RNA INTERFERENCE MEDIATED INHIBITION OF MUSCARINIC CHOLINERGIC RECEPTOR GENE EXPRESSION USING SHORT INTERFERING NUCLEIC ACID (siNA)

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Abstract

The present invention relates to compounds, compositions, and methods useful for modulating the expression of genes associated with respiratory and pulmonary disease, such as muscarinic cholinergic receptor genes, using short interfering nucleic acid (siNA) molecules. This invention also relates to compounds, compositions, and methods useful for modulating the expression and activity of muscarinic cholinergic receptor genes, or other genes involved in pathways of cholinergic muscarinic receptor gene expression and/or activity by RNA interference (RNAi) using small nucleic acid molecules. In particular, the instant invention features small nucleic acid molecules, such as short interfering nucleic acid (siNA), short interfering RNA (siRNA), double-stranded RNA (dsRNA), micro-RNA (miRNA), and short hairpin RNA (shRNA) molecules and methods used to modulate the expression of M3 muscarinic acetylcholine receptor or cholinergic receptor muscarinic 3 (CHRM3).
**Figure 1**

(1) FIRST STRAND
(2) SECOND STRAND

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DEPROTECTION

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PURIFICATION (DETRITYLATION)

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siRNA DUPLEX

= SOLID SUPPORT

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

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

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

**INVERTED DEOXYABASIC SUCCINATE LINKAGE**

**GLYCERYL SUCCINATE LINKAGE**
**Figure 4**

**A**

\[
\begin{align*}
\text{SENSE STRAND (SEQ ID NO 287)} \\
\text{ALL POSITIONS RIBONUCLEOTIDE EXCEPT POSITIONS (NN)} \\
\text{5'}- &\quad B-N NN NN NN NN NN NN NN NN NN NN NN NN NN NN NN NN NN NN-3' \\
3'- &\quad L-(N_2N) NN NN NN NN NN NN NN NN NN NN NN NN NN NN NN NN NN NN-5' \\
\text{ANTISENSE STRAND (SEQ ID NO 288)} \\
\text{ALL POSITIONS RIBONUCLEOTIDE EXCEPT POSITIONS (NN)}
\end{align*}
\]

**B**

\[
\begin{align*}
\text{SENSE STRAND (SEQ ID NO 289)} \\
\text{ALL PYRIMIDINES = 2'-FLUORO AND ALL PURINES = 2'-O-ME EXCEPT POSITIONS (NN)} \\
\text{5'}- &\quad N NN NN NN NN NN NN NN NN NN NN NN NN NN NN NN NN NN NN (N_2N)-3' \\
3'- &\quad L-(N_2N) NN NN NN NN NN NN NN NN NN NN NN NN NN NN NN NN NN NN-5' \\
\text{ANTISENSE STRAND (SEQ ID NO 290)} \\
\text{ALL PYRIMIDINES = 2'-FLUORO AND ALL PURINES = 2'-O-ME EXCEPT POSITIONS (NN)}
\end{align*}
\]

**C**

\[
\begin{align*}
\text{SENSE STRAND (SEQ ID NO 291)} \\
\text{ALL PYRIMIDINES = 2'-O-ME OR 2'-FLUORO EXCEPT POSITIONS (NN)} \\
\text{5'}- &\quad B-N NN NN NN NN NN NN NN NN NN NN NN NN NN NN NN NN NN NN (N_2N)-3' \\
3'- &\quad L-(N_2N) NN NN NN NN NN NN NN NN NN NN NN NN NN NN NN NN NN NN-5' \\
\text{ANTISENSE STRAND (SEQ ID NO 292)} \\
\text{ALL PYRIMIDINES = 2'-FLUORO EXCEPT POSITIONS (NN)}
\end{align*}
\]

**D**

\[
\begin{align*}
\text{SENSE STRAND (SEQ ID NO 293)} \\
\text{ALL PYRIMIDINES = 2'-FLUORO EXCEPT POSITIONS (NN) AND ALL PURINES = 2'-DEOXY} \\
\text{5'}- &\quad B-N NN NN NN NN NN NN NN NN NN NN NN NN NN NN NN NN NN NN (N_2N)-3' \\
3'- &\quad L-(N_2N) NN NN NN NN NN NN NN NN NN NN NN NN NN NN NN NN NN NN-5' \\
\text{ANTISENSE STRAND (SEQ ID NO 290)} \\
\text{ALL PYRIMIDINES = 2'-FLUORO AND ALL PURINES = 2'-O-ME EXCEPT POSITIONS (NN)}
\end{align*}
\]

**E**

\[
\begin{align*}
\text{SENSE STRAND (SEQ ID NO 294)} \\
\text{ALL PYRIMIDINES = 2'-FLUORO EXCEPT POSITIONS (NN)} \\
\text{5'}- &\quad B-N NN NN NN NN NN NN NN NN NN NN NN NN NN NN NN NN NN NN (N_2N)-3' \\
3'- &\quad L-(N_2N) NN NN NN NN NN NN NN NN NN NN NN NN NN NN NN NN NN NN-5' \\
\text{ANTISENSE STRAND (SEQ ID NO 290)} \\
\text{ALL PYRIMIDINES = 2'-FLUORO AND ALL PURINES = 2'-O-ME EXCEPT POSITIONS (NN)}
\end{align*}
\]

**F**

\[
\begin{align*}
\text{SENSE STRAND (SEQ ID NO 295)} \\
\text{ALL PYRIMIDINES = 2'-FLUORO EXCEPT POSITIONS (NN) AND ALL PURINES = 2'-DEOXY} \\
\text{5'}- &\quad B-N NN NN NN NN NN NN NN NN NN NN NN NN NN NN NN NN NN NN (N_2N)-3' \\
3'- &\quad L-(N_2N) NN NN NN NN NN NN NN NN NN NN NN NN NN NN NN NN NN NN-5' \\
\text{ANTISENSE STRAND (SEQ ID NO 290)} \\
\text{ALL PYRIMIDINES = 2'-FLUORO EXCEPT POSITIONS (NN) AND ALL PURINES = 2'-DEOXY}
\end{align*}
\]

**Positions (NN) Can Comprise Any Nucleotide, Such As Deoxynucleotides (Eg. Thymidine) Or Universal Bases
B = Abasic, Inverted Abasic, Inverted Nucleotide Or Other Terminal Cap That Is Optionally Present
L = Glyceryl Or B That Is Optionally Present
S = Phosphorothioate Or Phosphorodithioate That Is Optionally Absent**
Figure 5

A

\[
\begin{align*}
5' &- \text{iB-GACAAAUGACUGCU}TT\text{-iB} & -3' \\
3' &- L-T_{ST}CUUGUUACUAGCA & -5'
\end{align*}
\]

\text{SENSE STRAND (SEQ ID NO 296)}

\text{ANTISENSE STRAND (SEQ ID NO 297)}

B

\[
\begin{align*}
5' &- \text{gcaaacagauugcu}TT & -3' \\
3' &- L-T_{ST}Cuuguuacucacga & -5'
\end{align*}
\]

\text{SENSE STRAND (SEQ ID NO 298)}

\text{ANTISENSE STRAND (SEQ ID NO 299)}

C

\[
\begin{align*}
5' &- \text{iB-GAACAAAGAUAGCGCu}TT\text{-iB} & -3' \\
3' &- L-T_{ST}CuuguAACGA & -5'
\end{align*}
\]

\text{SENSE STRAND (SEQ ID NO 300)}

\text{ANTISENSE STRAND (SEQ ID NO 301)}

D

\[
\begin{align*}
5' &- \text{iB-GAACAAAGAUAGCGCu}TT\text{-iB} & -3' \\
3' &- L-T_{ST}CuuguAACGA & -5'
\end{align*}
\]

\text{SENSE STRAND (SEQ ID NO 302)}

\text{ANTISENSE STRAND (SEQ ID NO 299)}

E

\[
\begin{align*}
5' &- \text{iB-GAACAAAGAUAGCGCu}TT\text{-iB} & -3' \\
3' &- L-T_{ST}CuuguAACGA & -5'
\end{align*}
\]

\text{SENSE STRAND (SEQ ID NO 303)}

\text{ANTISENSE STRAND (SEQ ID NO 299)}

F

\[
\begin{align*}
5' &- \text{iB-GAACAAAGAUAGCGCu}TT\text{-iB} & -3' \\
3' &- L-T_{ST}CuuguAACGA & -5'
\end{align*}
\]

\text{SENSE STRAND (SEQ ID NO 302)}

\text{ANTISENSE STRAND (SEQ ID NO 304)}

\text{ITALIC UPPER CASE} = \text{DEOXY}

\text{iB} = \text{INVERTED DEOXYABASIC}

\text{L} = \text{GLYCERYL MOIETY or iB OPTIONALLY PRESENT}

\text{S} = \text{PHOSPHOROTHIOATE OR PHOSPHORODITHIOATE OPTIONALLY PRESENT}

lower case = 2'-O-Methyl or 2'-deoxy-2'-fluoro

italic lower case = 2'-deoxy-2'-fluoro

underline = 2'-O-methyl
Figure 6

1. SENSE 2. SENSE 3. SENSE 4. SENSE

n = 0, 1, 2, 3, 4
Figure 8

A  
5'- R1 NNNNNNNNNNNNNNNNNNNNNNNNNNN R2 X X X  
   ▼  3'-EXTENSION  
   ▼  CLEAVAGE WITH RESTRICTION ENZYMES 1 AND 2  

B  
5'- R1 NNNNNNNNNNNNNNNNNNNNNNNNNN R2 X X  
3' - R1 NNNNNNNNNNNNNNNNNNNNNNNNNN R2 X X  
   ▼  CLEAVAGE WITH RESTRICTION ENZYMES 1 AND 2  

C  
5'- R1 NNNNNNNNNNNNNNNNNNNNNNNNNN  
3' - R1 NNNNNNNNNNNNNNNNNNNNNNNNNN  
   ▼  CLONE  

U6 snRNA PROMOTER  
R1 = RESTRICTION SITE #1  
R2 = RESTRICTION SITE #2  
N = A, G, C, or T  
X = A, G, C, or T
Figure 9: Target site Selection using siRNA

A: Synthesize oligos encoding siRNA against Target RNA sequence
B: Clone oligos into vector
C: Transduce target cells
D: Sequence siRNA
E: Identify efficacious target sites based on siRNA sequence

Select cells exhibiting desired phenotype
R = O, S, N, alkyl, substituted alkyl, C-alkyl, S-alkyl, alkaryl, aralkyl, or arylalkyl
B = independently any nucleic base.
Figure 11: Modification Strategy

1. Test for activity in luciferase reporter system
2. Compare stability and activity vs unmodified construct
3. Test for nuclease stability in human serum
4. Make an educated modification

The process is cyclical, allowing for iterative refinement and analysis.
Figure 12: Phosphorylated siRNA constructs

Asymmetric hairpin siRNA

Asymmetric duplex siRNA

(n) = number of base pairs (e.g. 3-18 bp)

Phosphates can be modified as described herein
Figure 13: 5'-phosphate modifications
Figure 14A: Duplex forming oligonucleotide constructs that utilize palindrome or repeat sequences

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

(ii) 3' ●●●●●●●●●●● Design Complementary Sequence to the Target Nucleic Acid sequence of (i) above

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

(iv) 3' ●●●●●●●●●●● Self assembly of self complementary strands to form duplex construct

5' ●●●●●●●●●●●
Figure 14B: Example of a duplex forming oligonucleotide sequence that utilizes a palindrome or repeat sequence

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

(ii) AUUUAU GAUAAGC
   3' ······
   Design Complementary Sequence to the Target Nucleic Acid sequence of (i) above

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

(iv) 3' GCUUUAUC AUUUAU GAUAAGC
     5' CGAAUAAG AUUUAU CUAUUCG
     Self assembly of self complementary strands to form duplex construct (blunt ends)
Figure 14C: Example of a duplex forming oligonucleotide sequence that utilizes a palindrome or repeat sequence, self assembly.
Figure 14D: Example of a duplex forming oligonucleotide sequence that utilizes a palindromic or repeat sequence, self assembly and inhibition of Target Sequence Expression.
Figure 15: Duplex forming oligonucleotide constructs that utilize artificial palindrome or repeat sequences

Identify Target Nucleic Acid sequence (e.g., 14 to 24 nucleotides in length)

Design Complementary Sequence and utilize modified nucleotides (shown as X, Y) that interact with a portion of the target sequence and result in the formation of a palindrome/repeat sequence (e.g., 2 to 12 nucleotides) at 3'-end (dashed portion)

Append inverse sequence of Complementary region to 3'-end of palindrome/repeat sequence

Hybridize self complementary strands to form duplex siRNA construct
Figure 16: Examples of double stranded multifunctional siRNA constructs with distinct complementary regions
Figure 17: Examples of hairpin multifunctional siRNA constructs with distinct complementary regions.
Figure 18: Examples of double stranded multifunctional siRNA constructs with distinct complementary regions and a self complementary/palindrome region.

Complementary Region 1

Self Complementary/Palindrome Region

Complementary Region 2

Complementary Region 1

Complementary Region 2

5' 3'

5' 3'

A

B
Figure 19: Examples of hairpin multifunctional siRNA constructs with distinct complementary regions and a self complementary/palindrome region.
Figure 20: Example of multifunctional sRNA targeting two separate
Target nucleic acid sequences
Figure 21: Example of multifunctional siRNA targeting two regions within the same target nucleic acid sequence.
RNA INTERFERENCE MEDIATED INHIBITION OF MUSCARinic CHOLINERGIC RECEPTOR GENE EXPRESSION USING SHORT INTERFERING NUCLEIC ACID (SINA)


FIELD OF THE INVENTION

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

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; Lia 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, 558). 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 U.S. Pat. Nos. 6,107,098; 5,898,031; Clemens et al., 1997, J. Interferon & Cytokine Res., 17, 503-524; Adah et al., 2001, Curr. Med. Chem., 8, 1189).


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 RNAi activity and that ATP is utilized to maintain the 5'-phosphate moiety on the siRNA (Nykänen et al., 2001, Cell, 107, 309).

[0007] Studies have shown that replacing the 3'-terminal nucleotide overhang 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. 2,535,180, 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 deoxy-nucleotides 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-aminoallyluracil 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-aminoallyluracil in the antisense strand resulted in a substantial decrease in RNAi activity as well.


SUMMARY OF THE INVENTION

[0013] In one embodiment, the invention features one or more siRNA molecules and methods that independently or in combination modulate the expression of cholinergic muscarinic receptor genes encoding proteins, such as proteins comprising cholinergic muscarinic receptors and ligands thereof, associated with the maintenance and/or development of respiratory and/or pulmonary diseases, such as genes encoding sequences comprising those sequences referred to by GenBank Accession Nos. shown in Table I, referred to herein generally as CHRM3. The description below of the various aspects and embodiments of the invention is provided with reference to exemplary M3 muscarinic acetylcholine receptor referred to herein as CHRM3. However, the various aspects and embodiments are also directed to other muscarinic acetylcholine receptor genes, such as CHRM1, CHRM2, CHRM4, CHRM5 and polymorphisms (e.g., SNPs) associated with such muscarinic acetylcholine receptor genes (including CHRM3 polymorphisms, see for example Choi et al., WO 01/029176 and Hall, 2002, Respir. Res., 3, 10). As such, the various aspects and embodiments are also directed to other genes that are involved in CHRM3 mediated pathways of signal transduction or gene expression that are involved in the progression, development, and/or maintenance of disease (e.g., chronic obstructive pulmonary disease or "COPD", asthma, allergic rhinitis, sinusitis, pulmonary vasoconstriction, inflammation, allergies, impeded respiration, respiratory distress syndrome, cystic fibrosis, pulmonary hypertension, pulmonary vasoconstriction, emphysema, irritable bowel syndrome, Alzheimer’s disease, urinary incontinence), including genes encoding CHRM3 ligands and agonists. These additional genes can be analyzed for target sites using the methods described for CHRM3 genes herein. Thus, the modulation of other genes and the effects of such modulation of the other genes can be performed, determined, and measured as described herein.

[0014] In one embodiment, the invention features a double-stranded short interfering nucleic acid (siRNA) molecule that down-regulates expression of a cholinergic muscarinic receptor (e.g., CHRM3) gene, wherein said siRNA molecule comprises about 19 to about 21 base pairs.

[0015] In one embodiment, the invention features a siRNA molecule that down-regulates expression of a CHRM3 gene, for example, wherein the CHRM3 gene comprises CHRM3 encoding sequence. In one embodiment, the invention features a siRNA molecule that down-regulates expression of a CHRM3 gene, for example, wherein the CHRM3 gene comprises CHRM3 non-coding sequence or regulatory elements involved in CHRM3 gene expression.

A siRNA of the invention can be unmodified or chemically-modified. A siRNA of the instant invention can be chemically synthesized, expressed from a vector or enzymatically synthesized. The instant invention also features various chemically-modified synthetic short interfering nucleic acid (siNA) molecules capable of modulating repeat expansion 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 retains its RNAi activity. The siNA molecules of the instant invention provide useful reagents and methods for a variety of therapeutic, diagnostic, target validation, genomic discovery, genetic engineering, and pharmacogenomic applications.
In one embodiment, the invention features a siRNA molecule having RNAi activity against CHRM3 RNA, wherein the siRNA molecule comprises a sequence complementary to any RNA having CHRM3 encoding sequence, such as those sequences having GenBank Accession Nos. shown in Table I. In another embodiment, the invention features a siRNA molecule having RNAi activity against CHRM3 RNA, wherein the siRNA molecule comprises a sequence complementary to an RNA having other CHRM3 encoding sequence, for example other mutant CHRM3 genes not shown in Table I but known in the art to be associated with respiratory or pulmonary disease (e.g., chronic obstructive pulmonary disease or COPD, asthma, allergic rhinitis, sinusitis, pulmonary vasconstriction, inflammation, allergies, impeded respiration, respiratory distress syndrome, cystic fibrosis, pulmonary hypertension, pulmonary vasconstriction, emphysema, irritable bowel syndrome, Alzheimer’s disease, urinary incontinence). Chemical modifications as shown in Tables III and IV or otherwise described herein can be applied to any siRNA construct of the invention. In another embodiment, a siRNA molecule of the invention includes nucleotide sequence that can interact with nucleotide sequence of a CHRM3 gene and thereby mediate silencing of CHRM3 gene expression, for example, wherein the siRNA mediates regulation of CHRM3 gene expression by cellular processes that modulate the chromatin structure of the CHRM3 gene and prevent transcription of the CHRM3 gene.

In one embodiment, siRNA molecules of the invention are used to down regulate or inhibit the expression of CHRM3 proteins arising from CHRM3 haplotype polymorphisms that are associated with disease, (e.g., associated with a gain of function). Analysis of CHRM3 genes, or CHRM3 protein or RNA levels can be used to identify subjects with such polymorphisms or those subjects who are at risk of developing diseases described herein. These subjects are amenable to treatment, for example, treatment with siRNA molecules of the invention and any other composition useful in treating diseases related to CHRM3 gene expression. As such, analysis of CHRM3 protein or RNA levels can be used to determine treatment type and the course of therapy in treating a subject. Monitoring of CHRM3 protein or RNA levels can be used to predict treatment outcome and to determine the efficacy of compounds and compositions that modulate the level and/or activity of certain CHRM3 proteins associated with disease.

In another embodiment, the invention features a siRNA molecule comprising nucleotide sequence, for example, nucleotide sequence in the antisense region of the siRNA molecule that is complementary to a nucleotide sequence or portion of sequence of a CHRM3 gene. In another embodiment, the invention features a siRNA molecule comprising a region, for example, the antisense region of the siRNA construct, complementary to a sequence comprising a CHRM3 gene sequence or a portion thereof.

In another embodiment, the antisense region of CHRM3 siRNA constructs can comprise a sequence complementary to sequence having any of SEQ ID Nos. 1-99, 199-214, 223-230, 239-246, 255-262, 271-278, 296, 298, 300, 302, or 303. The sense region can comprise a sequence of SEQ ID NO. 287 and the antisense region can comprise a sequence of SEQ ID NO. 288. The sense region can comprise a sequence of SEQ ID NO. 289 and the antisense region can comprise a sequence of SEQ ID NO. 290. The sense region can comprise a sequence of SEQ ID NO. 291 and the antisense region can comprise a sequence of SEQ ID NO. 292. The sense region can comprise a sequence of SEQ ID NO. 293 and the antisense region can comprise a sequence of SEQ ID NO. 290. The sense region can comprise a sequence of SEQ ID NO. 294 and the antisense region can comprise a sequence of SEQ ID NO. 290. The sense region can comprise a sequence of SEQ ID NO. 293 and the antisense region can comprise a sequence of SEQ ID NO. 295.

In one embodiment, a siRNA molecule of the invention comprises any of SEQ ID Nos. 1-304. The sequences shown in SEQ ID Nos: 1-304 are not limiting. A siRNA molecule of the invention can comprise any contiguous CHRM3 sequence (e.g., about 19 to about 25, or about 19, 20, 21, 22, 23, 24 or 25 contiguous CHRM3 nucleotides).

In yet another embodiment, the invention features a siRNA molecule comprising a sequence, for example, the antisense sequence of the siRNA construct, complementary to a sequence or portion of sequence comprising sequence represented by GenBank Accession Nos. shown in Table I. Chemical modifications in Tables III and IV and described herein can be applied to any siRNA construct of the invention.

In one embodiment of the invention a siRNA molecule comprises an antisense strand having about 19 to about 29 (e.g., about 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, or 29) nucleotides, wherein the antisense strand is complementary to a RNA sequence encoding a CHRM3 protein, and wherein said siRNA further comprises a sense strand having about 19 to about 29 (e.g., about 19, 20, 21, 22, 23, 24, 25, 26, 27, 28 or 29) nucleotides, wherein said sense strand and said antisense strand are distinct nucleotide sequences with at least about 19 complementary nucleotides.

In another embodiment of the invention a siRNA molecule of the invention comprises an antisense region having about 19 to about 29 (e.g., about 19, 20, 21, 22, 23, 24, 25, 26, 27, 28 or 29) nucleotides, wherein the antisense region is complementary to a RNA sequence encoding a CHRM3 protein, and wherein said siRNA further comprises a sense region having about 19 to about 29 (e.g., about 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29 or more) nucleotides, wherein said sense region and said antisense region comprise a linear molecule with at least about 19 complementary nucleotides.

In one embodiment of the invention a siRNA molecule comprises an antisense strand comprising a nucleotide sequence that is complementary to a nucleotide sequence or a portion thereof encoding a CHRM3 protein. The siRNA further comprises a sense strand, wherein said sense strand comprises a nucleotide sequence of a CHRM3 gene or a portion thereof.

In another embodiment, a siRNA molecule comprises an antisense region comprising a nucleotide sequence that is complementary to a nucleotide sequence encoding a CHRM3 protein or a portion thereof. The siRNA molecule...
further comprises a sense region, wherein said sense region comprises a nucleotide sequence of a CHRM3 gene or a portion thereof.

