RNA INTERFERENCE MEDIATED INHIBITION OF EPITHELIAL SODIUM CHANNEL (ENaC) GENE EXPRESSION USING SHORT INTERFERING NUCLEIC ACID (siNA)

[0001] This application claims the benefit of U.S. Provisional Application No. 61/158,316 filed March 6, 2009, U.S. Provisional Application No. 61/118,160 filed November 26, 2008, U.S. Provisional Application No. 61/118,157 filed November 26, 2008, U.S. Provisional Application No. 61/118,150 filed November 26, 2008, U.S. Provisional Application No. 61/118,144 filed November 26, 2008. The instant application claims the benefit of all the listed applications, which are hereby incorporated by reference herein in their entireties, including the drawings.

SEQUENCE LISTING

[0002] The sequence listing submitted via EFS, in compliance with 37 CFR §1.52(e)(5), is incorporated herein by reference. The sequence listing text file submitted via EFS contains the file "SequenceListing72WPCT", created on November 18 2009, which is 98,183 bytes in size.

FIELD OF THE INVENTION

[0003] The present invention relates to compounds, compositions, and methods for the study, diagnosis, and treatment of traits, diseases and conditions that respond to the modulation of epithelial sodium channel (hereinafter ENaC), also known as sodium channel non-neuronal 1 (SCNN1) or amiloride sensitive sodium channel (ASSC), gene expression and/or activity.

[0004] The present invention is also directed to compounds, compositions, and methods relating to traits, diseases and conditions that respond to the modulation of expression and/or activity of genes involved in epithelial sodium channel (ENaC) gene expression pathways or other cellular processes that mediate the maintenance or development of such traits, diseases and conditions. Specifically, the invention relates to double stranded nucleic acid molecules including small nucleic acid molecules, such as short interfering nucleic acid (siNA), short interfering RNA (siRNA), double-stranded RNA (dsRNA), micro-RNA (miRNA), and short hairpin RNA (shRNA) molecules capable of mediating or that mediate RNA interference

(RNAi) against epithelial sodium channel (ENaC) gene expression, including cocktails of such small nucleic acid molecules and lipid nanoparticle (LNP) formulations of such small nucleic acid molecules. The present invention also relates to small nucleic acid molecules, such as siNA, siRNA, and others that can inhibit the function of endogenous RNA molecules, such as endogenous ENaC micro-RNA (miRNA) (e.g, miRNA inhibitors) or endogenous ENaC short interfering RNA (siRNA), (e.g., siRNA inhibitors) or that can inhibit the function of RISC (e.g., RISC inhibitors), to modulate ENaC gene expression by interfering with the regulatory function of such endogenous RNAs or proteins associated with such endogenous RNAs (e.g., RISC), including cocktails of such small nucleic acid molecules and lipid nanoparticle (LNP) formulations of such small nucleic acid molecules. Such small nucleic acid molecules are useful, for example, in providing compositions for treatment of traits, diseases and conditions that can respond to modulation of ENaC gene expression in a subject or organism, such respiratory diseases, traits, and conditions, including but not limited to COPD, asthma, eosinophilic cough, bronchitis, cystic fibrosis, sarcoidosis, pulmonary fibrosis, rhinitis, sinusitis, and/or other disease states associated with ENaC gene expression or activity in a subject or organism.

BACKGROUND OF THE INVENTION

[0005] 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.

[0006] RNA interference refers to the process of sequence-specific post-transcriptional gene silencing in animals mediated by short interfering RNAs (siRNAs) (Zamore *et al.,* 2000, Cell, 101, 25-33; Fire *et al.,* 1998, *Nature,* 391, 806; Hamilton *et al.,* 1999, *Science*, 286, 950-951; Lin *et al.,* 1999, *Nature,* 402, 128-129; Sharp, 1999, Genes & Dev., 13:139-141; and Strauss, 1999, *Science*, 286, 886). The corresponding process in plants (Heifetz *et al.,* International PCT Publication No. WO 99/61631) is commonly referred to as post-transcriptional gene silencing or RNA silencing and is also referred to as quelling in fungi. The process of post-transcriptional gene silencing is thought to be an evolutionarily-conserved cellular defense mechanism used to prevent the expression of foreign genes and is commonly shared by diverse flora and phyla (Fire *et al.,* 1999, *Trends Genet.,* 15, 358). Such protection from foreign gene expression can have evolved in response to the production of double-stranded RNAs (dsRNAs) derived from viral infection or from the random integration

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

[0007] The presence of long dsRNAs in cells stimulates the activity of a ribonuclease III enzyme referred to as dicer (Bass, 2000, Cell, 101, 235; Zamore *et al.,* 2000, Cell, 101, 25-33; Hammond *et al.*, 2000, Nature, 404, 293). Dicer is involved in the processing of the dsRNA into short pieces of dsRNA known as short interfering RNAs (siRNAs) (Zamore *et al.,* 2000, Cell, 101, 25-33; Bass, 2000, Cell, 101, 235; Berstein *et al.,* 2001, *Nature,* 409, 363). Short interfering RNAs derived from dicer activity are typically about 21 to about 23 nucleotides in length and comprise about 19 base pair duplexes (Zamore *et al.,* 2000, Cell, 101, 25-33; Elbashir *et al.,* 2001, *Genes Dev.,* 15, 188). Dicer has also been implicated in the excision of 21- and 22-nucleotide small temporal RNAs (stRNAs) from precursor RNA of conserved structure that are implicated in translational control (Hutvagner *et al.,* 2001, *Science,* 293, 834). The RNAi response also features an endonuclease complex, commonly referred to as an RNA-induced silencing complex (RISC), which mediates cleavage of single-stranded RNA having sequence complementary to the antisense strand of the siRNA duplex. Cleavage of the target RNA takes place in the middle of the region complementary to the antisense strand of the siRNA duplex (Elbashir *et al.,* 2001, *Genes Dev.,* 15, 188).

[0008] RNAi has been studied in a variety of systems. Fire *et al.,* 1998, *Nature,* 391, 806, were the first to observe RNAi in *C. elegans*. Bahramian and Zarbl, 1999, *Molecular and Cellular Biology*, 19, 274-283 and Wianny and Goetz, 1999, *Nature Cell Biol.,* 2, 70, describe RNAi mediated by dsRNA in mammalian systems. Hammond *et al.,* 2000, *Nature,* 404, 293, describe RNAi in *Drosophila* cells transfected with dsRNA. Elbashir *et al.,* 2001, *Nature,* 411, 494 and Tuschl *et al.,* International PCT Publication No. WO 01/75164, describe RNAi induced by introduction of duplexes of synthetic 21-nucleotide RNAs in cultured mammalian cells including human embryonic kidney and HeLa cells. Recent work

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

[0009] Studies have shown that replacing the 3'-terminal nucleotide overhanging segments of a 21-mer siRNA duplex having two-nucleotide 3'-overhangs with deoxyribonucleotides does not have an adverse effect on RNAi activity. Replacing up to four nucleotides on each end of the siRNA with deoxyribonucleotides has been reported to be well tolerated, whereas complete substitution with deoxyribonucleotides results in no RNAi activity (Elbashir *et al.,* 2001, *EMBO J.*, 20, 6877 and Tuschl *et al.,* International PCT Publication No. WO 01/75164). In addition, Elbashir *et al., supra*, also report that substitution of siRNA with 2'-O-methyl nucleotides completely abolishes RNAi activity. Li *et al.,* International PCT Publication No. WO 00/44914, and Beach *et al.,* International PCT Publication No. WO 01/68836 preliminarily suggest that siRNA can include modifications to either the phosphate-sugar backbone or the nucleoside to include at least one of a nitrogen or sulfur heteroatom, however, neither application postulates to what extent such modifications would be tolerated in siRNA molecules, nor provides any further guidance or examples of such modified siRNA. Kreutzer *et al.*, Canadian Patent Application No. 2,359,180, also describe certain chemical modifications for use in dsRNA constructs in order to counteract activation of double-stranded RNA-dependent protein kinase PKR, specifically 2'-amino or 2'-O-methyl nucleotides, and nucleotides containing a 2'-O or 4'-C methylene bridge. However, Kreutzer

et al. similarly fails to provide examples or guidance as to what extent these modifications would be tolerated in dsRNA molecules.

[0010] Parrish *et al.*, 2000, *Molecular Cell*, 6, 1077-1087, tested certain chemical modifications targeting the unc-22 gene in *C. elegans* using long (>25 nt) siRNA transcripts. The authors describe the introduction of thiophosphate residues into these siRNA transcripts by incorporating thiophosphate nucleotide analogs with T7 and T3 RNA polymerase and observed that RNAs with two phosphorothioate modified bases also had substantial decreases in effectiveness as RNAi. Further, Parrish *et al.* reported that phosphorothioate modification of more than two residues greatly destabilized the RNAs *in vitro* such that interference activities could not be assayed. *Id.* at 1081. The authors also tested certain modifications at the 2'-position of the nucleotide sugar in the long siRNA transcripts and found that substituting deoxynucleotides for ribonucleotides produced a substantial decrease in interference activity, especially in the case of Uridine to Thymidine and/or Cytidine to deoxy-Cytidine substitutions. *Id.* In addition, the authors tested certain base modifications, including substituting, in sense and antisense strands of the siRNA, 4-thiouracil, 5-bromouracil, 5-iodouracil, and 3-(aminoallyl)uracil for uracil, and inosine for guanosine. Whereas 4-thiouracil and 5-bromouracil substitution appeared to be tolerated, Parrish reported that inosine produced a substantial decrease in interference activity when incorporated in either strand. Parrish also reported that incorporation of 5-iodouracil and 3-(aminoallyl)uracil in the antisense strand resulted in a substantial decrease in RNAi activity as well.

[0011] The use of longer dsRNA has been described. For example, Beach *et al.*, International PCT Publication No. WO 01/68836, describes specific methods for attenuating gene expression using endogenously-derived dsRNA. Tuschl *et al.*, International PCT Publication No. WO 01/75164, describe a *Drosophila in vitro* RNAi system and the use of specific siRNA molecules for certain functional genomic and certain therapeutic applications; although Tuschl, 2001, *Chem. Biochem.*, 2, 239-245, doubts that RNAi can be used to cure genetic diseases or viral infection due to the danger of activating interferon response. Li *et al.*, International PCT Publication No. WO 00/44914, describe the use of specific long (141 bp-488 bp) enzymatically synthesized or vector expressed dsRNAs for attenuating the expression of certain target genes. Zernicka-Goetz *et al.*, International PCT Publication No. WO 01/36646, describe certain methods for inhibiting the expression of particular genes in

mammalian cells using certain long (550 bp-714 bp), enzymatically synthesized or vector expressed dsRNA molecules. Fire *et al.,* International PCT Publication No. WO 99/32619, describe particular methods for introducing certain long dsRNA molecules into cells for use in inhibiting gene expression in nematodes. Plaetinck *et al.,* International PCT Publication No. WO 00/01846, describe certain methods for identifying specific genes responsible for conferring a particular phenotype in a cell using specific long dsRNA molecules. Mello *et al.,* International PCT Publication No. WO 01/29058, describe the identification of specific genes involved in dsRNA-mediated RNAi. Pachuck *et al.*, International PCT Publication No. WO 00/63364, describe certain long (at least 200 nucleotide) dsRNA constructs. Deschamps Depaillette *et al.,* International PCT Publication No. WO 99/07409, describe specific compositions consisting of particular dsRNA molecules combined with certain anti-viral agents. Waterhouse *et al.,* International PCT Publication No. 99/53050 and 1998, *PNAS*, 95, 13959-13964, describe certain methods for decreasing the phenotypic expression of a nucleic acid in plant cells using certain dsRNAs. Driscoll *et al.,* International PCT Publication No. WO 01/49844, describe specific DNA expression constructs for use in facilitating gene silencing in targeted organisms.

[0012] Others have reported on various RNAi and gene-silencing systems. For example, Parrish *et al.,* 2000, *Molecular Cell*, 6, 1077-1087, describe specific chemically-modified dsRNA constructs targeting the unc-22 gene of *C. elegans.* Grossniklaus, International PCT Publication No. WO 01/38551, describes certain methods for regulating polycomb gene expression in plants using certain dsRNAs. Churikov *et al.,* International PCT Publication No. WO 01/42443, describe certain methods for modifying genetic characteristics of an organism using certain dsRNAs. Cogoni *et al,* International PCT Publication No. WO 01/53475, describe certain methods for isolating a Neurospora silencing gene and uses thereof. Reed *et al.,* International PCT Publication No. WO 01/68836, describe certain methods for gene silencing in plants. Honer *et al.,* International PCT Publication No. WO 01/70944, describe certain methods of drug screening using transgenic nematodes as Parkinson's Disease models using certain dsRNAs. Deak *et al.,* International PCT Publication No. WO 01/72774, describe certain *Drosophila*-derived gene products that can be related to RNAi in *Drosophila.* Arndt *et al.,* International PCT Publication No. WO 01/92513 describe certain methods for mediating gene suppression by using factors that enhance RNAi. Tuschl *et al.,* International PCT Publication No. WO 02/44321, describe certain synthetic siRNA constructs. Pachuk *et al.,* International PCT Publication No. WO 00/63364, and

Satishchandran *et al.,* International PCT Publication No. WO 01/04313, describe certain methods and compositions for inhibiting the function of certain polynucleotide sequences using certain long (over 250 bp), vector expressed dsRNAs. Echeverri *et al.,* International PCT Publication No. WO 02/38805, describe certain *C. elegans* genes identified via RNAi. Kreutzer *et al.,* International PCT Publications Nos. WO 02/055692, WO 02/055693, and EP 1144623 B1 describes certain methods for inhibiting gene expression using dsRNA. Graham *et al.,* International PCT Publications Nos. WO 99/49029 and WO 01/70949, and AU 4037501 describe certain vector expressed siRNA molecules. Fire *et al.,* US 6,506,559, describe certain methods for inhibiting gene expression in vitro using certain long dsRNA (299 bp-1033 bp) constructs that mediate RNAi. Martinez *et al.,* 2002, *Cell*, 110, 563-574, describe certain single stranded siRNA constructs, including certain 5'-phosphorylated single stranded siRNAs that mediate RNA interference in Hela cells. Harborth *et al.,* 2003, Antisense & Nucleic Acid Drug Development, 13, 83-105, describe certain chemically and structurally modified siRNA molecules. Chiu and Rana, 2003, RNA, 9, 1034-1048, describe certain chemically and structurally modified siRNA molecules. Woolf *et al.,* International PCT Publication Nos. WO 03/064626 and WO 03/064625 describe certain chemically modified dsRNA constructs. Hornung *et al.,* 2005, *Nature Medicine,* 11, 263 – 270, describe the sequence-specific potent induction of IFN-alpha by short interfering RNA in plasmacytoid dendritic cells through TLR7. Judge *et al.,* 2005, Nature Biotechnology, Published online: 20 March 2005, describe the sequence-dependent stimulation of the mammalian innate immune response by synthetic siRNA. Yuki *et al.,* International PCT Publication Nos. WO 05/049821 and WO 04/048566, describe certain methods for designing short interfering RNA sequences and certain short interfering RNA sequences with optimized activity. Saigo *et al.,* US Patent Application Publication No. US20040539332, describe certain methods of designing oligo- or polynucleotide sequences, including short interfering RNA sequences, for achieving RNA interference. Tei *et al.,* International PCT Publication No. WO 03/044188, describe certain methods for inhibiting expression of a target gene, which comprises transfecting a cell, tissue, or individual organism with a double-stranded polynucleotide comprising DNA and RNA having a substantially identical nucleotide sequence with at least a partial nucleotide sequence of the target gene.

[0013] Mattick, 2005, *Science*, 309, 1527-1528; Claverie, 2005, *Science*, 309, 1529-1530; Sethupathy *et al.,* 2006, *RNA,* 12, 192-197; and Czech, 2006 *NEJM*, 354, 11: 1194-1195; Hutvagner *et al.,* US 20050227256, and Tuschl *et al.,* US 20050182005, all describe

antisense molecules that can inhibit miRNA function via steric blocking and are all incorporated by reference herein in their entirety.

[0014] The following U.S. Patent Application Publications provide basic descriptions of siRNA molecules and phosphodiesterases in general: US-20050287551; US-20050164220; US-20050191627; US-20050118594; US-20050153919; US-20050085486; and US-20030158133; all incorporated by reference herein in their entirety.

SUMMARY OF THE INVENTION

[0015] This invention relates to compounds, compositions, and methods useful for modulating the expression of epithelial sodium channel (ENaC) genes, such as those ENaC genes associated with the development or maintenance of inflammatory and/or respiratory diseases and conditions by RNA interference (RNAi) using short interfering nucleic acid (siNA) molecules. This invention also relates to compounds, compositions, and methods useful for modulating the expression and activity of other genes involved in pathways of ENaC 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 ENaC genes and/or other genes involved in pathways of ENaC gene expression and/or activity.

[0016] The instant invention also relates to small nucleic acid molecules, such as siNA, siRNA, and others that can inhibit the function of endogenous RNA molecules, such as endogenous micro-RNA (miRNA) (e.g, miRNA inhibitors) or endogenous short interfering RNA (siRNA), (e.g., siRNA inhibitors) or that can inhibit the function of RISC (e.g., RISC inhibitors), to modulate ENaC gene expression by interfering with the regulatory function of such endogenous RNAs or proteins associated with such endogenous RNAs (e.g., RISC). Such molecules are collectively referred to herein as RNAi inhibitors.

[0017] A siNA or RNAi inhibitor of the invention can be unmodified or chemically-modified. A siNA or RNAi inhibitor of the instant invention can be chemically synthesized, expressed from a vector or enzymatically synthesized. The instant invention also features various chemically-modified synthetic short interfering nucleic acid (siNA) molecules capable of modulating ENaC gene expression or activity in cells by RNA interference

(RNAi). The instant invention also features various chemically-modified synthetic short nucleic acid (siNA) molecules capable of modulating RNAi activity in cells by interacting with miRNA, siRNA, or RISC, and hence down regulating or inhibiting RNA interference (RNAi), translational inhibition, or transcriptional silencing in a cell or organism. The use of chemically-modified siNA and/or RNAi inhibitors improves various properties of native siNA molecules and/or RNAi inhibitors through increased resistance to nuclease degradation *in vivo* and/or through improved cellular uptake. Further, contrary to earlier published studies, siNA molecules of the invention having multiple chemical modifications, including fully modified siNA, has retained or improved RNAi activity over minimally modified or unmodified siRNA. Therefore, Applicant teaches herein chemically modified siRNA (generally referred to herein as siNA) that retains or improves upon the activity of native siRNA. The siNA molecules of the instant invention provide useful reagents and methods for a variety of therapeutic, prophylactic, cosmetic, veterinary, diagnostic, target validation, genomic discovery, genetic engineering, and pharmacogenomic applications.

[0018] The epithelial sodium channel (ENaC, or sodium channel non-neuronal 1 (SCNN1) or amiloride sensitive sodium channel (ASSC)) is a membrane-bound ion-channel that is permeable for Li^+, protons and especially Na^+. It is a 'constitutively active' channel, ie. does not require a gating stimulus and is open at rest. ENaC is a heteromeric protein comprised of three different subunits - α (SCNN1A), β (SCNN1B), and γ (SCNN1G). The exact stoichiometry was until recently unclear, but based on homology to ASIC channels, is almost certainly a heterotrimer (Jasti, J. et al (2007) Nature 449 pp316 to 323). Each subunit consists of two transmembrane helices and an extracellular loop. The amino- and carboxy-termini of all polypeptides are located in the cytosol. In addition there is a fourth, so-called δ-subunit, that shares significant homology with the α-subunit and can form a functional ion-channel together with the β- and γ-subunits.

[0019] In one embodiment, the invention features one or more siNA molecules and/or RNAi inhibitors and methods that independently or in combination modulate the expression of ENaC gene(s) encoding epithelial sodium channel (ENaC) such as genes encoding the α (SCNN1A), β (SCNN1B), or γ (SCNN1G) subunit sequences comprising those sequences referred to by GenBank Accession Nos. shown in **Table 7**. References herein to "ENaC" include any or all of the α (SCNN1A), β (SCNN1B), or γ (SCNN1G) subunit sequences. In a preferred embodiment the invention features one or more siNA molecules and/or RNAi

inhibitors and methods that independently or in combination modulate the expression of ENaC gene(s) encoding the α (SCNN1A) subunit. The description below of the various aspects and embodiments of the invention is provided with reference to exemplary encoding epithelial sodium channel (ENaC) genes. The present invention is also directed to compounds, compositions, and methods relating to traits, diseases and conditions that respond to the modulation of expression and/or activity of genes involved in encoding epithelial sodium channel (ENaC) gene expression pathways or other cellular processes that mediate the maintenance or development of such traits, diseases and conditions. However, such reference is meant to be exemplary only and the various aspects and embodiments of the invention are also directed to other genes that express alternate ENaC genes, such as mutant ENaC genes, isotypes of ENaC genes, ENaC variants from species to species or subject to subject and alternatively spliced variants of the ENaC mRNA ("splice variants"). Such additional genes can be analyzed for target sites using the methods described herein for exemplary ENaC genes and sequences herein. Thus, the modulation and the effects of such modulation of the other genes can be performed as described herein. In other words, the term "ENaC" as it is defined herein below and recited in the described embodiments, is meant to encompass genes associated with the development and/or maintenance of diseases, traits and conditions herein, such as genes which encode ENaC polypeptides, ENaC regulatory polynucleotides (e.g., ENaC miRNAs and siRNAs), mutant ENaC genes, and isotypes of ENaC genes, as well as other genes involved in ENaC pathways of gene expression and/or activity. Thus, each of the embodiments described herein with reference to the term "ENaC" are applicable to all of the protein, peptide, polypeptide, and/or polynucleotide molecules covered by the term "ENaC", as that term is defined herein. Comprehensively, such gene targets are also referred to herein generally as "target" sequences.

