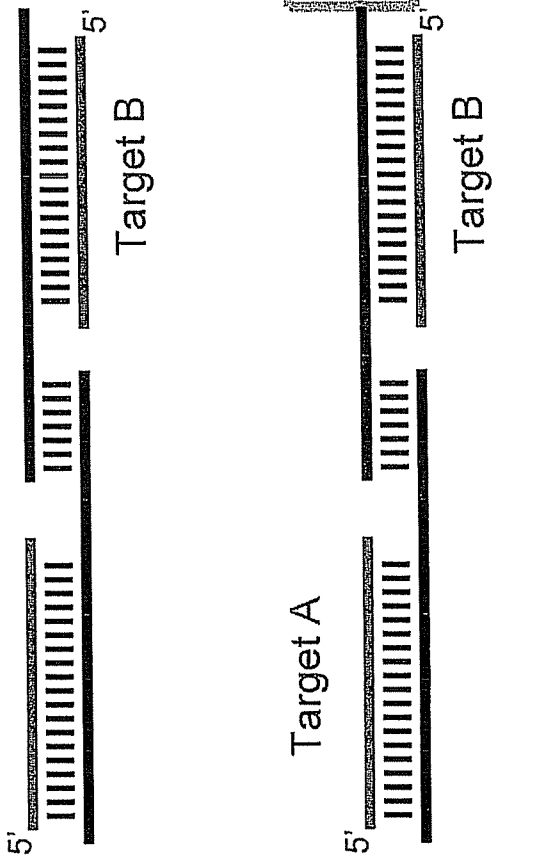


Figure 23: Additional Multifunctional siNA designs

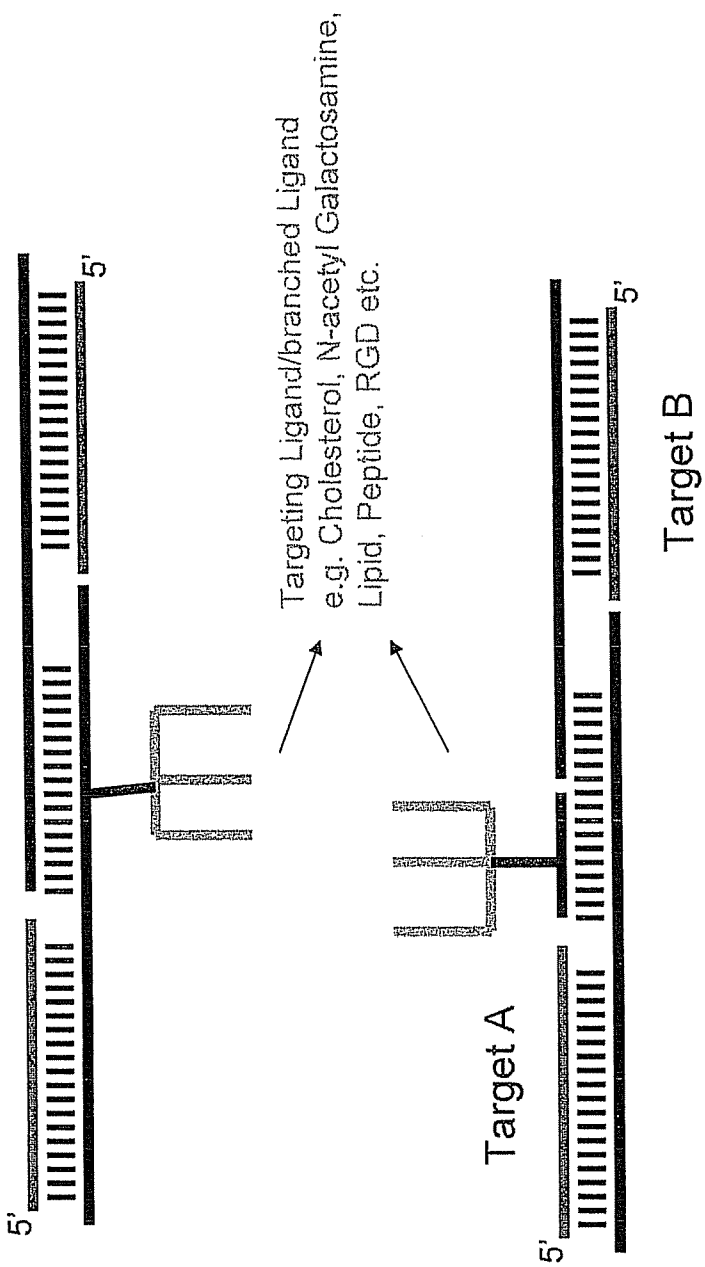
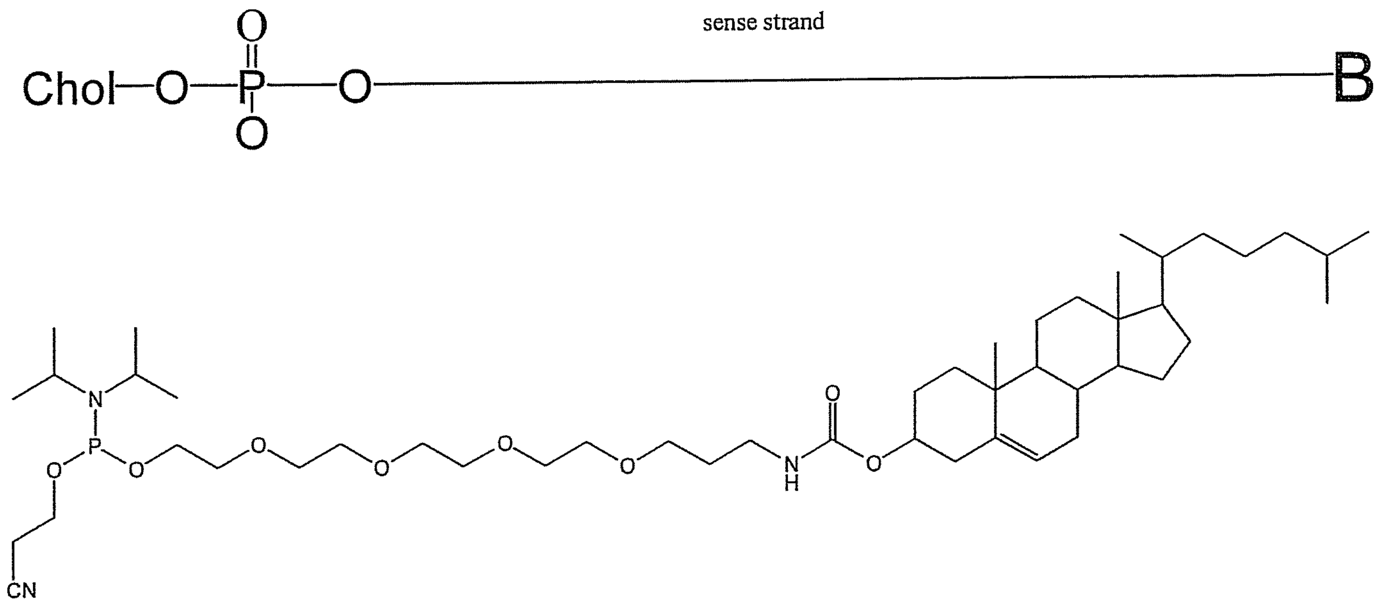
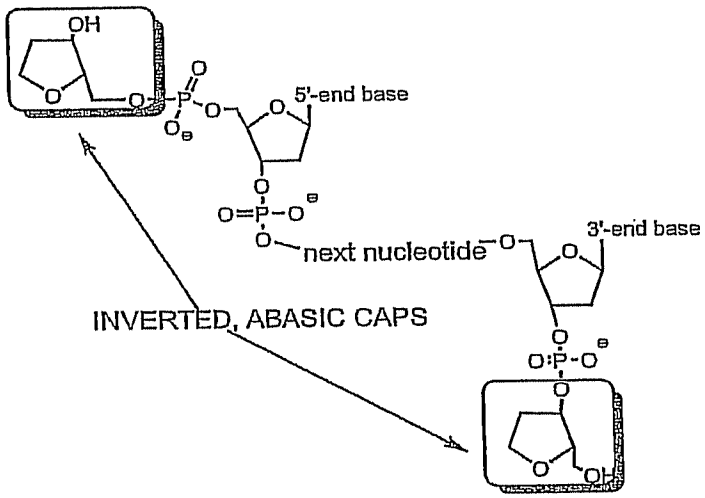


Figure 24: Cholesterol Conjugate Approach



$C_{48}H_{86}N_3O_8P$
Exact Mass: 863.62
Mol. Wt.: 864.19
C, 66.71; H, 10.03; N, 4.86; O, 14.81; P, 3.58

Figure 25


OH
5'-end base
next nucleotide
3'-end base
INVERTED, ABASIC CAPS
OH