[0026] In one embodiment, a siNA molecule of the invention has RNAi activity that modulates expression of RNA encoded by a CHRM3 gene. Because CHRM3 genes can share some degree of sequence homology with each other, siNA molecules can be designed to target a class of CHRM3 genes or alternately specific CHRM3 genes (e.g., polymorphic variants) by selecting sequences that are either shared amongst different CHRM3 targets or alternatively that are unique for a specific CHRM3 target. Therefore, in one embodiment, the siNA molecule can be designed to target conserved regions of CHRM3 RNA sequence having homology between several CHRM3 gene variants so as to target a class of CHRM3 genes with one siNA molecule. Accordingly, in one embodiment, the siNA molecule of the invention modulates the expression of one or both CHRM3 alleles in a subject. In another embodiment, the siNA molecule can be designed to target a sequence that is unique to a specific CHRM3 RNA sequence (e.g., a single CHRM3 allele or CHRM3 SNP) due to the high degree of specificity that the siNA molecule requires to mediate RNAi activity.

[0027] In one embodiment, nucleic acid molecules of the invention that act as mediators of the RNA interference gene silencing response are double-stranded nucleic acid molecules. In another embodiment, the siNA molecules of the invention consist of duplexes containing about 19 base pairs between oligonucleotides comprising about 19 to about 25 (e.g., about 19, 20, 21, 22, 23, 24 or 25) nucleotides. In yet another embodiment, siNA molecules of the invention comprise duplexes 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.

[0028] In one embodiment, the invention features one or more chemically-modified siNA constructs having specificity for CHRM3 expressing nucleic acid molecules, such as RNA encoding a CHRM3 protein. Non-limiting examples of such chemical modifications include without limitation phosphorothioate internucleotide linkages, 2'-deoxyribo-nucleotides, 2'-O-methyl ribonucleotides, 2'-deoxy-2'-fluoro ribonucleotides, “universal base” nucleotides, “acyclic” nucleotides, 5-C-methyl nucleotides, and terminal glyceryl and/or inverted deoxy abasic residue incorporation. These chemical modifications, when used in various siNA constructs, are shown to preserve RNAi activity in cells while at the same time, dramatically increasing the serum stability of these compounds. Furthermore, contrary to the data published by Parrish et al., supra, applicant demonstrates that multiple (greater than one) phosphorothioate substitutions are well-tolerated and confer substantial increases in serum stability for modified siNA constructs.

[0029] In one embodiment, a siNA molecule of the invention comprises modified nucleotides while maintaining the ability to mediate RNAi. The modified nucleotides can be used to improve in vitro or in vivo characteristics such as stability, activity, and/or bioavailability. For example, a siNA molecule of the invention can comprise modified nucleotides as a percentage of the total number of nucleotides present in the siNA molecule. As such, a siNA molecule of the invention can generally comprise about 5% to about 100% modified nucleotides (e.g., 5%, 10%, 15%, 20%, 25%, 30%, 35%, 40%, 45%, 50%, 55%, 60%, 65%, 70%, 75%, 80%, 85%, 90%, 95% or 100% modified nucleotides). The actual percentage of modified nucleotides present in a given siNA molecule will depend on the total number of nucleotides present in the siNA. If the siNA molecule is single stranded, the percent modification can be based upon the total number of nucleotides present in the single stranded siNA molecule. Likewise, if the siNA molecule is double stranded, the percent modification can be based upon the total number of nucleotides present in the sense strand, antisense strand, or both the sense and antisense strands.

[0030] One aspect of the invention features a double-stranded short interfering nucleic acid (siNA) molecule that down-regulates expression of a CHRM3 gene. In one embodiment, a double stranded siNA molecule comprises one or more chemical modifications and each strand of the double-stranded siNA is about 21 nucleotides long. In one embodiment, the double-stranded siNA molecule does not contain any ribonucleotides. In another embodiment, the double-stranded siNA molecule comprises one or more ribonucleotides. In one embodiment, each strand of the double-stranded siNA molecule comprises about 19 to about 29 (e.g., about 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, or 29) nucleotides, wherein each strand comprises about 19 nucleotides that are complementary to the nucleotides of the other strand. In one embodiment, one of the strands of the double-stranded siNA molecule comprises a nucleotide sequence that is complementary to a nucleotide sequence or a portion thereof of the CHRM3 gene, and the second strand of the double-stranded siNA molecule comprises a nucleotide sequence substantially similar to the nucleotide sequence of the CHRM3 gene or a portion thereof.

[0031] In another embodiment, the invention features a double-stranded short interfering nucleic acid (siNA) molecule that down-regulates expression of a CHRM3 gene comprising an antisense region, wherein the antisense region comprises a nucleotide sequence that is complementary to a nucleotide sequence of the CHRM3 gene or a portion thereof, and a sense region, wherein the sense region comprises a nucleotide sequence substantially similar to the nucleotide sequence of the CHRM3 gene or a portion thereof. In one embodiment, the antisense region and the sense region each comprise about 19 to about 29 (e.g., about 19, 20, 21, or 23) nucleotides, wherein the antisense region comprises about 19 nucleotides that are complementary to nucleotides of the sense region.

[0032] In another embodiment, the invention features a double-stranded short interfering nucleic acid (siNA) molecule that down-regulates expression of a CHRM3 gene comprising a sense region and an antisense region, wherein the antisense region comprises a nucleotide sequence that is complementary to a nucleotide sequence of RNA encoded by the CHRM3 gene or a portion thereof and the sense region comprises a nucleotide sequence that is complementary to the antisense region.

[0033] In one embodiment, a siNA molecule of the invention comprises blunt ends, i.e., ends that do not include any overhanging nucleotides. For example, a siNA molecule of the invention comprising modifications described herein
(e.g., comprising nucleotides having Formulae I-VII or siRNA constructs comprising Stab00-Stab22 or any combination thereof (see Table IV) and/or any length described herein can comprise blunt ends or ends with no overhanging nucleotides.

[0034] In one embodiment, any siRNA molecule of the invention can comprise one or more blunt ends, i.e. where a blunt end does not have any overhanging nucleotides. In a non-limiting example, a blunt ended siRNA molecule has a number of base pairs equal to the number of nucleotides present in each strand of the siRNA molecule. In another example, a siRNA molecule comprises one blunt end, for example wherein the 5'-end of the antisense strand and the 3'-end of the sense strand do not have any overhanging nucleotides. In another example, a siRNA molecule comprises one blunt end, for example wherein the 3'-end of the antisense strand and the 5'-end of the sense strand do not have any overhanging nucleotides. In another example, a siRNA molecule comprises two blunt ends, for example wherein the 3'-end of the antisense strand and the 5'-end of the sense strand as well as the 5'-end of the antisense strand and 3'-end of the sense strand do not have any overhanging nucleotides. A blunt ended siRNA molecule can comprise, for example, from about 18 to about 30 nucleotides (e.g., about 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, or 30 nucleotides). Other nucleotides present in a blunt ended siRNA molecule can comprise mismatches, bulges, loops, or wobble base pairs, for example, to modulate the activity of the siRNA molecule to mediate RNA interference.

[0035] By “blunt ends” is meant symmetric termini or termini of a double stranded siRNA molecule having no overhanging nucleotides. The two strands of a double stranded siRNA molecule align with each other without over-hanging nucleotides at the termini. For example, a blunt ended siRNA construct comprises terminal nucleotides that are complementary between the sense and antisense regions of the siRNA molecule.

[0036] In one embodiment, the invention features a double-stranded short interfering nucleic acid (siRNA) molecule that down-regulates expression of a CHRM3 gene, wherein the siRNA molecule is assembled from two separate oligonucleotide fragments wherein one fragment comprises the sense region and the second fragment comprises the antisense region of the siRNA molecule. The sense region can be connected to the antisense region via a linker molecule, such as a polynucleotide linker or a non-nucleotide linker.

[0037] In one embodiment, the invention features double-stranded short interfering nucleic acid (siRNA) molecule that down-regulates expression of a CHRM3 gene, wherein the siRNA molecule comprises about 19 to about 21 base pairs, and wherein each strand of the siRNA molecule comprises one or more chemical modifications. In another embodiment, one of the strands of the double-stranded siRNA molecule comprises a nucleotide sequence that is complementary to a nucleotide sequence of a CHRM3 gene or a portion thereof, and the second strand of the double-stranded siRNA molecule comprises a nucleotide sequence substantially similar to the nucleotide sequence or a portion thereof of the CHRM3 gene. In another embodiment, one of the strands of the double-stranded siRNA molecule comprises a nucleotide sequence that is complementary to a nucleotide sequence of a CHRM3 gene or a portion thereof, and the second strand of the double-stranded siRNA molecule comprises a nucleotide sequence substantially similar to the nucleotide sequence or a portion thereof of the CHRM3 gene. In another embodiment, each strand of the siRNA molecule comprises about 19 to about 23 nucleotides, and each strand comprises at least about 19 nucleotides that are complementary to the nucleotides of the other strand. The CHRM3 gene can comprise, for example, sequences referred to Table I.

[0038] In one embodiment, a siRNA molecule of the invention comprises no ribonucleotides. In another embodiment, a siRNA molecule of the invention comprises ribonucleotides.

[0039] In one embodiment, a siRNA molecule of the invention comprises an antisense region comprising a nucleotide sequence that is complementary to a nucleotide sequence of a CHRM3 gene or a portion thereof, and the siRNA further comprises a sense region comprising a nucleotide sequence substantially similar to the nucleotide sequence of the CHRM3 gene or a portion thereof. In another embodiment, the antisense region and the sense region each comprise about 19 to about 23 nucleotides and the antisense region comprises at least about 19 nucleotides that are complementary to nucleotides of the sense region. The CHRM3 gene can comprise, for example, sequences referred to Table I.

[0040] In one embodiment, a siRNA molecule of the invention comprises a sense region and an antisense region, wherein the antisense region comprises a nucleotide sequence that is complementary to a nucleotide sequence of RNA encoded by a CHRM3 gene, or a portion thereof, and the sense region comprises a nucleotide sequence that is complementary to the antisense region. In another embodiment, the siRNA molecule is assembled from two separate oligonucleotide fragments wherein one fragment comprises the sense region and the second fragment comprises the antisense region of the siRNA molecule. In another embodiment, the sense region is connected to the antisense region via a linker molecule. In another embodiment, the sense region is connected to the antisense region via a linker molecule, such as a nucleotide or non-nucleotide linker. The CHRM3 gene can comprise, for example, sequences referred to Table I.

[0041] In one embodiment, the invention features a double-stranded short interfering nucleic acid (siRNA) molecule that down-regulates expression of a CHRM3 gene comprising a sense region and an antisense region, wherein the antisense region comprises a nucleotide sequence that is complementary to a nucleotide sequence of RNA encoded by the CHRM3 gene or a portion thereof and the sense region comprises a nucleotide sequence that is complementary to the antisense region, and wherein the siRNA molecule has one or more modified pyrimidine and/or purine nucleotides. In one embodiment, the pyrimidine nucleotides in the sense region are 2'-O-methyl pyrimidine nucleotides or 2'-deoxy-2'-fluoro pyrimidine nucleotides and the purine nucleotides present in the sense region are 2'-deoxy purine nucleotides. In another embodiment, the pyrimidine nucleotides in the sense region are 2'-deoxy-2'-fluoro pyrimidine nucleotides and the purine nucleotides present in the sense region are 2'-O-methyl purine nucleotides. In another embodiment, the pyrimidine nucleotides in the sense region are 2'-deoxy-2'-fluoro pyrimidine nucleotides and the purine nucleotides present in the sense region are 2'-deoxy purine nucleotides. In one embodiment, the pyrimidine nucleotides
in the antisense region are 2'-deoxy-2'-fluoro pyrimidine nucleotides and the purine nucleotides present in the antisense region are 2'-O-methyl or 2'-deoxy purine nucleotides. In another embodiment of any of the above-described siNA molecules, any nucleotides present in a non-complementary region of the sense strand (e.g. overhang region) are 2'-deoxy nucleotides.

[0042] In one embodiment, the invention features a double-stranded short interfering nucleic acid (siNA) molecule that down-regulates expression of a CHRM3 gene, wherein the siNA molecule is assembled from two separate oligonucleotide fragments wherein one fragment comprises the sense region and the second fragment comprises the antisense region of the siNA molecule, and wherein the fragment comprising the sense region includes a terminal cap moiety at the 5'-end, the 3'-end, or both of the 5' and 3' ends of the fragment. In another embodiment, the terminal cap moiety is an inverted deoxy abasic moiety or glyceryl moiety. In another embodiment, each of the two fragments of the siNA molecule comprise about 21 nucleotides.

[0043] In one embodiment, the invention features a siNA molecule comprising at least one modified nucleotide, wherein the modified nucleotide is a 2'-deoxy-2'-fluoro nucleotide. The siNA can be, for example, of length between about 12 and about 36 nucleotides. In another embodiment, all pyrimidine nucleotides present in the siNA are 2'-deoxy-2'-fluoro pyrimidine nucleotides. In another embodiment, the modified nucleotides in the siNA include at least one 2'-deoxy-2'-fluoro cytidine or 2'-deoxy-2'-fluoro uridine nucleotide. In another embodiment, the modified nucleotides in the siNA include at least one 2'-fluoro cytidine and at least one 2'-deoxy-2'-fluoro uridine nucleotides. In another embodiment, all uridine nucleotides present in the siNA are 2'-deoxy-2'-fluoro uridine nucleotides. In another embodiment, all cytidine nucleotides present in the siNA are 2'-deoxy-2'-fluoro cytidine nucleotides. In another embodiment, all adenosine nucleotides present in the siNA are 2'-deoxy-2'-fluoro adenosine nucleotides. In another embodiment, all guanosine nucleotides present in the siNA are 2'-deoxy-2'-fluoro guanosine nucleotides. The siNA can further comprise at least one modified internucleotide linkage, such as phosphorothioate linkage. In another embodiment, the 2'-deoxy-2'-fluorouracil nucleotides are present at specifically selected locations in the siNA that are sensitive to cleavage by ribonucleases, such as locations having pyrimidine nucleotides.

[0044] In one embodiment, the invention features a method of increasing the stability of a siNA molecule against cleavage by ribonucleases comprising introducing at least one modified nucleotide into the siNA molecule, wherein the modified nucleotide is a 2'-deoxy-2'-fluoro nucleotide. In another embodiment, all pyrimidine nucleotides present in the siNA are 2'-deoxy-2'-fluoro pyrimidine nucleotides. In another embodiment, the modified nucleotides in the siNA include at least one 2'-deoxy-2'-fluoro cytidine or 2'-deoxy-2'-fluoro uridine nucleotide. In another embodiment, the modified nucleotides in the siNA include at least one 2'-fluoro cytidine and at least one 2'-deoxy-2'-fluoro uridine nucleotides. In another embodiment, all uridine nucleotides present in the siNA are 2'-deoxy-2'-fluoro uridine nucleotides. In another embodiment, all cytidine nucleotides present in the siNA are 2'-deoxy-2'-fluoro cytidine nucleotides. In another embodiment, all adenosine nucleotides present in the siNA are 2'-deoxy-2'-fluoro adenosine nucleotides. In another embodiment, all guanosine nucleotides present in the siNA are 2'-deoxy-2'-fluoro guanosine nucleotides. The siNA can further comprise at least one modified internucleotide linkage, such as phosphorothioate linkage. In another embodiment, the 2'-deoxy-2'-fluorouracil nucleotides are present at specifically selected locations in the siNA that are sensitive to cleavage by ribonucleases, such as locations having pyrimidine nucleotides.

[0045] In one embodiment, the invention features a double-stranded short interfering nucleic acid (siNA) molecule that down-regulates expression of a CHRM3 gene comprising a sense region and an antisense region, wherein the antisense region comprises a nucleotide sequence that is complementary to a nucleotide sequence of RNA encoded by the CHRM3 gene or a portion thereof and the sense region comprises a nucleotide sequence that is complementary to the antisense region, and wherein the purine nucleotides present in the antisense region comprise 2'-deoxy-purine nucleotides. In another embodiment, the purine nucleotides present in the antisense region comprise 2'-O-methyl purine nucleotides. In either of the above embodiments, the antisense region can comprise a glyceryl modification at the 3' end of the antisense region. In another embodiment of any of the above-described siNA molecules, any nucleotides present in a non-complementary region of the antisense strand (e.g. overhang region) are 2'-deoxy nucleotides.

[0046] In one embodiment, the antisense region of a siNA molecule of the invention comprises sequence complementary to a portion of a CHRM3 transcript having sequence unique to a particular CHRM3 disease related allele, such as sequence comprising a SNP associated with the disease specific allele. As such, the antisense region of a siNA molecule of the invention can comprise sequence complementary to sequences that are unique to a particular allele to provide specificity in mediating selective RNAi against the disease related allele.

[0047] In one embodiment, the invention features a double-stranded short interfering nucleic acid (siNA) molecule that down-regulates expression of a CHRM3 gene, wherein the siNA molecule is assembled from two separate oligonucleotide fragments wherein one fragment comprises the sense region and the second fragment comprises the antisense region of the siNA molecule. In another embodiment about 19 nucleotides of each fragment of the siNA molecule are base-paired to the complementary nucleotides of the other fragment of the siNA molecule and wherein at least two 3' terminal nucleotides of each fragment of the siNA molecule are not base-paired to the nucleotides of the other fragment of the siNA molecule. In one embodiment, each of the two 3' terminal nucleotides of each fragment of the siNA molecule is a 2'-deoxy-pyrimidine nucleotide, such as a 2'-deoxy-thymidine. In another embodiment, all 21 nucleotides of each fragment of the siNA molecule are base-paired to the complementary nucleotides of the other fragment of the siNA molecule. In another embodiment, about 19 nucleotides of the antisense region are base-paired to the nucleotide sequence or a portion thereof of the RNA encoded by the CHRM3 gene. In another embodiment,
about 21 nucleotides of the antisense region are base-paired to the nucleotide sequence or a portion thereof of the RNA encoded by the CHRM3 gene. In any of the above embodiments, the 3'-end of the fragment comprising said antisense region can optionally include a phosphatase group.

[0048] In one embodiment, the invention features a double-stranded short interfering nucleic acid (siRNA) molecule that inhibits the expression of a CHRM3 RNA sequence (e.g., wherein said target RNA sequence is encoded by a CHRM3 gene involved in the CHRM3 pathway), wherein the siRNA molecule does not contain any ribonucleotides and wherein each strand of the double-stranded siRNA molecule is about 21 nucleotides long. Examples of non-ribonucleotide containing siRNA constructs are combinations of stabilization chemistries shown in Table IV in any combination of Sense/Antisense chemistries, such as Stab 7/8, Stab 7/11, Stab 8/8, Stab 18/8, Stab 18/11, Stab 18/12/13, Stab 7/13, Stab 18/13, Stab 7/19, Stab 8/19, Stab 18/19, Stab 7/20, Stab 8/20, or Stab 18/20.

[0049] In one embodiment, the invention features a chemically synthesized double stranded RNA molecule that directs cleavage of a CHRM3 RNA via RNA interference, wherein each strand of said RNA molecule is about 21 to about 23 nucleotides in length; one strand of the RNA molecule comprises nucleotide sequence having sufficient complementarity to the CHRM3 RNA for the RNA molecule to direct cleavage of the CHRM3 RNA via RNA interference; and wherein at least one strand of the RNA molecule comprises one or more chemically modified nucleotides described herein, such as deoxynucleotides, 2'-O-methyl nucleotides, 2'-deoxy-2'-fluoro nucleotides, 2'-O-methoxyethyl nucleotides etc.

[0050] In one embodiment, the invention features a medicament comprising a siRNA molecule of the invention.

[0051] In one embodiment, the invention features an active ingredient comprising a siRNA molecule of the invention.

[0052] In one embodiment, the invention features the use of a double-stranded short interfering nucleic acid (siRNA) molecule to down-regulate expression of a CHRM3 gene, wherein the siRNA molecule comprises one or more chemical modifications and each strand of the double-stranded siRNA is about 18 to about 28 or more (e.g., 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28 or more) nucleotides long.

[0053] In one embodiment, the invention features the use of a double-stranded short interfering nucleic acid (siRNA) molecule that inhibits expression of a CHRM3 gene, wherein one of the strands of the double-stranded siRNA molecule is an antisense strand which comprises nucleotide sequence that is complementary to nucleotide sequence of CHRM3 RNA or a portion thereof, the other strand is a sense strand which comprises nucleotide sequence that is complementary to a nucleotide sequence of the antisense strand and wherein a majority of the pyrimidine nucleotides present in the double-stranded siRNA molecule comprises a sugar modification.

[0054] In one embodiment, the invention features a double-stranded short interfering nucleic acid (siRNA) molecule that inhibits expression of a CHRM3 gene, wherein one of the strands of the double-stranded siRNA molecule is an antisense strand which comprises nucleotide sequence that is complementary to nucleotide sequence of CHRM3 RNA or a portion thereof, wherein the other strand is a sense strand which comprises nucleotide sequence that is complementary to a nucleotide sequence of the antisense strand and wherein a majority of the pyrimidine nucleotides present in the double-stranded siRNA molecule comprises a sugar modification.

[0055] In one embodiment, the invention features a double-stranded short interfering nucleic acid (siRNA) molecule that inhibits expression of a CHRM3 gene, wherein one of the strands of the double-stranded siRNA molecule is an antisense strand which comprises nucleotide sequence that is complementary to nucleotide sequence of CHRM3 RNA that encodes a protein or portion thereof, the other strand is a sense strand which comprises nucleotide sequence that is complementary to a nucleotide sequence of the antisense strand and wherein a majority of the pyrimidine nucleotides present in the double-stranded siRNA molecule comprises a sugar modification. In one embodiment, the invention features a double-stranded short interfering nucleic acid (siRNA) molecule that inhibits expression of a CHRM3 gene, wherein one of the strands of the double-stranded siRNA molecule is an antisense strand which comprises nucleotide sequence that is complementary to nucleotide sequence of CHRM3 RNA or a portion thereof, wherein the other strand is a sense strand which comprises nucleotide sequence that is complementary to a nucleotide sequence of the antisense strand and wherein a majority of the pyrimidine nucleotides present in the double-stranded siRNA molecule comprises a sugar modification. In one embodiment, the invention features a double-stranded short interfering nucleic acid (siRNA) molecule that inhibits expression of a CHRM3 gene, wherein one of the strands of the double-stranded siRNA molecule is an antisense strand which comprises nucleotide sequence that is complementary to nucleotide sequence of CHRM3 RNA or a portion thereof, wherein the other strand is a sense strand which comprises nucleotide sequence that is complementary to a nucleotide sequence of the antisense strand and wherein a majority of the pyrimidine nucleotides present in the double-stranded siRNA molecule comprises a sugar modification.
present at the 5'-end, the 3'-end, or both of the 5' and 3' ends of the sense strand. In another embodiment, the antisense strand comprises a phosphorothioate internucleotide linkage at the 3' end of the antisense strand. In another embodiment, the antisense strand comprises a glyceryl modification at the 3' end. In another embodiment, the 5'-end of the antisense strand optionally includes a phosphate group.