[0020] In one embodiment, the invention features a composition comprising two or more different siNA molecules and/or RNAi inhibitors of the invention (e.g., siNA, duplex forming siNA, or multifunctional siNA or any combination thereof) targeting different polynucleotide targets, such as different regions of ENaC RNA or DNA (e.g., two different target sites herein or any combination of ENaC targets such as different isotypes) or both coding and non-coding targets. Such pools of siNA molecules can provide increased therapeutic effect.

[0021] In one embodiment, the invention features a pool of two or more different siNA molecules of the invention (e.g., siNA, duplex foming siNA, or multifunctional siNA or any

combination thereof) that have specificity for different polynucleotide targets, such as different regions of target ENaC RNA or DNA (e.g., two different target sites herein or any combination of ENaC targets or pathway targets such as different ENaC isotypes) or both coding and non-coding targets, wherein the pool comprises siNA molecules targeting about 2, 3, 4, 5, 6, 7, 8, 9, 10 or more different ENaC targets.

[0022] In one embodiment, the invention features a pool of two or more different siNA molecules and/or RNAi inhibitors that have specificity for a ENaC target, such as different ENaC isotype targets or any combination thereof. In one embodiment, the invention features a pool of two or more different siNA molecules and/or RNAi inhibitors that have specificity for ENaC. In one embodiment, the invention features a pool of two or more different siNA molecules and/or RNAi inhibitors that have specificity for ENaC. In one embodiment, the invention features a pool of two or more different siNA molecules and/or RNAi inhibitors that have specificity for ENaC and a isotype thereof.

[0023] Due to the potential for sequence variability of the ENaC gene across different organisms or different subjects, selection of siNA molecules for broad therapeutic applications likely involve the conserved regions of the ENaC gene. In one embodiment, the present invention relates to siNA molecules and/or RNAi inhibitors that target conserved regions of the ENaC gene or regions that are conserved across different ENaC targets. siNA molecules and/or RNAi inhibitors designed to target conserved regions of various ENaC targets enable efficient inhibition of ENaC target gene expression in diverse patient populations. Due to variations in enzymatic activity and cell-specific expression patterns of ENaC isoforms, selection of siNA molecules for treatment of target therapeutic applications likely involve specific ENaC isotypes. In one embodiment, the present invention relates to siNA molecules and/or RNAi inhibitors that target conserved regions of the ENaC gene or regions that are conserved across different ENaC targets. In another embodiment, the invention features a double-stranded siNA that down regulates expression of a target ENaC gene or directs cleavage of an ENaC target RNA, without affecting ENaC expression. siNA molecules and/or RNAi inhibitors designed to target conserved regions of various ENaC targets enable efficient inhibition of ENaC isotype expression in diverse patient populations.

[0024] In one embodiment, the invention features a double stranded nucleic acid molecule, such as an siNA molecule, where one of the strands comprises nucleotide sequence having complementarity to a predetermined nucleotide sequence in an ENaC target nucleic

acid molecule, or a portion thereof. In one embodiment, the predetermined nucleotide sequence is a nucleotide ENaC target sequence described herein. In another embodiment, the predetermined nucleotide sequence is a ENaC target sequence as is known in the art.

[0025] In one embodiment, the invention features a double-stranded short interfering nucleic acid (siNA) molecule that down-regulates expression of a ENaC target gene or that directs cleavage of a ENaC target RNA, wherein said siNA molecule comprises about 15 to about 30 base pairs.

[0026] In one embodiment, the invention features a double-stranded short interfering nucleic acid (siNA) molecule that directs cleavage of an ENaC target RNA, wherein said siNA molecule comprises about 15 to about 30 base pairs.

[0027] In one embodiment, the invention features a double stranded short interfering nucleic acid (siNA) molecule that directs cleavage of a target ENaC RNA via RNA interference (RNAi), wherein the double stranded siNA molecule comprises a first and a second strand, each strand of the siNA molecule is about 15 to about 30 (e.g., about 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, or 30) nucleotides in length, the first strand of the siNA molecule comprises nucleotide sequence having sufficient complementarity to the target ENaC RNA for the siNA molecule to direct cleavage of the target ENaC RNA via RNA interference, and the second strand of said siNA molecule comprises nucleotide sequence that is complementary to the first strand. In one specific embodiment, for example, each strand of the siNA molecule is about 15 to about 30 nucleotides in length.

[0028] In one embodiment, the invention features a double stranded short interfering nucleic acid (siNA) molecule that directs cleavage of a ENaC target RNA via RNA interference (RNAi), wherein the double stranded siNA molecule comprises a first and a second strand, each strand of the siNA molecule is about 18 to about 23 (e.g., about 18, 19, 20, 21, 22, or 23) nucleotides in length, the first strand of the siNA molecule comprises nucleotide sequence having sufficient complementarity to the ENaC target RNA for the siNA molecule to direct cleavage of the ENaC target RNA via RNA interference, and the second strand of said siNA molecule comprises nucleotide sequence that is complementary to the first strand.

[0029] In one embodiment, the invention features a chemically synthesized double stranded short interfering nucleic acid (siNA) molecule that directs cleavage of an ENaC

target RNA via RNA interference (RNAi), wherein each strand of the siNA molecule is about 15 to about 30 nucleotides in length; and one strand of the siNA molecule comprises nucleotide sequence having sufficient complementarity to the ENaC target RNA for the siNA molecule to direct cleavage of the ENaC target RNA via RNA interference.

[0030] In one embodiment, the invention features a chemically synthesized double stranded short interfering nucleic acid (siNA) molecule that directs cleavage of an ENaC target RNA via RNA interference (RNAi), wherein each strand of the siNA molecule is about 18 to about 23 nucleotides in length; and one strand of the siNA molecule comprises nucleotide sequence having sufficient complementarity to the ENaC target RNA for the siNA molecule to direct cleavage of the ENaC target RNA via RNA interference.

[0031] In one embodiment, the invention features a siNA molecule that down-regulates expression of an ENaC target gene or that directs cleavage of a ENaC target RNA, for example, wherein the ENaC target gene or RNA comprises protein encoding sequence. In one embodiment, the invention features a siNA molecule that down-regulates expression of a ENaC target gene or that directs cleavage of a ENaC target RNA, for example, wherein the ENaC target gene or RNA comprises non-coding sequence or regulatory elements involved in ENaC target gene expression (*e.g.*, non-coding RNA, miRNA, stRNA etc.).

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

genes. In this approach, a single siNA can be used to inhibit expression of more than one gene instead of using more than one siNA molecule to target the different genes.

[0033] In one embodiment, the invention features a siNA molecule having RNAi activity against ENaC target RNA (*e.g.*, coding or non-coding RNA), wherein the siNA molecule comprises a sequence complementary to any ENaC RNA sequence, such as those sequences having ENaC GenBank Accession Nos. shown in **Table 7** herein. In another embodiment, the invention features a siNA molecule having RNAi activity against ENaC target RNA, wherein the siNA molecule comprises a sequence complementary to an RNA having ENaC variant encoding sequence, for example other mutant ENaC genes known in the art to be associated with the maintenance and/or development of diseases, traits, disorders, and/or conditions described herein or otherwise known in the art. Chemical modifications as shown in **Table 8** or otherwise described herein can be applied to any siNA construct of the invention. In another embodiment, a siNA molecule of the invention includes a nucleotide sequence that can interact with nucleotide sequence of a ENaC target gene and thereby mediate silencing of ENaC target gene expression, for example, wherein the siNA mediates regulation of ENaC target gene expression by cellular processes that modulate the chromatin structure or methylation patterns of the ENaC target gene and prevent transcription of the ENaC target gene.

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

[0035] In one embodiment of the invention a siNA molecule comprises an antisense strand comprising a nucleotide sequence that is complementary to an ENaC nucleotide

sequence or a portion thereof encoding an ENaC target protein. The siNA further comprises a sense strand, wherein said sense strand comprises a nucleotide sequence of an ENaC target gene or a portion thereof.

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

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

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

[0039] In yet another embodiment, the invention features a siNA molecule comprising a sequence, for example, the antisense sequence of the siNA construct, complementary to a sequence or portion of sequence comprising sequence represented by GenBank Accession Nos. shown in **Table 7**. Chemical modifications in **Tables 1b and 8** and described herein can be applied to any siNA construct of the invention. LNP formulations described in **Table 10** can be applied to any siNA molecule or combination of siNA molecules herein.

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

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

[0042] In one embodiment, a siNA molecule of the invention (e.g., a double stranded nucleic acid molecule) comprises a sense (passenger) strand having about 15 to about 30 (*e.g.*, about 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, or 30) nucleotides that comprise sequence of an ENaC RNA or a portion thereof. In one embodiment, at least 15 nucleotides (e.g., about 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, or 30) nucleotides of an ENaC RNA sequence comprise the sense (passenger) strand of a siNA molecule of the invention.

[0043] In another embodiment of the invention a siNA molecule of the invention comprises an antisense region having about 15 to about 30 (*e.g.*, about 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, or 30) nucleotides, wherein the antisense region is complementary to an ENaC target DNA sequence, and wherein said siNA further comprises a sense region having about 15 to about 30 (*e.g.*, about 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, or 30) nucleotides, wherein said sense region and said antisense region are comprised in a linear molecule where the sense region comprises at least about 15 nucleotides that are complementary to the antisense region.

[0044] In one embodiment, a siNA molecule of the invention has RNAi activity that modulates expression of ENaC RNA encoded by one or more ENaC genes. Because ENaC genes can share some degree of sequence homology with each other, siNA molecules can be designed to target a class of ENaC genes, by selecting sequences that are either shared amongst different ENaC targets, alternatively that are unique for a specific ENaC target (e.g., unique for any ENaC isotype). Therefore, in one embodiment, the siNA molecule can be designed to target conserved regions of ENaC polynucleotide sequences having homology among several ENaC gene variants so as to target a class of ENaC genes with one siNA molecule. Accordingly, in one embodiment, the siNA molecule of the invention modulates the expression of one or more ENaC isoforms in a subject or organism. In another

embodiment, the siNA molecule can be designed to target a sequence that is unique to a specific ENaC polynucleotide sequence (*e.g.*, a single ENaC isoform or ENaC single nucleotide polymorphism (SNP)) due to the high degree of specificity that the siNA molecule requires to mediate RNAi activity.

[0045] 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 duplex nucleic acid molecules containing about 15 to about 30 base pairs between oligonucleotides comprising about 15 to about 30 (*e.g.*, about 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, or 30) nucleotides. In yet another embodiment, siNA molecules of the invention comprise duplex nucleic acid molecules with overhanging ends of about 1 to about 3 (*e.g.*, about 1, 2, or 3) nucleotides, for example, about 21-nucleotide duplexes with about 19 base pairs and 3'-terminal mononucleotide, dinucleotide, or trinucleotide overhangs. In yet another embodiment, siNA molecules of the invention comprise duplex nucleic acid molecules with blunt ends, where both ends are blunt, or alternatively, where one of the ends is blunt.

[0046] In one embodiment, a double stranded nucleic acid (*e.g.*, siNA) molecule comprises nucleotide or non-nucleotide overhangs. By "overhang" is meant a terminal portion of the nucleotide sequence that is not base paired between the two strands of a double stranded nucleic acid molecule (see for example **Figure 6**). In one embodiment, a double stranded nucleic acid molecule of the invention can comprise nucleotide or non-nucleotide overhangs at the 3'-end of one or both strands of the double stranded nucleic acid molecule. For example, a double stranded nucleic acid molecule of the invention can comprise a nucleotide or non-nucleotide overhang at the 3'-end of the guide strand or antisense strand/region, the 3'-end of the passenger strand or sense strand/region, or both the guide strand or antisense strand/region and the passenger strand or sense strand/region of the double stranded nucleic acid molecule. In another embodiment, the nucleotide overhang portion of a double stranded nucleic acid (siNA) molecule of the invention comprises 2'-O-methyl, 2'-deoxy, 2'-deoxy-2'-fluoro, 2'-deoxy-2'-fluoroarabino (FANA), 4'-thio, 2'-O-trifluoromethyl, 2'-O-ethyl-trifluoromethoxy, 2'-O-difluoromethoxy-ethoxy, universal base, acyclic, or 5-C-methyl nucleotides. In another embodiment, the non-nucleotide overhang portion of a double stranded nucleic acid (siNA) molecule of the invention comprises glyceryl, abasic, or inverted deoxy abasic non-nucleotides.

[0047] In one embodiment, the nucleotides comprising the overhang portions of a double stranded nucleic acid (*e.g.*, siNA) molecule of the invention correspond to the nucleotides comprising the ENaC target polynucleotide sequence of the siNA molecule. Accordingly, in such embodiments, the nucleotides comprising the overhang portion of a siNA molecule of the invention comprise sequence based on the ENaC target polynucleotide sequence in which nucleotides comprising the overhang portion of the guide strand or antisense strand/region of a siNA molecule of the invention can be complementary to nucleotides in the ENaC target polynucleotide sequence and nucleotides comprising the overhang portion of the passenger strand or sense strand/region of a siNA molecule of the invention can comprise the nucleotides in the ENaC target polynucleotide sequence. Such nucleotide overhangs comprise sequence that would result from Dicer processing of a native dsRNA into siRNA.

[0048] In one embodiment, the nucleotides comprising the overhang portion of a double stranded nucleic acid (*e.g.*, siNA) molecule of the invention are complementary to the ENaC target polynucleotide sequence and are optionally chemically modified as described herein. As such, in one embodiment, the nucleotides comprising the overhang portion of the guide strand or antisense strand/region of a siNA molecule of the invention can be complementary to nucleotides in the ENaC target polynucleotide sequence, i.e. those nucleotide positions in the ENaC target polynucleotide sequence that are complementary to the nucleotide positions of the overhang nucleotides in the guide strand or antisense strand/region of a siNA molecule. In another embodiment, the nucleotides comprising the overhang portion of the passenger strand or sense strand/region of a siNA molecule of the invention can comprise the nucleotides in the ENaC target polynucleotide sequence, i.e. those nucleotide positions in the ENaC target polynucleotide sequence that correspond to same the nucleotide positions of the overhang nucleotides in the passenger strand or sense strand/region of a siNA molecule. In one embodiment, the overhang comprises a two nucleotide (*e.g.*, 3'-GA; 3'-GU; 3'-GG; 3'GC; 3'-CA; 3'-CU; 3'-CG; 3'CC; 3'-UA; 3'-UU; 3'-UG; 3'UC; 3'-AA; 3'-AU; 3'-AG; 3'-AC; 3'-TA; 3'-TU; 3'-TG; 3'-TC; 3'-AT; 3'-UT; 3'-GT; 3'-CT) overhang that is complementary to a portion of the ENaC target polynucleotide sequence. In one embodiment, the overhang comprises a two nucleotide (*e.g.*, 3'-GA; 3'-GU; 3'-GG; 3'GC; 3'-CA; 3'-CU; 3'-CG; 3'CC; 3'-UA; 3'-UU; 3'-UG; 3'UC; 3'-AA; 3'-AU; 3'-AG; 3'-AC; 3'-TA; 3'-TU; 3'-TG; 3'-TC; 3'-AT; 3'-UT; 3'-GT; 3'-CT) overhang that is not complementary to a portion of the ENaC target polynucleotide sequence. In another embodiment, the overhang nucleotides of a siNA molecule of the invention are 2'-O-methyl nucleotides, 2'-deoxy-2'-

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

[0049] In one embodiment, the nucleotides comprising the overhang portion of a double stranded nucleic acid (*e.g.*, siNA) molecule of the invention are not complementary to the ENaC target polynucleotide sequence and are optionally chemically modified as described herein. In one embodiment, the overhang comprises a 3'-UU overhang that is not complementary to a portion of the ENaC target polynucleotide sequence. In another embodiment, the nucleotides comprising the overhanging portion of a siNA molecule of the invention are 2'-O-methyl nucleotides, 2'-deoxy-2'-fluoroarabino and/or 2'-deoxy-2'-fluoro nucleotides.

[0050] In one embodiment, the double stranded nucleic molecule (*e.g.* siNA) of the invention comprises a two or three nucleotide overhang, wherein the nucleotides in the overhang are the same or different. In one embodiment, the double stranded nucleic molecule (*e.g.* siNA) of the invention comprises a two or three nucleotide overhang, wherein the nucleotides in the overhang are the same or different and wherein one or more nucleotides in the overhang are chemically modified at the base, sugar and/or phosphate backbone.

[0051] In one embodiment, the invention features one or more chemically-modified siNA constructs having specificity for ENaC target nucleic acid molecules, such as DNA, or RNA encoding a protein or non-coding RNA associated with the expression of ENaC target genes. In one embodiment, the invention features a RNA based siNA molecule (*e.g.*, a siNA comprising 2'-OH nucleotides) having specificity for nucleic acid molecules that includes one or more chemical modifications described herein. Non-limiting examples of such chemical modifications include without limitation phosphorothioate internucleotide linkages, 2'-deoxyribonucleotides, 2'-O-methyl ribonucleotides, 2'-deoxy-2'-fluoro ribonucleotides, 4'-thio ribonucleotides, 2'-O-trifluoromethyl nucleotides, 2'-O-ethyl-trifluoromethoxy

nucleotides, 2'-O-difluoromethoxy-ethoxy nucleotides (see for example USSN 10/981,966 filed November 5, 2004, incorporated by reference herein), "universal base" nucleotides, "acyclic" nucleotides, 5-C-methyl nucleotides, 2'-deoxy-2'-fluoroarabino (FANA, see for example Dowler et al., 2006, Nucleic Acids Research, 34, 1669-1675) and terminal glyceryl and/or inverted deoxy abasic residue incorporation. These chemical modifications, when used in various siNA constructs, (*e.g.*, RNA based siNA constructs), are shown to preserve RNAi activity in cells while at the same time, dramatically increasing the serum stability of these compounds.

[0052] In one embodiment, a siNA molecule of the invention comprises chemical modifications described herein (e.g., 2'-O-methyl ribonucleotides, 2'-deoxy-2'-fluoro ribonucleotides, 4'-thio ribonucleotides, 2'-O-trifluoromethyl nucleotides, 2'-O-ethyl-trifluoromethoxy nucleotides, 2'-O-difluoromethoxy-ethoxy nucleotides, LNA) at the internal positions of the siNA molecule. By "internal position", is meant the base paired positions of a siNA duplex.

[0053] In one embodiment, the invention features one or more chemically-modified siNA constructs having specificity for target ENaC nucleic acid molecules, such as ENaC DNA, or ENaC RNA encoding an ENaC protein or non-coding RNA associated with the expression of target ENaC genes.

[0054] In one embodiment, the invention features a RNA based siNA molecule (e.g., a siNA comprising 2'-OH nucleotides) having specificity for nucleic acid molecules that includes one or more chemical modifications described herein. Non-limiting examples of such chemical modifications include without limitation phosphorothioate internucleotide linkages, 2'-deoxyribonucleotides, 2'-O-methyl ribonucleotides, 2'-deoxy-2'-fluoro ribonucleotides, 4'-thio ribonucleotides, 2'-O-trifluoromethyl nucleotides, 2'-O-ethyl-trifluoromethoxy nucleotides, 2'-O-difluoromethoxy-ethoxy nucleotides (see for example USSN 10/981,966 filed November 5, 2004, incorporated by reference herein), "universal base" nucleotides, "acyclic" nucleotides, 5-C-methyl nucleotides, and terminal glyceryl and/or inverted deoxy abasic residue incorporation. These chemical modifications, when used in various siNA constructs, (e.g., RNA based siNA constructs), are shown to preserve RNAi activity in cells while at the same time, dramatically increasing the serum stability of these compounds. 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.

[0055] In one embodiment, a siNA molecule of the invention comprises modified nucleotides while maintaining the ability to mediate RNAi. The modified nucleotides can be used to improve *in vitro* or *in vivo* characteristics such as stability, activity, toxicity, immune response, and/or bioavailability. For example, a siNA molecule of the invention can comprise modified nucleotides as a percentage of the total number of nucleotides present in the siNA molecule. As such, a siNA molecule of the invention can generally comprise about 5% to about 100% modified nucleotides (*e.g.*, about 5%, 10%, 15%, 20%, 25%, 30%, 35%, 40%, 45%, 50%, 55%, 60%, 65%, 70%, 75%, 80%, 85%, 90%, 95% or 100% modified nucleotides). For example, in one embodiment, between about 5% to about 100% (*e.g.*, about 5%, 10%, 15%, 20%, 25%, 30%, 35%, 40%, 45%, 50%, 55%, 60%, 65%, 70%, 75%, 80%, 85%, 90%, 95% or 100% modified nucleotides) of the nucleotide positions in a siNA molecule of the invention comprise a nucleic acid sugar modification, such as a 2'-sugar modification, *e.g.*, 2'-O-methyl nucleotides, 2'-deoxy-2'-fluoro nucleotides, 2'-deoxy-2'-fluoroarabino, 2'-O-methoxyethyl nucleotides, 2'-O-trifluoromethyl nucleotides, 2'-O-ethyl-trifluoromethoxy nucleotides, 2'-O-difluoromethoxy-ethoxy nucleotides, or 2'-deoxy nucleotides. In another embodiment, between about 5% to about 100% (*e.g.*, about 5%, 10%, 15%, 20%, 25%, 30%, 35%, 40%, 45%, 50%, 55%, 60%, 65%, 70%, 75%, 80%, 85%, 90%, 95% or 100% modified nucleotides) of the nucleotide positions in a siNA molecule of the invention comprise a nucleic acid base modification, such as inosine, purine, pyridin-4-one, pyridin-2-one, phenyl, pseudouracil, 2, 4, 6-trimethoxy benzene, 3-methyl uracil, dihydrouridine, naphthyl, aminophenyl, 5-alkylcytidines (*e.g.*, 5-methylcytidine), 5-alkyluridines (*e.g.*, ribothymidine), 5-halouridine (*e.g.*, 5-bromouridine) or 6-azapyrimidines or 6-alkylpyrimidines (*e.g.* 6-methyluridine), or propyne modifications. In another embodiment, between about 5% to about 100% (*e.g.*, about 5%, 10%, 15%, 20%, 25%, 30%, 35%, 40%, 45%, 50%, 55%, 60%, 65%, 70%, 75%, 80%, 85%, 90%, 95% or 100% modified nucleotides) of the nucleotide positions in a siNA molecule of the invention comprise a nucleic acid backbone modification, such as a backbone modification having Formula I herein. In another embodiment, between about 5% to about 100% (*e.g.*, about 5%, 10%, 15%, 20%, 25%, 30%, 35%, 40%, 45%, 50%, 55%, 60%, 65%, 70%, 75%, 80%, 85%, 90%, 95% or 100% modified nucleotides) of the nucleotide positions in a siNA molecule of the invention comprise a nucleic acid sugar, base, or backbone modification or any

combination thereof (*e.g.*, any combination of nucleic acid sugar, base, backbone or non-nucleotide modifications herein). In one embodiment, a siNA molecule of the invention comprises at least about 20%, 25%, 30%, 35%, 40%, 45%, 50%, 55%, 60%, 65%, 70%, 75%, 80%, 85%, 90%, 95% or 100% modified nucleotides. The actual percentage of modified nucleotides present in a given siNA molecule will depend on the total number of nucleotides present in the siNA. If the siNA molecule is single stranded, the percent modification can be based upon the total number of nucleotides present in the single stranded siNA molecules. Likewise, if the siNA molecule is double stranded, the percent modification can be based upon the total number of nucleotides present in the sense strand, antisense strand, or both the sense and antisense strands.

[0056] A siNA molecule of the invention can comprise modified nucleotides at various locations within the siNA molecule. In one embodiment, a double stranded siNA molecule of the invention comprises modified nucleotides at internal base paired positions within the siNA duplex. For example, internal positions can comprise positions from about 3 to about 19 nucleotides from the 5'-end of either sense or antisense strand or region of a 21 nucleotide siNA duplex having 19 base pairs and two nucleotide 3'-overhangs. In another embodiment, a double stranded siNA molecule of the invention comprises modified nucleotides at non-base paired or overhang regions of the siNA molecule. By "non-base paired" is meant, the nucleotides are not base paired between the sense strand or sense region and the antisense strand or antisense region or the siNA molecule. The overhang nucleotides can be complementary or base paired to a corresponding ENaC target polynucleotide sequence (see for example **Figure 6C**). For example, overhang positions can comprise positions from about 20 to about 21 nucleotides from the 5'-end of either sense or antisense strand or region of a 21 nucleotide siNA duplex having 19 base pairs and two nucleotide 3'-overhangs. In another embodiment, a double stranded siNA molecule of the invention comprises modified nucleotides at terminal positions of the siNA molecule. For example, such terminal regions include the 3'-position, 5'-position, for both 3' and 5'-positions of the sense and/or antisense strand or region of the siNA molecule. In another embodiment, a double stranded siNA molecule of the invention comprises modified nucleotides at base-paired or internal positions, non-base paired or overhang regions, and/or terminal regions, or any combination thereof.

[0057] One aspect of the invention features a double-stranded short interfering nucleic acid (siNA) molecule that down-regulates expression of an ENaC target gene or that directs

cleavage of an ENaC target RNA. In one embodiment, the 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 independently comprises about 15 to about 30 (*e.g.*, about 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, or 30) nucleotides, wherein each strand comprises about 15 to about 30 (*e.g.*, about 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, or 30) nucleotides that are complementary to the nucleotides of the other strand. In one embodiment, one of the strands of the double-stranded siNA molecule comprises a nucleotide sequence that is complementary to a nucleotide sequence or a portion thereof of the ENaC target gene, and the second strand of the double-stranded siNA molecule comprises a nucleotide sequence substantially similar to the nucleotide sequence of the ENaC target gene or a portion thereof.

[0058] In another embodiment, the invention features a double-stranded short interfering nucleic acid (siNA) molecule that down-regulates expression of an ENaC target gene or that directs cleavage of an ENaC target RNA, comprising an antisense region, wherein the antisense region comprises a nucleotide sequence that is complementary to a nucleotide sequence of the ENaC target gene or a portion thereof, and a sense region, wherein the sense region comprises a nucleotide sequence substantially similar to the nucleotide sequence of the ENaC target gene or a portion thereof. In one embodiment, the antisense region and the sense region independently comprise about 15 to about 30 (*e.g.* about 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, or 30) nucleotides, wherein the antisense region comprises about 15 to about 30 (*e.g.* about 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, or 30) nucleotides that are complementary to nucleotides of the sense region.

[0059] In another embodiment, the invention features a double-stranded short interfering nucleic acid (siNA) molecule that down-regulates expression of an ENaC target gene or that directs cleavage of an ENaC target RNA, comprising a sense region and an antisense region, wherein the antisense region comprises a nucleotide sequence that is complementary to a nucleotide sequence of RNA encoded by the ENaC target gene or a portion thereof and the sense region comprises a nucleotide sequence that is complementary to the antisense region.

[0060] 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 comprising modifications described herein (*e.g.*, comprising nucleotides having Formulae I-VII or siNA constructs comprising "Stab 00"-"Stab 36" or "Stab 3F"-"Stab 36F" (**Table 8**) or any combination thereof (see **Table 8**)) and/or any length described herein can comprise blunt ends or ends with no overhanging nucleotides.

[0061] In one embodiment, any siNA molecule of the invention can comprise one or more blunt ends, i.e. where a blunt end does not have any overhanging nucleotides. In one embodiment, the blunt ended siNA molecule has a number of base pairs equal to the number of nucleotides present in each strand of the siNA molecule. In another embodiment, the siNA molecule comprises one blunt end, for example wherein the 5'-end of the antisense strand and the 3'-end of the sense strand do not have any overhanging nucleotides. In another example, the siNA molecule comprises one blunt end, for example wherein the 3'-end of the antisense strand and the 5'-end of the sense strand do not have any overhanging nucleotides. In another example, a siNA molecule comprises two blunt ends, for example wherein the 3'-end of the antisense strand and the 5'-end of the sense strand as well as the 5'-end of the antisense strand and 3'-end of the sense strand do not have any overhanging nucleotides. A blunt ended siNA molecule can comprise, for example, from about 15 to about 30 nucleotides (*e.g.*, about 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, or 30 nucleotides). Other nucleotides present in a blunt ended siNA molecule can comprise, for example, mismatches, bulges, loops, or wobble base pairs to modulate the activity of the siNA molecule to mediate RNA interference.

[0062] By "blunt ends" is meant symmetric termini or termini of a double stranded siNA molecule having no overhanging nucleotides. The two strands of a double stranded siNA molecule align with each other without over-hanging nucleotides at the termini. For example, a blunt ended siNA construct comprises terminal nucleotides that are complementary between the sense and antisense regions of the siNA molecule.

[0063] In one embodiment, the invention features a double-stranded short interfering nucleic acid (siNA) molecule that down-regulates expression of an ENaC target gene or that directs cleavage of an ENaC target RNA, wherein the siNA molecule is assembled from two separate oligonucleotide fragments wherein one fragment comprises the sense region and the second fragment comprises the antisense region of the siNA molecule. The sense region can

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

[0064] In one embodiment, a double stranded nucleic acid molecule (*e.g.*, siNA) molecule of the invention comprises ribonucleotides at positions that maintain or enhance RNAi activity. In one embodiment, ribonucleotides are present in the sense strand or sense region of the siNA molecule, which can provide for RNAi activity by allowing cleavage of the sense strand or sense region by an enzyme within the RISC (*e.g.*, ribonucleotides present at the position of passenger strand, sense strand or sense region cleavage, such as position 9 of the passenger strand of a 19 base-pair duplex, which is cleaved in the RISC by AGO2 enzyme, see, for example, Matranga *et al.,* 2005, *Cell*, 123:1-114 and Rand *et al.*, 2005, *Cell,* 123:621-629). In another embodiment, one or more (for example 1, 2, 3, 4 or 5) nucleotides at the 5'-end of the guide strand or guide region (also known as antisense strand or antisense region) of the siNA molecule are ribonucleotides.

[0065] In one embodiment, a double stranded nucleic acid molecule (*e.g.*, siNA) molecule of the invention comprises one or more ribonucleotides at positions within the passenger strand or passenger region (also known as the sense strand or sense region) that allows cleavage of the passenger strand or passenger region by an enzyme in the RISC complex, (*e.g.*, ribonucleotides present at the position of passenger strand, such as position 9 of the passenger strand of a 19 base-pair duplex that is cleaved in the RISC, such as by AGO2 enzyme, see, for example, Matranga *et al.,* 2005, *Cell*, 123:1-114 and Rand *et al.*, 2005, *Cell,* 123:621-629).

[0066] In one embodiment, a siNA molecule of the invention contains at least 2, 3, 4, 5, or more chemical modifications that can be the same of different. In one embodiment, a siNA molecule of the invention contains at least 2, 3, 4, 5, or more different chemical modifications.

[0067] In one embodiment, a siNA molecule of the invention is a double-stranded short interfering nucleic acid (siNA), wherein the double stranded nucleic acid molecule comprises about 15 to about 30 (*e.g.* about 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, or 30) base pairs, and wherein one or more (*e.g.*, at least 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, or 30) of the nucleotide positions

in each strand of the siNA molecule comprises a chemical modification. In another embodiment, the siNA contains at least 2, 3, 4, 5, or more different chemical modifications.

[0068] In one embodiment, the invention features double-stranded short interfering nucleic acid (siNA) molecule that down-regulates expression of an ENaC target gene or that directs cleavage of an ENaC target RNA, wherein the siNA molecule comprises about 15 to about 30 (*e.g.* about 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, or 30) base pairs, and wherein each strand of the siNA molecule comprises one or more chemical modifications. In one embodiment, each strand of the double stranded siNA molecule comprises at least two (*e.g.*, 2, 3, 4, 5, or more) different chemical modifications, *e.g.*, different nucleotide sugar, base, or backbone modifications. In another embodiment, one of the strands of the double-stranded siNA molecule comprises a nucleotide sequence that is complementary to a nucleotide sequence of an ENaC target gene or a portion thereof, and the second strand of the double-stranded siNA molecule comprises a nucleotide sequence substantially similar to the nucleotide sequence or a portion thereof of the ENaC target gene. In another embodiment, one of the strands of the double-stranded siNA molecule comprises a nucleotide sequence that is complementary to a nucleotide sequence of an ENaC target gene or portion thereof, and the second strand of the double-stranded siNA molecule comprises a nucleotide sequence substantially similar to the nucleotide sequence or portion thereof of the ENaC target gene. In another embodiment, each strand of the siNA molecule comprises about 15 to about 30 (*e.g.* about 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, or 30) nucleotides, and each strand comprises at least about 15 to about 30 (*e.g.* about 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, or 30) nucleotides that are complementary to the nucleotides of the other strand. The ENaC target gene can comprise, for example, sequences referred to herein or incorporated herein by reference. The ENaC gene can comprise, for example, sequences referred to by GenBank Accession number herein, such as in **Table 7**.

[0069] In one embodiment, each strand of a double stranded siNA molecule of the invention comprises a different pattern of chemical modifications, such as any "Stab 00"-"Stab 36" or "Stab 3F"-"Stab 36F" (**Table 8**) modification patterns herein or any combination thereof (see **Table 8**). Non-limiting examples of sense and antisense strands of such siNA molecules having various modification patterns are shown in **Figures 4 and 5**.

[0070] In one embodiment, a siNA molecule of the invention comprises no ribonucleotides. In another embodiment, a siNA molecule of the invention comprises one or more ribonucleotides (e.g., 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, or more ribonucleotides).

[0071] In one embodiment, a siNA molecule of the invention comprises an antisense region comprising a nucleotide sequence that is complementary to a nucleotide sequence of an ENaC target gene or a portion thereof, and the siNA further comprises a sense region comprising a nucleotide sequence substantially similar to the nucleotide sequence of the ENaC target gene or a portion thereof. In another embodiment, the antisense region and the sense region each comprise about 15 to about 30 (*e.g.* about 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, or 30) nucleotides and the antisense region comprises at least about 15 to about 30 (*e.g.* about 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, or 30) nucleotides that are complementary to nucleotides of the sense region. In one embodiment, each strand of the double stranded siNA molecule comprises at least two (*e.g.*, 2, 3, 4, 5, or more) different chemical modifications, *e.g.*, different nucleotide sugar, base, or backbone modifications. The ENaC target gene can comprise, for example, sequences referred to herein or incorporated by reference herein. In another embodiment, the siNA is a double stranded nucleic acid molecule, where each of the two strands of the siNA molecule independently comprise about 15 to about 40 (*e.g.* about 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 23, 33, 34, 35, 36, 37, 38, 39, or 40) nucleotides, and where one of the strands of the siNA molecule comprises at least about 15 (*e.g.* about 15, 16, 17, 18, 19, 20, 21, 22, 23, 24 or 25 or more) nucleotides that are complementary to the nucleic acid sequence of the ENaC target gene or a portion thereof.

[0072] In one embodiment, a siNA molecule of the invention comprises a sense region and an antisense region, wherein the antisense region comprises a nucleotide sequence that is complementary to a nucleotide sequence of RNA encoded by an ENaC target gene, or a portion thereof, and the sense region comprises a nucleotide sequence that is complementary to the antisense region. In one embodiment, the siNA molecule is assembled from two separate oligonucleotide fragments, wherein one fragment comprises the sense region and the second fragment comprises the antisense region of the siNA molecule. In another embodiment, the sense region is connected to the antisense region via a linker molecule. In another embodiment, the sense region is connected to the antisense region via a linker molecule, such as a nucleotide or non-nucleotide linker. In one embodiment, each strand of

the double stranded siNA molecule comprises at least two (*e.g.*, 2, 3, 4, 5, or more) different chemical modifications, *e.g.*, different nucleotide sugar, base, or backbone modifications. The ENaC target gene can comprise, for example, sequences referred herein or incorporated by reference herein.

[0073] In one embodiment, a siNA molecule of the invention comprises one or more (e.g., 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20 or more) 2'-deoxy-2'-fluoro pyrimidine modificatons (e.g., where one or more or all pyrimidine (e.g., U or C) positions of the siNA are modified with 2'-deoxy-2'-fluoro nucleotides). In one embodiment, the 2'-deoxy-2'-fluoro pyrimidine modifications are present in the sense strand. In one embodiment, the 2'-deoxy-2'-fluoro pyrimidine modifications are present in the antisense strand. In one embodiment, the 2'-deoxy-2'-fluoro pyrimidine modifications are present in both the sense strand and the antisense strand of the siNA molecule.

[0074] In one embodiment, a siNA molecule of the invention comprises one or more (e.g., 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20 or more) 2'-O-methyl purine modificatons (e.g., where one or more or all purine (e.g., A or G) positions of the siNA are modified with 2'-O-methyl nucleotides). In one embodiment, the 2'-O-methyl purine modifications are present in the sense strand. In one embodiment, the 2'-O-methyl purine modifications are present in the antisense strand. In one embodiment, the 2'-O-methyl purine modifications are present in both the sense strand and the antisense strand of the siNA molecule.

[0075] In one embodiment, a siNA molecule of the invention comprises one or more (e.g., 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20 or more) 2'-deoxy purine modificatons (e.g., where one or more or all purine (e.g., A or G) positions of the siNA are modified with 2'-deoxy nucleotides). In one embodiment, the 2'-deoxy purine modifications are present in the sense strand. In one embodiment, the 2'-deoxy purine modifications are present in the antisense strand. In one embodiment, the 2'-deoxy purine modifications are present in both the sense strand and the antisense strand of the siNA molecule.

[0076] In one embodiment, the invention features a double-stranded short interfering nucleic acid (siNA) molecule that down-regulates expression of an ENaC target gene or that directs cleavage of an ENaC target RNA, comprising a sense region and an antisense region, wherein the antisense region comprises a nucleotide sequence that is complementary to a

nucleotide sequence of RNA encoded by the ENaC target gene or a portion thereof and the sense region comprises a nucleotide sequence that is complementary to the antisense region, and wherein the siNA molecule has one or more modified pyrimidine and/or purine nucleotides. In one embodiment, each strand of the double stranded siNA molecule comprises at least two (*e.g.*, 2, 3, 4, 5, or more) different chemical modifications, *e.g.*, different nucleotide sugar, base, or backbone modifications. In one embodiment, the pyrimidine nucleotides in the sense region are 2'-O-methyl pyrimidine nucleotides or 2'-deoxy-2'-fluoro pyrimidine nucleotides and the purine nucleotides present in the sense region are 2'-deoxy purine nucleotides. In another embodiment, the pyrimidine nucleotides in the sense region are 2'-deoxy-2'-fluoro pyrimidine nucleotides and the purine nucleotides present in the sense region are 2'-O-methyl purine nucleotides. In another embodiment, the pyrimidine nucleotides in the sense region are 2'-deoxy-2'-fluoro pyrimidine nucleotides and the purine nucleotides present in the sense region are 2'-deoxy purine nucleotides. In one embodiment, the pyrimidine nucleotides in the antisense region are 2'-deoxy-2'-fluoro pyrimidine nucleotides and the purine nucleotides present in the antisense region are 2'-O-methyl or 2'-deoxy purine nucleotides. In another embodiment of any of the above-described siNA molecules, any nucleotides present in a non-complementary region of the sense strand (*e.g.* overhang region) are 2'-deoxy nucleotides.

[0077] In one embodiment, the invention features a double-stranded short interfering nucleic acid (siNA) molecule that down-regulates expression of an ENaC target gene or that directs cleavage of an ENaC target RNA, wherein the siNA molecule is assembled from two separate oligonucleotide fragments wherein one fragment comprises the sense region and the second fragment comprises the antisense region of the siNA molecule, and wherein the fragment comprising the sense region includes a terminal cap moiety at the 5'-end, the 3'-end, or both of the 5' and 3' ends of the fragment. In one embodiment, the terminal cap moiety is an inverted deoxy abasic moiety or glyceryl moiety. In one embodiment, each of the two fragments of the siNA molecule independently comprise about 15 to about 30 (*e.g.* about 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, or 30) nucleotides. In another embodiment, each of the two fragments of the siNA molecule independently comprise about 15 to about 40 (*e.g.* about 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 23, 33, 34, 35, 36, 37, 38, 39, or 40) nucleotides. In a non-limiting example, each of the two fragments of the siNA molecule comprise about 21 nucleotides.

[0078] 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, 2'-deoxy-2'-fluoroarabino, 2'-O-trifluoromethyl nucleotide, 2'-O-ethyl-trifluoromethoxy nucleotide, or 2'-O-difluoromethoxy-ethoxy nucleotide or any other modified nucleoside/nucleotide described herein and in USSN 10/981,966, filed November 5, 2004, incorporated by reference herein. In one embodiment, the invention features a siNA molecule comprising at least two (*e.g.*, 2, 3, 4, 5, 6, 7, 8 , 9 ,10, or more) modified nucleotides, wherein the modified nucleotide is selected from the group consisting of 2'-deoxy-2'-fluoro nucleotide, 2'-deoxy-2'-fluoroarabino, 2'-O-trifluoromethyl nucleotide, 2'-O-ethyl-trifluoromethoxy nucleotide, or 2'-O-difluoromethoxy-ethoxy nucleotide or any other modified nucleoside/nucleotide described herein and in USSN 10/981,966, filed November 5, 2004, incorporated by reference herein. The modified nucleotide/nucleoside can be the same or different. The siNA can be, for example, about 15 to about 40 nucleotides in length. In one embodiment, all pyrimidine nucleotides present in the siNA are 2'-deoxy-2'-fluoro, 2'-deoxy-2'-fluoroarabino, 2'-O-trifluoromethyl, 2'-O-ethyl-trifluoromethoxy, or 2'-O-difluoromethoxy-ethoxy, 4'-thio pyrimidine nucleotides. In one embodiment, the modified nucleotides in the siNA include at least one 2'-deoxy-2'-fluoro cytidine or 2'-deoxy-2'-fluoro uridine nucleotide. In another embodiment, the modified nucleotides in the siNA include at least one 2'-deoxy-2'-fluoro cytidine and at least one 2'-deoxy-2'-fluoro uridine nucleotides. In one embodiment, all uridine nucleotides present in the siNA are 2'-deoxy-2'-fluoro uridine nucleotides. In one embodiment, all cytidine nucleotides present in the siNA are 2'-deoxy-2'-fluoro cytidine nucleotides. In one embodiment, all adenosine nucleotides present in the siNA are 2'-deoxy-2'-fluoro adenosine nucleotides. In one embodiment, all guanosine nucleotides present in the siNA are 2'-deoxy-2'-fluoro guanosine nucleotides. The siNA can further comprise at least one modified internucleotidic linkage, such as phosphorothioate linkage. In one embodiment, the 2'-deoxy-2'-fluoronucleotides are present at specifically selected locations in the siNA that are sensitive to cleavage by ribonucleases, such as locations having pyrimidine nucleotides.