In one embodiment, the invention features a double-stranded short interfering nucleic acid (siNA) molecule that inhibits expression of a CHRM3 gene, wherein one of the strands of the double-stranded siNA molecule is an antisense strand which comprises nucleotide sequence that is complementary to nucleotide sequence of CHRM3 RNA or a portion thereof, wherein the other strand is a sense strand which comprises nucleotide sequence that is complementary to a nucleotide sequence of the antisense strand and wherein a majority of the pyrimidine nucleotides present in the double-stranded siNA molecule comprises a sugar modification, and wherein each of the two strands of the siNA molecule comprises about 21 nucleotides. In one embodiment, about 21 nucleotides of each strand of the siNA molecule are base-paired to the complementary nucleotides of the other strand of the siNA molecule. In another embodiment, about 19 nucleotides of each strand of the siNA molecule are base-paired to the complementary nucleotides of the other strand of the siNA molecule, wherein at least two 3' terminal nucleotides of each strand of the siNA molecule are not base-paired to the nucleotides of the other strand of the siNA molecule. In another embodiment, each of the two 3' terminal nucleotides of each fragment of the siNA molecule is a 2'-deoxy-pyrimidine, such as 2'-deoxy-thymidine. In another embodiment, each strand of the siNA molecule is base-paired to the complementary nucleotides of the other strand of the siNA molecule. In another embodiment, about 19 nucleotides of the antisense strand are base-paired to the nucleotide sequence of the CHRM3 RNA or a portion thereof. In another embodiment, about 21 nucleotides of the antisense strand are base-paired to the nucleotide sequence of the CHRM3 RNA or a portion thereof.

In one embodiment, the invention features a double-stranded short interfering nucleic acid (siNA) molecule that inhibits expression of a CHRM3 gene, wherein one of the strands of the double-stranded siNA molecule is an antisense strand which comprises nucleotide sequence that is complementary to nucleotide sequence of CHRM3 RNA or a portion thereof, the other strand is a sense strand which comprises nucleotide sequence that is complementary to a nucleotide sequence of the antisense strand and wherein a majority of the pyrimidine nucleotides present in the double-stranded siNA molecule comprises a sugar modification, and wherein the 5'-end of the antisense strand optionally includes a phosphate group.

In one embodiment, the invention features a double-stranded short interfering nucleic acid (siNA) molecule that inhibits expression of a CHRM3 gene, wherein one of the strands of the double-stranded siNA molecule is an antisense strand which comprises nucleotide sequence that is complementary to nucleotide sequence of CHRM3 RNA or a portion thereof, the other strand is a sense strand which comprises nucleotide sequence that is complementary to a nucleotide sequence of the antisense strand and wherein a majority of the pyrimidine nucleotides present in the double-stranded siNA molecule comprises a sugar modification, and wherein the nucleotide sequence or a portion thereof of the antisense strand is complementary to a nucleotide sequence of the untranslated region or a portion thereof of the CHRM3 RNA.

In one embodiment, the invention features a double-stranded short interfering nucleic acid (siNA) molecule that inhibits expression of a CHRM3 gene, wherein one of the strands of the double-stranded siNA molecule is an antisense strand which comprises nucleotide sequence that is complementary to nucleotide sequence of CHRM3 RNA or a portion thereof, wherein the other strand is a sense strand which comprises nucleotide sequence that is complementary to a nucleotide sequence of the antisense strand, wherein a majority of the pyrimidine nucleotides present in the double-stranded siNA molecule comprises a sugar modification, and wherein the nucleotide sequence of the antisense strand is complementary to a nucleotide sequence of the CHRM3 RNA or a portion thereof that is present in the CHRM3 RNA.

In one embodiment, the invention features a composition comprising a siNA molecule of the invention in a pharmaceutically acceptable carrier or diluent.

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 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 in humans.

In any of the embodiments of siNA molecules described herein, the antisense region of a siNA molecule of the invention can comprise a phosphorothioate internucleotide linkage at the 3'-end of said antisense region. In any of the embodiments of siNA molecules described herein, the antisense region can comprise about one to about five phosphorothioate internucleotide linkages at the 5'-end of said antisense region. In any of the embodiments of siNA molecules described herein, the 3'-terminal nucleotide overhangs of a siNA molecule of the invention can comprise ribonucleotides or deoxyribonucleotides that are chemically-modified at a nucleic acid sugar, base, or backbone. In any of the embodiments of siNA molecules described herein, the 3'-terminal nucleotide overhangs can comprise one or more universal base ribonucleotides. In any of the embodiments of siNA molecules described herein, the 3'-terminal nucleotide overhangs can comprise one or more acyclic nucleotides.
One embodiment of the invention provides an expression vector comprising a nucleic acid sequence encoding at least one siRNA molecule of the invention in a manner that allows expression of the nucleic acid molecule. Another embodiment of the invention provides a mammalian cell comprising such an expression vector. The mammalian cell can be a human cell. The siRNA molecule of the expression vector can comprise a sense region and an antisense region. The antisense region can comprise sequence complementary to a RNA or DNA sequence encoding CHRM3 and the sense region can comprise sequence complementary to the antisense region. The siRNA molecule can comprise two distinct strands having complementary sense and antisense regions. The siRNA molecule can comprise a single strand having complementary sense and antisense regions.

In one embodiment, the invention features a chemically-modified short interfering nucleic acid (siRNA) molecule capable of mediating RNA interference (RNAi) against CHRM3 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, 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, 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).

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 siRNA duplex, for example, in the sense strand, the antisense strand, or both strands. The siRNA 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 1 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 siRNA molecule of the invention can comprise about 1 to about 5 or more (e.g., about 1, 2, 3, 4, 5, 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 siRNA 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 siRNA molecule of the invention having internucleotide linkage(s) of Formula I also comprises a chemically-modified nucleotide or non-nucleotide having any of Formulas I-VII.

In one embodiment, the invention features a chemically-modified short interfering nucleic acid (siRNA) molecule capable of mediating RNA interference (RNAi) against CHRM3 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:

[0068] 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-OSH, O-alkyl-SH, S-alkyl-OH, S-alkyl-SH, alkyl-S-alkyl, alkyl-O-alkyl, O-alkyl-OH, ONO2, NO2, N3, NIH2, aminoalkyl, aminoacid, aminoacyl, ONH2, O-aminoalkyl, O-aminoacid, O-aminoacyl, heterocycloalkyl, heterocycloalkenyl, aminoalkenylamino, polyalkylamino, substituted silyl, or group having Formula I or II; R9 is O, S, CH2, S==O, CHF, or CF2, and B is a nucleosidic base such as adene, 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-nitropyrrrole, 5-nitroindole, nebularine, pyridone, pyridinone, or any other non-naturally occurring universal base that can be complementary or non-complementary to target RNA.

The chemically-modified nucleotide or non-nucleotide of Formula II can be present in one or both oligonucleotide strands of the siRNA duplex, for example in the sense strand, the antisense strand, or both strands. The siRNA molecules of the invention can comprise one or more chemically-modified nucleotide or non-nucleotide 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 siRNA molecule of the invention can comprise about 1 to about 5 or more (e.g., about 1, 2, 3, 4, 5, or more) chemically-modified nucleotides or non-nucleotides of Formula II at the 5'-end of the sense strand, the antisense strand, or both strands. In another non-limiting example, an exemplary siRNA 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. 

[0070] In one embodiment, the invention features a chemically-modified short interfering nucleic acid (siNA) molecule capable of mediating RNA interference (RNAi) against CHRM3 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:

\[
\begin{align*}
\text{R} & \quad \text{R} \quad \text{R} \quad \text{R} \\
\text{R} & \quad \text{R} \quad \text{R} \quad \text{R}
\end{align*}
\]

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-OSH, O-alkyl-SH, S-alkyl-OH, S-alkyl-SH, alkyl-S-alkyl, alkyl-O-alkyl, ON02, NO2, N3, NH2, aminoalkyl, aminoacid, aminoacetyl, ONH2, O-aminoalkyl, O-aminoacid, O-aminoacetyl, heterocycloalkyl, heterocycloalkylamin, aminoislylamino, polyalkylamino, substituted silyl, or group having Formula I or II; R9 is O, S, CH2, S=S=O, CHF, or CF2, and B is a nucleoside 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-nucleoside base such as phenyl, naphthyl, 3-nitropyrole, 5-nitroindole, nebularine, pyridone, pyridinone, or any other non-naturally occurring universal base that can be complementary or non-complementary to target RNA.

[0071] 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 nucleotide or non-nucleotide 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, for 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 3'-end of the sense strand, the antisense strand, or both strands. In another non-limiting example, an exemplary siNA molecule of the invention can comprise about 1 to about 5 or more (e.g., about 1, 2, 3, 4, 5, or more) chemically-modified nucleotide or non-nucleotide of Formula III at the 3'-end of the sense strand, the antisense strand, or both strands.

[0072] 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.

[0073] In another embodiment, the invention features a chemically-modified short interfering nucleic acid (siNA) molecule capable of mediating RNA interference (RNAi) against CHRM3 inside a cell or reconstituted in vitro system, wherein the chemical modification comprises a 5'-terminal phosphate group having Formula IV:

\[
\begin{align*}
\text{X} & \quad \text{P} \quad \text{Y} \\
\text{Z} & \quad \text{W}
\end{align*}
\]

wherein each X and Y is independently O, S, N, alkyl, substituted alkyl, alkaryl; wherein each Z and W is independently O, S, N, alkyl, substituted alkyl, O-alkyl, S-alkyl, alkaryl, alkaryl, alkaryl, or acetyl; and wherein W, X, Y and Z are not all O.

[0074] In one embodiment, the invention features a chemically-modified short interfering nucleic acid (siNA) molecule capable of mediating RNA interference (RNAi) against CHRM3 inside a cell or reconstituted in vitro system, wherein the chemical modification comprises a 5'-terminal phosphate group having Formula IV:

\[
\begin{align*}
\text{X} & \quad \text{P} \quad \text{Y} \\
\text{Z} & \quad \text{W}
\end{align*}
\]

wherein each X and Y is independently O, S, N, alkyl, substituted alkyl, alkaryl; wherein each Z and W is independently O, S, N, alkyl, substituted alkyl, O-alkyl, S-alkyl, alkaryl, alkaryl, alkaryl, or acetyl; and wherein W, X, Y and Z are not all O.

[0075] In one embodiment, the invention features a siNA molecule having a 5'-terminal phosphate group having Formula IV on the target-complementary strand, for example, a strand complementary to a target RNA, wherein the siNA molecule comprises an all RNA siNA molecule. In another embodiment, the invention features a siNA molecule having a 5'-terminal phosphate group having Formula IV on the target-complementary strand wherein the siNA molecule also comprises about 1 to about 3 (e.g., about 1, 2, or 3) nucleotide 3'-terminal nucleotide overhangs having about 1 to about 4 (e.g., about 1, 2, 3, or 4) deoxynucleonucleotides on the 3'-end of one or both strands. In another embodiment, a 5'-terminal phosphate group having Formula IV is present on the target-complementary strand of a siNA molecule of the invention, for example a siNA molecule having chemical modifications having any of Formulas I-VII.

[0076] In one embodiment, the invention features a chemically-modified short interfering nucleic acid (siNA) molecule capable of mediating RNA interference (RNAi) against CHRM3 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.

In one embodiment, the invention features a SiNA molecule wherein the sense strand comprises one or more, for example, about 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, or more phosphorothioate internucleotide linkages, and/or one or more (e.g., about 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, or more) 2'-deoxy, 2'-O-methyl, 2'-deoxy-2'-fluoro, and/or one or more (e.g., about 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, or more) universal base modified nucleotides, and optionally a terminal cap molecule at the 3'-end, the 5'-end, or both of the 3' and 5'-ends of the sense strand and wherein the antisense strand comprises about 1 to about 10 or more, specifically about 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, or more phosphorothioate internucleotide linkages, and/or one or more (e.g., about 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, or more) 2'-deoxy, 2'-O-methyl, 2'-deoxy-2'-fluoro, and/or one or more (e.g., about 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, or more) universal base modified nucleotides, and optionally a terminal cap molecule at the 3'-end, the 5'-end, or both of the 3' and 5'-ends of the antisense strand. In another embodiment, one or more, for example about 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, or more, pyrimidine nucleotides of the sense and/or antisense SiNA strand are chemically-modified with 2'-deoxy, 2'-O-methyl and/or 2'-deoxy-2'-fluoro nucleotides, with or without one or more, for example about 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, or more phosphorothioate internucleotide linkages and/or a terminal cap molecule at the 3'-end, the 5'-end, or both of the 3' and 5'-ends.
(0084) In another embodiment, a chemically-modified siNA molecule of the invention comprises a duplex having two strands, one or both of which can be chemically-modified, wherein each strand is about 18 to about 27 (e.g., about 18, 19, 20, 21, 22, 23, 24, 25, 26, or 27) nucleotides in length, wherein the duplex has about 18 to about 23 (e.g., about 18, 19, 20, 21, 22, or 23) base pairs, and wherein the chemical modification comprises a structure having any of Formulae I-VII. For example, an exemplary chemically-modified siNA molecule of the invention comprises a duplex having two strands, one or both of which can be chemically-modified with a chemical modification having any of Formulae I-VII or any combination thereof, wherein each strand consists of about 21 nucleotides, each having a 2-nucleotide 3′-terminal nucleotide overhang, and wherein the duplex has about 19 base pairs. In another embodiment, a siNA molecule of the invention comprises a single stranded hairpin structure, wherein the siNA is about 36 to about 70 (e.g., about 36, 40, 45, 50, 55, 60, 65, or 70) nucleotides in length having about 18 to about 23 (e.g., about 18, 19, 20, 21, 22, or 23) base pairs, and wherein the siNA can include a chemical modification comprising a structure having any of Formulae I-VII or any combination thereof. For example, an exemplary chemically-modified siNA molecule of the invention comprises a linear oligonucleotide having about 42 to about 50 (e.g., about 42, 43, 44, 45, 46, 47, 48, 49, or 50) nucleotides that is chemically-modified with a chemical modification having any of Formulae I-VII or any combination thereof, wherein the linear oligonucleotide forms a hairpin structure having about 19 base pairs and a 2-nucleotide 3′-terminal nucleotide overhang. In another embodiment, a linear hairpin siNA molecule of the invention contains a stem loop motif, wherein the loop portion of the siNA molecule is biodegradable. For example, an exemplary chemically-modified siNA molecule of the invention comprises a linear oligonucleotide having about 25 to about 35 (e.g., about 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36, 37, 38, 39, 40, 41, 42, 43, 44, 45, 46, 47, 48, 49, or 50) nucleotides in length having about 3 to about 23 (e.g., about 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 20, 21, or 22) base pairs, and wherein the siNA can include one or more chemical modifications comprising a structure having any of Formulae I-VII or any combination thereof. For example, an exemplary chemically-modified siNA molecule of the invention comprises a linear oligonucleotide having about 25 to about 35 (e.g., about 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, or 35) nucleotides that is chemically-modified with one or more chemical modifications having any of Formulae I-VII or any combination thereof, wherein the linear oligonucleotide forms a hairpin structure having about 3 to about 23 (e.g., about 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, or 23) base pairs and a 5′-terminal phosphate group that can be chemically modified as described herein (for example a 5′-terminal phosphate group having Formula IV). In another embodiment, a linear hairpin siNA molecule of the invention comprises a loop portion comprising a non-nucleotide linker.
can be chemically modified as described herein (for example a 5'-terminal phosphate group having Formula IV).

In another embodiment, a siRNA molecule of the invention comprises a circular nucleic acid molecule, wherein the siRNA is about 38 to about 70 (e.g., about 38, 40, 45, 50, 55, 60, 65, or 70) nucleotides in length having about 18 to about 23 (e.g., about 18, 19, 20, 21, 22, or 23) base pairs, and wherein the siRNA can include a chemical modification, which comprises a structure having any of Formulas I-VII or any combination thereof. For example, an exemplary chemically-modified siRNA molecule of the invention comprises a circular oligonucleotide having about 42 to about 50 (e.g., about 42, 43, 44, 45, 46, 47, 48, 49, or 50) nucleotides that is chemically-modified with a chemical modification having any of Formulas I-VII or any combination thereof, wherein the circular oligonucleotide forms a dumbbell shaped structure having about 19 base pairs and 2 loops.

In another embodiment, a circular siRNA molecule of the invention contains two loop motifs, wherein one or both loop portions of the siRNA molecule is biodegradable. For example, a circular siRNA molecule of the invention is designed such that degradation of the loop portions of the siRNA molecule in vivo can generate a double-stranded siRNA molecule with 3'-terminal overhangs, such as 3'-terminal nucleotide overhangs comprising about 2 nucleotides.

In one embodiment, a siRNA 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, OF3, OCN, O-alkyl, S-alkyl, N-alkyl, O-alkenyl, S-alkenyl, N-alkenyl, SO-alkyl, alkyl-OSH, alkoxy-alkyl, O-alkyl-OSH, O-alkenyl-OSH, S-alkenyl-OSH, alkyl-S-alkyl, alkyl-O-alkyl, ONO2, NO2, N3, NH2, aminoalkyl, aminoacid, aminooxacyl, ONH2, O-aminoalkyl, O-aminoisocyan, O-aminoacyl, heterocycloalkyl, heterocycloalkaryl, aminooxalamin, polyalkylamin, substituted silyl, or a group having Formula I or II; R9 is O, S, CH2, S==O, CHF, or CF2.

In one embodiment, a siRNA 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, OF3, OCN, O-alkyl, S-alkyl, N-alkyl, O-alkenyl, S-alkenyl, N-alkenyl, SO-alkyl, alkyl-OSH, alkoxy-alkyl, O-alkyl-OSH, O-alkenyl-OSH, S-alkenyl-OSH, alkyl-S-alkyl, alkyl-O-alkyl, ONO2, NO2, N3, NH2, aminoalkyl, aminoacid, aminooxacyl, ONH2, O-aminoalkyl, O-aminoisocyan, O-aminoacyl, heterocycloalkyl, heterocycloalkaryl, aminooxalamin, polyalkylamin, substituted silyl, or a group having Formula I, and R1, R2 or R3 serves as points of attachment to the siRNA molecule of the invention.

In another embodiment, the invention features a compound having Formula VII, wherein each R4 is 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 siRNA molecule of the invention or to a single-stranded siRNA molecule of the invention. This modification is referred to herein as “glyceryl” (for example modification 6 in FIG. 10).

In another embodiment, 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 siRNA molecule of the invention. For example, 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 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.

[0098] 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.

[0099] 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.

[0100] 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.

[0101] In one embodiment, the invention features a chemically-modified short interfering nucleic acid (siNA) molecule of the invention comprising a sense region, wherein any (e.g., one or more or all) pyrimidine nucleotides present in the sense region are 2'-deoxy-2'-fluoro pyrimidine nucleotides (e.g., wherein all pyrimidine nucleotides are 2'-deoxy-2'-fluoro pyrimidine nucleotides or alternately a plurality of pyrimidine nucleotides are 2'-deoxy-2'-fluoro pyrimidine nucleotides), and wherein any (e.g., one or more or all) purine nucleotides present in the sense region are 2'-deoxy purine nucleotides (e.g., wherein all purine nucleotides are 2'-deoxy purine nucleotides or alternately a plurality of purine nucleotides are 2'-deoxy purine nucleotides).

[0102] In one embodiment, the invention features a chemically-modified short interfering nucleic acid (siNA) molecule of the invention comprising a sense region, wherein any (e.g., one or more or all) pyrimidine nucleotides present in the sense region are 2'-deoxy-2'-fluoro pyrimidine nucleotides (e.g., wherein all pyrimidine nucleotides are 2'-deoxy-2'-fluoro pyrimidine nucleotides or alternately a plurality of pyrimidine nucleotides are 2'-deoxy-2'-fluoro pyrimidine nucleotides), and wherein any (e.g., one or more or all) purine nucleotides present in the sense region are 2'-deoxy purine nucleotides (e.g., wherein all purine nucleotides are 2'-deoxy purine nucleotides or alternately a plurality of purine nucleotides are 2'-deoxy purine nucleotides), wherein any nucleotides comprising a 3'-terminal nucleotide overhang that are present in said sense region are 2'-deoxy nucleotides.

[0103] In one embodiment, the invention features a chemically-modified short interfering nucleic acid (siNA) molecule of the invention comprising a sense region, wherein any (e.g., one or more or all) pyrimidine nucleotides present in the sense region are 2'-deoxy-2'-fluoro pyrimidine nucleotides (e.g., wherein all pyrimidine nucleotides are 2'-deoxy-2'-fluoro pyrimidine nucleotides or alternately a plurality of pyrimidine nucleotides are 2'-deoxy-2'-fluoro pyrimidine nucleotides), and wherein any (e.g., one or more or all) purine nucleotides present in the sense region are 2'-O-methyl purine nucleotides (e.g., wherein all purine nucleotides are 2'-O-methyl purine nucleotides or alternately a plurality of purine nucleotides are 2'-O-methyl purine nucleotides).

[0104] In one embodiment, the invention features a chemically-modified short interfering nucleic acid (siNA) molecule of the invention comprising a sense region, wherein any (e.g., one or more or all) pyrimidine nucleotides present in the sense region are 2'-deoxy-2'-fluoro pyrimidine nucleotides (e.g., wherein all pyrimidine nucleotides are 2'-deoxy-2'-fluoro pyrimidine nucleotides or alternately a plurality of pyrimidine nucleotides are 2'-deoxy-2'-fluoro pyrimidine nucleotides), and wherein any (e.g., one or more or all) purine nucleotides present in the sense region are 2'-O-methyl purine nucleotides (e.g., wherein all purine nucleotides are 2'-O-methyl purine nucleotides or alternately a plurality of purine nucleotides are 2'-O-methyl purine nucleotides), and wherein any nucleotides comprising a 3'-terminal nucleotide overhang that are present in said sense region are 2'-deoxy nucleotides.

[0105] In one embodiment, the invention features a chemically-modified short interfering nucleic acid (siNA) molecule of the invention comprising an antisense region, wherein any (e.g., one or more or all) pyrimidine nucleotides present in the antisense region are 2'-deoxy-2'-fluoro pyrimidine nucleotides (e.g., wherein all pyrimidine nucleotides are 2'-deoxy-2'-fluoro pyrimidine nucleotides or alternately a plurality of pyrimidine nucleotides are 2'-deoxy-2'-fluoro pyrimidine nucleotides), and wherein any (e.g., one or more or all) purine nucleotides present in the antisense region are 2'-O-methyl purine nucleotides (e.g., wherein all purine nucleotides are 2'-O-methyl purine nucleotides or alternately a plurality of purine nucleotides are 2'-O-methyl purine nucleotides).