[0079] 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 one embodiment, all pyrimidine nucleotides present in the siNA are 2'-deoxy-2'-fluoro pyrimidine nucleotides. In one embodiment, the modified nucleotides in

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

[0080] 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'-fluoroarabino nucleotide. In one embodiment, all pyrimidine nucleotides present in the siNA are 2'-deoxy-2'-fluoroarabino pyrimidine nucleotides. In one embodiment, the modified nucleotides in the siNA include at least one 2'-deoxy-2'-fluoroarabino cytidine or 2'-deoxy-2'-fluoroarabino uridine nucleotide. In another embodiment, the modified nucleotides in the siNA include at least one 2'-fluoro cytidine and at least one 2'-deoxy-2'-fluoroarabino uridine nucleotides. In one embodiment, all uridine nucleotides present in the siNA are 2'-deoxy-2'-fluoroarabino uridine nucleotides. In one embodiment, all cytidine nucleotides present in the siNA are 2'-deoxy-2'-fluoroarabino cytidine nucleotides. In one embodiment, all adenosine nucleotides present in the siNA are 2'-deoxy-2'-fluoroarabino adenosine nucleotides. In one embodiment, all guanosine nucleotides present in the siNA are 2'-deoxy-2'-fluoroarabino guanosine nucleotides. The siNA can further comprise at least one modified internucleotidic linkage, such as a phosphorothioate linkage. In one embodiment, the 2'-deoxy-2'-fluoroarabinonucleotides are present at specifically selected locations in the siNA that are sensitive to cleavage by ribonucleases, such as locations having pyrimidine nucleotides.

[0081] In one embodiment, the invention features a double-stranded short interfering nucleic acid (siNA) molecule that down-regulates expression of an ENaC target gene or that

directs cleavage of an ENaC target RNA, comprising a sense region and an antisense region, wherein the antisense region comprises a nucleotide sequence that is complementary to a nucleotide sequence of RNA encoded by the ENaC target gene or a portion thereof and the sense region comprises a nucleotide sequence that is complementary to the antisense region, and wherein the purine nucleotides present in the antisense region comprise 2'-deoxy- purine nucleotides. In an alternative embodiment, the purine nucleotides present in the antisense region comprise 2'-O-methyl purine nucleotides. In either of the above embodiments, the antisense region can comprise a phosphorothioate internucleotide linkage at the 3' end of the antisense region. Alternatively, in either of the above embodiments, the antisense region can comprise a glyceryl modification at the 3' end of the antisense region. In another embodiment of any of the above-described siNA molecules, any nucleotides present in a non-complementary region of the antisense strand (*e.g.* overhang region) are 2'-deoxy nucleotides.

[0082] In one embodiment, the antisense region of a siNA molecule of the invention comprises sequence complementary to a portion of an endogenous transcript having sequence unique to a particular disease or trait related allele in a subject or organism, such as sequence comprising a single nucleotide polymorphism (SNP) associated with the disease or trait specific allele. As such, the antisense region of a siNA molecule of the invention can comprise sequence complementary to sequences that are unique to a particular allele to provide specificity in mediating selective RNAi against the disease, condition, or trait related allele.

[0083] In one embodiment, the invention features a double-stranded short interfering nucleic acid (siNA) molecule that down-regulates expression of an ENaC target gene or that directs cleavage of an ENaC target RNA, wherein the siNA molecule is assembled from two separate oligonucleotide fragments wherein one fragment comprises the sense region and the second fragment comprises the antisense region of the siNA molecule. In one embodiment, each strand of the double stranded siNA molecule is about 21 nucleotides long and about 19 nucleotides of each fragment of the siNA molecule are base-paired to the complementary nucleotides of the other fragment of the siNA molecule, wherein at least two 3' terminal nucleotides of each fragment of the siNA molecule are not base-paired to the nucleotides of the other fragment of the siNA molecule. In another embodiment, the siNA molecule is a double stranded nucleic acid molecule, where each strand is about 19 nucleotide long and

where the nucleotides of each fragment of the siNA molecule are base-paired to the complementary nucleotides of the other fragment of the siNA molecule to form at least about 15 (*e.g.*, 15, 16, 17, 18, or 19) base pairs, wherein one or both ends of the siNA molecule are blunt ends. In one embodiment, each of the two 3' terminal nucleotides of each fragment of the siNA molecule is a 2'-deoxy-pyrimidine nucleotide, such as a 2'-deoxy-thymidine. In one embodiment, each of the two 3' terminal nucleotides of each fragment of the siNA molecule is a 2'-O-methyl pyrimidine nucleotide, such as a 2'-O-methyl uridine, cytidine, or thymidine. In another embodiment, all nucleotides of each fragment of the siNA molecule are base-paired to the complementary nucleotides of the other fragment of the siNA molecule. In another embodiment, the siNA molecule is a double stranded nucleic acid molecule of about 19 to about 25 base pairs having a sense region and an antisense region, where about 19 nucleotides of the antisense region are base-paired to the nucleotide sequence or a portion thereof of the RNA encoded by the ENaC target gene. In another embodiment, about 21 nucleotides of the antisense region are base-paired to the nucleotide sequence or a portion thereof of the RNA encoded by the ENaC target gene. In any of the above embodiments, the 5'-end of the fragment comprising said antisense region can optionally include a phosphate group.

[0084] In one embodiment, the invention features a double-stranded short interfering nucleic acid (siNA) molecule that inhibits the expression of an ENaC target RNA sequence, wherein the siNA molecule does not contain any ribonucleotides and wherein each strand of the double-stranded siNA molecule is about 15 to about 30 nucleotides. In one embodiment, the siNA molecule is 21 nucleotides in length. Examples of non-ribonucleotide containing siNA constructs are combinations of stabilization chemistries shown in **Table 8** in any combination of Sense/Antisense chemistries, such as Stab 7/8, Stab 7/11, Stab 8/8, Stab 18/8, Stab 18/11, Stab 12/13, Stab 7/13, Stab 18/13, Stab 7/19, Stab 8/19, Stab 18/19, Stab 7/20, Stab 8/20, Stab 18/20, Stab 7/32, Stab 8/32, or Stab 18/32 (*e.g.*, any siNA having Stab 7, 8, 11, 12, 13, 14, 15, 17, 18, 19, 20, or 32 sense or antisense strands or any combination thereof). Herein, numeric Stab chemistries can include both 2'-fluoro and 2'-OCF3 versions of the chemistries shown in **Table 8**. For example, "Stab 7/8" refers to both Stab 7/8 and Stab 7F/8F etc. In one embodiment, the invention features a chemically synthesized double stranded RNA molecule that directs cleavage of an ENaC target RNA via RNA interference, wherein each strand of said RNA molecule is about 15 to about 30 nucleotides in length; one strand of the RNA molecule comprises nucleotide sequence having sufficient

complementarity to the ENaC target RNA for the RNA molecule to direct cleavage of the ENaC target RNA via RNA interference; and wherein at least one strand of the RNA molecule optionally comprises one or more chemically modified nucleotides described herein, such as without limitation deoxynucleotides, 2'-O-methyl nucleotides, 2'-deoxy-2'-fluoro nucleotides, 2'-deoxy-2'-fluoroarabino, 2'-O-methoxyethyl nucleotides, 4'-thio nucleotides, 2'-O-trifluoromethyl nucleotides, 2'-O-ethyl-trifluoromethoxy nucleotides, 2'-O-difluoromethoxy-ethoxy nucleotides, etc. or any combination thereof. The chemically modified nucleotides can be the same or different.

[0085] In one embodiment, an ENaC target RNA of the invention comprises sequence encoding an ENaC protein.

[0086] In one embodiment, an ENaC target RNA of the invention comprises non-coding RNA sequence (*e.g.*, miRNA, snRNA, siRNA etc.), see for example Mattick, 2005, *Science*, 309, 1527-1528; Claverie, 2005, *Science*, 309, 1529-1530; Sethupathy et al., 2006, RNA, 12, 192-197; and Czech, 2006 NEJM, 354, 11: 1194-1195.

[0087] In one embodiment, the invention features a medicament comprising a siNA molecule of the invention.

[0088] In one embodiment, the invention features an active ingredient comprising a siNA molecule of the invention.

[0089] In one embodiment, the invention features the use of a double-stranded short interfering nucleic acid (siNA) molecule to inhibit, down-regulate, or reduce expression of an ENaC target gene, wherein the siNA molecule comprises one or more chemical modifications that can be the same or different and each strand of the double-stranded siNA is independently about 15 to about 30 or more (*e.g.*, about 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29 or 30 or more) nucleotides long. In one embodiment, the siNA molecule of the invention is a double stranded nucleic acid molecule comprising one or more chemical modifications, where each of the two fragments of the siNA molecule independently comprise about 15 to about 40 (*e.g.* about 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 23, 33, 34, 35, 36, 37, 38, 39, or 40) nucleotides and where one of the strands comprises at least 15 nucleotides that are complementary to nucleotide sequence of ENaC target encoding RNA or a portion thereof. In a non-limiting example, each of the two fragments of the siNA molecule comprise about 21 nucleotides. In another embodiment, the

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

[0090] In one embodiment, the invention features the use of a double-stranded short interfering nucleic acid (siNA) molecule that inhibits, down-regulates, or reduces expression of an ENaC target gene, wherein one of the strands of the double-stranded siNA molecule is an antisense strand which comprises nucleotide sequence that is complementary to nucleotide sequence of ENaC target RNA or a portion thereof, the other strand is a sense strand which comprises nucleotide sequence that is complementary to a nucleotide sequence of the antisense strand. In one embodiment, each strand has at least two (*e.g.*, 2, 3, 4, 5, or more) chemical modifications, which can be the same or different, such as nucleotide, sugar, base, or backbone modifications. In one embodiment, a majority of the pyrimidine nucleotides

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

[0091] In one embodiment, the invention features a double-stranded short interfering nucleic acid (siNA) molecule that inhibits, down-regulates, or reduces expression of an ENaC target gene, wherein one of the strands of the double-stranded siNA molecule is an antisense strand which comprises nucleotide sequence that is complementary to nucleotide sequence of ENaC target RNA or a portion thereof, wherein the other strand is a sense strand which comprises nucleotide sequence that is complementary to a nucleotide sequence of the antisense strand. In one embodiment, each strand has at least two (*e.g.*, 2, 3, 4, 5, or more) chemical modifications, which can be the same or different, such as nucleotide, sugar, base, or backbone modifications. In one embodiment, a majority of the pyrimidine nucleotides present in the double-stranded siNA molecule comprises a sugar modification. In one embodiment, a majority of the purine nucleotides present in the double-stranded siNA molecule comprises a sugar modification.

[0092] In one embodiment, the invention features a double-stranded short interfering nucleic acid (siNA) molecule that inhibits, down-regulates, or reduces expression of an ENaC target gene, wherein one of the strands of the double-stranded siNA molecule is an antisense strand which comprises nucleotide sequence that is complementary to nucleotide sequence of ENaC target RNA that encodes a protein or portion thereof, the other strand is a sense strand which comprises nucleotide sequence that is complementary to a nucleotide sequence of the antisense strand and wherein a majority of the pyrimidine nucleotides present in the double-stranded siNA molecule comprises a sugar modification. In one embodiment, each strand of the siNA molecule comprises about 15 to about 30 or more (*e.g.*, about 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, or 30 or more) nucleotides, wherein each strand comprises at least about 15 nucleotides that are complementary to the nucleotides of the other strand. In one embodiment, the siNA molecule is assembled from two oligonucleotide fragments, wherein one fragment comprises the nucleotide sequence of the antisense strand of the siNA molecule and a second fragment comprises nucleotide sequence of the sense region of the siNA molecule. In one embodiment, the sense strand is connected to the antisense strand via a linker molecule, such as a polynucleotide linker or a non-nucleotide linker. In a further embodiment, the pyrimidine nucleotides present in the sense strand are 2'-deoxy-2'fluoro

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

[0093] In any of the above-described embodiments of a double-stranded short interfering nucleic acid (siNA) molecule that inhibits expression of an ENaC target gene, wherein a majority of the pyrimidine nucleotides present in the double-stranded siNA molecule comprises a sugar modification, each of the two strands of the siNA molecule can comprise about 15 to about 30 or more (*e.g.*, about 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, or 30 or more) nucleotides. In one embodiment, about 15 to about 30 or more (*e.g.*, about 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, or 30 or more) nucleotides of each strand of the siNA molecule are base-paired to the complementary nucleotides of the other strand of the siNA molecule. In another embodiment, about 15 to about 30 or more (*e.g.*, about 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, or 30 or more) nucleotides of each strand of the siNA molecule are base-paired to the complementary nucleotides of the other strand of the siNA molecule, wherein at least two 3' terminal nucleotides of each strand of the siNA molecule are not base-paired to the nucleotides of the other strand of the siNA molecule. In another embodiment, each of the two 3' terminal nucleotides of each fragment of the siNA molecule is a 2'-deoxy-pyrimidine, such as 2'-deoxy-thymidine. In one

embodiment, each strand of the siNA molecule is base-paired to the complementary nucleotides of the other strand of the siNA molecule. In one embodiment, about 15 to about 30 (*e.g.*, about 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, or 30) nucleotides of the antisense strand are base-paired to the nucleotide sequence of the ENaC target RNA or a portion thereof. In one embodiment, about 18 to about 25 (*e.g.*, about 18, 19, 20, 21, 22, 23, 24, or 25) nucleotides of the antisense strand are base-paired to the nucleotide sequence of the ENaC target RNA or a portion thereof.

[0094] In one embodiment, the invention features a double-stranded short interfering nucleic acid (siNA) molecule that inhibits expression of an ENaC target gene, wherein one of the strands of the double-stranded siNA molecule is an antisense strand which comprises nucleotide sequence that is complementary to nucleotide sequence of ENaC target RNA or a portion thereof, the other strand is a sense strand which comprises nucleotide sequence that is complementary to a nucleotide sequence of the antisense strand. In one embodiment, each strand has at least two (*e.g.*, 2, 3, 4, 5, or more) different chemical modifications, such as nucleotide sugar, base, or backbone modifications. In one embodiment, a majority of the pyrimidine nucleotides present in the double-stranded siNA molecule comprises a sugar modification. In one embodiment, a majority of the purine nucleotides present in the double-stranded siNA molecule comprises a sugar modification. In one embodiment, the 5'-end of the antisense strand optionally includes a phosphate group.

[0095] In one embodiment, the invention features a double-stranded short interfering nucleic acid (siNA) molecule that inhibits expression of an ENaC target gene, wherein one of the strands of the double-stranded siNA molecule is an antisense strand which comprises nucleotide sequence that is complementary to nucleotide sequence of ENaC target RNA or a portion thereof, the other strand is a sense strand which comprises nucleotide sequence that is complementary to a nucleotide sequence of the antisense strand and wherein a majority of the pyrimidine nucleotides present in the double-stranded siNA molecule comprises a sugar modification, and wherein the nucleotide sequence or a portion thereof of the antisense strand is complementary to a nucleotide sequence of the untranslated region or a portion thereof of the ENaC target RNA.

[0096] In one embodiment, the invention features a double-stranded short interfering nucleic acid (siNA) molecule that inhibits expression of an ENaC target gene, wherein one of the strands of the double-stranded siNA molecule is an antisense strand which comprises

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

[0097] In one embodiment, the invention features a composition comprising a siNA molecule of the invention in a pharmaceutically acceptable carrier or diluent. In another embodiment, the invention features two or more differing siNA molecules of the invention (e.g. siNA molecules that target different regions of ENaC target RNA or siNA molecules that target ENaC pathway RNA) in a pharmaceutically acceptable carrier or diluent.

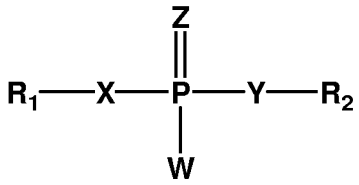
[0098] In a non-limiting example, the introduction of chemically-modified nucleotides into nucleic acid molecules provides a powerful tool in overcoming potential limitations of *in vivo* stability and bioavailability inherent to native RNA molecules that are delivered exogenously. For example, the use 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 ENaC targeting particular cells or tissues and/or improving cellular uptake of the nucleic acid molecule. Therefore, even if the activity of a chemically-modified nucleic acid molecule is reduced as compared to a native nucleic acid molecule, for example, when compared to an all-RNA nucleic acid molecule, the overall activity of the modified nucleic acid molecule can be greater than that of the native molecule due to improved stability and/or delivery of the molecule. Unlike native unmodified siNA, chemically-modified siNA can also minimize the possibility of activating interferon activity or immunostimulation in humans. These properties therefore improve upon native siRNA or minimally modified siRNA's ability to mediate RNAi in various in vitro and in vivo settings, including use in both research and therapeutic applications. Applicant describes herein chemically modified siNA molecules with improved RNAi activity compared to corresponding unmodified or minimally modified siRNA molecules. The chemically modified siNA motifs disclosed herein provide the capacity to maintain RNAi activity that is substantially similar to

unmodified or minimally modified active siRNA (see for example Elbashir et al., 2001, EMBO J., 20:6877-6888) while at the same time providing nuclease resistance and pharmacoketic properties suitable for use in therapeutic applications.

[0099] 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.

[0100] One embodiment of the invention provides an expression vector comprising a nucleic acid sequence encoding at least one siNA molecule of the invention in a manner that allows expression of the nucleic acid molecule. Another embodiment of the invention provides a mammalian cell comprising such an expression vector. The mammalian cell can be a human cell. The siNA molecule of the expression vector can comprise a sense region and an antisense region. The antisense region can comprise sequence complementary to a RNA or DNA sequence encoding an ENaC target and the sense region can comprise sequence complementary to the antisense region. The siNA molecule can comprise two distinct strands having complementary sense and antisense regions. The siNA molecule can comprise a single strand having complementary sense and antisense regions.

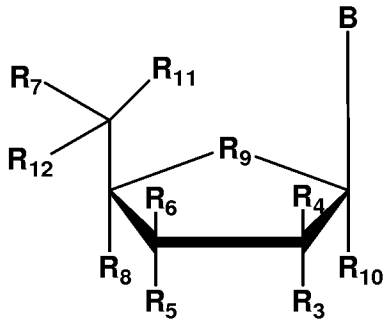
[0101] In one embodiment, the invention features a chemically-modified short interfering nucleic acid (siNA) molecule capable of mediating RNA interference (RNAi) inside a cell or reconstituted *in vitro* system, wherein the chemical modification comprises one or more (*e.g.*, about 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, or more) nucleotides comprising a backbone modified internucleotide linkage having Formula I:



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

[0102] The chemically-modified internucleotide linkages having Formula I, for example, wherein any Z, W, X, and/or Y independently comprises a sulphur atom, can be present in one or both oligonucleotide strands of the siNA duplex, for example, in the sense strand, the antisense strand, or both strands. The siNA molecules of the invention can comprise one or more (*e.g.*, about 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, or more) chemically-modified internucleotide linkages having Formula I at the 3'-end, the 5'-end, or both of the 3' and 5'-ends of the sense strand, the antisense strand, or both strands. For example, an exemplary siNA molecule of the invention can comprise about 1 to about 5 or more (*e.g.*, about 1, 2, 3, 4, 5, or more) chemically-modified internucleotide linkages having Formula I at the 5'-end of the sense strand, the antisense strand, or both strands. In another non-limiting example, an exemplary siNA molecule of the invention can comprise one or more (*e.g.*, about 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, or more) pyrimidine nucleotides with chemically-modified internucleotide linkages having Formula I in the sense strand, the antisense strand, or both strands. In yet another non-limiting example, an exemplary siNA molecule of the invention can comprise one or more (*e.g.*, about 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, or more) purine nucleotides with chemically-modified internucleotide linkages having Formula I in the sense strand, the antisense strand, or both strands. In another embodiment, a siNA molecule of the invention having internucleotide linkage(s) of Formula I also comprises a chemically-modified nucleotide or non-nucleotide having any of Formulae I-VII.

[0103] In one embodiment, the invention features a chemically-modified short interfering nucleic acid (siNA) molecule capable of mediating RNA interference (RNAi) inside a cell or reconstituted *in vitro* system, wherein the chemical modification comprises one or more (*e.g.*, about 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, or more) nucleotides or non-nucleotides having Formula II:

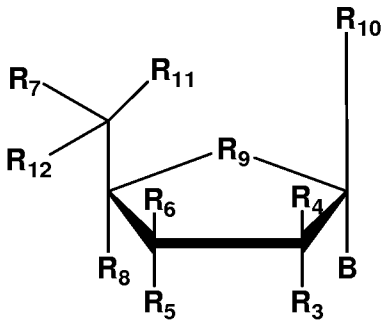


wherein each R3, R4, R5, R6, R7, R8, R10, R11 and R12 is independently H, OH, alkyl, substituted alkyl, alkaryl or aralkyl, F, Cl, Br, CN, CF3, OCF3, OCH3, OCN, O-alkyl, S-alkyl, N-alkyl, O-alkenyl, S-alkenyl, N-alkenyl, SO-alkyl, alkyl-OSH, alkyl-OH, O-alkyl-OH, O-alkyl-SH, S-alkyl-OH, S-alkyl-SH, alkyl-S-alkyl,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

nucleotide. In one embodiment, a nucleotide of the invention having Formula II is a 2'-O-methyl nucleotide. In one embodiment, a nucleotide of the invention having Formula II is a 2'-deoxy nucleotide.

[0104] The chemically-modified nucleotide or non-nucleotide of Formula II can be present in one or both oligonucleotide strands of the siNA duplex, for example in the sense strand, the antisense strand, or both strands. The siNA molecules of the invention can comprise one or more chemically-modified nucleotides or non-nucleotides of Formula II at the 3'-end, the 5'-end, or both of the 3' and 5'-ends of the sense strand, the antisense strand, or both strands. For example, an exemplary siNA molecule of the invention can comprise about 1 to about 5 or more (*e.g.*, about 1, 2, 3, 4, 5, or more) chemically-modified nucleotides or non-nucleotides of Formula II at the 5'-end of the sense strand, the antisense strand, or both strands. In anther non-limiting example, an exemplary siNA molecule of the invention can comprise about 1 to about 5 or more (*e.g.*, about 1, 2, 3, 4, 5, or more) chemically-modified nucleotides or non-nucleotides of Formula II at the 3'-end of the sense strand, the antisense strand, or both strands.