[0106] In one embodiment, the invention features a chemically-modified short interfering nucleic acid (siNA) molecule of the invention comprising an antisense region, wherein any (e.g., one or more or all) pyrimidine nucleotides present in the antisense region are 2'-deoxy-2'-fluoro pyrimidine nucleotides (e.g., wherein all pyrimidine nucleotides are 2'-deoxy-2'-fluoro pyrimidine nucleotides or alternately a plurality of pyrimidine nucleotides are 2'-deoxy-2'-fluoro pyrimidine nucleotides), and wherein any (e.g., one or more or all) purine nucleotides present in the antisense region are 2'-O-methyl purine nucleotides (e.g., wherein all purine nucleotides are 2'-O-methyl purine nucleotides or alternately a plurality of purine nucleotides are 2'-O-methyl purine nucleotides), and wherein any nucleotides comprising a 3'-terminal nucleotide overhang that are present in said antisense region are 2'-deoxy nucleotides.

[0107] In one embodiment, the invention features a chemically-modified short interfering nucleic acid (siNA) molecule of the invention comprising an antisense region, wherein any (e.g., one or more or all) pyrimidine nucleotides present in the antisense region are 2'-deoxy-2'-fluoro pyrimidine nucleotides (e.g., wherein all pyrimidine nucleotides are 2'-deoxy-2'-fluoro pyrimidine nucleotides or alternately a plurality of pyrimidine nucleotides are 2'-deoxy-2'-fluoro pyrimidine nucleotides), and wherein any (e.g., one or more or all) purine nucleotides present in the antisense region are 2'-deoxy purine nucleotides (e.g., wherein all purine nucleotides are 2'-deoxy purine nucleotides or alternately a plurality of purine nucleotides are 2'-deoxy purine nucleotides).
In one embodiment, the invention features a chemically-modified short interfering nucleic acid (siNA) molecule of the invention comprising an antisense region, wherein any (e.g., one or more) or all pyrimidine nucleotides present in the antisense region are 2'-deoxy-2'-fluoro pyrimidine nucleotides (e.g., wherein all pyrimidine nucleotides are 2'-deoxy-2'-fluoro pyrimidine nucleotides or alternately a plurality of pyrimidine nucleotides are 2'-deoxy-2'-fluoro pyrimidine nucleotides), and wherein any (e.g., one or more) or all purine nucleotides present in the antisense region are 2'-O-methyl purine nucleotides (e.g., wherein all purine nucleotides are 2'-O-methyl purine nucleotides or alternately a plurality of purine nucleotides are 2'-O-methyl purine nucleotides).

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

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

In one embodiment, the sense strand of a double stranded siNA molecule of the invention comprises a terminal cap moiety, (see for example FIG. 10) such as an inverted deoxyabasic moiety, at the 3'-end, 5'-end, or both 3' and 5'-ends of the sense strand.

In one embodiment, the invention features a chemically-modified short interfering nucleic acid molecule (siNA) capable of mediating RNA interference (RNAi) against CHRM3 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 Vargese et al., U.S. Ser. No. 10/427,160, filed Apr. 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 polyethylene glycol, human serum albumin, or a ligand for a cellular receptor that can mediate cellular uptake. Examples of specific conjugate molecules contemplated by the instant invention that can be attached to chemically-modified SiNA molecules are described in Vargese et al., U.S. Ser. No. 10/201,394, filed Jul. 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.

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

In yet another embodiment, a non-nucleotide linker of the invention comprises anabase, nucleoside, polyether, polyamine, pyridazine, 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 Scuda and Kaiser, Nucleic Acids Res. 1990, 18:6333 and Nucleic Acids Res. 1987, 15:3113; Cloud and Schepartz, J. Am. Chem. Soc. 1991, 113:4024; Richardson and Schepartz, J. Am. Chem. Soc. 1991, 113:2013; Ma et al., Nucleic Acids Res. 1993, 21:2585 and Biochemistry 1993, 32:1751; Durand et al., Nucleic Acids Res. 1990, 18:6333; McCurdy et al., Nucleosides & Nucleotides 1991, 10:267; Jersch 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 95/0731; Dudycz et al., International Publication No. WO 95/11910 and Ferentz and Verdin, 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.

In one embodiment, the invention features a short interfering nucleic acid (siNA) molecule capable of mediating RNA interference (RNAi) inside a cell or reconstituted in vitro system, wherein one or both strands of the siNA molecule that are assembled from two separate oligonucleotides do not comprise any ribonucleotides. For example, a siNA molecule can be assembled from a single oligonucleotide where the sense and antisense regions of the siNA comprise separate oligonucleotides not having any ribonucleotides (e.g., nucleotides having a 2'-OH group) present in the oligonucleotides. In another example, a siNA molecule can be assembled from a single oligonucleotide where the sense and antisense regions of the siNA are linked or circularized by a nucleotide or non-nucleotide linker as described herein, wherein the oligonucleotide does not have any ribonucleotides (e.g., nucleotides having a 2'-OH group) present in the oligonucleotide. Applicant has surprisingly found that the presence 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.
cally-modified nucleotides such as nucleotides having any of Formulas I-VII, or any combination thereof to the extent that the ability of the siNA molecule to support RNAi activity in a cell is maintained.

[0117] In one embodiment, a siNA molecule of the invention is a single stranded siNA molecule that mediates RNAi activity in a cell or reconstituted in vitro system comprising a single stranded polynucleotide having complementarity to a target nucleic acid sequence, wherein one or more pyrimidine nucleotides present in the siNA are 2-deoxy-2-fluoro pyrimidine nucleotides (e.g., wherein all pyrimidine nucleotides are 2-deoxy-2-fluoro pyrimidine nucleotides or alternately a plurality of pyrimidine nucleotides are 2-deoxy-2-fluoro pyrimidine nucleotides), and wherein any purine nucleotides present in the antisense region are 2'-O-methyl purine nucleotides (e.g., wherein all purine nucleotides are 2'-O-methyl purine nucleotides or alternately a plurality of purine nucleotides are 2'-O-methyl purine nucleotides), and a terminal cap modification, such as any modification described herein or shown in FIG. 10, that is optionally present at the 3'-end, the 5'-end, or both of the 3' and 5'-ends of the antisense sequence. The siNA optionally further comprises about 1 to about 4 or more (e.g., about 1, 2, 3, 4 or more) terminal 2'-deoxyribose nucleotides at the 3'-end of the siNA molecule, wherein the terminal nucleotides can further comprise one or more (e.g., 1, 2, 3, 4 or more) phosphorothioate, phosphonoacetate, and/or thiophosphonoacetate internucleotide linkages, and wherein the siNA optionally further comprises a terminal phosphate group, such as a 5'-terminal phosphate group. In any of these embodiments, any purine nucleotides present in the antisense region are alternatively 2'-deoxy purine nucleotides (e.g., wherein all purine nucleotides are 2'-deoxy purine nucleotides or alternately a plurality of purine nucleotides are 2'-deoxy purine nucleotides). Also, in any of these embodiments, any purine nucleotides present in the siNA (i.e., purine nucleotides present in the sense and/or antisense region) can alternatively be locked nucleic acid (LNA) nucleotides (e.g., wherein all purine nucleotides are LNA nucleotides or alternately a plurality of purine nucleotides are LNA nucleotides). Also, in any of these embodiments, any purine nucleotides present in the siNA are alternatively 2'-methoxymethyl purine nucleotides (e.g., wherein all purine nucleotides are 2'-methoxymethyl purine nucleotides or alternately a plurality of purine nucleotides are 2'-methoxymethyl purine nucleotides). In another embodiment, any modified nucleotides present in the single stranded siNA molecule of the invention comprise modified nucleotides having properties or characteristics similar to naturally occurring ribonucleotides. For example, the invention features siNA molecules including modified nucleotides having a Northern conformation (e.g., Northern pseudorotation cycle, see for example Saenger, Principles of Nucleic Acid Structure, Springer-Verlag ed., 1984). As such, chemically modified nucleotides present in the single stranded siNA molecules of the invention are preferably resistant to nuclease degradation while at the same time maintaining the capacity to mediate RNAi.

[0118] In one embodiment, the invention features a method for modulating the expression of a CHRM3 gene within a cell comprising: (a) synthesizing a siNA molecule of the invention, which can be chemically-modified, wherein one of the siNA strands comprises a sequence complementary to RNA of the CHRM3 gene; and (b) introducing the siNA molecule into a cell under conditions suitable to modulate the expression of the CHRM3 gene in the cell.

[0119] In one embodiment, the invention features a method for modulating the expression of a CHRM3 gene within a cell comprising: (a) synthesizing a siNA molecule of the invention, which can be chemically-modified, wherein one of the siNA strands comprises a sequence complementary to RNA of the CHRM3 gene and wherein the sense strand sequence of the siNA comprises a sequence identical or substantially similar to the sequence of the target RNA; and (b) introducing the siNA molecule into a cell under conditions suitable to modulate the expression of the CHRM3 gene in the cell.

[0120] In another embodiment, the invention features a method for modulating the expression of more than one CHRM3 gene within a cell comprising: (a) synthesizing siNA molecules of the invention, which can be chemically-modified, wherein one of the siNA strands comprises a sequence complementary to RNA of the CHRM3 genes; and (b) introducing the siNA molecules into a cell under conditions suitable to modulate the expression of the CHRM3 genes in the cell.

[0121] In another embodiment, the invention features a method for modulating the expression of two or more CHRM3 genes within a cell comprising: (a) synthesizing one or more siNA molecules of the invention, which can be chemically-modified, wherein the siNA strands comprise sequences complementary to RNA of the CHRM3 genes and wherein the sense strand sequences of the siNAS comprise sequences identical or substantially similar to the sequences of the target RNAAs; and (b) introducing the siNA molecules into a cell under conditions suitable to modulate the expression of the CHRM3 genes in the cell.

[0122] In another embodiment, the invention features a method for modulating the expression of more than one CHRM3 gene within a cell comprising: (a) synthesizing a siNA molecule of the invention, which can be chemically-modified, wherein one of the siNA strands comprises a sequence complementary to RNA of the CHRM3 gene and wherein the sense strand sequence of the siNA comprises a sequence identical or substantially similar to the sequences of the target RNAAs; and (b) introducing the siNA molecule into a cell under conditions suitable to modulate the expression of the CHRM3 genes in the cell.

[0123] In one embodiment, siNA molecules of the invention are used as reagents in ex vivo applications. For example, siNAreagents are introduced into tissue or cells that are transplanted into a subject for therapeutic effect. The cells and/or tissue can be derived from an organism or subject that later receives the explant, or can be derived from another organism or subject prior to transplantation. The siNA molecules can be used to modulate the expression of one or more genes in the cells or tissue, such that the cells or tissue obtain a desired phenotype or are able to perform a function when transplanted in vivo. In one embodiment, certain target cells from a patient are extracted. These extracted cells are contacted with siNAS targeting a specific nucleotide sequence within the cells under conditions suitable for uptake of the siNAS by these cells (e.g. using delivery reagents such as cationic lipids, liposomes and the like or using techniques such as electroporation to facilitate the delivery of siNAS into cells). The cells are then reintro-
duced back into the same patient or other patients. In one embodiment, the invention features a method of modulating the expression of a CHRM3 gene in a tissue explant comprising: (a) synthesizing a siNA molecule of the invention, which can be chemically-modified, wherein one of the siNA strands comprises a sequence complementary to RNA of the CHRM3 gene; and (b) introducing the siNA molecule into a cell of the tissue explant derived from a particular organism under conditions suitable to modulate the expression of the CHRM3 gene in the tissue explant. In another embodiment, the method further comprises introducing the tissue explant back into the organism the tissue was derived from or into another organism under conditions suitable to modulate the expression of the CHRM3 gene in that organism.

[0124] In one embodiment, the invention features a method of modulating the expression of a CHRM3 gene in a tissue explant comprising: (a) synthesizing a siNA molecule of the invention, which can be chemically-modified, wherein one of the siNA strands comprises a sequence complementary to RNA of the CHRM3 gene and wherein the sense strand sequence of the siNA comprises a sequence identical or substantially similar to the sequence of the target RNA; and (b) introducing the siNA molecule into a cell of the tissue explant derived from a particular organism under conditions suitable to modulate the expression of the CHRM3 gene in the tissue explant. In another embodiment, the method further comprises introducing the tissue explant back into the organism the tissue was derived from or into another organism under conditions suitable to modulate the expression of the CHRM3 gene in that organism.

[0125] In another embodiment, the invention features a method of modulating the expression of more than one CHRM3 gene in a tissue explant comprising: (a) synthesizing siNA molecules of the invention, which can be chemically-modified, wherein one of the siNA strands comprises a sequence complementary to RNA of the CHRM3 genes; and (b) introducing the siNA molecules into a cell of the tissue explant derived from a particular organism under conditions suitable to modulate the expression of the CHRM3 genes in the tissue explant. In another embodiment, the method further comprises introducing the tissue explant back into the organism the tissue was derived from or into another organism under conditions suitable to modulate the expression of the CHRM3 genes in that organism.

[0126] In one embodiment, the invention features a method of modulating the expression of a CHRM3 gene in an organism comprising: (a) synthesizing a siNA molecule of the invention, which can be chemically-modified, wherein one of the siNA strands comprises a sequence complementary to RNA of the CHRM3 gene; and (b) introducing the siNA molecule into the organism under conditions suitable to modulate the expression of the CHRM3 gene in the organism. The level of CHRM3 protein or RNA can be determined as is known in the art.

[0127] In another embodiment, the invention features a method of modulating the expression of more than one CHRM3 gene in an organism comprising: (a) synthesizing siNA molecules of the invention, which can be chemically-modified, wherein one of the siNA strands comprises a sequence complementary to RNA of the CHRM3 genes; and (b) introducing the siNA molecules into the organism under conditions suitable to modulate the expression of the CHRM3 genes in the organism. The level of CHRM3 protein or RNA can be determined as is known in the art.

[0128] In one embodiment, the invention features a method for modulating the expression of a CHRM3 gene within a cell comprising: (a) synthesizing a siNA molecule of the invention, which can be chemically-modified, wherein the siNA comprises a single stranded sequence having complementarity to RNA of the CHRM3 gene; and (b) introducing the siNA molecule into a cell under conditions suitable to modulate the expression of the CHRM3 gene in the cell.

[0129] In another embodiment, the invention features a method for modulating the expression of more than one CHRM3 gene within a cell comprising: (a) synthesizing siNA molecules of the invention, which can be chemically-modified, wherein the siNA comprises a single stranded sequence having complementarity to RNA of the CHRM3 gene; and (b) contacting the cell in vitro or in vivo with the siNA molecule under conditions suitable to modulate the expression of the CHRM3 genes in the cell.

[0130] In one embodiment, the invention features a method of modulating the expression of a CHRM3 gene in a tissue explant comprising: (a) synthesizing a siNA molecule of the invention, which can be chemically-modified, wherein the siNA comprises a single stranded sequence having complementarity to RNA of the CHRM3 gene; and (b) contacting the cell of the tissue explant derived from a particular organism with the siNA molecule under conditions suitable to modulate the expression of the CHRM3 gene in the tissue explant. In another embodiment, the method further comprises introducing the tissue explant back into the organism the tissue was derived from or into another organism under conditions suitable to modulate the expression of the CHRM3 gene in that organism.

[0131] In another embodiment, the invention features a method of modulating the expression of more than one CHRM3 gene in a tissue explant comprising: (a) synthesizing siNA molecules of the invention, which can be chemically-modified, wherein the siNA comprises a single stranded sequence having complementarity to RNA of the CHRM3 gene; and (b) introducing the siNA molecules into a cell of the tissue explant derived from a particular organism under conditions suitable to modulate the expression of the CHRM3 genes in the tissue explant. In another embodiment, the method further comprises introducing the tissue explant back into the organism the tissue was derived from or into another organism under conditions suitable to modulate the expression of the CHRM3 genes in that organism.

[0132] In one embodiment, the invention features a method of modulating the expression of a CHRM3 gene in an organism comprising: (a) synthesizing a siNA molecule of the invention, which can be chemically-modified, wherein the siNA comprises a single stranded sequence having complementarity to RNA of the CHRM3 gene; and (b) introducing the siNA molecule into the organism under conditions suitable to modulate the expression of the CHRM3 gene in the organism.

[0133] In another embodiment, the invention features a method of modulating the expression of more than one CHRM3 gene in an organism comprising: (a) synthesizing siNA molecules of the invention, which can be chemically-
modified, wherein the siNA comprises a single stranded sequence having complementarity to RNA of the CHRM3 gene; and (b) introducing the siNA molecules into the organism under conditions suitable to modulate the expression of the CHRM3 genes in the organism.

[0134] In one embodiment, the invention features a method of modulating the expression of a CHRM3 gene in an organism comprising contacting the organism with a siNA molecule of the invention under conditions suitable to modulate the expression of the CHRM3 gene in the organism.

[0135] In another embodiment, the invention features a method of modulating the expression of more than one CHRM3 gene in an organism comprising contacting the organism with one or more siNA molecules of the invention under conditions suitable to modulate the expression of the CHRM3 genes in the organism.

[0136] The siNA molecules of the invention can be designed to down regulate or inhibit target (e.g., CHRM3) gene expression through RNAi targeting of a variety of RNA molecules. In one embodiment, the siNA molecules of the invention are used to target various RNAs corresponding to a target gene. Non-limiting examples of such RNAs include messenger RNA (mRNA), alternative RNA splice variants of target gene(s), post-transcriptionally modified RNA of target gene(s), pre-mRNA of target gene(s), and/or RNA templates. If alternative splicing produces a family of transcripts that are distinguished by usage of appropriate exons, the instant invention can be used to inhibit gene expression through the appropriate exons to specifically inhibit or to distinguish among the functions of gene family members. For example, a protein that contains an alternatively spliced transmembrane domain can be expressed in both membrane bound and secreted forms. Use of the invention to target the exon containing the transmembrane domain can be used to determine the functional consequences of pharmaceutical targeting of the membrane bound as opposed to the secreted form of the protein. Non-limiting examples of applications of the invention relating to targeting these RNA molecules include therapeutic pharmaceutical applications, pharmaceutical discovery applications, molecular diagnostic and gene function applications, and gene mapping, for example using single nucleotide polymorphism mapping with siNA molecules of the invention. Such applications can be implemented using known gene sequences or from partial sequences available from an expressed sequence tag (EST).

[0137] In another embodiment, the siNA molecules of the invention are used to target conserved sequences corresponding to a gene family or gene families such as CHRM3 family genes. As such, siNA molecules targeting multiple CHRM3 targets can provide increased therapeutic effect. In addition, siNA can be used to characterize pathways of gene function in a variety of applications. For example, the present invention can be used to inhibit the activity of target gene(s) in a pathway to determine the function of uncharacterized gene(s) in gene function analysis, mRNA function analysis, or translational analysis. The invention can be used to determine potential target gene pathways involved in various diseases and conditions toward pharmaceutical development. The invention can be used to understand pathways of gene expression involved in, for example, the progression and/or maintenance of cancer.

[0138] In one embodiment, siNA molecule(s) and/or methods of the invention are used to down regulate the expression of gene(s) that encode RNA referred to by Genbank Accession, for example CHRM3 genes encoding RNA sequence(s) referred to herein by Genbank Accession number, for example, Genbank Accession Nos. shown in Table I.

[0139] In one embodiment, the invention features a method comprising: (a) generating a library of siNA constructs having a predetermined complexity; and (b) assaying the siNA constructs of (a) above, under conditions suitable to determine RNAi target sites within the target RNA sequence. In one embodiment, the siNA molecules of (a) have strands of a fixed length, for example, about 23 nucleotides in length. In another embodiment, the siNA molecules of (a) are of differing length, for example having strands of about 19 to about 25 (e.g., about 19, 20, 21, 22, 23, 24, or 25) nucleotides in length. In one embodiment, the assay can comprise a reconstituted in vitro siNA assay as described herein. In another embodiment, the assay can comprise a cell culture system in which target RNA is expressed. In another embodiment, fragments of target RNA are analyzed for detectable levels of cleavage, for example by gel electrophoresis, northern blot analysis, or RNase protection assays, to determine the most suitable target site(s) within the target RNA sequence. The target RNA sequence can be obtained as is known in the art, for example, by cloning and/or transcription for in vitro systems, and by cellular expression in in vivo systems.

[0140] In one embodiment, the invention features a method comprising: (a) generating a randomized library of siNA constructs having a predetermined complexity, such as of 4N, where N represents the number of base paired nucleotides in each of the siNA construct strands (e.g., for a siNA construct having 21 nucleotide sense and antisense strands with 19 base pairs, the complexity would be 419); and (b) assaying the siNA constructs of (a) above, under conditions suitable to determine RNAi target sites within the target CHRM3 RNA sequence. In another embodiment, the siNA molecules of (a) have strands of a fixed length, for example about 23 nucleotides in length. In yet another embodiment, the siNA molecules of (a) are of differing length, for example having strands of about 19 to about 25 (e.g., about 19, 20, 21, 22, 23, 24, or 25) nucleotides in length. In one embodiment, the assay can comprise a reconstituted in vitro siNA assay as described in Example 7 herein. In another embodiment, the assay can comprise a cell culture system in which target RNA is expressed. In another embodiment, fragments of CHRM3 RNA are analyzed for detectable levels of cleavage, for example by gel electrophoresis, northern blot analysis, or RNase protection assays, to determine the most suitable target site(s) within the target CHRM3 RNA sequence. The target CHRM3 RNA sequence can be obtained as is known in the art, for example, by cloning and/or transcription for in vitro systems, and by cellular expression in in vivo systems.

[0141] In another embodiment, the invention features a method comprising: (a) analyzing the sequence of a RNA target encoded by a target gene; (b) synthesizing one or more sets of siNA molecules having sequence complementary to one or more regions of the RNA of (a); and (c) assaying the siNA molecules of (b) under conditions suitable to determine RNAi targets within the target RNA sequence. In one
embodiment, the siRNA molecules of (b) have strands of a fixed length, for example about 23 nucleotides in length. In another embodiment, the siRNA molecules of (b) are of differing length, for example having strands of about 19 to about 25 (e.g., about 19, 20, 21, 22, 23, 24, or 25) nucleotides in length. In one embodiment, the assay can comprise a reconstituted in vitro siRNA assay as described herein. In another embodiment, the assay can comprise a cell culture system in which target RNA is expressed. Fragments of target RNA are analyzed for detectable levels of cleavage, for example by gel electrophoresis, northern blot analysis, or RNAase protection assays, to determine the most suitable target site(s) within the target RNA sequence. The target RNA sequence can be obtained as is known in the art, for example, by cloning and/or transcription for in vitro systems, and by expression in in vivo systems.

[0142] By “target site” is meant a sequence within a target RNA that is “targeted” for cleavage mediated by a siRNA construct which contains sequences within its antisense region that are complementary to the target sequence.