[0105] In one embodiment, the invention features a chemically-modified short interfering nucleic acid (siNA) molecule capable of mediating RNA interference (RNAi) inside a cell or reconstituted *in vitro* system, wherein the chemical modification comprises one or more (*e.g.*, about 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, or more) nucleotides or non-nucleotides having Formula III:



wherein each R3, R4, R5, R6, R7, R8, R10, R11 and R12 is independently H, OH, alkyl, substituted alkyl, alkaryl or aralkyl, F, Cl, Br, CN, CF3, OCF3, OCH3, OCN, O-alkyl, S-alkyl, N-alkyl, O-alkenyl, S-alkenyl, N-alkenyl, SO-alkyl, alkyl-OSH, alkyl-OH, O-alkyl-OH, O-alkyl-SH, S-alkyl-OH, S-alkyl-SH, alkyl-S-alkyl, alkyl-O-alkyl, ONO2, NO2, N3, NH2, aminoalkyl, aminoacid, aminoacyl, ONH2, O-aminoalkyl, O-aminoacid, O-aminoacyl, heterocycloalkyl, heterocycloalkaryl, aminoalkylamino, polyalklylamino, substituted silyl, or

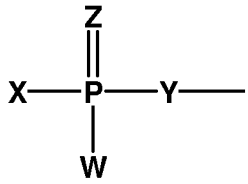
a group having any of Formula I, II, III, IV, V, VI and/or VII, any of which can be included in the structure of the siNA molecule or serve as a point of attachment to the siNA molecule; R9 is O, S, CH2, S=O, CHF, or CF2, and B is a nucleosidic base such as adenine, guanine, uracil, cytosine, thymine, 2-aminoadenosine, 5-methylcytosine, 2,6-diaminopurine, or any other non-naturally occurring base that can be employed to be complementary or non-complementary to target RNA or a non-nucleosidic base such as phenyl, naphthyl, 3-nitropyrrole, 5-nitroindole, nebularine, pyridone, pyridinone, or any other non-naturally occurring universal base that can be complementary or non-complementary to target RNA. In one embodiment, R3 and/or R7 comprises a conjugate moiety and a linker (*e.g.*, a nucleotide or non-nucleotide linker as described herein or otherwise known in the art). Non-limiting examples of conjugate moieties include ligands for cellular receptors, such as peptides derived from naturally occurring protein ligands; protein localization sequences, including cellular ZIP code sequences; antibodies; nucleic acid aptamers; vitamins and other co-factors, such as folate and N-acetylgalactosamine; polymers, such as polyethyleneglycol (PEG); phospholipids; cholesterol; steroids, and polyamines, such as PEI, spermine or spermidine.

[0106] The chemically-modified nucleotide or non-nucleotide of Formula III can be present in one or both oligonucleotide strands of the siNA duplex, for example, in the sense strand, the antisense strand, or both strands. The siNA molecules of the invention can comprise one or more chemically-modified nucleotides or non-nucleotides of Formula III at the 3'-end, the 5'-end, or both of the 3' and 5'-ends of the sense strand, the antisense strand, or both strands. For example, an exemplary siNA molecule of the invention can comprise about 1 to about 5 or more (*e.g.*, about 1, 2, 3, 4, 5, or more) chemically-modified nucleotide(s) or non-nucleotide(s) of Formula III at the 5'-end of the sense strand, the antisense strand, or both strands. In anther non-limiting example, an exemplary siNA molecule of the invention can comprise about 1 to about 5 or more (*e.g.*, about 1, 2, 3, 4, 5, or more) chemically-modified nucleotide or non-nucleotide of Formula III at the 3'-end of the sense strand, the antisense strand, or both strands.

[0107] 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.

[0108] In one embodiment, the invention features a chemically-modified short interfering nucleic acid (siNA) molecule capable of mediating RNA interference (RNAi) inside a cell or reconstituted *in vitro* system, wherein the chemical modification comprises a 5'-terminal phosphate group having Formula IV:



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

[0109] In one embodiment, the invention features a siNA molecule having a 5'-terminal phosphate group having Formula IV on the ENaC target-complementary strand, for example, a strand complementary to an ENaC 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 PD Nongrafted corneas and syngeneic (Lewis-Lewis) E4 target-complementary strand wherein the siNA molecule also comprises about 1 to about 3 (*e.g.*, about 1, 2, or 3) nucleotide 3'-terminal nucleotide overhangs having about 1 to about 4 (*e.g.*, about 1, 2, 3, or 4) deoxyribonucleotides on the 3'-end of one or both strands. In another embodiment, a 5'-terminal phosphate group having Formula IV is present on the ENaC target-complementary strand of a siNA molecule of the invention, for example a siNA molecule having chemical modifications having any of Formulae I-VII.

[0110] In one embodiment, the invention features a chemically-modified short interfering nucleic acid (siNA) molecule capable of mediating RNA interference (RNAi) inside a cell or reconstituted *in vitro* system, wherein the chemical modification comprises one or more phosphorothioate internucleotide linkages. For example, in a non-limiting example, the invention features a chemically-modified short interfering nucleic acid (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.

[0111] Each strand of the double stranded siNA molecule can have one or more chemical modifications such that each strand comprises a different pattern of chemical modifications. Several non-limiting examples of modification schemes that could give rise to different patterns of modifications are provided herein.

[0112] 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, 2'-O-trifluoromethyl, 2'-O-ethyl-trifluoromethoxy, 2'-O-difluoromethoxy-ethoxy and/or about one or more (*e.g.*, about 1, 2, 3, 4, 5, 6, 7, 8, 9, 10 or more) universal base modified nucleotides, and optionally a terminal cap molecule at the 3'-end, the 5'-end, or both of the 3'- and 5'-ends of the sense strand; and wherein the antisense strand comprises about 1 to about 10 or more, specifically about 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, or more phosphorothioate internucleotide linkages, and/or one or more (*e.g.*, about 1, 2, 3, 4, 5, 6, 7, 8, 9, 10 or more) 2'-deoxy, 2'-O-methyl, 2'-deoxy-2'-fluoro, 2'-O-trifluoromethyl, 2'-O-ethyl-trifluoromethoxy, 2'-O-difluoromethoxy-ethoxy, 4'-thio and/or

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

[0113] In another embodiment, the invention features a siNA molecule, wherein the sense strand comprises about 1 to about 5, specifically about 1, 2, 3, 4, or 5 phosphorothioate internucleotide linkages, and/or one or more (*e.g.*, about 1, 2, 3, 4, 5, or more) 2'-deoxy, 2'-O-methyl, 2'-deoxy-2'-fluoro, 2'-O-trifluoromethyl, 2'-O-ethyl-trifluoromethoxy, 2'-O-difluoromethoxy-ethoxy, 4'-thio and/or one or more (*e.g.*, about 1, 2, 3, 4, 5, or more) universal base modified nucleotides, and optionally a terminal cap molecule at the 3-end, the 5'-end, or both of the 3'- and 5'-ends of the sense strand; and wherein the antisense strand comprises about 1 to about 5 or more, specifically about 1, 2, 3, 4, 5, or more phosphorothioate internucleotide linkages, and/or one or more (*e.g.*, about 1, 2, 3, 4, 5, 6, 7, 8, 9, 10 or more) 2'-deoxy, 2'-O-methyl, 2'-deoxy-2'-fluoro, 2'-O-trifluoromethyl, 2'-O-ethyl-trifluoromethoxy, 2'-O-difluoromethoxy-ethoxy, 4'-thio and/or one or more (*e.g.*, about 1, 2, 3, 4, 5, 6, 7, 8, 9, 10 or more) universal base modified nucleotides, and optionally a terminal cap molecule at the 3'-end, the 5'-end, or both of the 3'- and 5'-ends of the antisense strand. In another embodiment, one or more, for example about 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, or more, pyrimidine nucleotides of the sense and/or antisense siNA strand are chemically-modified with 2'-deoxy, 2'-O-methyl, 2'-O-trifluoromethyl, 2'-O-ethyl-trifluoromethoxy, 2'-O-difluoromethoxy-ethoxy, 4'-thio and/or 2'-deoxy-2'-fluoro nucleotides, with or without about 1 to about 5 or more, for example about 1, 2, 3, 4, 5, or more phosphorothioate internucleotide linkages and/or a terminal cap molecule at the 3'-end, the 5'-end, or both of the 3'- and 5'-ends, being present in the same or different strand.

[0114] In one embodiment, the invention features a siNA molecule, wherein the antisense strand comprises one or more, for example, about 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, or more phosphorothioate internucleotide linkages, and/or about one or more (*e.g.*, about 1, 2, 3, 4, 5,

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

[0115] In another embodiment, the invention features a siNA molecule, wherein the antisense strand comprises about 1 to about 5 or more, specifically about 1, 2, 3, 4, 5 or more phosphorothioate internucleotide linkages, and/or one or more (*e.g.*, about 1, 2, 3, 4, 5, 6, 7, 8, 9, 10 or more) 2'-deoxy, 2'-O-methyl, 2'-deoxy-2'-fluoro, 2'-O-trifluoromethyl, 2'-O-ethyl-trifluoromethoxy, 2'-O-difluoromethoxy-ethoxy, 4'-thio and/or one or more (*e.g.*, about 1, 2, 3, 4, 5, 6, 7, 8, 9, 10 or more) universal base modified nucleotides, and optionally a terminal cap molecule at the 3'-end, the 5'-end, or both of the 3'- and 5'-ends of the sense strand; and wherein the antisense strand comprises about 1 to about 5 or more, specifically about 1, 2, 3, 4, 5 or more phosphorothioate internucleotide linkages, and/or one or more (*e.g.*, about 1, 2, 3, 4, 5, 6, 7, 8, 9, 10 or more) 2'-deoxy, 2'-O-methyl, 2'-deoxy-2'-fluoro, 2'-O-trifluoromethyl, 2'-O-ethyl-trifluoromethoxy, 2'-O-difluoromethoxy-ethoxy, 4'-thio and/or one or more (*e.g.*, about 1, 2, 3, 4, 5, 6, 7, 8, 9, 10 or more) universal base modified nucleotides, and optionally a terminal cap molecule at the 3'-end, the 5'-end, or both of the 3'- and 5'-ends of the antisense strand. In another embodiment, one or more, for example about 1, 2, 3, 4, 5, 6, 7, 8, 9, 10 or more pyrimidine nucleotides of the sense and/or antisense siNA strand are

chemically-modified with 2'-deoxy, 2'-O-methyl, 2'-O-trifluoromethyl, 2'-O-ethyl-trifluoromethoxy, 2'-O-difluoromethoxy-ethoxy, 4'-thio and/or 2'-deoxy-2'-fluoro nucleotides, with or without about 1 to about 5, for example about 1, 2, 3, 4, 5 or more phosphorothioate internucleotide linkages and/or a terminal cap molecule at the 3'-end, the 5'-end, or both of the 3'- and 5'-ends, being present in the same or different strand.

[0116] In one embodiment, the invention features a chemically-modified short interfering nucleic acid (siNA) molecule having about 1 to about 5 or more (specifically about 1, 2, 3, 4, 5 or more) phosphorothioate internucleotide linkages in each strand of the siNA molecule.

[0117] In another embodiment, the invention features a siNA molecule comprising 2'-5' internucleotide linkages. The 2'-5' internucleotide linkage(s) can be at the 3'-end, the 5'-end, or both of the 3'- and 5'-ends of one or both siNA sequence strands. In addition, the 2'-5' internucleotide linkage(s) can be present at various other positions within one or both siNA sequence strands, for example, about 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, or more including every internucleotide linkage of a pyrimidine nucleotide in one or both strands of the siNA molecule can comprise a 2'-5' internucleotide linkage, or about 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, or more including every internucleotide linkage of a purine nucleotide in one or both strands of the siNA molecule can comprise a 2'-5' internucleotide linkage.

[0118] 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 independently about 15 to about 30 (*e.g.*, about 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, or 30) nucleotides in length, wherein the duplex has about 15 to about 30 (*e.g.*, about 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, or 30) base pairs, and wherein the chemical modification comprises a structure having any of Formulae I-VII. For example, an exemplary chemically-modified siNA molecule of the invention comprises a duplex having two strands, one or both of which can be chemically-modified with a chemical modification having any of Formulae I-VII or any combination thereof, wherein each strand consists of about 21 nucleotides, each having a 2-nucleotide 3'-terminal nucleotide overhang, and wherein the duplex has about 19 base pairs. In another embodiment, a siNA molecule of the invention comprises a single stranded hairpin structure, wherein the siNA is about 36 to about 70 (*e.g.*, about 36, 40, 45, 50, 55, 60, 65, or 70) nucleotides in length having about 15 to about 30 (*e.g.*, about 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, or 30) base pairs, and wherein the siNA can include a chemical

modification comprising a structure having any of Formulae I-VII or any combination thereof. For example, an exemplary chemically-modified siNA molecule of the invention comprises a linear oligonucleotide having about 42 to about 50 (*e.g.*, about 42, 43, 44, 45, 46, 47, 48, 49, or 50) nucleotides that is chemically-modified with a chemical modification having any of Formulae I-VII or any combination thereof, wherein the linear oligonucleotide forms a hairpin structure having about 19 to about 21 (*e.g.*, 19, 20, or 21) base pairs and a 2-nucleotide 3'-terminal nucleotide overhang. In another embodiment, a linear hairpin siNA molecule of the invention contains a stem loop motif, wherein the loop portion of the siNA molecule is biodegradable. For example, a linear hairpin siNA molecule of the invention is designed such that degradation of the loop portion of the siNA molecule *in vivo* can generate a double-stranded siNA molecule with 3'-terminal overhangs, such as 3'-terminal nucleotide overhangs comprising about 2 nucleotides.

[0119] In another embodiment, a siNA molecule of the invention comprises a hairpin structure, wherein the siNA is about 25 to about 50 (*e.g.*, about 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36, 37, 38, 39, 40, 41, 42, 43, 44, 45, 46, 47, 48, 49, or 50) nucleotides in length having about 3 to about 25 (*e.g.*, about 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, or 25) base pairs, and wherein the siNA can include one or more chemical modifications comprising a structure having any of Formulae I-VII or any combination thereof. For example, an exemplary chemically-modified siNA molecule of the invention comprises a linear oligonucleotide having about 25 to about 35 (*e.g.*, about 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, or 35) nucleotides that is chemically-modified with one or more chemical modifications having any of Formulae I-VII or any combination thereof, wherein the linear oligonucleotide forms a hairpin structure having about 3 to about 25 (*e.g.*, about 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, or 25) base pairs and a 5'-terminal phosphate group that can be chemically modified as described herein (for example a 5'-terminal phosphate group having Formula IV). In another embodiment, a linear hairpin siNA molecule of the invention contains a stem loop motif, wherein the loop portion of the siNA molecule is biodegradable. In one embodiment, a linear hairpin siNA molecule of the invention comprises a loop portion comprising a non-nucleotide linker.

[0120] In another embodiment, a siNA molecule of the invention comprises an asymmetric hairpin structure, wherein the siNA is about 25 to about 50 (*e.g.*, about 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36, 37, 38, 39, 40, 41, 42, 43, 44, 45, 46, 47, 48, 49, or 50)

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

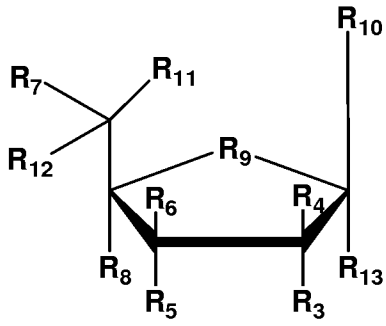
[0121] In another embodiment, a siNA molecule of the invention comprises an asymmetric double stranded structure having separate polynucleotide strands comprising sense and antisense regions, wherein the antisense region is about 15 to about 30 (*e.g.*, about 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, or 30) nucleotides in length, wherein the sense region is about 3 to about 25 (*e.g.*, about 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, or 25) nucleotides in length, wherein the sense region and the antisense region have at least 3 complementary nucleotides, and wherein the siNA can include one or more chemical modifications comprising a structure having any of Formulae I-VII or any combination thereof. For example, an exemplary chemically-modified siNA molecule of the invention comprises an asymmetric double stranded structure having separate polynucleotide strands comprising sense and antisense regions, wherein the antisense region is about 18 to about 23 (*e.g.*, about 18, 19, 20, 21, 22, or 23) nucleotides in length and wherein the sense region is about 3 to about 15 (*e.g.*, about 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, or 15) nucleotides in length, wherein the sense region the antisense region have at least 3 complementary nucleotides, and wherein the siNA can include one or more chemical modifications comprising a structure having any of Formulae I-VII or any combination thereof. In another embodiment, the asymmetric double stranded siNA molecule can also

have a 5'-terminal phosphate group that can be chemically modified as described herein (for example a 5'-terminal phosphate group having Formula IV).

[0122] In another embodiment, a siNA molecule of the invention comprises a circular nucleic acid molecule, wherein the siNA is about 38 to about 70 (*e.g.*, about 38, 40, 45, 50, 55, 60, 65, or 70) nucleotides in length having about 15 to about 30 (*e.g.*, about 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, or 30) base pairs, and wherein the siNA can include a chemical modification, which comprises a structure having any of Formulae I-VII or any combination thereof. For example, an exemplary chemically-modified siNA molecule of the invention comprises a circular oligonucleotide having about 42 to about 50 (*e.g.*, about 42, 43, 44, 45, 46, 47, 48, 49, or 50) nucleotides that is chemically-modified with a chemical modification having any of Formulae I-VII or any combination thereof, wherein the circular oligonucleotide forms a dumbbell shaped structure having about 19 base pairs and 2 loops.

[0123] In another embodiment, a circular siNA molecule of the invention contains two loop motifs, wherein one or both loop portions of the siNA molecule is biodegradable. For example, a circular siNA molecule of the invention is designed such that degradation of the loop portions of the siNA molecule *in vivo* can generate a double-stranded siNA molecule with 3'-terminal overhangs, such as 3'-terminal nucleotide overhangs comprising about 2 nucleotides.

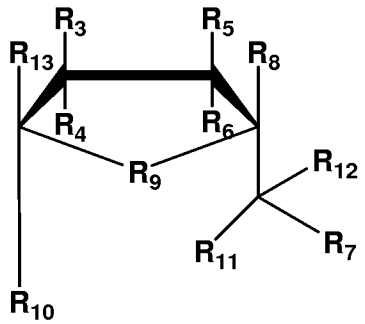
[0124] In one embodiment, a siNA molecule of the invention comprises at least one (*e.g.*, about 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, or more) abasic moiety, for example a compound having Formula V:



wherein each R3, R4, R5, R6, R7, R8, R10, R11, R12, and R13 is independently H, OH, alkyl, substituted alkyl, alkaryl or aralkyl, F, Cl, Br, CN, CF3, OCF3, OCH3, OCN, O-alkyl, S-alkyl, N-alkyl, O-alkenyl, S-alkenyl, N-alkenyl, SO-alkyl, alkyl-OSH, alkyl-OH, O-alkyl-

OH, O-alkyl-SH, S-alkyl-OH, S-alkyl-SH, alkyl-S-alkyl, alkyl-O-alkyl, ONO2, NO2, N3, NH2, aminoalkyl, aminoacid, aminoacyl, ONH2, O-aminoalkyl, O-aminoacid, O-aminoacyl, heterocycloalkyl, heterocycloalkaryl, aminoalkylamino, polyalklylamino, substituted silyl, or a group having any of Formula I, II, III, IV, V, VI and/or VII, any of which can be included in the structure of the siNA molecule or serve as a point of attachment to the siNA molecule; R9 is O, S, CH2, S=O, CHF, or CF2. In one embodiment, R3 and/or R7 comprises a conjugate moiety and a linker (*e.g.*, a nucleotide or non-nucleotide linker as described herein or otherwise known in the art). Non-limiting examples of conjugate moieties include ligands for cellular receptors, such as peptides derived from naturally occurring protein ligands; protein localization sequences, including cellular ZIP code sequences; antibodies; nucleic acid aptamers; vitamins and other co-factors, such as folate and N-acetylgalactosamine; polymers, such as polyethyleneglycol (PEG); phospholipids; cholesterol; steroids, and polyamines, such as PEI, spermine or spermidine.

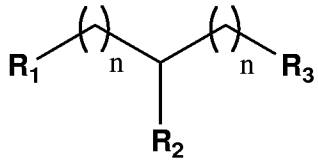
[0125] In one embodiment, a siNA molecule of the invention comprises at least one (*e.g.*, about 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, or more) inverted abasic moiety, for example a compound having Formula VI:



wherein each R3, R4, R5, R6, R7, R8, R10, R11, R12, and R13 is independently H, OH, alkyl, substituted alkyl, alkaryl or aralkyl, F, Cl, Br, CN, CF3, OCF3, OCH3, OCN, O-alkyl, S-alkyl, N-alkyl, O-alkenyl, S-alkenyl, N-alkenyl, SO-alkyl, alkyl-OSH, alkyl-OH, O-alkyl-OH, O-alkyl-SH, S-alkyl-OH, S-alkyl-SH, alkyl-S-alkyl, alkyl-O-alkyl, ONO2, NO2, N3, NH2, aminoalkyl, aminoacid, aminoacyl, ONH2, O-aminoalkyl, O-aminoacid, O-aminoacyl, heterocycloalkyl, heterocycloalkaryl, aminoalkylamino, polyalklylamino, substituted silyl, or a group having any of Formula I, II, III, IV, V, VI and/or VII, any of which can be included in the structure of the siNA molecule or serve as a point of attachment to the siNA molecule; R9 is O, S, CH2, S=O, CHF, or CF2, and either R2, R3, R8 or R13 serve as points of attachment to the siNA molecule of the invention. In one embodiment, R3 and/or R7

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

[0126] In another embodiment, a siNA molecule of the invention comprises at least one (*e.g.*, about 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, or more) substituted polyalkyl moieties, for example a compound having Formula VII:



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

[0127] By "ZIP code" sequences is meant, any peptide or protein sequence that is involved in cellular topogenic signaling mediated transport (see for example Ray *et al.*, 2004, *Science*, 306(1501): 1505).