[0143] 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.

[0144] In one embodiment, the invention features a composition comprising a siRNA 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 siRNA 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 or condition in a subject comprising administering to the subject a composition of the invention under conditions suitable for the diagnosis of the disease or condition in the subject. In another embodiment, the invention features a method for treating or preventing a disease or condition in a subject, comprising administering to the subject a composition of the invention under conditions suitable for the treatment or prevention of the disease or condition in the subject, alone or in conjunction with one or more other therapeutic compounds. In yet another embodiment, the invention features a method for reducing or preventing tissue rejection in a subject comprising administering to the subject a composition of the invention under conditions suitable for the reduction or prevention of tissue rejection in the subject.

[0145] In another embodiment, the invention features a method for validating a CHRM3 gene target, comprising: (a) synthesizing a siRNA molecule of the invention, which can be chemically-modified, wherein one of the siRNA strands includes a sequence complementary to RNA of a CHRM3 target gene; (b) introducing the siRNA molecule into a cell, tissue, or organism under conditions suitable for modulating expression of the CHRM3 target gene in the cell, tissue, or organism; and (c) determining the function of the gene by assaying for any phenotypic change in the cell, tissue, or organism.

[0146] In another embodiment, the invention features a method for validating a CHRM3 target comprising: (a) synthesizing a siRNA molecule of the invention, which can be chemically-modified, wherein one of the siRNA strands includes a sequence complementary to RNA of a CHRM3 target gene; (b) introducing the siRNA molecule into a biological system under conditions suitable for modulating expression of the CHRM3 target gene in the biological system; and (c) determining the function of the gene by assaying for any phenotypic change in the biological system.

[0147] 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, or organism, or extract thereof. The term biological system also includes reconstituted RNAi systems that can be used in an in vitro setting.

[0148] 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., siRNA). 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 Fluorescent Protein (GFP) or various tags that are used to identify an expressed protein or any other cellular component that can be assayed.

[0149] In one embodiment, the invention features a kit containing a siRNA molecule of the invention, which can be chemically-modified, that can be used to modulate the expression of a CHRM3 target gene in a biological system, including, for example, in a cell, tissue, or organism. In another embodiment, the invention features a kit containing more than one siRNA molecule of the invention, which can be chemically-modified, that can be used to modulate the expression of more than one CHRM3 target gene in a biological system, including, for example, in a cell, tissue, or organism.

[0150] In one embodiment, the invention features a cell containing one or more siRNA molecules of the invention, which can be chemically-modified. In another embodiment, the cell containing a siRNA molecule of the invention is a mammalian cell. In yet another embodiment, the cell containing a siRNA molecule of the invention is a human cell.

[0151] In one embodiment, the synthesis of a siRNA molecule of the invention, which can be chemically-modified, comprises: (a) synthesis of two complementary strands of the siRNA molecule; (b) annealing the two complementary strands together under conditions suitable to obtain a double-stranded siRNA molecule. In another embodiment, synthesis of the two complementary strands of the siRNA molecule is by solid phase oligonucleotide synthesis. In yet another embodiment, synthesis of the two complementary strands of the siRNA molecule is by solid phase tandem oligonucleotide synthesis.

[0152] In one embodiment, the invention features a method for synthesizing a siRNA duplex molecule comprising: (a) synthesizing a first oligonucleotide sequence strand of the siRNA 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 methylaniline. 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.

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.

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.

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.

In another embodiment, the method of synthesis of siNA molecules of the invention comprises the teachings of Scharing et al., U.S. Pat. Nos. 5,889,136; 6,008,400; and 6,111,086, incorporated by reference herein in their entirety.

In one embodiment, the invention features siNA constructs that mediate RNAi against CHRM3, wherein the siNA construct comprises one or more chemical modifications, for example, one or more chemical modifications having any of Formula I-VII or any combination thereof that increases the nucleic resistance of the siNA construct.

In another embodiment, the invention features a method for generating siNA molecules with increased nucleic 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 nucleic resistance.

In one embodiment, the invention features siNA constructs that mediate RNAi against CHRM3, 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.

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.

In one embodiment, the invention features siNA constructs that mediate RNAi against CHRM3, 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.

[0162] In one embodiment, the invention features siNA constructs that mediate RNAi against CHRM3, 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.

[0163] 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.

[0164] 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.

[0165] In one embodiment, the invention features siNA constructs that mediate RNAi against CHRM3, 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.

[0166] 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.

[0167] In one embodiment, the invention features chemically-modified siNA constructs that mediate RNAi against CHRM3 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.

[0168] In another embodiment, the invention features a method for generating siNA molecules with improved RNAi activity against CHRM3 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.

[0169] In yet another embodiment, the invention features a method for generating siNA molecules with improved RNAi activity against CHRM3 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.

[0170] In yet another embodiment, the invention features a method for generating siNA molecules with improved RNAi activity against CHRM3 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.

[0171] In one embodiment, the invention features siNA constructs that mediate RNAi against CHRM3, wherein the siNA construct comprises one or more chemical modifications described herein that modulates the cellular uptake of the siNA construct.

[0172] In another embodiment, the invention features a method for generating siNA molecules against CHRM3 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.

[0173] In one embodiment, the invention features siNA constructs that mediate RNAi against CHRM3, 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. Ser. No. 10/201,394 incorporated by reference herein.

[0174] 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; and/or molecules such as folate and N-acetylglactosamine; polymers, such as polyethyleneglycol (PEG); phospholipids; cholesterol; polyamines, such as spermine or spermidine; and others.

[0175] 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.

[0176] 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. 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.

[0177] 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.

[0178] 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.

[0179] 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 FIG. 10, an alkyl or cycloalkyl group, a heterocycle, or any other group that prevents RNAi activity in which the second sequence serves as a guide sequence or template for RNAi.

[0180] 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 FIG. 10, an alkyl or cycloalkyl group, a heterocycle, or any other group that prevents RNAi activity in which the second sequence serves as a guide sequence or template for RNAi.

[0181] 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 FIG. 10 (e.g., inverted deoxyabasic moieties) or any other chemical modification that renders a portion of the siNA molecule (e.g., the sense strand) incapable of mediating RNA interference against an off target nucleic acid sequence. 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 wherein 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” and “Stab 17/22” chemistries and variants thereof (see Table IV) wherein the 5'-end and 3'-end of the sense strand of the siNA do not comprise a hydroxyl group or phosphate group.

[0182] 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” and “Stab 17/22” chemistries and variants thereof (see Table IV) wherein the 5'-end and 3'-end of the sense strand of the siNA do not comprise a hydroxyl group or phosphate group.

[0183] In one embodiment, the invention features a method for screening siNA molecules that are active in mediating RNA interference against a target nucleic acid sequence comprising (a) generating a plurality of unmodified siNA molecules, (b) screening the siNA molecules of step (a) under conditions suitable for isolating siNA mol-
molecules that are active in mediating RNA interference against the target nucleic acid sequence, and (c) introducing chemical modifications (e.g., chemical modifications as described herein or as otherwise known in the art) into the active sNiA molecules of (b). In one embodiment, the method further comprises re-screening the chemically modified sNiA molecules of step (c) under conditions suitable for isolating chemically modified sNiA molecules that are active in mediating RNA interference against the target nucleic acid sequence.

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

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.

In another embodiment, the invention features a method for generating sNiA molecules of the invention with improved bioavailability comprising (a) introducing nucleotides having any of Formulae I-VII or any combination thereof into a sNiA molecule, and (b) assaying the sNiA molecule of step (a) under conditions suitable for isolating sNiA molecules having improved bioavailability.

In another embodiment, the invention features a method for generating sNiA molecules of the invention with improved bioavailability comprising (a) introducing nucleotides having any of Formulae I-VII or any combination thereof into a sNiA molecule, and (b) assaying the sNiA molecule of step (a) under conditions suitable for isolating sNiA molecules having improved bioavailability.

In another embodiment, polyethylene glycol (PEG) can be covalently attached to sNiA compounds of the present invention. The attached PEG can be any molecular weight, preferably from about 2,000 to about 50,000 daltons (Da).

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 sNiA molecule of the invention and a vehicle that promotes introduction of the sNiA 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., U.S. Pat. No. 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., U.S. Ser. No. 60/402,996). Such a kit can also include instructions to allow a user of the kit to practice the invention.

The term “short interfering nucleic acid”, “sSiNA”, “short interfering RNA”, “sSiRNA”, “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, for example by mediating RNA interference “RNAi” or gene silencing in a sequence-specific manner; see for example 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; Plasitic et al., International PCT Publication No. WO 00/01846; Mello and Fire, International PCT Publication No. WO 00/29058; Deschamps-Delpaille, 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; Hvizdov 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). Non limiting examples of sNiA molecules of the invention are shown in FIGS. 4-6, and Tables II and III herein. For example the sNiA can be a double-stranded polynucleotide 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 sNiA 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 19 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. Alternatively, the sNiA is assembled from a single oligonucleotide, where the self-complementary sense and antisense regions of the sNiA are linked by means of a nucleic acid based or non-nucleic acid-based linker(s). The sNiA 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 sNiA 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 siRNA molecule capable of mediating RNAi. The siRNA 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 siRNA molecule does not require the presence within the siRNA 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, 537-548), or 5',3'-diphosphate. In certain embodiments, the siRNA 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 siRNA molecules of the invention comprise nucleotide sequence that is complementary to nucleotide sequence of a target gene. In another embodiment, the siRNA 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, siRNA 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 siRNA molecules that do not require the presence of ribonucleotides within the siRNA 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, siRNA molecules may 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 siRNA 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 (pgsRNA), and others. In addition, as used herein, the term RNAi: means to be equivalent to other terms used to describe sequence specific RNA interference, such as post transcriptional gene silencing, translational inhibition, or epigenetics. For example, siRNA 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 regulation of gene expression by siRNA molecules of the invention can result from siRNA mediated modification of chromatin structure 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; Jennewein, 2002, Science, 297, 2215-2218; and Hall et al., 2002, Science, 297, 2232-2237).

[0191] In one embodiment, a siRNA molecule of the invention is a duplex forming oligonucleotide “DF0”, (see for example FIGS. 14-15 and Vaish et al., U.S. Ser. No. 10/727,780 filed Dec. 3, 2003).

[0192] In one embodiment, a siRNA molecule of the invention is a multifunctional siRNA, (see for example FIGS. 16-22 and Jadhav et al., U.S. Ser. No. 60/543,480, filed Feb. 10, 2004). The multifunctional siRNA of the invention can comprise sequence targeting, for example, two regions of CHRM3 RNA (see for example target sequences in Tables II and III).

[0193] By “asymmetric hairpin” as used herein is meant a linear siRNA 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 siRNA 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 19 to about 22 (e.g., about 19, 20, 21, or 22) nucleotides) and a loop region comprising about 4 to about 8 (e.g., about 4, 5, 6, 7, or 8) nucleotides, and a sense region having about 3 to about 18 (e.g., about 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, or 18) nucleotides that are complementary to the antisense region. The asymmetric hairpin siRNA molecule can also comprise a 5'-terminal phosphate group that can be chemically modified. The loop portion of the asymmetric hairpin siRNA molecule can comprise nucleotides, non-nucleotides, linker molecules, or conjugate molecules as described herein.

[0194] By “asymmetric duplex” as used herein is meant a siRNA 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 siRNA 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 19 to about 22 (e.g., about 19, 20, 21, or 22) nucleotides) and a sense region having about 3 to about 18 (e.g., about 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, or 18) nucleotides that are complementary to the antisense region.

[0195] By “modulate” is meant that the expression of the gene, or level of 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 upregulated or downregulated, 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.

[0196] 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, 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.

[0197] By “gene”, or “target gene”, 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 IRN or ncRNA involved in functional or regulatory cellular processes. Aberrant RNA or ncRNA activity leading to disease can therefore be modulated by siNA molecules of the invention. siNA molecules targeting RNA and ncRNA can also be used to manipulate or alter the genotype or phenotype of an 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.

[0198] By “CHRM3” as used herein is meant, any cholinergic receptor muscarinic 3 protein, peptide, or polypeptide, for example encoded by sequences referred to by accession numbers shown in Table I. The term “CHRM3” also refers to nucleic acid sequences encoding any cholinergic receptor muscarinic 3 protein, peptide, or polypeptide, such as CHRM3 RNA or CHRM3 DNA.

[0199] 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 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.).

[0200] 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 or organism to another biological system or organism. The polynucleotide can include both coding and non-coding DNA and RNA.

[0201] 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.

[0202] 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.

[0203] By “target nucleic acid” is meant any nucleic acid sequence whose expression or activity is to be modulated. The target nucleic acid can be DNA or RNA.

[0204] 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. 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. LI 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). “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.
[0205] The siNA molecules of the invention represent a novel therapeutic approach to treat a variety of disease and conditions such as chronic obstructive pulmonary disease or “COPD”, asthma, allergic rhinitis, sinusitis, pulmonary vasoconstriction, inflammation, allergies, impeded respiration, respiratory distress syndrome, cystic fibrosis, pulmonary hypertension, pulmonary vasoconstriction, emphysema, irritable bowel syndrome, Alzheimer’s disease, urinary incontinence and any other diseases or conditions that are related to or will respond to the levels of CHRM3 in a cell or tissue, alone or in combination with other therapies. The reduction of CHRM3 expression and thus reduction in the level of the respective protein relieves, to some extent, the symptoms of the disease or condition.

[0206] In one embodiment of the present invention, each sequence of a siNA molecule of the invention is independently about 18 to about 24 nucleotides in length, in specific embodiments about 18, 19, 20, 21, 22, 23, or 24 nucleotides in length. In another embodiment, the siNA duplexes of the invention independently comprise about 17 to about 23 base pairs (e.g., about 17, 18, 19, 20, 21, 22 or 23). In yet another embodiment, siNA molecules of the invention comprising hairpin or circular structures are at least 35 to at least 55 (e.g., about 35, 40, 45, 50 or 55) nucleotides in length, or at least 38 to at least 44 (e.g., 38, 39, 40, 41, 42, 43 or 44) nucleotides in length and comprising about 16 to about 22 (e.g., about 16, 17, 18, 19, 20, 21 or 22) base pairs. Exemplary siNA molecules of the invention are shown in Table II. Exemplary synthetic siNA molecules of the invention are shown in Table III and/or FIGS. 4-5.

[0207] 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.

[0208] 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 injection, infusion pump or stent, with or without their incorporation in biopolymers. In particular embodiments, the nucleic acid molecules of the invention comprise sequences shown in Tables II-III and/or FIGS. 4-5. Examples of such nucleic acid molecules consist essentially of sequences defined in these tables and figures. Furthermore, the chemically modified constructs described in Table IV can be applied to any siNA sequence of the invention.

[0209] 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.

[0210] 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 P-D-ribo-furanose 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.

[0211] 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.

[0212] The term “phosphorothioate” as used herein refers to an internucleotide linkage having Formula I, wherein Z and/or W comprises a sulfur atom. Hence, the term phosphorothioate refers to both phosphorothioate and phosphorodithioate internucleotide linkages.

[0213] The term “phosphonoacetate” as used herein refers to an internucleotide linkage having Formula I, wherein Z and/or W comprises an acetylated or protected acetyl group.

[0214] The term “thiophosphonoacetate” as used herein refers to an internucleotide linkage having Formula I, wherein Z comprises an acetylated or protected acetyl group and W comprises a sulfur atom or alternatively W comprises an acetylated or protected acetyl group and Z comprises a sulfur atom.

[0215] 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-nitropyrolole, 4-nitroindole, 5-nitroindole, and 6-nitroindole as known in the art (see for example Loakes, 2001, Nucleic Acids Research, 29, 2437-2447).

[0216] 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.

[0217] The nucleic acid molecules of the instant invention, individually, or in combination or in conjunction with other drugs, can be used to treat diseases or conditions discussed herein (e.g., cancers and other proliferative conditions). For example, to treat a particular disease or condition, the siNA molecules can be administered to a subject or can be administered to other appropriate cells evident to those skilled in the art, individually or in combination with one or more drugs under conditions suitable for the treatment.

[0218] In a further embodiment, the siNA molecules can be used in combination with other known treatments to treat conditions or diseases discussed above. For example, the described molecules could be used in combination with one
or more known therapeutic agents to treat a disease or condition. Non-limiting examples of other therapeutic agents that can be readily combined with a siNA molecule of the invention are enzymatic nucleic acid molecules, allosteric nucleic acid molecules, antisense, decoy, or aptamer nucleic acid molecules, antibodies such as monoclonal antibodies, small molecules, and other organic and/or inorganic compounds including metals, salts and ions.

In one embodiment, the invention features an expression vector comprising a nucleic acid sequence encoding at least one siNA molecule of the invention, in a manner which allows expression of the siNA molecule. For example, the vector can contain sequence(s) encoding both strands of a siNA molecule comprising a duplex. The vector can also contain sequence(s) encoding a single nucleic acid molecule that is self-complementary and thus forms a siNA molecule. Non-limiting examples of such expression vectors are described in Paul et al., 2002, Nature Biotechnology, 19, 505; Miyagishi and Taïra, 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.

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

In yet another embodiment, the expression vector of the invention comprises a sequence for a siNA molecule having complementarity to a RNA molecule referred to by a Genbank Accession numbers, for example Genbank Accession Nos. shown in Table I.

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

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

By “vectors” is meant any nucleic acid- and/or viral-based technique used to deliver a desired nucleic acid.

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

BRIEF DESCRIPTION OF THE DRAWINGS

FIG. 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.

FIG. 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.

FIG. 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 siRNA can be introduced directly into a cell by appropriate means. An active siRNA 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 siRNA molecules, thereby amplifying the RNAi response.

FIGS. 4A-F shows non-limiting examples of chemically-modified siNA constructs of the present invention. In the figure, N stands for any nucleotide (adenosine, guanosine, cytosine, oruridine, or optionally thymidine, for example thymidine can be substituted in the overhanging regions designated by parenthesis (N)). Various modifications are shown for the sense and antisense strands of the siNA constructs.

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

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

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

FIGS. 5A-F shows non-limiting examples of specific chemically-modified siRNA sequences of the invention. A-F applies the chemical modifications described in FIGS. 4A-F to a CHRM3 siRNA sequence. Such chemical modifications can be applied to any CHRM3 sequence and/or CHRM3 polymorphism sequence.

FIG. 6 shows non-limiting examples of different siRNA constructs of the invention. The examples shown (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 siRNA construct 2 in vivo and/or in vitro, which can optionally utilize another biodegradable linker to generate the active siRNA construct 1 in vivo and/or in vitro. As such, the stability and/or activity of the siRNA constructs can be modulated based on the design of the siRNA construct for use in vivo or in vitro.

[0238] FIGS. 7A-C is a diagrammatic representation of a scheme utilized in generating an expression cassette to generate siRNA hairpin constructs.

[0239] FIG. 7A: A DNA oligomer is synthesized with a 5'-restriction site (RI) sequence followed by a region having sequence identical (sense region of siRNA) to a predetermined CHRM3 target sequence, wherein the sense region comprises, for example, about 19, 20, 21, or 22 nucleotides (N) in length, which is followed by a loop sequence of defined sequence (X), comprising, for example, about 3 to about 10 nucleotides.

[0240] FIG. 7B: The synthetic construct is then extended by DNA polymerase to generate a hairpin structure having self-complementary sequence that will result in a siRNA transcript having specificity for a CHRM3 target sequence and having self-complementary sense and antisense regions.

[0241] FIG. 7C: The construct is heated (for example to about 95°C) to linearize the sequence, thus allowing extension of a complementary second DNA strand using a primer to the 3'-restriction sequence of the first strand. The double-stranded DNA is then inserted into an appropriate vector for expression in cells. The construct can be designed such that a 3'-terminal nucleotide overhangs results from the transcription, for example by engineering restriction sites and/or utilizing a poly-U termination region as described in Paul et al., 2002, Nature Biotechnology, 29, 505-508.

[0242] FIGS. 8A-C is a diagrammatic representation of a scheme utilized in generating an expression cassette to generate double-stranded siRNA constructs.

[0243] FIG. 8A: A DNA oligomer is synthesized with a 5'-restriction (RI) site sequence followed by a region having sequence identical (sense region of siRNA) to a predetermined CHRM3 target sequence, wherein the sense region comprises, for example, about 19, 20, 21, or 22 nucleotides (N) in length, and which is followed by a 3'-restriction site (R2) which is adjacent to a loop sequence of defined sequence (X).

[0244] FIG. 8B: The synthetic construct is then extended by DNA polymerase to generate a hairpin structure having self-complementary sequence.

[0245] FIG. 8C: The construct is processed by restriction enzymes specific to R1 and R2 to generate a double-stranded DNA which is then inserted into an appropriate vector for expression in cells. The transcription cassette is designed such that a U6 promoter region flanks each side of the dsDNA which generates the separate sense and antisense strands of the siRNA. Poly T termination sequences can be added to the constructs to generate U overhangs in the resulting transcript.

[0246] FIGS. 9A-E is a diagrammatic representation of a method used to determine target sites for siRNA mediated RNAI within a particular target nucleic acid sequence, such as messenger RNA.

[0247] FIG. 9A: A pool of siRNA oligonucleotides are synthesized wherein the antisense region of the siRNA constructs has complementarity to target sites across the target nucleic acid sequence, and wherein the sense region comprises sequence complementary to the antisense region of the siRNA.

[0248] FIGS. 9B-C. FIG. 9B) The sequences are pooled and are inserted into vectors such that (FIG. 9C) transfection of a vector into cells results in the expression of the siRNA.

[0249] FIG. 9D: Cells are sorted based on phenotypic change that is associated with modulation of the target nucleic acid sequence.

[0250] FIG. 9E: The siRNA is isolated from the sorted cells and is sequenced to identify efficacious target sites within the target nucleic acid sequence.

[0251] FIG. 10 shows non-limiting examples of different stabilization chemistries (1-10) that can be used, for example, to stabilize the 3'-end of siRNA sequences of the invention, including (1) [3'-3']-inverted deoxyribose; (2) deoxyribofuranoside; (3) [5'-3']-3'-deoxyribofuranoside; (4) [5'-3']-ribose; (5) [5'-3']-3'-O-methyl ribonucleoside; (6) 3'-glyceryl; (7) [3'-5']-3'-deoxyribofuranoside; (8) [3'-3']-deoxyribofuranoside; (9) [5'-2']-deoxyribonucleotide; and (10) [5'-3']-deoxyribonucleotide. 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'-deoxyribose 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 Formulas I-VII or any combination thereof.

[0252] FIG. 11 shows a non-limiting example of a strategy used to identify chemically modified siRNA constructs of the invention that are nucleasase resistant while preserving the ability to mediate RNAi activity. Chemical modifications are introduced into the siRNA construct based on educated design parameters (e.g., introducing 2'-modifications, base modifications, backbone modifications, terminal cap modifications etc). The modified construct is tested in an appropriate system (e.g., human serum for nucleasase resistance, shown, or an animal model for PK/delivery parameters). In parallel, the siRNA construct is tested for RNAi activity, for example in a cell culture system such as a luciferase reporter assay). Lead siRNA 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 siRNA-conjugate molecules with improved pharmacokinetic profiles, delivery, and RNAi activity.

[0253] FIG. 12 shows non-limiting examples of phosphorolylated siRNA molecules of the invention, including linear and duplex constructs and asymmetric derivatives thereof.
FIG. 13 shows non-limiting examples of chemically modified terminal phosphate groups of the invention.

FIG. 14A shows a non-limiting example of methodology used to design self complementary DFO constructs utilizing palindrome and/or repeat nucleic acid sequences that are identified in a target nucleic acid sequence. (i) A palindrome or repeat sequence is identified in a nucleic acid target sequence. (ii) A sequence is designed that is complementary to the target nucleic acid sequence and the palindrome sequence. (iii) An inverse repeat sequence of the non-palindrome/repeat portion of the complementary sequence is appended to the 3'-end of the complementary sequence to generate a self complimentary DFO molecule comprising sequence complementary to the nucleic acid target. (iv) The DFO molecule can self-assemble to form a double stranded oligonucleotide.

FIG. 14B shows a non-limiting representative example of a duplex forming oligonucleotide sequence.

FIG. 14C shows a non-limiting example of the self assembly schematic of a representative duplex forming oligonucleotide sequence.

FIG. 14D shows a non-limiting example of the self assembly schematic of a representative duplex forming oligonucleotide sequence followed by interaction with a target nucleic acid sequence resulting in modulation of gene expression.

FIG. 15 shows a non-limiting example of the design of self complementary DFO constructs utilizing palindrome and/or repeat nucleic acid sequences that are incorporated into the DFO constructs that have sequence complementary to any target nucleic acid sequence of interest. Incorporation of these palindrome/repeat sequences allow the design of DFO constructs that form duplexes in which each strand is capable of mediating modulation of target gene expression, for example by RNAi. First, the target sequence is identified. A complementary sequence is then generated in which nucleotide or non-nucleotide modifications (shown as X or Y) are introduced into the complementary sequence that generate an artificial palindrome (shown as XYZXY 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 complimentary DFO comprising sequence complementary to the nucleic acid target. The DFO can self-assemble to form a double stranded oligonucleotide.

FIG. 16 shows non-limiting examples of multifunctional siNA molecules of the invention comprising two separate polynucleotide sequences that are each capable of mediating RNAi directed cleavage of differing target nucleic acid sequences.

FIG. 16A shows a non-limiting example of a multifunctional siNA molecule having a first region that is complementary to a first target nucleic acid sequence (complementary region 1) and a second region that is complementary to a second target nucleic acid sequence (complementary region 2), wherein the first and second complementary regions are situated at the 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.

FIG. 16B shows a non-limiting example of a multifunctional siNA molecule having a first region that is complementary to a first target nucleic acid sequence (complementary region 1) and a second region that is complementary to a second target nucleic acid sequence (complementary region 2), wherein the first and second complementary regions are situated at the 5'-ends of each polynucleotide sequence in the multifunctional siNA. The dashed portions of each polynucleotide sequence of the multifunctional siNA construct have complementarity with regard to corresponding portions of the siNA duplex, but do not have complementarity to the target nucleic acid sequences.

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

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

FIG. 17B shows a non-limiting example of a multifunctional siNA molecule having a first region that is complementary to a first target nucleic acid sequence (complementary region 1) and a second region that is complementary to a second target nucleic acid sequence (complementary region 2), wherein the first complementary region is situated at the 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. In one embodiment, these multifunctional siNA constructs are processed in vivo or in vitro to generate multifunctional siNA constructs as shown in FIG. 16.

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

FIG. 18A shows a non-limiting example of a multifunctional siNA molecule having a first region that is complementary to a first target nucleic acid sequence (complementary region 1) and a second region that is
complementary to a second target nucleic acid sequence (complementary region 2), wherein the first and second complementary regions are situated at the 3'-ends of each polynucleotide sequence in the multifunctional siNA, and wherein the first and second complementary regions further comprise a self complementary, palindromic, 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.

[0268] FIG. 18B shows a non-limiting example of a multifunctional siNA molecule having a first region that is complementary to a first target nucleic acid sequence (complementary region 1) and a second region that is complementary to a second target nucleic acid sequence (complementary region 2), wherein the first and second complementary regions are situated at the 5'-ends of each polynucleotide sequence in the multifunctional siNA, and wherein the first and second complementary regions further comprise a self complementary, palindromic, 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.

[0269] FIG. 19 shows non-limiting examples of multifunctional siNA molecules of the invention comprising a single polynucleotide sequence comprising distinct regions that are each capable of mediating RNAi directed cleavage of differing target nucleic acid sequences and wherein the multifunctional siNA construct further comprises a self complementary, palindromic, or repeat region, thus enabling shorter bifunctional siNA constructs that can mediate RNA interference against differing target nucleic acid sequences.

[0270] FIG. 19A shows a non-limiting example of a multifunctional siNA molecule having a first region that is complementary to a first target nucleic acid sequence (complementary region 1) and a second region that is complementary to a second target nucleic acid sequence (complementary region 2), wherein the second complementary region is situated at the 3'-end of the polynucleotide sequence in the multifunctional siNA, and wherein the first and second complementary regions further comprise a self complementary, palindromic, 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.

[0271] FIG. 19B shows a non-limiting example of a multifunctional siNA molecule having a first region that is complementary to a first target nucleic acid sequence (complementary region 1) and a second region that is complementary to a second target nucleic acid sequence (complementary region 2), wherein the first complementary region is situated at the 5'-end of the polynucleotide sequence in the multifunctional siNA, and wherein the first and second complementary regions further comprise a self complementary, palindromic, 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 FIG. 18.

[0272] FIG. 20 shows a non-limiting example of how multifunctional siNA molecules of the invention can target two separate target nucleic acid molecules, such as separate RNA molecules encoding differing proteins, 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.

[0273] FIG. 21 shows a non-limiting example of how multifunctional siNA molecules of the invention can target two separate target nucleic acid sequences within the same target nucleic acid molecule, such as alternate coding regions of a RNA, coding and non-coding regions of a RNA, or alternate splice variant regions of a RNA. Each strand of the multifunctional siNA construct comprises a region having complementarity to the separate regions of the target nucleic acid molecule. The multifunctional siNA molecule is designed such that each strand of the siNA can be utilized by the RISC complex to initiate RNA interference mediated cleavage of its corresponding target region. These design parameters can include destabilization of each end of the siNA construct (see for example Schwarz et al., 2003, Cell, 115, 199-208). Such destabilization can be accomplished for example by using guanosine-cytidine base pairs, alternate base pairs (e.g., wobbles), or destabilizing chemically modified nucleotides at terminal nucleotide positions as is known in the art.

DETAILED DESCRIPTION OF THE INVENTION

[0274] Mechanism of Action of Nucleic Acid Molecules of the Invention

[0275] 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 siRNA 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 siRNA to mediate RNAi and the stability of the siRNAs 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 siRNA containing a plurality of ribonucleotides. In some cases, the activity or stability of the siRNA molecule can be decreased (i.e., less than ten-fold), but the overall activity of the siRNA molecule is enhanced in vitro and/or in vivo.

**[0276]** RNA interference refers to the process of sequence specific post-transcriptional gene silencing in animals mediated by short interfering RNAs (siRNAs) (Fire et al., 1998, *Nature*, 391, 806). The corresponding process in plants is commonly referred to as post-transcriptional gene silencing or RNA silencing and is also referred to as quelling in fungi. The process of post-transcriptional gene silencing is thought to be an evolutionarily-conserved cellular defense mechanism used to prevent the expression of foreign genes which is commonly shared by diverse flora and phyla (Fire et al., 1999, *Trends Genet.*, 15, 358). Such protection from foreign gene expression may have evolved in response to the production of double-stranded RNAs (dsRNAs) derived from viral infection or the random integration of transposon elements into a host genome via a cellular response that specifically destroys homologous single-stranded RNA or viral genomic RNA. The presence of dsRNA in cells triggers the RNAi response through a mechanism that has yet to be fully characterized. This mechanism appears to be different from the interferon response that results from dsRNA-mediated activation of protein kinase PKR and 2′,5′-oligoadenylate synthetase resulting in non-specific cleavage of mRNA by ribonuclease L.

**[0277]** 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) (Berkstein et al., 2001, *Nature*, 409, 363). Short interfering RNAs derived from Dicer activity are typically about 21 to about 23 nucleotides in length and comprise about 19 base pair duplexes. Dicer has also been implicated in the excision of 21- and 22-nucleotide small temporal RNAs (stRNAs) from precursor RNA of conserved structure that are implicated in translational control (Hutvagner et al., 2001, *Science*, 293, 834). The RNAi response also features an endonuclease complex containing a siRNA, commonly referred to as an RNA-induced silencing complex (RISC), which mediates cleavage of single-stranded RNA having sequence homologous to the siRNA. Cleavage of the target RNA takes place in the middle of the region complementary to the guide sequence of the siRNA duplex (Elbashir et al., 2001, *Genes Dev.*, 15, 188). In addition, RNA interference can also involve small RNA (e.g., micro-RNA or miRNA) mediated gene silencing, presumably through cellular mechanisms that regulate chromatin structure and thereby prevent transcription of target gene sequences (see for example Allshire, 2002, *Science*, 297, 1818-1819; Volpe et al., 2002, *Science*, 297, 1833-1837; Jenuwein, 2002, *Science*, 297, 2215-2218; and Hall et al., 2002, *Science*, 297, 2232-2237). As such, siRNA molecules of the invention can be used to mediate gene silencing via interaction with RNA transcripts or alternately by interaction with particular gene sequences, wherein such interaction results in gene silencing either at the transcriptional level or post-transcriptional level.

**[0278]** 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*. Winn 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 (Nykänen 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.

**[0279]** Synthesis of Nucleic Acid Molecules

**[0280]** 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 siRNA oligonucleotide sequences or siRNA sequences synthesized in tandem) are preferably used for exogenous delivery. The simple structure of these molecules increases the ability of the nucleic acid to invade targeted regions of protein and/or RNA structure. Exemplary molecules of the instant invention are chemically synthesized, and others can similarly be synthesized.

**[0281]** 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 V outlines the amounts and the contact times of the reagents used in the synthesis cycle. Alternatively, syntheses at the 0.2 µmol scale can be performed on a 96-well plate synthesizer, such as the instrument produced by Protegente (Palo Alto, Calif.) 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-methylimidazole in THF (ABI) and 10% acetic anhydride/10% 2,6-lutidine in THF (ABI); and oxidation solution is 15.9 mM I2, 49 mM pyridine, 9% water in THF (PerSeptive Biosystems, Inc.). Burdick & Jackson Synthesis Grade acetonitrile is used directly from the reagent bottle. S-Ethyltetrazole solution (0.25 M in acetonitrile) is made up from the solid obtained from American International Chemical, Inc. Alternatively, for the introduction of phosphorothioate linkages, Beaucage reagent (3H-1,2-Benzodithiol-3-one 1,1-dioxide, 0.05 M in acetonitrile) is used.

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 methanolamine (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.

The method of synthesis used for RNA including certain siRNA molecules of the invention follows the procedure as described in Usman et al., 1987, J. Am. Chem. Soc., 109, 7845; Scarringe et al., 1990, Nucleic Acids Res., 18, 5433; and Wincott et al., 1995, Nucleic Acids Res. 23, 2677-2684. Wincott et al., 1997, Methods Mol. Bio., 74, 59, and makes use of common nucleic acid protecting and coupling groups, such as dimethoxytrityl at the 5'-end, and phosphoramidites at the 3'-end. In a non-limiting example, small scale syntheses are conducted on a 394 Applied Biosystems, Inc. synthesizer using a 0.2 µmol scale protocol with a 7.5 min coupling step for alkylsilyl protected nucleotides and a 2.5 min coupling step for 2'-O-methylated nucleotides. Table V outlines the amounts and the contact times of the reagents used in the synthesis cycle. Alternatively, syntheses at the 0.2 µmol scale can be done on a 96-well plate synthesizer, such as the instrument produced by Protegente (Palo Alto, Calif.) 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-methylimidazole in THF (ABI) and 10% acetic anhydride/10% 2,6-lutidine in THF (ABI); oxidation solution is 16.9 mM I2, 49 mM pyridine, 9% water in THF (PerSeptive Biosystems, Inc.). Burdick & Jackson Synthesis Grade acetonitrile is used directly from the reagent bottle. S-Ethyltetrazole solution (0.25 M in acetonitrile) is made up from the solid obtained from American International Chemical, Inc. Alternatively, for the introduction of phosphorothioate linkages, Beaucage reagent (3H-1,2-Benzodithiol-3-one 1,1-dioxide 0.05 M in acetonitrile) is used.
together post-synthetically, for example, by ligation (Moore et al., 1992, Science 256, 9923; Draper et al., International PCT publication No. WO 93/23569; Shabrova 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.

[0289] 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 multowell/multispot 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.

[0290] 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.

[0291] The nucleic acid molecules of the present invention can be modified extensively to enhance stability by modification with nucleoside 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.

[0292] 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.

[0293] Optimizing Activity of the Nucleic Acid Molecule of the Invention.

[0294] 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; Perrell et al., 1990 Nature 344, 565; Picken 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; Sprott, 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.

[0295] 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 nucleic stability and efficacy. For example, oligonucleotides are modified to enhance stability and/or enhance biological activity by modification with nucleic acid 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, 14900). Sugar modification of nucleic acid molecules have been extensively described in the art (see Eckstein et al., International Publication PCT No. WO 92/07065; Perrell et al. Nature, 1990, 344, 565-568; Picken 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; Sprott, 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., U.S. Ser. No. 60/082,404 which was filed on Apr. 20, 1998; Karpeisky et al., 1998, Tetrahedron Lett., 39, 1131; Earnshaw and Gaal, 1998, Biopolymers (Nucleic Acid Sciences), 48, 39-55; Verma and Eckstein, 1998, Annu. Rev. Biochem., 67, 99-134; and Burlika et al., 1997, Bioorg. Med. Chem., 5, 1999-2016; 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 RNAs is cells is not significantly inhibited.

[0296] While chemical modification of oligonucleotide internucleotide linkages with phosphorothioate, phosphorodiithioate, 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.

[0297] 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 nuclease 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 DNA and RNA (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 nucleae stability, as described above.

[0298] 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 methylidene bicyclo nucleotide (see for example Wengel et al., International PCT Publication No. WO 00/06604 and WO 99/14220).

[0299] In another embodiment, the invention features conjugates and/or complexes of siRNA molecules of the invention. Such conjugates and/or complexes can be used to facilitate delivery of siRNA 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.

[0300] 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 siRNA molecule of the invention or the sense and antisense strands of a siRNA 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 dimmer, 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.

[0301] The term “biodegradable” as used herein, refers to degradation in a biological system, for example enzymatic degradation or chemical degradation.

[0302] 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 siRNA 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, triple complex forming oligonucleotides, 2,5-A chimera, siRNA, dsRNA, aldehydes, 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, polyanides, polyethylene glycol and other polyethers.

[0303] 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.

[0304] Therapeutic nucleic acid molecules (e.g., siRNA 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 nucleae stability as described above.

[0305] In yet another embodiment, siRNA 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.

[0306] Use of the nucleic acid-based molecules of the invention will lead to better treatment of the disease pro-
gression by affording the possibility of combination thera-
pies (e.g., multiple siNA molecules targeted to different
genes; nucleic acid molecules coupled with known small
molecule modulators; or intermittent treatment with combi-
nations 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.

[0307] In another aspect a siNA molecule of the invention
comprises one or more 5’ and/or a 3’-cap structure, for
example only the sense siNA strand, the antisense siNA
strand, or both siNA strands.

[0308] By “cap structure” is meant chemical modifica-
tions, 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-erythrofuranylosyl) nucleotide; 4-thio nucleotide;
carboxyclic nucleotide; 5-aminocarboxylic nucleotide;
1-nucleotides; alpha-nucleotides; modified base nucleotides;
phosphorothioate linkage; three- or furanose nucleotide;
4,5-deoxy 3’,4’-seco nucleotide; 5-aminodeoxy 3’,4’-deoxy-2-
butyl nucleotide; acyclic 3,5-dihydroxypentyl nucleotide;
3,3’-inverted nucleotide moiety; 3,3’-inverted abasic moi-
ety; 3,2’-inverted nucleotide moiety; 3,2’-inverted abasic
moiety; 1,4-butanediol phosphate; 3-phosphate;
hexaphosphate; aminohexyl phosphate; 3-phosphate;
3-phosphorothioate; phosphorothioate; or bridging or
non-bridging methylphosphonate moiety.

[0309] Non-limiting examples of the 3’-cap include, but
are not limited to, glyceryl, inverted deoxy abasic residue
(moiey), 4, 5-methylene nucleotide; 1-(beta-D-erythro-
furanylosyl) nucleotide; 4-thio nucleotide; carboxyclic nucle-
otide; 5-amino-alkyl phosphate; 1,3-diamino-2-propyl
phosphate; 3-aminopropyl phosphate; 6-aminoalkyl phospho-
ate; 1,2-aminoalkyl phosphate; hydroxypropyl phosphate;
1,5-anhydroxylitol nucleotide; L-nucleotide; alpha-
nucleotide; modified base nucleotide; phosphorothioate;
threo-4-furanosyl nucleotide; acyclic 3’,4’,seco nucle-
otide; 3,4-dihydroxybutyl nucleotide; 3,5-dihydroxypentyl
nucleotide; 5,5-inverted nucleotide moiety; 5,5-inverted
abasic moiety; 5-phosphorhamidate; 5-phosphorothioate;
1,4-butanediol phosphate; 5-amino; bridging and/or non-
bridging 5-phosphorhamidate; phosphorothioate and/or
phosphorothioate, bridging or non bridging methyl-
phosphonate and 5-mercapto moieties (for more details see
Beaucage and Iyer, 1993, Tetrahedron 49, 1925; incorpo-
rated by reference herein).

[0310] 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.

[0311] 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 sub-
stituted group(s) is preferably, hydroxyl, cyano, alkoxy,
==O, ==S, NO2 or N(CH3)2, amino, or SH. The term also
includes alkyl groups that are unsaturated hydrocarbon
groups containing at least one carbon-carbon double bond,
including straight-chain, branched-chain, and cyclic 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 may be
substituted or unsubstituted. When substituted the substit-
uted group(s) is preferably, hydroxyl, cyano, alkoxy, ==O,
==S, NO2, halogen, N(CH3)2, amino, or SH. The term
“alkyl” also includes alkyl 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 alkyl group has
1 to 12 carbons. More preferably, it is a lower alkynyl of
from 1 to 7 carbons, more preferably 1 to 4 carbons. The
alkynyl group may be substituted or unsubstituted. When
substituted the substituted group(s) is preferably, hydroxyl,
cyano, alkoxy, ==O, ==S, NO2, or N(CH3)2, amino or SH.

[0312] 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 substitu-
ent(s) of aryl groups are halogen, trihalomethyl, hydroxyl,
SH, OH, cyano, alkoxy, alkyl, alkenyl, and amino groups.
An “alkylaryl” group refers to an alkyl group (as
described above) covalently joined to an aryl group (as
described above). Carboxyclic aryl groups are groups
whirering the ring atoms on the aromatic ring are all carbon
atoms. The carbon atoms are optionally substituted. Hetero-
cyclic 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
oxigen, sulfur, and nitrogen, and include furanyli, thiethyl,
pyridyl, pyrrollyl, N-lower alkyl pyrrolo, pyrimidyl, pyrazyl,
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.

[0313] 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 interchange-
ably as nucleotide analogs, modified nucleotides, non-natu-
ral 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 Rev. 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-balouridine (e.g., 5-bromouridine) or 6-azapirimidines 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.

[0314] In one embodiment, the invention features modified siRNA molecules, with phosphate backbone modifications comprising one or more phosphorothioate, phosphoro-

[0315] By “abasic” is meant sugar moieties lacking a base or having other chemical groups in place of a base at the 1’ position, see for example Adamic et al., U.S. Pat. No. 5,998,203.

[0316] 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.

[0317] 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 Formulas I-VII and/or other modifications described herein.

[0318] In connection with 2’-modified nucleotides as described for the present invention, by “amino” is meant 2’-NH or 2’-O—NH₂, which can be modified or unmodified. Such modified groups are described, for example, in Eckstein et al., U.S. Pat. No. 5,672,695 and Matulic-Adamic et al., U.S. Pat. No. 6,248,876, which are both incorporated by reference in their entirety.

[0319] Various modifications to nucleic acid siRNA 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.

[0320] Administration of Nucleic Acid Molecules

[0321] A siRNA molecule of the invention can be adapted for use to treat, for example, chronic obstructive pulmonary disease or “COPD”, asthma, allergic rinitis, sinusitis, pulmonary vasoconstriction, inflammation, allergies, impeded respiration, respiratory distress syndrome, cystic fibrosis, pulmonary hypertension, pulmonary vasoconstriction, emphysema, irritable bowel syndrome, Alzheimer’s disease, urinary incontinence and any other diseases or conditions that are related to or will respond to the levels of CHRM3 in a cell or tissue, alone or in combination with other therapies. For example, a siRNA molecule 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 U.S. Pat. No. 6,447,796 and U.S. Pat. No. Application Publication No. US 20021304350), 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-polyethylene glycol-N-acetylglactosamine (PEI-PEG-GAL) or polyethyleneimine-polyethylene glycol-tri-N-acetylglactosamine (PEI-PEG-triGAL) derivatives. Alternatively, the nucleic acid/vehicle combination is locally delivered by direct injection or by use of an infusion pump.
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 U.S. Pat. No. 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. One illustrative type of solid particulate aerosol generator is an insufflator. Suitable formulations for administration by insufflation include finely comminuted powders which can be delivered by means of an insufflator. 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 liquefied 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 U.S. patent application No. 20040037780, and U.S. Pat. Nos. 6,592,904; 6,582,728; 6,565,885.

In one embodiment, a siRNA molecule of the invention is complexed with membrane disruptive agents such as those described in U.S. patent application Publication No. 20010076666, incorporated by reference herein in its entirety including the drawings. In another embodiment, the membrane disruptive agent or agents and the siRNA molecule are also complexed with a cationic lipid or helper lipid molecule, such as those lipids described in U.S. Pat. No. 6,235,310, incorporated by reference herein in its entirety including the drawings.

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 into 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 tablets, capsules or elixirs for oral administration, suppositories for rectal administration, sterile solutions, suspensions for injectable administration, and the other compositions known in the art.

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.

A pharmacological composition or formulation refers to a composition or formulation in a form suitable for administration, e.g., systemic 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.

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, intraperitoneal, inhalation, oral, intrapulmonary and intramuscular. Each of these administration routes exposes the siRNA molecules of the invention to an accessible diseased tissue. 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, such as cells producing excess repeat expansion genes.