[0128] Each nucleotide within the double stranded siNA molecule can independently have a chemical modification comprising the structure of any of Formulae I-VIII. Thus, in one embodiment, one or more nucleotide positions of a siNA molecule of the invention comprises a chemical modification having structure of any of Formulae I-VII or any other modification herein. In one embodiment, each nucleotide position of a siNA molecule of the invention comprises a chemical modification having structure of any of Formulae I-VII or any other modification herein.

[0129] In one embodiment, one or more nucleotide positions of one or both strands of a double stranded siNA molecule of the invention comprises a chemical modification having structure of any of Formulae 1-VII or any other modification herein. In one embodiment, each nucleotide position of one or both strands of a double stranded siNA molecule of the invention comprises a chemical modification having structure of any of Formulae I-VII or any other modification herein.

[0130] In another embodiment, the invention features a compound having Formula VII, wherein R1 and R2 are hydroxyl (OH) groups, n = 1, and R3 comprises O and is the point of attachment to the 3'-end, the 5'-end, or both of the 3' and 5'-ends of one or both strands of a double-stranded siNA molecule of the invention or to a single-stranded siNA molecule of the invention. This modification is referred to herein as "glyceryl" (for example modification 6 in **Figure 7**).

[0131] In another embodiment, a chemically modified nucleoside or non-nucleoside (*e.g.* a moiety having any of Formula V, VI or VII) of the invention is at the 3'-end, the 5'-end, or both of the 3' and 5'-ends of a siNA molecule of the invention. For example, chemically modified nucleoside or non-nucleoside (*e.g.*, a moiety having Formula V, VI or VII) can be present at the 3'-end, the 5'-end, or both of the 3' and 5'-ends of the antisense strand, the sense strand, or both antisense and sense strands of the siNA molecule. In one embodiment, the chemically modified nucleoside or non-nucleoside (*e.g.*, a moiety having Formula V, VI or VII) is present at the 5'-end and 3'-end of the sense strand and the 3'-end of the antisense strand of a double stranded siNA molecule of the invention. In one embodiment, the

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

[0132] 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.

[0133] 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.

[0134] In one embodiment, a siNA molecule of the invention comprises one or more (*e.g.*, about 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, or more) 4'-thio nucleotides, for example, at the 5'-end, the 3'-end, both of the 5' and 3'-ends, or any combination thereof, of the siNA molecule.

[0135] 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.

[0136] In one embodiment, a chemically-modified short interfering nucleic acid (siNA) molecule of the invention comprises a sense strand or sense region having one or more (e.g., 1, 2, 3, 4, 5, 6, 7, 8, 9, 10 , 11, 12, 13 ,14 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30 or more) 2'-O-alkyl (e.g. 2'-O-methyl), 2'-deoxy-2'-fluoro, 2'-deoxy, FANA, or abasic chemical modifications or any combination thereof.

[0137] In one embodiment, a chemically-modified short interfering nucleic acid (siNA) molecule of the invention comprises an antisense strand or antisense region having one or more (e.g., 1, 2, 3, 4, 5, 6, 7, 8, 9, 10 , 11, 12, 13 ,14 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30 or more) 2'-O-alkyl (e.g. 2'-O-methyl), 2'-deoxy-2'-fluoro, 2'-deoxy, FANA, or abasic chemical modifications or any combination thereof.

[0138] In one embodiment, a chemically-modified short interfering nucleic acid (siNA) molecule of the invention comprises a sense strand or sense region and an antisense strand or antisense region, each having one or more (e.g., 1, 2, 3, 4, 5, 6, 7, 8, 9, 10 , 11, 12, 13 ,14 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30 or more) 2'-O-alkyl (e.g. 2'-O-methyl), 2'-deoxy-2'-fluoro, 2'-deoxy, FANA, or abasic chemical modifications or any combination thereof.

[0139] 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 (ie. more than one) of pyrimidine nucleotides are 2'-deoxy-2'-fluoro pyrimidine nucleotides).

[0140] 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 FANA pyrimidine nucleotides (*e.g.*, wherein all pyrimidine nucleotides are FANA pyrimidine nucleotides or alternately a plurality (ie. more than one) of pyrimidine nucleotides are FANA pyrimidine nucleotides).

[0141] 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 (ie. more than one) of pyrimidine nucleotides are 2'-deoxy-2'-fluoro pyrimidine nucleotides).

[0142] In one embodiment, the invention features a chemically-modified short interfering nucleic acid (siNA) molecule of the invention comprising a sense region and an antisense

region, wherein any (*e.g.,* one or more or all) pyrimidine nucleotides present in the sense region and the antisense region are 2'-deoxy-2'-fluoro pyrimidine nucleotides (*e.g.*, wherein all pyrimidine nucleotides are 2'-deoxy-2'-fluoro pyrimidine nucleotides or alternately a plurality (ie. more than one) of pyrimidine nucleotides are 2'-deoxy-2'-fluoro pyrimidine nucleotides).

[0143] 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) purine nucleotides present in the sense region are 2'-deoxy purine nucleotides (*e.g.*, wherein all purine nucleotides are 2'-deoxy purine nucleotides or alternately a plurality (ie. more than one) of purine nucleotides are 2'-deoxy purine nucleotides).

[0144] 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) purine nucleotides present in the antisense region are 2'-O-methyl purine nucleotides (*e.g.*, wherein all purine nucleotides are 2'-O-methyl purine nucleotides or alternately a plurality (ie. more than one) of pyrimidine nucleotides are 2'-O-methyl purine nucleotides).

[0145] 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 (ie. more than one) of pyrimidine nucleotides are 2'-deoxy-2'-fluoro pyrimidine nucleotides), and wherein any (*e.g.,* one or more or all) purine nucleotides present in the sense region are 2'-deoxy purine nucleotides (*e.g.*, wherein all purine nucleotides are 2'-deoxy purine nucleotides or alternately a plurality (ie. more than one) of purine nucleotides are 2'-deoxy purine nucleotides).

[0146] 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, 4'-thio, 2'-O-trifluoromethyl, 2'-O-ethyl-trifluoromethoxy, or 2'-O-difluoromethoxy-ethoxy pyrimidine nucleotides (*e.g.*, wherein all pyrimidine nucleotides are 2'-deoxy-2'-fluoro, 4'-thio, 2'-O-trifluoromethyl, 2'-O-ethyl-trifluoromethoxy, or 2'-O-difluoromethoxy-ethoxy

pyrimidine nucleotides or alternately a plurality (ie. more than one) of pyrimidine nucleotides are 2'-deoxy-2'-fluoro, 4'-thio, 2'-O-trifluoromethyl, 2'-O-ethyl-trifluoromethoxy, or 2'-O-difluoromethoxy-ethoxy pyrimidine nucleotides), and wherein any (*e.g.,* one or more or all) purine nucleotides present in the sense region are 2'-deoxy purine nucleotides (*e.g.*, wherein all purine nucleotides are 2'-deoxy purine nucleotides or alternately a plurality (ie. more than one) of purine nucleotides are 2'-deoxy purine nucleotides), wherein any nucleotides comprising a 3'-terminal nucleotide overhang that are present in said sense region are 2'-deoxy nucleotides.

[0147] 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, 4'-thio, 2'-O-trifluoromethyl, 2'-O-ethyl-trifluoromethoxy, or 2'-O-difluoromethoxy-ethoxy pyrimidine nucleotides (*e.g.*, wherein all pyrimidine nucleotides are 2'-deoxy-2'-fluoro, 4'-thio, 2'-O-trifluoromethyl, 2'-O-ethyl-trifluoromethoxy, or 2'-O-difluoromethoxy-ethoxy pyrimidine nucleotides or alternately a plurality (ie. more than one) of pyrimidine nucleotides are 2'-deoxy-2'-fluoro, 4'-thio, 2'-O-trifluoromethyl, 2'-O-ethyl-trifluoromethoxy, or 2'-O-difluoromethoxy-ethoxy pyrimidine nucleotides), and wherein any (*e.g.,* one or more or all) purine nucleotides present in the sense region are 2'-O-methyl purine nucleotides (*e.g.*, wherein all purine nucleotides are 2'-O-methyl, 4'-thio, 2'-O-trifluoromethyl, 2'-O-ethyl-trifluoromethoxy, or 2'-O-difluoromethoxy-ethoxy purine nucleotides or alternately a plurality (ie. more than one) of purine nucleotides are 2'-O-methyl, 4'-thio, 2'-O-trifluoromethyl, 2'-O-ethyl-trifluoromethoxy, or 2'-O-difluoromethoxy-ethoxy purine nucleotides).

[0148] 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, 4'-thio, 2'-O-trifluoromethyl, 2'-O-ethyl-trifluoromethoxy, or 2'-O-difluoromethoxy-ethoxy pyrimidine nucleotides (*e.g.*, wherein all pyrimidine nucleotides are 2'-deoxy-2'-fluoro, 4'-thio, 2'-O-trifluoromethyl, 2'-O-ethyl-trifluoromethoxy, or 2'-O-difluoromethoxy-ethoxy pyrimidine nucleotides or alternately a plurality (ie. more than one) of pyrimidine nucleotides are 2'-deoxy-2'-fluoro, 4'-thio, 2'-O-trifluoromethyl, 2'-O-ethyl-trifluoromethoxy, or 2'-O-difluoromethoxy-ethoxy pyrimidine nucleotides), wherein any

(*e.g.*, one or more or all) purine nucleotides present in the sense region are 2'-O-methyl, 4'-thio, 2'-O-trifluoromethyl, 2'-O-ethyl-trifluoromethoxy, or 2'-O-difluoromethoxy-ethoxy purine nucleotides (*e.g.*, wherein all purine nucleotides are 2'-O-methyl, 4'-thio, 2'-O-trifluoromethyl, 2'-O-ethyl-trifluoromethoxy, or 2'-O-difluoromethoxy-ethoxy purine nucleotides or alternately a plurality (ie. more than one) of purine nucleotides are 2'-O-methyl, 4'-thio, 2'-O-trifluoromethyl, 2'-O-ethyl-trifluoromethoxy, or 2'-O-difluoromethoxy-ethoxy purine nucleotides), and wherein any nucleotides comprising a 3'-terminal nucleotide overhang that are present in said sense region are 2'-deoxy nucleotides.

[0149] 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, 4'-thio, 2'-O-trifluoromethyl, 2'-O-ethyl-trifluoromethoxy, or 2'-O-difluoromethoxy-ethoxy pyrimidine nucleotides (*e.g.*, wherein all pyrimidine nucleotides are 2'-deoxy-2'-fluoro, 4'-thio, 2'-O-trifluoromethyl, 2'-O-ethyl-trifluoromethoxy, or 2'-O-difluoromethoxy-ethoxy pyrimidine nucleotides or alternately a plurality (ie. more than one) of pyrimidine nucleotides are 2'-deoxy-2'-fluoro, 4'-thio, 2'-O-trifluoromethyl, 2'-O-ethyl-trifluoromethoxy, or 2'-O-difluoromethoxy-ethoxy pyrimidine nucleotides), and wherein any (*e.g.*, one or more or all) purine nucleotides present in the antisense region are 2'-O-methyl, 4'-thio, 2'-O-trifluoromethyl, 2'-O-ethyl-trifluoromethoxy, or 2'-O-difluoromethoxy-ethoxy purine nucleotides (*e.g.*, wherein all purine nucleotides are 2'-O-methyl, 4'-thio, 2'-O-trifluoromethyl, 2'-O-ethyl-trifluoromethoxy, or 2'-O-difluoromethoxy-ethoxy purine nucleotides or alternately a plurality (ie. more than one) of purine nucleotides are 2'-O-methyl, 4'-thio, 2'-O-trifluoromethyl, 2'-O-ethyl-trifluoromethoxy, or 2'-O-difluoromethoxy-ethoxy purine nucleotides).

[0150] 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, 4'-thio, 2'-O-trifluoromethyl, 2'-O-ethyl-trifluoromethoxy, or 2'-O-difluoromethoxy-ethoxy pyrimidine nucleotides (*e.g.*, wherein all pyrimidine nucleotides are 2'-deoxy-2'-fluoro, 4'-thio, 2'-O-trifluoromethyl, 2'-O-ethyl-trifluoromethoxy, or 2'-O-difluoromethoxy-ethoxy pyrimidine nucleotides or alternately a plurality (ie. more than one) of pyrimidine nucleotides are 2'-deoxy-2'-fluoro, 4'-thio, 2'-O-trifluoromethyl, 2'-O-ethyl-

trifluoromethoxy, or 2'-O-difluoromethoxy-ethoxy pyrimidine nucleotides), wherein any (*e.g.,* one or more or all) purine nucleotides present in the antisense region are 2'-O-methyl, 4'-thio, 2'-O-trifluoromethyl, 2'-O-ethyl-trifluoromethoxy, or 2'-O-difluoromethoxy-ethoxy purine nucleotides (*e.g.*, wherein all purine nucleotides are 2'-O-methyl, 4'-thio, 2'-O-trifluoromethyl, 2'-O-ethyl-trifluoromethoxy, or 2'-O-difluoromethoxy-ethoxy purine nucleotides or alternately a plurality (ie. more than one) of purine nucleotides are 2'-O-methyl, 4'-thio, 2'-O-trifluoromethyl, 2'-O-ethyl-trifluoromethoxy, or 2'-O-difluoromethoxy-ethoxy purine nucleotides), and wherein any nucleotides comprising a 3'-terminal nucleotide overhang that are present in said antisense region are 2'-deoxy nucleotides.

[0151] 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, 4'-thio, 2'-O-trifluoromethyl, 2'-O-ethyl-trifluoromethoxy, or 2'-O-difluoromethoxy-ethoxy pyrimidine nucleotides (*e.g.*, wherein all pyrimidine nucleotides are 2'-deoxy-2'-fluoro, 4'-thio, 2'-O-trifluoromethyl, 2'-O-ethyl-trifluoromethoxy, or 2'-O-difluoromethoxy-ethoxy pyrimidine nucleotides or alternately a plurality (ie. more than one) of pyrimidine nucleotides are 2'-deoxy-2'-fluoro, 4'-thio, 2'-O-trifluoromethyl, 2'-O-ethyl-trifluoromethoxy, or 2'-O-difluoromethoxy-ethoxy pyrimidine nucleotides), and wherein any (*e.g.,* one or more or all) purine nucleotides present in the antisense region are 2'-deoxy purine nucleotides (*e.g.*, wherein all purine nucleotides are 2'-deoxy purine nucleotides or alternately a plurality (ie. more than one) of purine nucleotides are 2'-deoxy purine nucleotides).

[0152] 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, 4'-thio, 2'-O-trifluoromethyl, 2'-O-ethyl-trifluoromethoxy, or 2'-O-difluoromethoxy-ethoxy pyrimidine nucleotides (*e.g.*, wherein all pyrimidine nucleotides are 2'-deoxy-2'-fluoro, 4'-thio, 2'-O-trifluoromethyl, 2'-O-ethyl-trifluoromethoxy, or 2'-O-difluoromethoxy-ethoxy pyrimidine nucleotides or alternately a plurality (ie. more than one) of pyrimidine nucleotides are 2'-deoxy-2'-fluoro, 4'-thio, 2'-O-trifluoromethyl, 2'-O-ethyl-trifluoromethoxy, or 2'-O-difluoromethoxy-ethoxy pyrimidine nucleotides), and wherein any (*e.g.,* one or more or all) purine nucleotides present in the antisense region are 2'-O-methyl,

4'-thio, 2'-O-trifluoromethyl, 2'-O-ethyl-trifluoromethoxy, or 2'-O-difluoromethoxy-ethoxy purine nucleotides (*e.g.*, wherein all purine nucleotides are 2'-O-methyl, 4'-thio, 2'-O-trifluoromethyl, 2'-O-ethyl-trifluoromethoxy, or 2'-O-difluoromethoxy-ethoxy purine nucleotides or alternately a plurality (ie. more than one) of purine nucleotides are 2'-O-methyl, 4'-thio, 2'-O-trifluoromethyl, 2'-O-ethyl-trifluoromethoxy, or 2'-O-difluoromethoxy-ethoxy purine nucleotides).

[0153] In one embodiment, the invention features a chemically-modified short interfering nucleic acid (siNA) molecule of the invention capable of mediating RNA interference (RNAi) inside a cell or reconstituted *in vitro* system comprising a sense region, wherein one or more pyrimidine nucleotides present in the sense region are 2'-deoxy-2'-fluoro, 4'-thio, 2'-O-trifluoromethyl, 2'-O-ethyl-trifluoromethoxy, or 2'-O-difluoromethoxy-ethoxy pyrimidine nucleotides (*e.g.*, wherein all pyrimidine nucleotides are 2'-deoxy-2'-fluoro, 4'-thio, 2'-O-trifluoromethyl, 2'-O-ethyl-trifluoromethoxy, or 2'-O-difluoromethoxy-ethoxy pyrimidine nucleotides or alternately a plurality (ie. more than one) of pyrimidine nucleotides are 2'-deoxy-2'-fluoro, 4'-thio, 2'-O-trifluoromethyl, 2'-O-ethyl-trifluoromethoxy, or 2'-O-difluoromethoxy-ethoxy pyrimidine nucleotides), and one or more purine nucleotides present in the sense region are 2'-deoxy purine nucleotides (*e.g.*, wherein all purine nucleotides are 2'-deoxy purine nucleotides or alternately a plurality (ie. more than one) of purine nucleotides are 2'-deoxy purine nucleotides), and an antisense region, wherein one or more pyrimidine nucleotides present in the antisense region are 2'-deoxy-2'-fluoro, 4'-thio, 2'-O-trifluoromethyl, 2'-O-ethyl-trifluoromethoxy, or 2'-O-difluoromethoxy-ethoxy pyrimidine nucleotides (*e.g.*, wherein all pyrimidine nucleotides are 2'-deoxy-2'-fluoro, 4'-thio, 2'-O-trifluoromethyl, 2'-O-ethyl-trifluoromethoxy, or 2'-O-difluoromethoxy-ethoxy pyrimidine nucleotides or alternately a plurality (ie. more than one) of pyrimidine nucleotides are 2'-deoxy-2'-fluoro, 4'-thio, 2'-O-trifluoromethyl, 2'-O-ethyl-trifluoromethoxy, or 2'-O-difluoromethoxy-ethoxy pyrimidine nucleotides), and one or more purine nucleotides present in the antisense region are 2'-O-methyl, 4'-thio, 2'-O-trifluoromethyl, 2'-O-ethyl-trifluoromethoxy, or 2'-O-difluoromethoxy-ethoxy purine nucleotides (*e.g.*, wherein all purine nucleotides are 2'-O-methyl, 4'-thio, 2'-O-trifluoromethyl, 2'-O-ethyl-trifluoromethoxy, or 2'-O-difluoromethoxy-ethoxy purine nucleotides or alternately a plurality (ie. more than one) of purine nucleotides are 2'-O-methyl, 4'-thio, 2'-O-trifluoromethyl, 2'-O-ethyl-trifluoromethoxy, or 2'-O-difluoromethoxy-ethoxy purine nucleotides). The sense region and/or the antisense region can have a terminal cap

modification, such as any modification described herein or shown in **Figure 7**, that is optionally present at the 3'-end, the 5'-end, or both of the 3' and 5'-ends of the sense and/or antisense sequence. The sense and/or antisense region can optionally further comprise a 3'-terminal nucleotide overhang having about 1 to about 4 (*e.g.*, about 1, 2, 3, or 4) 2'-deoxynucleotides. The overhang nucleotides can further comprise one or more (*e.g.*, about 1, 2, 3, 4 or more) phosphorothioate, phosphonoacetate, and/or thiophosphonoacetate internucleotide linkages. Non-limiting examples of these chemically-modified siNAs are shown in **Figures 4 and 5** and **Tables 1b and 8** herein. In any of these described embodiments, the purine nucleotides present in the sense region are alternatively 2'-O-methyl, 4'-thio, 2'-O-trifluoromethyl, 2'-O-ethyl-trifluoromethoxy, or 2'-O-difluoromethoxy-ethoxy purine nucleotides (*e.g.*, wherein all purine nucleotides are 2'-O-methyl, 4'-thio, 2'-O-trifluoromethyl, 2'-O-ethyl-trifluoromethoxy, or 2'-O-difluoromethoxy-ethoxy purine nucleotides or alternately a plurality of purine nucleotides are 2'-O-methyl, 4'-thio, 2'-O-trifluoromethyl, 2'-O-ethyl-trifluoromethoxy, or 2'-O-difluoromethoxy-ethoxy purine nucleotides) and one or more purine nucleotides present in the antisense region are 2'-O-methyl, 4'-thio, 2'-O-trifluoromethyl, 2'-O-ethyl-trifluoromethoxy, or 2'-O-difluoromethoxy-ethoxy purine nucleotides (*e.g.*, wherein all purine nucleotides are 2'-O-methyl, 4'-thio, 2'-O-trifluoromethyl, 2'-O-ethyl-trifluoromethoxy, or 2'-O-difluoromethoxy-ethoxy purine nucleotides or alternately a plurality (ie. more than one) of purine nucleotides are 2'-O-methyl, 4'-thio, 2'-O-trifluoromethyl, 2'-O-ethyl-trifluoromethoxy, or 2'-O-difluoromethoxy-ethoxy purine nucleotides). Also, in any of these embodiments, one or more purine nucleotides present in the sense region are alternatively purine ribonucleotides (*e.g.*, wherein all purine nucleotides are purine ribonucleotides or alternately a plurality (ie. more than one) of purine nucleotides are purine ribonucleotides) and any purine nucleotides present in the antisense region are 2'-O-methyl, 4'-thio, 2'-O-trifluoromethyl, 2'-O-ethyl-trifluoromethoxy, or 2'-O-difluoromethoxy-ethoxy purine nucleotides (*e.g.*, wherein all purine nucleotides are 2'-O-methyl, 4'-thio, 2'-O-trifluoromethyl, 2'-O-ethyl-trifluoromethoxy, or 2'-O-difluoromethoxy-ethoxy purine nucleotides or alternately a plurality (ie. more than one) of purine nucleotides are 2'-O-methyl, 4'-thio, 2'-O-trifluoromethyl, 2'-O-ethyl-trifluoromethoxy, or 2'-O-difluoromethoxy-ethoxy purine nucleotides). Additionally, in any of these embodiments, one or more purine nucleotides present in the sense region and/or present in the antisense region are alternatively selected from the group consisting of 2'-deoxy nucleotides, locked nucleic acid (LNA) nucleotides, 2'-methoxyethyl nucleotides, 4'-thionucleotides, 2'-O-trifluoromethyl nucleotides, 2'-O-ethyl-trifluoromethoxy nucleotides,

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

[0154] 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) otherwise known as a "ribo-like" or "A-form helix" configuration. Such nucleotides having a Northern conformation are generally considered to be "ribo-like" as they have a C3'-endo sugar pucker conformation. As such, chemically modified nucleotides present in the siNA molecules of the invention, preferably in the antisense strand of the siNA molecules of the invention, but also optionally in the sense and/or both antisense and sense strands, are resistant to nuclease degradation while at the same time maintaining the capacity to mediate RNAi. Non-limiting examples of nucleotides having a northern configuration include locked nucleic acid (LNA) nucleotides (*e.g.*, 2´-O, 4´-C-methylene-(D-ribofuranosyl) nucleotides); 2'-methoxyethoxy (MOE) nucleotides; 2'-methyl-thio-ethyl, 2'-deoxy-2'-fluoro nucleotides, 2'-deoxy-2'-chloro nucleotides, 2'-azido nucleotides, 2'-O-trifluoromethyl nucleotides, 2'-O-ethyl-trifluoromethoxy nucleotides, 2'-O-difluoromethoxy-ethoxy nucleotides, 4'-thio nucleotides and 2'-O-methyl nucleotides.

[0155] In one embodiment, the sense strand of a double stranded siNA molecule of the invention comprises a terminal cap moiety, (see for example **Figure 7**) such as an inverted deoxyabaisc moiety, at the 3'-end, 5'-end, or both 3' and 5'-ends of the sense strand.

[0156] In one embodiment, the invention features a chemically-modified short interfering nucleic acid molecule (siNA) capable of mediating RNA interference (RNAi) inside a cell or reconstituted *in vitro* system, wherein the chemical modification comprises a conjugate covalently attached to the chemically-modified siNA molecule. Non-limiting examples of conjugates contemplated by the invention include conjugates and ligands described in Vargeese *et al.,* USSN 10/427,160, filed April 30, 2003, incorporated by reference herein in its entirety, including the drawings. In another embodiment, the conjugate is covalently attached to the chemically-modified siNA molecule via a biodegradable linker. In one embodiment, the conjugate molecule is attached at the 3'-end of either the sense strand, the antisense strand, or both strands of the chemically-modified siNA molecule. In another embodiment, the conjugate molecule is attached at the 5'-end of either the sense strand, the antisense strand, or both strands of the chemically-modified siNA molecule. In yet another embodiment, the conjugate molecule is attached both the 3'-end and 5'-end of either the sense strand, the antisense strand, or both strands of the chemically-modified siNA molecule, or any combination thereof. In one embodiment, a conjugate molecule of the invention comprises a molecule that facilitates delivery of a chemically-modified siNA molecule into a biological system, such as a cell. In another embodiment, the conjugate molecule attached to the chemically-modified siNA molecule is a ligand for a cellular receptor, such as peptides derived from naturally occurring protein ligands; protein localization sequences, including cellular ZIP code sequences; antibodies; nucleic acid aptamers; vitamins and other co-factors, such as folate and N-acetylgalactosamine; polymers, such as polyethyleneglycol (PEG); phospholipids; cholesterol; steroids, and polyamines, such as PEI, spermine or spermidine. Examples of specific conjugate molecules contemplated by the instant invention that can be attached to chemically-modified siNA molecules are described in Vargeese *et al.,* U.S. Serial No. 10/201,394, filed July 22, 2002 incorporated by reference herein. The type of conjugates used and the extent of conjugation of siNA molecules of the invention can be evaluated for improved pharmacokinetic profiles, bioavailability, and/or stability of siNA constructs while at the same time maintaining the ability of the siNA to mediate RNAi activity. As such, one skilled in the art can screen siNA constructs that are modified with various conjugates to determine whether the siNA conjugate complex possesses improved properties while maintaining the ability to mediate RNAi, for example in animal models as are generally known in the art.

[0157] In one embodiment, the invention features a short interfering nucleic acid (siNA) molecule of the invention, wherein the siNA further comprises a nucleotide, non-nucleotide, or mixed nucleotide/non-nucleotide linker that joins the sense region of the siNA to the antisense region of the siNA. In one embodiment, a nucleotide, non-nucleotide, or mixed nucleotide/non-nucleotide linker is used, for example, to attach a conjugate moiety to the siNA. In one embodiment, a nucleotide linker of the invention can be a linker of ≥ 2 nucleotides in length, for example about 3, 4, 5, 6, 7, 8, 9, or 10 nucleotides in length. In another embodiment, the nucleotide linker can be a nucleic acid aptamer. By "aptamer" or "nucleic acid aptamer" as used herein is meant a nucleic acid molecule that binds specifically to an ENaC target molecule wherein the nucleic acid molecule has sequence that comprises a sequence recognized by the ENaC target molecule in its natural setting. Alternately, an aptamer can be a nucleic acid molecule that binds to an ENaC target molecule where the ENaC target molecule does not naturally bind to a nucleic acid. The ENaC target molecule can be any molecule of interest (e.g., EnaC or any isotype thereof). For example, the aptamer can be used to bind to a ligand-binding domain of a protein, thereby preventing interaction of the naturally occurring ligand with the protein. This is a non-limiting example and those in the art will recognize that other embodiments can be readily generated using techniques generally known in the art. (See, for example, Gold *et al.,* 1995, *Annu. Rev. Biochem.,* 64, 763; Brody and Gold, 2000, *J. Biotechnol.,* 74, 5; Sun, 2000, *Curr. Opin. Mol. Ther.,* 2, 100; Kusser, 2000, *J. Biotechnol.,* 74, 27; Hermann and Patel, 2000, *Science,* 287, 820; and Jayasena, 1999, *Clinical Chemistry,* 45, 1628.)

[0158] In yet another embodiment, a non-nucleotide linker of the invention comprises abasic nucleotide, polyether, polyamine, polyamide, peptide, carbohydrate, lipid, polyhydrocarbon, or other polymeric compounds (*e.g.* polyethylene glycols such as those having between 2 and 100 ethylene glycol units). Specific examples include those described by Seela and Kaiser, *Nucleic Acids Res.* 1990, *18*:6353 and *Nucleic Acids Res.* 1987, *15*:3113; Cload and Schepartz, *J. Am. Chem. Soc.* 1991, *113*:6324; Richardson and Schepartz, *J. Am. Chem. Soc.* 1991, *113*:5109; Ma *et al., Nucleic Acids Res.* 1993, *21*:2585 and *Biochemistry* 1993, *32*:1751; Durand *et al., Nucleic Acids Res.* 1990, *18*:6353; McCurdy *et al., Nucleosides & Nucleotides* 1991, *10*:287; Jschke *et al., Tetrahedron Lett.* 1993, *34*:301; Ono *et al., Biochemistry* 1991, *30*:9914; Arnold *et al.,* International Publication No. WO 89/02439; Usman *et al.,* International Publication No. WO 95/06731; Dudycz *et al.,* International Publication No. WO 95/11910 and Ferentz and Verdine, *J. Am. Chem. Soc.*

1991, *113*:4000, all hereby incorporated by reference herein. A "non-nucleotide" further means any group or compound that can be incorporated into a nucleic acid chain in the place of one or more nucleotide units, including either sugar and/or phosphate substitutions, and allows the remaining bases to exhibit their enzymatic activity. The group or compound can be abasic in that it does not contain a commonly recognized nucleotide base, such as adenosine, guanine, cytosine, uracil or thymine, for example at the C1 position of the sugar.

[0159] 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 oligonculeotide where the sense and antisense regions of the siNA comprise separate oligonucleotides that do not have any ribonucleotides (*e.g.*, nucleotides having a 2'-OH group) present in the oligonucleotides. In another example, a siNA molecule can be assembled from a single oligonculeotide where the sense and antisense regions of the siNA are linked or circularized by a nucleotide or non-nucleotide linker as described herein, wherein the oligonucleotide does not have any ribonucleotides (*e.g.*, nucleotides having a 2'-OH group) present in the oligonucleotide. Applicant has surprisingly found that the presense of ribonucleotides (*e.g.*, nucleotides having a 2'-hydroxyl group) within the siNA molecule is not required or essential to support RNAi activity. As such, in one embodiment, all positions within the siNA can include chemically modified nucleotides and/or non-nucleotides such as nucleotides and or non-nucleotides having Formula I, II, III, IV, V, VI, or VII or any combination thereof to the extent that the ability of the siNA molecule to support RNAi activity in a cell is maintained.

[0160] In one embodiment, a chemically-modified short interfering nucleic acid (siNA) molecule of the invention comprises a sense strand or sense region having two or more (e.g., 2, 3, 4, 5, 6, 7, 8, 9, 10 , 11, 12, 13 ,14 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30 or more) 2'-O-alkyl (e.g. 2'-O-methyl) modifications or any combination thereof. In another embodiment, the 2'-O-alkyl modification is at alternating position in the sense strand or sense region of the siNA, such as position 1, 3, 5, 7, 9, 11, 13, 15, 17, 19, 21 etc. or position 2, 4, 6, 8, 10, 12, 14, 16, 18, 20 etc.

[0161] In one embodiment, a chemically-modified short interfering nucleic acid (siNA) molecule of the invention comprises an antisense strand or antisense region having two or

more (e.g., 2, 3, 4, 5, 6, 7, 8, 9, 10 , 11, 12, 13 ,14 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30 or more) 2'-O-alkyl (e.g. 2'-O-methyl) modifications or any combination thereof. In another embodiment, the 2'-O-alkyl modification is at alternating position in the antisense strand or antisense region of the siNA, such as position 1, 3, 5, 7, 9, 11, 13, 15, 17, 19, 21 etc. or position 2, 4, 6, 8, 10, 12, 14, 16, 18, 20 etc.

[0162] In one embodiment, a chemically-modified short interfering nucleic acid (siNA) molecule of the invention comprises a sense strand or sense region and an antisense strand or antisense region, each having two or more (e.g., 2, 3, 4, 5, 6, 7, 8, 9, 10 , 11, 12, 13 ,14 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30 or more) 2'-O-alkyl (e.g. 2'-O-methyl), 2'-deoxy-2'-fluoro, 2'-deoxy, or abasic chemical modifications or any combination thereof. In another embodiment, the 2'-O-alkyl modification is at alternating position in the sense strand or sense region of the siNA, such as position 1, 3, 5, 7, 9, 11, 13, 15, 17, 19, 21 etc. or position 2, 4, 6, 8, 10, 12, 14, 16, 18, 20 etc. In another embodiment, the 2'-O-alkyl modification is at alternating position in the antisense strand or antisense region of the siNA, such as position 1, 3, 5, 7, 9, 11, 13, 15, 17, 19, 21 etc. or position 2, 4, 6, 8, 10, 12, 14, 16, 18, 20 etc.

[0163] In one embodiment, a siNA molecule of the invention comprises chemically modified nucleotides or non-nucleotides (*e.g.*, having any of Formulae I-VII, such as 2'-deoxy, 2'-deoxy-2'-fluoro, 4'-thio, 2'-O-trifluoromethyl, 2'-O-ethyl-trifluoromethoxy, 2'-O-difluoromethoxy-ethoxy or 2'-O-methyl nucleotides) at alternating positions within one or more strands or regions of the siNA molecule. For example, such chemical modifications can be introduced at every other position of a RNA based siNA molecule, starting at either the first or second nucleotide from the 3'-end or 5'-end of the siNA. In a non-limiting example, a double stranded siNA molecule of the invention in which each strand of the siNA is 21 nucleotides in length is featured wherein positions 1, 3, 5, 7, 9, 11, 13, 15, 17, 19 and 21 of each strand are chemically modified (*e.g.*, with compounds having any of Formulae I-VII, such as such as 2'-deoxy, 2'-deoxy-2'-fluoro, 4'-thio, 2'-O-trifluoromethyl, 2'-O-ethyl-trifluoromethoxy, 2'-O-difluoromethoxy-ethoxy or 2'-O-methyl nucleotides). In another non-limiting example, a double stranded siNA molecule of the invention in which each strand of the siNA is 21 nucleotides in length is featured wherein positions 2, 4, 6, 8, 10, 12, 14, 16, 18, and 20 of each strand are chemically modified (*e.g.*, with compounds having any of Formulae I-VII, such as such as 2'-deoxy, 2'-deoxy-2'-fluoro, 4'-thio, 2'-O-trifluoromethyl,

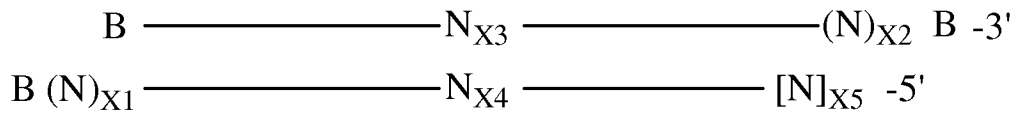
2'-O-ethyl-trifluoromethoxy, 2'-O-difluoromethoxy-ethoxy or 2'-O-methyl nucleotides). In one embodiment, one strand of the double stranded siNA molecule comprises chemical modifications at positions 2, 4, 6, 8, 10, 12, 14, 16, 18, and 20 and chemical modifications at positions 1, 3, 5, 7, 9, 11, 13, 15, 17, 19 and 21. Such siNA molecules can further comprise terminal cap moieties and/or backbone modifications as described herein.

[0164] In one embodiment, a siNA molecule of the invention comprises the following features: if purine nucleotides are present at the 5'-end (*e.g.*, at any of terminal nucleotide positions 1, 2, 3, 4, 5, or 6 from the 5'-end) of the antisense strand or antisense region (otherwise referred to as the guide sequence or guide strand) of the siNA molecule then such purine nucleosides are ribonucleotides. In another embodiment, the purine ribonucleotides, when present, are base paired to nucleotides of the sense strand or sense region (otherwise referred to as the passenger strand) of the siNA molecule. Such purine ribonucleotides can be present in a siNA stabilization motif that otherwise comprises modified nucleotides.

[0165] In one embodiment, a siNA molecule of the invention comprises the following features: if pyrimidine nucleotides are present at the 5'-end (*e.g.*, at any of terminal nucleotide positions 1, 2, 3, 4, 5, or 6 from the 5'-end) of the antisense strand or antisense region (otherwise referred to as the guide sequence or guide strand) of the siNA molecule then such pyrimidine nucleosides are ribonucleotides. In another embodiment, the pyrimidine ribonucleotides, when present, are base paired to nucleotides of the sense strand or sense region (otherwise referred to as the passenger strand) of the siNA molecule. Such pyrimidine ribonucleotides can be present in a siNA stabilization motif that otherwise comprises modified nucleotides.

[0166] In one embodiment, a siNA molecule of the invention comprises the following features: if pyrimidine nucleotides are present at the 5'-end (*e.g.*, at any of terminal nucleotide positions 1, 2, 3, 4, 5, or 6 from the 5'-end) of the antisense strand or antisense region (otherwise referred to as the guide sequence or guide strand) of the siNA molecule then such pyrimidine nucleosides are modified nucleotides. In another embodiment, the modified pyrimidine nucleotides, when present, are base paired to nucleotides of the sense strand or sense region (otherwise referred to as the passenger strand) of the siNA molecule. Non-limiting examples of modified pyrimidine nucleotides include those having any of Formulae I-VII, such as such as 2'-deoxy, 2'-deoxy-2'-fluoro, 4'-thio, 2'-O-trifluoromethyl, 2'-O-ethyl-trifluoromethoxy, 2'-O-difluoromethoxy-ethoxy or 2'-O-methyl nucleotides.

[0167] In one embodiment, the invention features a double stranded nucleic acid (siNA) molecule having structure SI:



SI

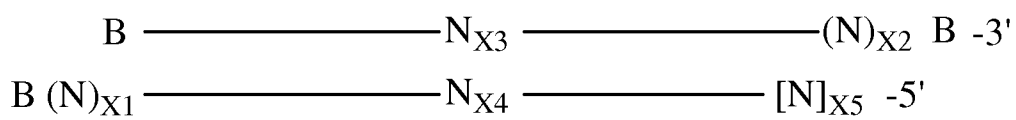
wherein each N is independently a nucleotide which can be unmodified or chemically modified; each B is a terminal cap moiety that can be present or absent; (N) represents non-base paired or overhanging nucleotides which can be unmodified or chemically modified; [N] represents nucleotide positions wherein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

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

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

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

[0168] In one embodiment, the invention features a double stranded nucleic acid (siNA) molecule having structure SII:



SII

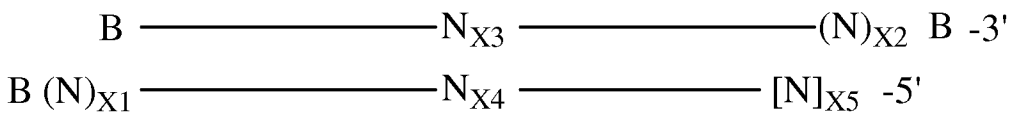
wherein each N is independently a nucleotide which can be unmodified or chemically modified; each B is a terminal cap moiety that can be present or absent; (N) represents non-base paired or overhanging nucleotides which can be unmodified or chemically modified; [N] represents nucleotide positions wherein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

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

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

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

[0169] In one embodiment, the invention features a double stranded nucleic acid (siNA) molecule having structure SIII:



SIII

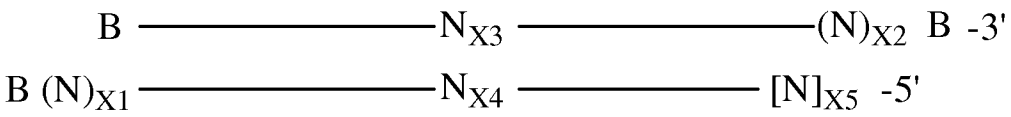
wherein each N is independently a nucleotide which can be unmodified or chemically modified; each B is a terminal cap moiety that can be present or absent; (N) represents non-base paired or overhanging nucleotides which can be unmodified or chemically modified; [N] represents nucleotide positions wherein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

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

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

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

[0170] In one embodiment, the invention features a double stranded nucleic acid (siNA) molecule having structure SIV:



SIV

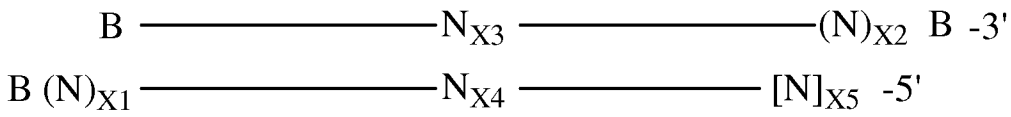
wherein each N is independently a nucleotide which can be unmodified or chemically modified; each B is a terminal cap moiety that can be present or absent; (N) represents non-base paired or overhanging nucleotides which can be unmodified or chemically modified; [N] represents nucleotide positions wherein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

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

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

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

[0171] In one embodiment, the invention features a double stranded nucleic acid (siNA) molecule having structure SV:



SV

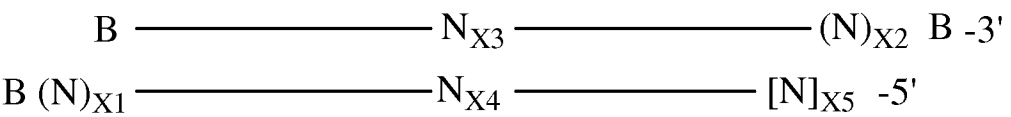
wherein each N is independently a nucleotide which can be unmodified or chemically modified; each B is a terminal cap moiety that can be present or absent; (N) represents non-base paired or overhanging nucleotides which can be unmodified or chemically modified; [N] represents nucleotide positions wherein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

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

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

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

[0172] In one embodiment, the invention features a double stranded nucleic acid (siNA) molecule having structure SVI:



SVI

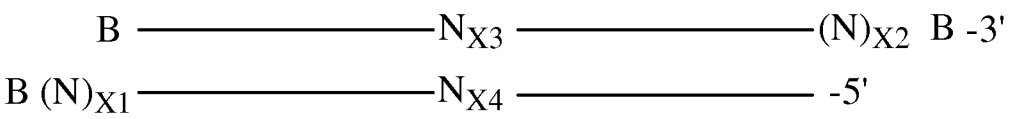
wherein each N is independently a nucleotide which can be unmodified or chemically modified; each B is a terminal cap moiety that can be present or absent; (N) represents non-base paired or overhanging nucleotides which can be unmodified or chemically modified; [N] represents nucleotide positions comprising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

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

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

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

[0173] In one embodiment, the invention features a double stranded nucleic acid (siNA) molecule having structure SVII:

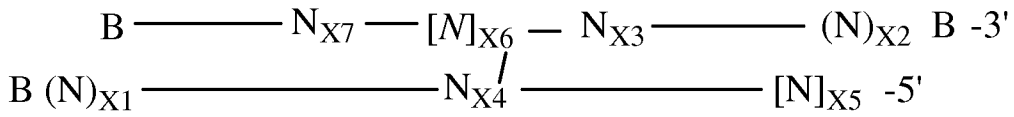


SVII

wherein each N is independently a nucleotide which can be unmodified or chemically modified; each B is a terminal cap moiety that can be present or absent; (N) represents non-base paired or overhanging nucleotides; X1 and X2 are independently

integers from about 0 to about 4; X3 is an integer from about 9 to about 30; X4 is an integer from about 11 to about 30; NX3 is complementary to NX4, and any (N) nucleotides are 2'-O-methyl and/or 2'-deoxy-2'-fluoro nucleotides.