By “pharmaceutically acceptable formulation” 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 suit-
able for formulation with the nucleic acid molecules of the instant invention include: P-glycoprotein inhibitors (such as Pluronic P85); biodegradable polymers, such as poly (DL-lactide-co-glycolide) microspheres for sustained release delivery (Emerich, D F 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; Pardi et al., 1995, PNAS USA., 92, 5592-5596; Boado, 1995, Adv Drug Delivery Rev. 15, 73-107; Al-Ahmad and others, 1998, Nucleic Acids Res., 26, 4910-4916; and Tyler et al., 1999, PNAS USA., 96, 7053-7058.

[0330] The invention also features the use of the composition comprising surface-modified liposomes containing poly (ethylene glycol) lipids (PEG-modified, or long-circulating liposomes or stealth liposomes). These formulations offer a method for increasing the accumulation of drugs 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 (Lasie et al. Chem. Rev: 1995, 95, 2611-2627; 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 (Lasie 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.

[0331] 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 edii. 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.

[0332] 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.

[0333] 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 pharmaceutical 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.

[0334] 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, color 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 alginate 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 gastrointesinal tract and thereby provide a sustained action over a longer period. For example, a time delay material such as glycercly monostearate or glycercly distearate can be employed.

[0335] 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.

[0336] 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, hydroxypropyl-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 hep-tadecaethyleneoxycetanol, or condensation products of ethy-lene oxide with partial esters derived from fatty acids and a hexitol such as polyoxyethylene sorbitol monooctanate, or condensation products of ethylene oxide with partial esters derived from fatty acids and hexitol anhydrides, for example polyethylene sorbitan monoleate. 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.

[0337] 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.

[0338] 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.

[0339] 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.

[0340] 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 parenterally 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.

[0341] 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.

[0342] 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.

[0343] 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.

[0344] 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.

[0345] 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.

[0346] 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.

[0347] 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, trienentary structures are bound with greater affinity than biantenary or monoentenary 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 mannose-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 Vargese et al., U.S. Ser. No. 10/201,394, filed Aug. 13, 2001; and Matulic-Adamic et al., U.S. Ser. No. 10/151,116, filed May 17, 2002. In one embodiment, nucleic acid molecules of the invention are complexed with or covalently attached to nanoparticles, such as Hepatitis B virus S, M, or L envelope proteins (see for example Yamado et al., 2003, Nature Biotechnology, 21, 885). In one embodiment, nucleic acid molecules of the invention are delivered with specificity for human tumor cells, specifically non-apoptotic human tumor cells including for example T-cells, hepatocytes, breast carcinoma cells, ovarian carcinoma cells, melanoma cells, intestinal epithelial cells, prostate cells, testicular cells, non-small cell lung cancers, small cell lung cancers, etc.


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. siRNA expressing viral vectors can be constructed based on, but not limited to, adenovirus-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. Pat. Nos. 5,902,880 and 6,146,886). The recombinant vectors capable of expressing the siRNA 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 siRNA molecule interacts with the target mRNA and generates an RNAi response. Delivery of siRNA molecule expressing vectors can be systemic, such as by intravenous or intra-muscular administration, by administration to target cells ex-planted from a subject following 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).

In one aspect the invention features an expression vector comprising a nucleic acid sequence encoding at least one siRNA molecule of the instant invention. The expression vector can encode one or both strands of a siRNA duplex, or a single self-complementary strand that self hybridizes into a siRNA duplex. The nucleic acid sequences encoding the siRNA molecules of the instant invention can be operably linked in a manner that allows expression of the siRNA molecule (see for example Paul et al., 2002, Nature Biotechnology, 19, 505; Miyagishii and Taara, 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).

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 siRNA 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 siRNA 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 siRNA of the invention; and/or an intron (intervening sequences).

Transcription of the siRNA 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. USA, 87, 6743-7; Gao and Huang 1993, Nucleic Acids Res., 21, 2867-72; Lieber et al., 1993, Methods Enzymol., 217, 47-60; 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. USA, 89, 10802-6; Chen et al., 1992, Nucleic Acids Res., 20, 4581-9; Yu et al., 1993, Proc. Natl. Acad. Sci. USA, 90, 6340-4; L’Huillier et al., 1992, EMBO J., 11, 4411-8; Liszewicz et al., 1993, Proc. Natl. Acad. Sci. U. S. A., 90, 8000-4; Thompson et al., 1995, Nucleic Acids Res., 23, 2259; Sulenger & 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 siRNA 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.,
smooth muscle contraction and its stimulation results in secretion of glandular tissue in the respiratory tract. M3 receptor stimulation activates a pertussis toxin insensitive G(q) protein that in turn activates phospholipase C (PLC) resulting in the release of inositol triphosphate (IP3) and diacylglycerol (DAG) (Spector Ed., 1999, Anticholinergic Agents in the Upper and Lower Airways, 1999, Volume 134, p 16-17). The release of IP3 mediates release of calcium whereas DAG activates protein kinase C, the combination of which leads to smooth muscle contraction in airway, gastrointestinal, urinary bladder, vascular, and other tissues. M3 receptors are distributed throughout the large and small airways and are highly localized to smooth muscle and submucosal gland cells. In these locations, M3 receptor activity induces contraction in smooth muscle cells and exocytosis in submucosal gland cells. Non-selective M3 receptor antagonists such as atropine, ipratropium bromide and selective M3 receptor antagonists such as darifenacin and rivastigmine all inhibit cholinergically induced smooth muscle contraction and exocytosis from smooth muscle cells and submucosal gland cells respectively (Spector Ed., 1999, Anticholinergic Agents in the Upper and Lower Airways, 1999, Volume 134, p 16-17).

The use of small interfering nucleic acid molecules targeting CHRM3, therefore provides a class of novel therapeutic agents that can be used in the treatment of diseases and conditions associated with airway M3 receptor induced smooth muscle constriction and glandular secretion such as chronic obstructive pulmonary disease or “COPD”, asthma, allergic rhinitis, sinusitis, irritable bowel syndrome, and any other diseases or conditions that are related to or will respond to the levels of CHRM3 in a cell or tissue (e.g. Alzheimer’s disease, urinary incontinence and other conditions, see for example Choi et al., WO 01,029176), alone or in combination with other therapies.

EXAMPLES

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

Exemplary siNA molecules of the invention are synthesized in tandem using a cleavable linker, for example, a succinyl-based linker. Tandem synthesis as described herein is followed by a one-step purification process that provides RNAi molecules in high yield. This approach is highly amenable to siNA synthesis in support of high throughput RNAi screening, and can be readily adapted to multi-column or multi-well synthesis platforms.

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.

[0363] 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 FIG. 1) or an equivalent cleavable linker. A non-limiting example of linker coupling conditions that can be used includes a hindered base such as disopropylammonium (DIPA) and/or DMAP in the presence of an activator reagent such as 2,6-dimethyl-4(3H)-pyrimidinone (PyBrOP). After the linker is coupled, standard synthesis chemistry is utilized to complete synthesis of the second sequence leaving the terminal 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 50 mM NaOAc or 1.5M NH₄HCO₃.

[0364] Purification of the siNA duplex can be readily accomplished using solid phase extraction, for example using a Waters C18 SepPak 1 g cartridge conditioned with 1 column volume (CV) of acetonitrile, 2 CV H₂O, and 2 CV 50 mM NaOAc. The sample is loaded and then washed with 1 CV H₂O or 50 mM NaOAc. Failure sequences are eluted with 1 CV 14% ACN (Aqueous with 50 mM NaOAc and 50 mM NaCl). The column is then washed, for example with 1 CV H₂O followed by on-column detritylation, for example by passing 1 CV of 1% aqueous trifluoroacetic acid (TFA) over the column, then adding a second CV of 1% aqueous TFA to the column and allowing to stand for approximately 10 minutes. The remaining TFA solution is removed and the column washed with H₂O followed by 1 CV 1M NaCl and additional H₂O. The siNA duplex product is then eluted, for example, using 1 CV 20% aqueous CAN.

[0365] FIG. 2 provides an example of MALDI-TOF mass spectrometry analysis of a purified siNA construct in which each peak corresponds to the calculated mass of an individual siNA strand of the siNA duplex. The same purified siNA provides three peaks when analyzed by capillary gel electrophoresis (CGE), one peak presumably corresponding to the duplex siNA, and two peaks presumably corresponding to the separate siNA sequence strands. Ion exchange HPLC analysis of the same siNA construct only shows a single peak. Testing of the purified siNA construct using a luciferase reporter assay described below demonstrated the same RNAi activity compared to siNA constructs generated from separately synthesized oligonucleotide sequence strands.

Example 2

Identification of Potential siNA Target Sites in Any RNA Sequence

[0366] The sequence of an RNA target of interest, such as a viral or human mRNA transcript, is screened for target sites, for example by using a computer folding algorithm. In a non-limiting example, the sequence of a gene or RNA gene transcript obtained from a database, such as Genbank, is used to generate siNA targets having complementarity to the target. Such sequences can be obtained from a database, or can be determined experimentally as known in the art. Target sites that are known, for example, those target sites determined to be effective target sites based on studies with other nucleic acid molecules, for example ribozymes or antisense, or those targets known to be associated with a disease or condition such as those sites containing mutations or deletions, can be used to design siNA molecules targeting those sites. Various parameters can be used to determine which sites are the most suitable target sites within the target RNA sequence. These parameters include but are not limited to secondary or tertiary RNA structure, the nucleotide base composition of the target sequence, the degree of homology between various regions of the target sequence, or the relative position of the target sequence within the RNA transcript. Based on these determinations, any number of target sites within the RNA transcript can be chosen to screen siNA molecules for efficacy, for example by using in vitro RNA cleavage assays, cell culture, or animal models. In a non-limiting example, anywhere from 1 to 1000 target sites are chosen within the transcript based on the size of the siNA construct to be used. High throughput screening assays can be developed for screening siNA molecules using methods known in the art, such as with multi-well or multi-plate assays to determine efficient reduction in target gene expression.

Example 3

Selection of siNA Molecule Target Sites in a RNA

[0367] The following non-limiting steps can be used to carry out the selection of siNAs targeting a given gene sequence or transcript.

[0368] 1. The target sequence is parsed in silico into a list of all fragments or subsequences of a particular length, for example 23 nucleotide fragments, contained within the target sequence. This step is typically carried out using a custom Perl script, but commercial sequence analysis programs such as Oligo, MacVector, or the GCG Wisconsin Package can be employed as well.

[0369] 2. In some instances the siNAs correspond to more than one target sequence; such would be the case for example in targeting different transcripts of the same gene, targeting different transcripts of more than one gene, or for targeting both the human gene and an animal homolog. In this case, a subsequence list of a particular length is generated for each of the targets, and then the lists are compared to find matching sequences in each list. The subsequences are then ranked according to the number of target sequences that contain the given subsequence; the goal is to find subsequences that are present in most or all of the target sequences. Alternately, the ranking can identify subsequences that are unique to a target sequence, such as a mutant target sequence. Such an approach would enable the use of siNA to target specifically the mutant sequence and not affect the expression of the normal sequence.

[0370] 3. In some instances the siNA subsequences are absent in one or more sequences while present in the desired target sequence; such would be the case if the siNA targets a gene with a paralogous family member that is to remain untargeted. As in case 2 above, a subsequence list of a particular length is generated for each of the targets, and then
the lists are compared to find sequences that are present in the target gene but are absent in the untargeted paralog.

[0371] 4. The ranked siRNA subsequences can be further analyzed and ranked according to GC content. A preference can be given to sites containing 30-70% GC, with a further preference to sites containing 40-60% GC.

[0372] 5. The ranked siRNA subsequences can be further analyzed and ranked according to self-folding and internal hairpins. Weaker internal folds are preferred; strong hairpin structures are to be avoided.

[0373] 6. The ranked siRNA subsequences can be further analyzed and ranked according to whether they have runs of GGG or CCC in the sequence. GGG (or even more Gs) in either strand can make oligonucleotide synthesis problematic and can potentially interfere with RNai activity, so it is avoided whenever better sequences are available. CCC is searched in the target strand because that will place GGG in the antisense strand.

[0374] 7. The ranked siRNA subsequences can be further analyzed and ranked according to whether they have the dinucleotide UU (uridine dinucleotide) on the 3'-end of the sequence, and/or AA on the 5'-end of the sequence (to yield 3' UU on the antisense sequence). These sequences allow one to design siRNA molecules with terminal TT thymidine dinucleotides.

[0375] 8. Four or five target sites are chosen from the ranked list of subsequences as described above. For example, in subsequences having 23 nucleotides, the right 21 nucleotides of each chosen 23-mer subsequence are then designed and synthesized for the upper (sense) strand of the siRNA duplex, while the reverse complement of the left 21 nucleotides of each chosen 23-mer subsequence are then designed and synthesized for the lower (antisense) strand of the siRNA duplex (see Tables II and III). If terminal TT residues are desired for the sequence (as described in paragraph 7), then the two 3' terminal nucleotides of both the sense and antisense strands are replaced by TT prior to synthesizing the oligos.

[0376] 9. The siRNA molecules are screened in an in vitro, cell culture or animal model system to identify the most active siRNA molecule or the most preferred target site within the target RNA sequence.


[0378] In an alternate approach, a pool of siRNA constructs specific to a CHRM3 target sequence is used to screen for target sites in cells expressing CHRM3 RNA, such as airway smooth muscle (ASM) or submucosal gland (e.g., serous or mucus) cells. The general strategy used in this approach is shown in FIG. 9. A non-limiting example of such is a pool comprising sequences having any of SEQ ID NOS 1-304. Cells expressing CHRM3 are transfected with the pool of siRNA constructs and cells that demonstrate a phenotype associated with CHRM3 inhibition are sorted. The pool of siRNA constructs can be expressed from transcription cassettes inserted into appropriate vectors (see for example FIG. 7 and FIG. 8). The siRNA from cells demonstrating a positive phenotypic change (e.g., decreased proliferation, decreased CHRM3 mRNA levels or decreased CHRM3 protein expression), are sequenced to determine the most suitable target site(s) within the target CHRM3 RNA sequence.

EXAMPLE 4

CHRM3 Targeted siRNA Design

[0379] siRNA target sites were chosen by analyzing sequences of the CHRM3 RNA target and optionally prioritizing the target sites on the basis of folding (structure of any given sequence analyzed to determine siRNA accessibility to the target), by using a library of siRNA molecules as described in Example 3, or alternately by using an in vitro siRNA system as described in Example 6 herein. siRNA molecules were designed that could bind each target and are optionally individually analyzed by computer folding to assess whether the siRNA molecule can interact with the target sequence. Varying the length of the siRNA molecules can be chosen to optimize activity. Generally, a sufficient number of complementary nucleotide bases are chosen to bind to, or otherwise interact with, the target RNA, but the degree of complementarity can be modulated to accommodate siRNA duplexes or varying length or base composition. By using such methodologies, siRNA molecules can be designed to target sites within any known RNA sequence, for example those RNA sequences corresponding to any gene transcript.

[0380] Chemically modified siRNA constructs are designed to provide nuclease stability for systemic administration in vivo and/or improved pharmacokinetic, localization, and delivery properties while preserving the ability to mediate RNai activity. Chemical modifications as described herein are introduced synthetically using synthetic methods described herein and those generally known in the art. The synthetic siRNA constructs are then assayed for nuclease stability in serum and/or cellular/tissue extracts (e.g., liver extracts). The synthetic siRNA constructs are also tested in parallel for RNai activity using an appropriate assay, such as a luciferase reporter assay as described herein or another suitable assay that can quantify RNai activity. Synthetic siRNA constructs that possess both nuclease stability and RNai activity can be further modified and re-evaluated in stability and activity assays. The chemical modifications of the stabilized active siRNA constructs can then be applied to any siRNA sequence targeting any chosen RNA and used, for example, in target screening assays to pick lead siRNA compounds for therapeutic development (see for example FIG. 11).

EXAMPLE 5

Chemical Synthesis and Purification of siRNA

[0381] siRNA molecules can be designed to interact with various sites in the RNA message, for example, target sequences within the RNA sequences described herein. The sequence of one strand of the siRNA molecule(s) is complementary to the target site sequences described above. The siRNA molecules can be chemically synthesized using methods described herein. Inactive siRNA molecules that are used as control sequences can be synthesized by scrambling the
sequence of the siNA molecules such that it is not complementary to the target sequence. Generally, siNA constructs can be synthesized using solid phase oligonucleotide synthesis methods as described herein (see for example Usman et al., U.S. Pat. Nos. 5,804,683; 5,831,071; 5,998,203; 6,117,657; 6,553,098; 6,362,323; 6,437,117; 6,469,158; Scaringe et al., U.S. Pat. Nos. 6,111,086; 6,008,400; 6,111,086 all incorporated by reference herein in their entirety).

[0382] In a non-limiting example, RNA oligonucleotides are synthesized in a stepwise fashion using the phosphorimidate chemistry as is known in the art. Standard phosphorimidate chemistry involves the use of nucleosides comprising any of 5'-O-dimethoxytrityl, 2',5'-O-tert-butyldimethylsilyl, 3',5'-O-2-Cyanoethyl N,N-disopropylphospho-phoramidite groups, and exocyclic amine protecting groups (e.g. N6-benzoyl adenosine, N4 acetyl cytidine, and N2-isobutyl guanosine). Alternately, 2'-O-Silyl Ethers can be used in conjunction with acid-labile 2'-O-orthoester protecting groups in the synthesis of RNA as described by Scaringe supra. Differing 2' chemistries can require different protecting groups, for example 2'-deoxy-2'-amino nucleosides can utilize N-phthaloyl protection as described by Usman et al., U.S. Pat. No. 5,631,360, incorporated by reference herein in its entirety).

[0383] During solid phase synthesis, each nucleotide is added sequentially (5'- to 3'-direction) to the solid support-bound oligonucleotide. The first nucleoside at the 3'-end of the chain is covalently attached to a solid support (e.g., controlled pore glass or polystyrene) using various linkers. The nucleotide precursor, a ribonucleoside phosphoramidite, and activator are combined resulting in the coupling of the second nucleoside phosphoramidite onto the 5'-end of the first nucleoside. The support is then washed and any unreacted 3'-hydroxyl groups are capped with a capping reagent such as acetic anhydride to yield inactive 5'-acetyl moieties. The trivalent phosphorus linkage is then oxidized to a more stable phosphate linkage. At the end of the nucleotide addition cycle, the 5'-protecting group is cleaved under suitable conditions (e.g., acidic conditions for trityl-based groups and Fluoride for silyl-based groups). The cycle is repeated for each subsequent nucleotide.

[0384] Modification of synthesis conditions can be used to optimize coupling efficiency, for example by using differing coupling times, differing reagent/phosphoramidite concentrations, differing contact times, differing solid supports and solid support linker chemistries depending on the particular chemical composition of the siNA to be synthesized. Deprotection and purification of the siNA can be performed as is generally described in Deprotection and purification of the siNA can be performed as is generally described in Usman et al., U.S. Pat. No. 5,831,071, U.S. Pat. No. 6,353,098, U.S. Pat. No. 6,437,117, and Bellon et al., U.S. Pat. No. 6,054,576, U.S. Pat. No. 6,162,909, U.S. Pat. No. 6,303,773, or Scaringe supra, incorporated by reference herein in their entirety. Additionally, deprotection conditions can be modified to provide the best possible yield and purity of siNA constructs. For example, applicant has observed that oligonucleotides comprising 2'-deoxy-2'-fluoro nucleotides can degrade under inappropriate deprotection conditions. Such oligonucleotides are deprotected using aqueous methylene at about 35° C. for 30 minutes. If the 2'-deoxy-2'-fluoro containing oligonucleotide also comprises ribonucleotides, after deprotection with aqueous methylene at about 35° C. for 30 minutes, TEA-HF is added and the reaction maintained at about 65° C. for an additional 15 minutes.

EXAMPLE 6

RNAi In Vitro Assay to Assess siNA Activity

[0385] An in vitro assay that recapitulates RNAi in a cell-free system is used to evaluate siNA constructs targeting CHRM3 RNA targets. The assay comprises the system described by Tuschi et al., 1999, *Genes and Development*, 13, 3191-3197 and Zamoore et al., 2000, *Cell*, 101, 25-33 adapted for use with CHRM3 target RNA. A *Drosophila* extract derived from syncytial blastoderm is used to reconstitute RNAi activity in vitro. Target RNA is generated via in vitro transcription from an appropriate CHRM3 expressing plasmid using T7 RNA polymerase or via chemical synthesis as described herein. Sense and antisense siRNA strands (for example 20 nt each) are annealed by incubation in buffer (such as 100 mM potassium acetate, 30 mM HEPES-KOH, pH 7.4, 2 mM magnesium acetate) for 1 minute at 90° C. followed by 1 hour at 37° C. then diluted in lysis buffer (for example 100 mM potassium acetate, 30 mM HEPES-KOH at pH 7.4, 2 mM magnesium acetate). Annealing can be monitored by gel electrophoresis on an agarose gel in TBE buffer and stained with ethidium bromide. The *Drosophila* lysate is prepared using zero to two-hour-old embryos from Oregon R flies collected on yeasted molasses agar that are dechorionated and lysed. The lysate is centrifuged and the supernatant isolated. The assay comprises a reaction mixture containing 50% lysate [vol/olv], RNA (10-50 pM final concentration), and 10% [vol/olv] lysis buffer containing siNA (10 nM final concentration). The reaction mixture also contains 10 mM creatine phosphate, 10 μg/ml creatine phosphokinase, 100 μM GTP, 100 μM UTP, 100 μM CTP, 500 μM ATP, 5 mM DTT, 0.1 U/μl RNAsin (Promega), and 100 μM of each amino acid. The final concentration of potassium acetate is adjusted to 100 mM. The reactions are pre-assembled on ice and preincubated at 25° C. for 10 minutes before adding RNA, then incubated at 25° C. for an additional 60 minutes. Reactions are quenched with 4 volumes of 1.25 x Passive Lysis Buffer (Promega). Target RNA cleavage is assayed by RT-PCR analysis or other methods known in the art and are compared to control reactions in which siNA is omitted from the reaction.

[0386] Alternately, internally-labeled target RNA for the assay is prepared by in vitro transcription in the presence of [α-32P] CTP, passed over a G 50 Sephadex column by spin chromatography and used as target RNA without further purification. Optionally, target RNA is 5'-32P-end labeled using T4 polynucleotide kinase enzyme. Assays are performed as described above and target RNA and the specific RNA cleavage products generated by RNAi are visualized on an autoradiograph of a gel. The percentage of cleavage is determined by PHOSPHOR IMAGE® software quantitation of bands representing intact control RNA or RNA from control reactions without siNA and the cleavage products generated by the assay.

[0387] In one embodiment, this assay is used to determine target sites the CHRM3 RNA target for siNA mediated RNAi cleavage, wherein a plurality of siNA constructs are screened for RNAi mediated cleavage of the CHRM3 RNA
target, for example, by analyzing the assay reaction by electrophoresis of labeled target RNA, or by northern blotting, as well as by other methodology well known in the art.

EXAMPLE 7

Nucleic Acid Inhibition of CHRM3 Target RNA In Vitro

[0388] siRNA molecules targeted to the human CHRM3 RNA are designed and synthesized as described above. These nucleic acid molecules can be tested for cleavage activity in vivo, for example, using the following procedure. The target sequences and the nucleotide location within the CHRM3 RNA are given in Table II and III.

[0389] Two formats are used to test the efficacy of siRNAs targeting CHRM3. First, the reagents are tested in cell culture using, for example, airway smooth muscle (ASM) or submucosal gland (e.g., serous or mucus) cells, to determine the extent of RNA and protein inhibition. siRNA reagents (e.g.; see Tables II and III) are selected against the CHRM3 target as described herein. RNA inhibition is measured after delivery of these reagents by a suitable transfection agent to, for example, airway smooth muscle (ASM) or submucosal gland (e.g., serous or mucus) cells. Relative amounts of target RNA are measured versus actin using real-time PCR monitoring of amplification (e.g., ABI 7700 TAQMAN®). A comparison is made to a mixture of oligonucleotide sequences made to unrelated targets or to a randomized siRNA control with the same overall length and chemistry, but randomly substituted at each position. Primary and secondary lead reagents are chosen for the target and optimization performed. After an optimal transfection agent concentration is chosen, a RNA time-course of inhibition is performed with the lead siRNA molecule. In addition, a cell-plating format can be used to determine RNA inhibition.

[0390] Delivery of siRNA to Cells

[0391] Cells (e.g., ASM or submucosal gland cells) are seeded, for example, at 1x10^5 cells per well of a six-well dish in EGM-2 (BioWhittaker) the day before transfection. siRNA (final concentration, for example 20 nM) and cationic lipid (e.g., final concentration 2 µg/ml) are complexed in EGM basal media (Bio Whittaker) at 37°C. For 30 minutes in polystyrene tubes. Following vortexing, the complexed siRNA is added to each well and incubated for the times indicated. For initial optimization experiments, cells are seeded, for example, at 1x10^5 in 96 well plates and siRNA complex added as described. Efficiency of delivery of siRNA to cells is determined using a fluorescent siRNA complexed with lipid. Cells in 6-well dishes are incubated with siRNA for 24 hours, rinsed with PBS and fixed in 2% paraformaldehyde for 15 minutes at room temperature. Uptake of siRNA is visualized using a fluorescent microscope.

[0392] TAQMAN® (Real-time PCR Monitoring of Amplification) and Lightcycler Quantification of mRNA

[0393] Total RNA is prepared from cells following siRNA delivery, for example, using Qiagen RNA purification kits for 6-well or RNeasy extraction kits for 96-well assays. For TAQMAN® analysis (real-time PCR monitoring of amplification), dual-labeled probes are synthesized with the reporter dye, FAM or JOE, covalently linked at the 5'-end and the quencher dye TAMRA conjugated to the 3'-end. One-step RT-PCR amplifications are performed on, for example, an ABI PRISM 7700 Sequence Detector using 50 µl reactions consisting of 10 µl total RNA, 100 nM forward primer, 900 nM reverse primer, 100 nM probe, 1x TAQMAN® PCR reaction buffer (PE-Applied Biosystems), 5.5 mM MgCl2, 300 µM each dATP, dCTP, dGTP, and dTTP, 10U RNase Inhibitor (Promega), 1.25U AMPLITaq GOLD® (DNA polymerase) (PE-Applied Biosystems) and 10U M-MLV Reverse Transcriptase (Promega). The thermal cycling conditions can consist of 30 minutes at 48°C, 10 minutes at 95°C, followed by 40 cycles of 15 seconds at 95°C and 1 minute at 60°C. Quantification of mRNA levels is determined relative to standards generated from serial dilution total cellular RNA (300, 100, 33, 11 ng/µl) and normalizing to β-actin or GAPDH mRNA in parallel TAQMAN® reactions (real-time PCR monitoring of amplification). For each gene of interest an upper and lower primer and a fluorescently labeled probe are designed. Real time incorporation of SYBR Green I dye into a specific PCR product can be measured in glass capillary tubes using a lightcycler. A standard curve is generated for each primer pair using control cRNA. Values are represented as relative expression to GAPDH in each sample.

[0394] Western Blotting

[0395] Nuclear extracts can be prepared using a standard micro preparation technique (see for example Andrews and Faller, 1991, Nucleic Acids Research, 19, 2499). Protein extracts from supernatants are prepared, for example using TCA precipitation. An equal volume of 20% TCA is added to the cell supernatant, incubated on ice for 1 hour and pelleted by centrifugation for 5 minutes. Pellets are washed in acetone, dried and resuspended in water. Cellular protein extracts are run on a 10% Bis-Tris NuPage (nuclear extracts) or 4-12% Tris-Glycine (supernatant extracts) polyacrylamide gel and transferred onto nitro-cellulose membranes. Non-specific binding can be blocked by incubation, for example, with 5% non-fat milk for 1 hour followed by primary antibody for 16 hour at 4°C. After washing, the secondary antibody is applied, for example (1:10,000 dilution) for 1 hour at room temperature and the signal detected with SuperSignal reagent (Pierce).

EXAMPLE 8

Animal Models Useful to Evaluate the Down-regulation of CHRM3 Gene Expression

various other animals including pig, sheep, and rabbits. Sugimoto et al., 2000, Immunopharmacology, 48, 1-7 and Shimizu et al., 2000, Am. J. Respir. Crit. Care Med., 161, 1648-54, describe rat models of allergic rhinitis. All of these models can be adapted for use for pre-clinical evaluation of the efficacy of nucleic acid compositions of the invention in modulating CHRM3 gene expression toward therapeutic use.

EXAMPLE 9

RNAi Mediated Inhibition of CHRM3 Expression in Cell Culture

The use of anticholinergic agents, anti-inflammatory, bronchodilators, adenosine inhibitors, adenosine A1 receptor inhibitors, non-selective M3 receptor antagonists such as atropine, ipratropium bromide and selective M3 receptor antagonists such as darifenacin and revatropate are all non-limiting examples of agents that can be combined with or used in conjunction with the nucleic acid molecules (e.g. siRNA molecules) of the instant invention. Those skilled in the art will recognize that other compounds and therapies used to treat the diseases and conditions described herein can similarly be combined with the nucleic acid molecules of the instant invention (e.g. siRNA molecules) and are hence within the scope of the instant invention.

EXAMPLE 11

Diagnostic Uses

The siRNA 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 siRNA molecules involves utilizing reconstructed RNAi systems, for example, using cellular lysates or partially purified cellular lysates. siRNA 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 siRNA 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 siRNA 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 siRNA 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 siRNA molecules targeted to different genes, siRNA molecules coupled with known small molecule inhibitors, or intermittent treatment with combinations siRNA molecules and/or other chemical or biological molecules). Other in vitro uses of siRNA 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 siRNA using standard methodologies, for example, fluorescence resonance emission transfer (FRET).

In a specific example, siRNA molecules that cleave only wild-type or mutant forms of the target RNA are used for the assay. The first siRNA 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 siRNA 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 siRNA molecules to demonstrate the relative siRNA 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 siRNA 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.

[0404] 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.

[0405] 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.

[0406] 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 siRNA molecules with improved RNAi activity.

[0407] 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.

[0408] 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>
<thead>
<tr>
<th>TABLE I</th>
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<tbody>
<tr>
<td>CHRMI Accession Numbers</td>
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<tr>
<td>Homo sapiens cholinergic receptor, muscarinic 3 (CHRMI), mRNA</td>
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The 3'-ends of the Upper sequence and the Lower sequence of the siRNA construct can include an overhang sequence, for example about 1, 2, 3, or 4 nucleotides in length, preferably 2 nucleotides in length, wherein the overhanging sequence of the Lower sequence is optionally complementary to a portion of the target sequence. The overhang can comprise the general structure B, BNN, NN, BNN, or NaN, where B stands for any terminal caps ology, N stands for any nucleotide (e.g., thymidine- and s stands for phosphorothioate or other internucleotide linkage as described herein (e.g. internucleotide linkage having Formula 1). The upper sequence is also referred to as the sense strand, whereas the lower sequence is also referred to as the antisense strand. The upper and lower sequences in the Table can further comprise a chemical modification having Formulae I-VII or any combination thereof (see for example chemical modifications as shown in Table V herein).

[0410]

TABLE III

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<td>GuGAgAuGGAccAAGAccAAT</td>
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<td>CHRM3:33L21 siRNA (315C) stab11 antisense</td>
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<td>Target Pos</td>
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**TABLE III-continued**

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<th>CHRM3 synthetic sRNA and Target Sequences</th>
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<th>Sequence</th>
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<tr>
<td>1713</td>
<td>B uGucucuGcuGGuAcuGcTcT</td>
<td>B 256</td>
</tr>
<tr>
<td>183</td>
<td>B cuuucuccuGGcGccccTcT</td>
<td>B 257</td>
</tr>
<tr>
<td>184</td>
<td>B uGucucuGcuGGuAcuGcTcT</td>
<td>B 258</td>
</tr>
<tr>
<td>185</td>
<td>B GCAGAUGGAcAAGAcccTcT</td>
<td>B 259</td>
</tr>
<tr>
<td>186</td>
<td>B AGCAGuAccAGCAGAGAcTcT</td>
<td>B 260</td>
</tr>
<tr>
<td>187</td>
<td>B AGCAGuAccAGCAGAGAcTcT</td>
<td>B 261</td>
</tr>
<tr>
<td>188</td>
<td>B uGucucuGcuGGuAcuGcTcT</td>
<td>B 262</td>
</tr>
<tr>
<td>189</td>
<td>B AACGAgGAgGGuGcUcTcT</td>
<td>B 263</td>
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<tr>
<td>190</td>
<td>B AACGAgGAgGGuGcUcTcT</td>
<td>B 264</td>
</tr>
<tr>
<td>191</td>
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<td>B 267</td>
</tr>
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<td>B uGucucuGcuGGuAcuGcTcT</td>
<td>B 268</td>
</tr>
<tr>
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<td>B 269</td>
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<td>B 270</td>
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<td>B AGCAGuAccAGCAGAGAcTcT</td>
<td>B 271</td>
</tr>
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<td>198</td>
<td>B uGucucuGcuGGuAcuGcTcT</td>
<td>B 272</td>
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<td>199</td>
<td>B GCAGAUGGAcAAGAcccTcT</td>
<td>B 273</td>
</tr>
<tr>
<td>200</td>
<td>B uGucucuGcuGGuAcuGcTcT</td>
<td>B 274</td>
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</tr>
<tr>
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<td>B uGucucuGcuGGuAcuGcTcT</td>
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Jan. 20, 2005
### TABLE III-continued

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<th>Sequence</th>
<th>Seq ID</th>
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<td>CHRM3;133L21 sIgNA (315C) stab10 antisense</td>
<td>GCCAGGCUCUAAAGAGAGTTT</td>
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<tr>
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<td>CUUCUCUUAAGCGGGCGCU</td>
<td>202</td>
<td>CHRM3;135L21 sIgNA (317C) stab10 antisense</td>
<td>CAGGCAGGCUCUAAAGAGTTT</td>
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<tr>
<td>973</td>
<td>GAGCAGAGUGCGAGACCCAG</td>
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<td>GUGGGCUUGGCUCCUCUGCTTT</td>
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<td>CHRM3;1732L21 sIgNA (1714C) stab10 antisense</td>
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<td>285</td>
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<td>CHRM3;1733L21 sIgNA (1715C) stab10 antisense</td>
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<td>286</td>
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### TABLE IV-continued

#### TABLE IV

<table>
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<tr>
<th>Chemistry</th>
<th>Pyrimidine</th>
<th>Purine</th>
<th>cap</th>
<th>p = S</th>
<th>Strand</th>
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<tbody>
<tr>
<td>“Stab 00”</td>
<td>Ribo</td>
<td>Ribo</td>
<td>3’-ends</td>
<td>S/AS</td>
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<tr>
<td>“Stab 1”</td>
<td>Ribo</td>
<td>Ribo</td>
<td>5 at 5’-end</td>
<td>S/AS</td>
<td></td>
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<tr>
<td>“Stab 2”</td>
<td>Ribo</td>
<td>Ribo</td>
<td>1 at 3’-end</td>
<td>S/AS</td>
<td></td>
</tr>
<tr>
<td>“Stab 3”</td>
<td>2’-fluoro</td>
<td>Ribo</td>
<td>Usual AS linkages</td>
<td>U</td>
<td>S</td>
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<tr>
<td>“Stab 4”</td>
<td>2’-fluoro</td>
<td>Ribo</td>
<td>5’-end</td>
<td>Usual S</td>
<td></td>
</tr>
<tr>
<td>“Stab 5”</td>
<td>2’-fluoro</td>
<td>Ribo</td>
<td>1 at 3’-end</td>
<td>Usual AS</td>
<td></td>
</tr>
<tr>
<td>“Stab 6”</td>
<td>2’-O-Methyl</td>
<td>Ribo</td>
<td>5’-end</td>
<td>Usual S</td>
<td></td>
</tr>
<tr>
<td>“Stab 7”</td>
<td>2’-fluoro</td>
<td>2’-deoxy</td>
<td>5’-end</td>
<td>Usual S</td>
<td></td>
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<tr>
<td>“Stab 8”</td>
<td>2’-fluoro</td>
<td>2’-O-</td>
<td>1 at 3’-end</td>
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<tr>
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<td>Ribo</td>
<td>5’-end</td>
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<tr>
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<td>5’-end</td>
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<tr>
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<td>LNA</td>
<td>1 at 3’-end</td>
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</table>

**CAP** = any terminal cap, see for example FIG. 10.

All Stab 1–12 chemistries can comprise 3’-terminal thymidine (TT) residues.

All Stab 1–22 chemistries typically comprise about 21 nucleotides, but can vary as described herein.

S = sense strand

AS = antisense strand

### TABLE V

<table>
<thead>
<tr>
<th>Reagent</th>
<th>Equivalents</th>
<th>Amount</th>
<th>Wait Time* DNA</th>
<th>Wait Time* 2’-O-methyl</th>
<th>Wait Time* RNA</th>
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<tbody>
<tr>
<td>Phosphonomimidates</td>
<td>6.5</td>
<td>163 µL</td>
<td>45 sec</td>
<td>2.5 min</td>
<td>7.5 min</td>
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<tr>
<td>S-Ethyl Tetrazole</td>
<td>23.8</td>
<td>238 µL</td>
<td>45 sec</td>
<td>2.5 min</td>
<td>7.5 min</td>
</tr>
<tr>
<td>Acetic Anhydride</td>
<td>100</td>
<td>233 µL</td>
<td>5 sec</td>
<td>5 sec</td>
<td>5 sec</td>
</tr>
<tr>
<td>N-Methyl Imidazole</td>
<td>186</td>
<td>233 µL</td>
<td>5 sec</td>
<td>5 sec</td>
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<td>TCA</td>
<td>176</td>
<td>2.3 mL</td>
<td>21 sec</td>
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A. 2.5 µmol Synthesis Cycle ABI 394 Instrument
TABLE V-continued

<table>
<thead>
<tr>
<th>Iodine</th>
<th>11.2</th>
<th>1.7 mL</th>
<th>45 sec</th>
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<tbody>
<tr>
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<td>12.9</td>
<td>645 μL</td>
<td>100 sec</td>
<td>300 sec</td>
<td>300 sec</td>
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<tr>
<td>Acetonitrile</td>
<td>NA</td>
<td>6.67 mL</td>
<td>NA</td>
<td>NA</td>
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</table>

B. 0.2 μmol Synthesis Cycle ABL 394 Instrument

| Phosphorimidites | 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 | 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 |
| Beauge | 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

<table>
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<tr>
<th></th>
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<td>180 sec</td>
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<td>S-Ethyl Tetrazole</td>
<td>70/105/210</td>
<td>40/60/120 μL</td>
<td>60 sec</td>
<td>180 min</td>
<td>360 sec</td>
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<td>Acetic Anhydride</td>
<td>265/265/265</td>
<td>50/50/50 μL</td>
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<td>10 sec</td>
<td>10 sec</td>
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<tr>
<td>N-Methyl</td>
<td>50/50/50/200</td>
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<td>10 sec</td>
<td>10 sec</td>
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<td>250/500/500 μL</td>
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<td>15 sec</td>
<td>15 sec</td>
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<td>Iodine</td>
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<td>80/80/80 μL</td>
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<td>30 sec</td>
<td>30 sec</td>
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<tr>
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<td>80/120/120</td>
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<td>200 sec</td>
<td>200 sec</td>
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<td>1150/1150/150 μL</td>
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Wait time does not include contact time during delivery.
Tandem synthesis utilizes double coupling of linker molecule

[0413] Sequence Listing:

[0414] Please incorporate the sequence listing into the application. The sequence listing is submitted herewith on CD in duplicate, in lieu of the paper copy, in compliance with 37 CFR §1.821(c) and 1.52 and on CD in compliance with 37 CFR § 1.821(c) (CRF copy). The sequence listing does not contain new matter.

[0415] As required by 37 CFR 1.821(f): the paper copy of the sequence listing submitted on CD-R (in lieu of paper copy) is the same as the CRF copy of the sequence listing, also submitted on CD-R.

What we claim is:

1. A chemically synthesized double stranded short interfering nucleic acid (siRNA) molecule that directs cleavage of a cholinergic receptor muscarinic 3 (CHRM3) RNA via RNA interference, wherein:
   a. each strand of said siRNA molecule is about 19 to about 23 nucleotides in length;
   b. one strand of said siRNA molecule comprises nucleotide sequence having sufficient complementarity to said CHRM3 RNA for the siRNA molecule to direct cleavage of the CHRM3 RNA via RNA interference; and
   c. said siRNA molecule does not require the presence of nucleotides having a 2'-hydroxy group for mediating RNA interference.

2. The siRNA molecule of claim 1, wherein said siRNA molecule comprises no ribonucleotides.

3. The siRNA molecule of claim 1, wherein said siRNA molecule comprises ribonucleotides.

4. The siRNA molecule of claim 1, wherein one strand of said double-stranded siRNA molecule comprises a nucleotide sequence that is complementary to a nucleotide sequence of a CHRM3 gene or a portion thereof, and wherein a second strand of said double-stranded siRNA molecule comprises a nucleotide sequence substantially similar to the nucleotide sequence or a portion thereof of said CHRM3 RNA.

5. The siRNA molecule of claim 4, wherein each strand of the siRNA molecule comprises about 19 to about 23 nucleotides, and wherein each strand comprises at least about 19 nucleotides that are complementary to the nucleotides of the other strand.

6. The siRNA molecule of claim 1, wherein said siRNA molecule comprises an antisense region comprising a nucleotide sequence that is complementary to a nucleotide sequence of a CHRM3 gene or a portion thereof, and wherein said antisense region comprises a nucleotide sequence substantially similar to the nucleotide sequence of said CHRM3 gene or a portion thereof.

7. The siRNA molecule of claim 6, wherein said antisense region and said sense region comprises about 19 to about 23 nucleotides, and wherein said antisense region comprises at least about 19 nucleotides that are complementary to nucleotides of the sense region.

8. The siRNA molecule of claim 1, wherein said siRNA molecule comprises a sense region and an antisense region, and wherein said antisense region comprises a nucleotide sequence that is complementary to a nucleotide sequence of RNA encoded by a CHRM3 gene, or a portion thereof, and said sense region comprises a nucleotide sequence that is complementary to said antisense region.

9. The siRNA molecule of claim 6, wherein said siRNA molecule is assembled from two separate oligonucleotide
fragments wherein one fragment comprises the sense region and a second fragment comprises the antisense region of said siNA molecule.

10. The siNA molecule of claim 6, wherein said sense region is connected to the antisense region via a linker molecule.

11. The siNA molecule of claim 10, wherein said linker molecule is a polyornucleotide linker.

12. The siNA molecule of claim 10, wherein said linker molecule is a non-nucleotide linker.

13. The siNA molecule of claim 6, wherein pyrimidine nucleotides in the sense region are 2′-O-methyl pyrimidine nucleotides.

14. The siNA molecule of claim 6, wherein purine nucleotides in the sense region are 2′-deoxy purine nucleotides.

15. The siNA molecule of claim 6, wherein pyrimidine nucleotides present in the sense region are 2′-deoxy-2′-fluoro pyrimidine nucleotides.

16. The siNA molecule of claim 9, wherein the fragment comprising said 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 comprising said sense region.

17. The siNA molecule of claim 16, wherein said terminal cap moiety is an inverted deoxy adenosine moiety.

18. The siNA molecule of claim 6, wherein pyrimidine nucleotides of said antisense region are 2′-deoxy-2′-fluoro pyrimidine nucleotides.

19. The siNA molecule of claim 6, wherein purine nucleotides of said antisense region are 2′-O-methyl purine nucleotides.

20. The siNA molecule of claim 6, wherein purine nucleotides present in said antisense region comprise 2′-deoxy-purine nucleotides.

21. The siNA molecule of claim 18, wherein said antisense region comprises a phosphorothioate internucleotide linkage at the 3′ end of said antisense region.

22. The siNA molecule of claim 6, wherein said antisense region comprises a glyceryl modification at the 3′ end of said antisense region.

23. The siNA molecule of claim 9, wherein each of the two fragments of said siNA molecule comprise 21 nucleotides.

24. The siNA molecule of claim 23, wherein about 19 nucleotides of each fragment of the siNA molecule are base-paired to the complementary nucleotides of the other fragment of the siNA molecule and wherein at least two 3′ terminal nucleotides of each fragment of the siNA molecule are not base-paired to the nucleotides of the other fragment of the siNA molecule.

25. The siNA molecule of claim 24, wherein each of the two 3′ terminal nucleotides of each fragment of the siNA molecule are 2′-deoxy-pyrimidines.

26. The siNA molecule of claim 25, wherein said 2′-deoxy-pyrimidine is 2′-deoxy-thymidine.

27. The siNA molecule of claim 23, wherein all 21 nucleotides of each fragment of the siNA molecule are base-paired to the complementary nucleotides of the other fragment of the siNA molecule.

28. The siNA molecule of claim 23, wherein about 19 nucleotides of the antisense region are base-paired to the nucleotide sequence of the RNA encoded by a CHRM3 gene or a portion thereof.

29. The siNA molecule of claim 23, wherein 21 nucleotides of the antisense region are base-paired to the nucleotide sequence of the RNA encoded by a CHRM3 gene or a portion thereof.

30. The siNA molecule of claim 9, wherein the 5′-end of the fragment comprising said antisense region optionally includes a phosphate group.

31. A pharmaceutical composition comprising the siNA molecule of claim 1 in an acceptable carrier or diluent.

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