[0174] In one embodiment, the invention features a double stranded nucleic acid (siNA) molecule having structure SVIII:



SVIII

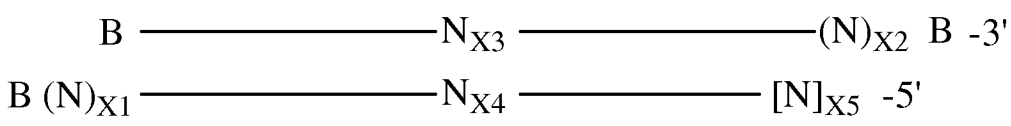
wherein each N is independently a nucleotide which can be unmodified or chemically modified; each B is a terminal cap moiety that can be present or absent; (N) represents non-base paired or overhanging nucleotides which can be unmodified or chemically modified; [N] represents nucleotide positions comprising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

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

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

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

[0175] In one embodiment, the invention features a double stranded nucleic acid (siNA) molecule having structure SIX:



SIX

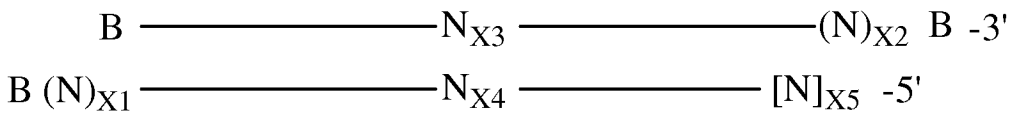
wherein each N is independently a nucleotide which can be unmodified or chemically modified; each B is a terminal cap moiety that can be present or absent; (N) represents non-base paired or overhanging nucleotides which can be unmodified or chemically modified; [N] represents nucleotide positions that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

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

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

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

[0176] In one embodiment, the invention features a double stranded nucleic acid (siNA) molecule having structure SX:



SX

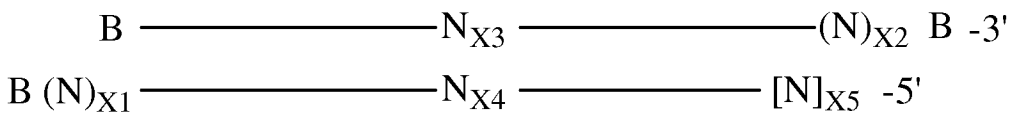
wherein each N is independently a nucleotide which can be unmodified or chemically modified; each B is a terminal cap moiety that can be present or absent; (N) represents non-base paired or overhanging nucleotides which can be unmodified or chemically modified; [N] represents nucleotide positions that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

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

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

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

[0177] In one embodiment, the invention features a double stranded nucleic acid (siNA) molecule having structure SXI:



SXI

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

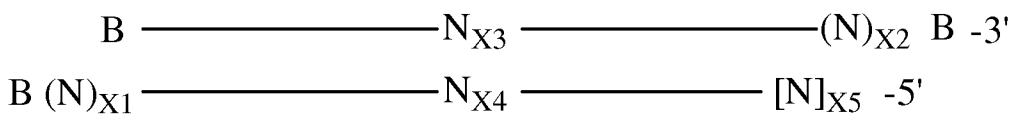
and X2 are independently integers from about 0 to about 4; X3 is an integer from about 9 to about 30; X4 is an integer from about 11 to about 30, provided that the sum of X4 and X5 is between 17-36; X5 is an integer from about 1 to about 6; NX3 is complementary to NX4 and NX5, and

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

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

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

[0178] In one embodiment, the invention features a double stranded nucleic acid (siNA) molecule having structure SXII:



SXII

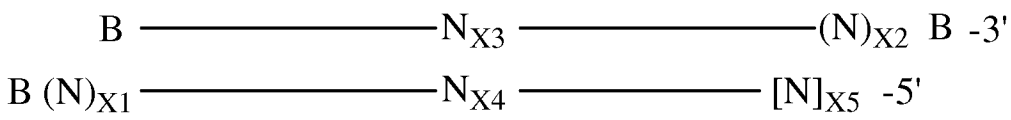
wherein each N is independently a nucleotide which can be unmodified or chemically modified; each B is a terminal cap moiety that can be present or absent; (N) represents non-base paired or overhanging nucleotides which can be unmodified or chemically modified; [N] represents nucleotide positions that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

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

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

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

[0179] In one embodiment, the invention features a double stranded nucleic acid (siNA) molecule having structure SXIII:



SXIII

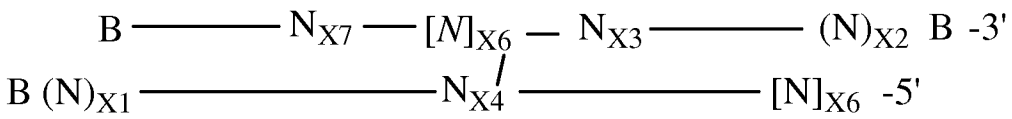
wherein each N is independently a nucleotide which can be unmodified or chemically modified; each B is a terminal cap moiety that can be present or absent; (N) represents non-base paired or overhanging nucleotides which can be unmodified or chemically modified; [N] represents nucleotide positions that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

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

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

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

[0180] In one embodiment, the invention features a double stranded nucleic acid (siNA) molecule having structure SXIV:



SXIV

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

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

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

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

[0181] In one embodiment, a double stranded nucleic acid (siNA) molecule having any of structure SI, SII, SIII, SIV, SV, SVI, SVII, SVIII, SIX, SX, SXI, SXII, SXIII, or SXIV

comprises a terminal phosphate group at the 5'-end of the antisense strand or antisense region of the nucleic acid molecule.

[0182] In one embodiment, a double stranded nucleic acid (siNA) molecule having any of structure SI, SII, SIII, SIV, SV, SVI, SVII ,SVIII, SIX, SX, SXI, SXII, SXIII, or SXIV comprises X5 = 1, 2, or 3; each X1 and X2 = 1 or 2; X3 = 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, or 30, and X4 = 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, or 30.

[0183] In one embodiment, a double stranded nucleic acid (siNA) molecule having any of structure SI, SII, SIII, SIV, SV, SVI, SVII ,SVIII, SIX, SX, SXI, SXII, SXIII, or SXIV comprises X5 = 1; each X1 and X2 = 2; X3 = 19, and X4 = 18.

[0184] In one embodiment, a double stranded nucleic acid (siNA) molecule having any of structure SI, SII, SIII, SIV, SV, SVI, SVII ,SVIII, SIX, SX, SXI, SXII, SXIII, or SXIV comprises X5 = 2; each X1 and X2 = 2; X3 = 19, and X4 = 17

[0185] In one embodiment, a double stranded nucleic acid (siNA) molecule having any of structure SI, SII, SIII, SIV, SV, SVI, SVII ,SVIII, SIX, SX, SXI, SXII, SXIII, or SXIV comprises X5 = 3; each X1 and X2 = 2; X3 = 19, and X4 = 16.

[0186] In one embodiment, a double stranded nucleic acid (siNA) molecule having any of structure SI, SII, SIII, SIV, SV, SVI, SVII ,SVIII, SIX, SX, SXI, SXII, SXIII, or SXIV comprises B at the 3' and 5' ends of the sense strand or sense region.

[0187] In one embodiment, a double stranded nucleic acid (siNA) molecule having any of structure SI, SII, SIII, SIV, SV, SVI, SVII ,SVIII, SIX, SX, SXI, SXII, SXIII, or SXIV comprises B at the 3'-end of the antisense strand or antisense region.

[0188] In one embodiment, a double stranded nucleic acid (siNA) molecule having any of structure SI, SII, SIII, SIV, SV, SVI, SVII ,SVIII, SIX, SX, SXI, SXII, SXIII, or SXIV comprises B at the 3' and 5' ends of the sense strand or sense region and B at the 3'-end of the antisense strand or antisense region.

[0189] In one embodiment, a double stranded nucleic acid (siNA) molecule having any of structure SI, SII, SIII, SIV, SV, SVI, SVII ,SVIII, SIX, SX, SXI, SXII, SXIII, or SXIV further comprises one or more phosphorothioate internucleotide linkages at the first terminal

(N) on the 3'end of the sense strand, antisense strand, or both sense strand and antisense strands of the nucleic acid molecule. For example, a double stranded nucleic acid molecule can comprise X1 and/or X2 = 2 having overhanging nucleotide positions with a phosphorothioate internucleotide linkage, *e.g.*, (NsN) where "s" indicates phosphorothioate.

[0190] In one embodiment, a double stranded nucleic acid (siNA) molecule having any of structure SI, SII, SIII, SIV, SV, SVI, SVII ,SVIII, SIX, SX, SXI, SXII, SXIII, or SXIV comprises (N) nucleotides that are 2'-O-methyl nucleotides.

[0191] In one embodiment, a double stranded nucleic acid (siNA) molecule having any of structure SI, SII, SIII, SIV, SV, SVI, SVII ,SVIII, SIX, SX, SXI, SXII, SXIII, or SXIV comprises (N) nucleotides that are 2'-deoxy nucleotides.

[0192] In one embodiment, a double stranded nucleic acid (siNA) molecule having any of structure SI, SII, SIII, SIV, SV, SVI, SVII ,SVIII, SIX, SX, SXI, SXII, SXIII, or SXIV comprises (N) nucleotides that are 2'-deoxy-2'-fluoro nucleotides.

[0193] In one embodiment, a double stranded nucleic acid (siNA) molecule having any of structure SI, SII, SIII, SIV, SV, SVI, SVII ,SVIII, SIX, SX, SXI, SXII, SXIII, or SXIV comprises (N) nucleotides in the antisense strand (lower strand) that are complementary to nucleotides in an ENaC target polynucleotide sequence having complementary to the N and [N] nucleotides of the antisense (lower) strand.

[0194] In one embodiment, a double stranded nucleic acid (siNA) molecule having any of structure SI, SII, SIII, SIV, SV, SVI, SVII ,SVIII, SIX, SX, SXI, SXII, SXIII, or SXIV comprises (N) nucleotides in the sense strand (upper strand) that comprise a contiguous nucleotide sequence of about 15 to about 30 nucleotides of an ENaC target polynucleotide sequence. In one embodiment, a double stranded nucleic acid (siNA) molecule having any of structure SI, SII, SIII, SIV, SV, SVI, SVII ,SVIII, SIX, SX, SXI, SXII, SXIII, or SXIV comprises (N) nucleotides in the sense strand (upper strand) that comprise nucleotide sequence corresponding an ENaC target polynucleotide sequence having complementary to the antisense (lower) strand such that the contiguous (N) and N nucleotide sequence of the sense strand comprises nucleotide sequence of the ENaC target nucleic acid sequence.

[0195] In one embodiment, a double stranded nucleic acid (siNA) molecule having any of structure SVIII or SXIV comprises B only at the 5'-end of the sense (upper) strand of the double stranded nucleic acid molecule.

[0196] In one embodiment, a double stranded nucleic acid (siNA) molecule having any of structure SI, SII, SIII, SIV, SV, SVI, SVII ,SVIII, SIX, SX, SXI, SXII, SXIII, or SXIV further comprises an unpaired terminal nucleotide at the 5'-end of the antisense (lower) strand. The unpaired nucleotide is not complementary to the sense (upper) strand. In one embodiment, the unpaired terminal nucleotide is complementary to an ENaC target polynucleotide sequence having complementary to the N and [**N**] nucleotides of the antisense (lower) strand. In another embodiment, the unpaired terminal nucleotide is not complementary to an ENaC target polynucleotide sequence having complementary to the N and [**N**] nucleotides of the antisense (lower) strand.

[0197] In one embodiment, a double stranded nucleic acid (siNA) molecule having any of structure SVIII or SXIV comprises X6 = 1 and X3 = 10.

[0198] In one embodiment, a double stranded nucleic acid (siNA) molecule having any of structure SVIII or SXIV comprises X6 = 2 and X3 = 9.

[0199] In one embodiment, the invention features a composition comprising a siNA molecule or double stranded nucleic acid molecule or RNAi inhibitor formulated as any of formulation LNP-051; LNP-053; LNP-054; LNP-069; LNP-073; LNP-077; LNP-080; LNP-082; LNP-083; LNP-060; LNP-061; LNP-086; LNP-097; LNP-098; LNP-099; LNP-100; LNP-101; LNP-102; LNP-103; or LNP-104 (see **Table 10**).

[0200] In one aspect, the invention comprises a double stranded nucleic acid (siNA) molecule having a first strand and a second strand that are complementary to each other, wherein at least one strand comprises:

5'- UGUGCAACCAGAACAAAUC -3' (SEQ ID NO: 10);

5'- GAUUUGUUCUGGUUGCACA -3' (SEQ ID NO: 107);

5'- UUAUGGAUGAUGGUGGCUU -3' (SEQ ID NO: 13);

5'- AAGCCACCAUCAUCCAUAA -3' (SEQ ID NO: 124);

5'- GUGUGGCUGUGCCUACAUC -3' (SEQ ID NO: 16);

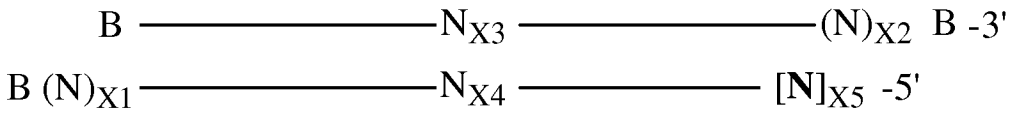
5'- GAUGUAGGCACAGCCACAC -3' (SEQ ID NO: 125)

5'- GCUGUGCCUACAUCUUCUA -3' (SEQ ID NO: 21); or

5'- UAGAAGAUGUAGGCACAGC -3' (SEQ ID NO: 126); and

wherein one or more of the nucleotides are optionally chemically modified. In one embodiment of this aspect, the double-stranded nucleic acid (siNA) molecule comprises nucleotides that are all unmodified. In one embodiment, the double-stranded nucleic acid (siNA) molecule comprises nucleotides that are all chemically modified.

[0201] In another aspect, the invention comprises a double stranded nucleic acid (siNA) molecule comprising structure SIX' having a sense strand and an antisense strand:



SIX'

wherein

the upper strand is the sense strand and the lower strand is the antisense strand of the double stranded nucleic acid molecule, and said sense strand comprises a sequence complementary to the antisense strand;

said antisense strand comprises sequence complementary to SEQ ID NO: 10, SEQ ID NO: 13, SEQ ID NO: 16, or SEQ ID NO: 21;

each N is independently a nucleotide which is unmodified or chemically modified;

each B is a terminal cap moiety that is present or absent;

(N) represents overhanging nucleotides, each of which is independently unmodified or a 2'-O-methyl nucleotide, 2'-deoxy-2'-fluoro nucleotide, or 2'-deoxyribonucleotide;

[N] represents nucleotides that are ribonucleotides;

X1 and X2 are independently integers from 0 to 4;

X3 is an integer from 9 to 30;

X4 is an integer from 11 to 30, provided that the sum of X4 and X5 is 17-36;

X5 is an integer from 1 to 6; and wherein

(a) each pyrimidine nucleotide in N_{X4} positions is independently a 2'-deoxy-2'-fluoro nucleotide or a 2'-O-methyl nucleotide;

each purine nucleotide in N_{X4} positions is independently a 2'-O-methyl nucleotide or a 2'-deoxyribonucleotide; and

(b) each pyrimidine nucleotide in N_{X3} positions is a 2'-deoxy-2'-fluoro nucleotide;

each purine nucleotide in N_{X3} positions is independently a 2'-deoxyribonucleotide or a 2'-O-methyl nucleotide.

In an embodiment, each B is an inverted abasic cap moiety as shown in **Figure 27**.

[0202] In another aspect, the invention also comprises a double-stranded nucleic acid (siNA) molecule wherein the siNA is:

```
5'-  BuGuGcAAccAGAAcAAAucTTB -3'      (Sense)     (SEQ ID NO:51)
     ||||||||||||||||||||
3'- UUAcAcGuuGGucuuGuuUAG    -5'      (Antisense) (SEQ ID NO:52)
```

wherein:

each B is an inverted abasic cap moiety;

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

u is 2'-deoxy-2'fluorouridine;

A is a 2'-deoxyadenosine;

G is a 2'deoxyguanosine;

T is a thymidine;

A is adenosine;

G is guanosine;

U is uridine

A is a 2'-O-methyl-adenosine;

G is a 2'-O-methyl-guanosine;

U is a 2'-O-methyl-uridine; and

the internucleotide linkages are chemically modified or unmodified.

In one embodiment of this aspect, the internucleotide linkages are unmodified.

[0203] In another aspect, the invention also comprises a double-stranded nucleic acid (siNA) molecule wherein the siNA is:

```
5'- BuuAuGGAuGAuGGuGGcuuTTB -3'      (Sense)     (SEQ ID NO:57)
    ||||||||||||||||||||
3'-UUAAuAccuAcuAccAccGAA    -5'      (Antisense) (SEQ ID NO:58)
```

wherein:

each B is an inverted abasic cap;

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

u is 2'-deoxy-2'fluorouridine;

A is a 2'-deoxyadenosine;

G is a 2'deoxyguanosine;

T is a thymidine;

A is adenosine;

G is guanosine;

A is a 2'-O-methyl-adenosine;

U is a 2'-O-methyl-uridine; and

the internucleotide linkages are chemically modified or unmodified.

In one embodiment of this aspect, the internucleotide linkages are unmodified.

[0204] In another aspect, the invention also comprises a double-stranded nucleic acid (siNA) molecule wherein the siNA is:

```
5'  BGuGuGGcuGuGccuAcAucTTB 3'(Sense) (SEQ ID NO:63)
     ||||||||||||||||||||
3' UUcAcAccGAcAcGGAuGUAG     5'(Antisense) (SEQ ID NO:64)
```

wherein:

each B is an inverted abasic cap moiety;

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

u is 2'-deoxy-2'fluorouridine;

A is a 2'-deoxyadenosine;

G is a 2'deoxyguanosine;

T is a thymidine;

A is adenosine;

G is guanosine;

U is uridine;

A is a 2'-O-methyl-adenosine;

G is a 2'-O-methyl-guanosine;

U is a 2'-O-methyl-uridine; and

the internucleotide linkages are chemically modified or unmodified.

In one embodiment of this aspect, the internucleotide linkages are unmodified.

[0205] In another aspect, the invention also comprises a double-stranded nucleic acid (siNA) molecule wherein the siNA is:

```
5'- BGcuGuGccuAcAucuucuATTB -3'     (Sense)     (SEQ ID NO:73)
     ||||||||||||||||||||
3' UUcGAcAcGGAuGuAGAAGAU    -5'     (Antisense) (SEQ ID NO:74)
```

wherein:

each B is an inverted abasic cap moiety;

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

u is 2'-deoxy-2'fluorouridine;

A is a 2'-deoxyadenosine;

G is a 2'deoxyguanosine;

T is a thymidine;

A is adenosine;

G is guanosine;

U is uridine;

A is a 2'-O-methyl-adenosine;

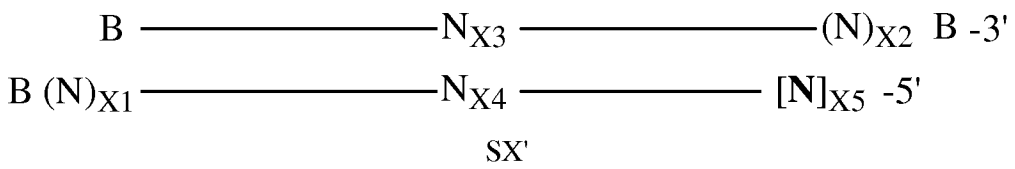
G is a 2'-O-methyl-guanosine;

U is a 2'-O-methyl-uridine; and

the internucleotide linkages are chemically modified or unmodified.

In one embodiment of this aspect, the internucleotide linkages are unmodified.

[0206] In another aspect, the invention comprises a double stranded nucleic acid (siNA) molecule comprising structure SX' having a sense strand and an antisense strand:



wherein

the upper strand is the sense strand and the lower strand is the antisense strand of the double stranded nucleic acid molecule; said antisense strand comprises sequence having complementarity to SEQ ID NO: 10, SEQ ID NO: 13, SEQ ID NO: 16, or SEQ ID NO: 21, and said sense strand comprises a sequence having complementarity to the antisense strand;

each N is independently a nucleotide which is unmodified or chemically modified;

each B is a terminal cap moiety that is present or absent;

(N) represents overhanging nucleotides, each of which is independently unmodified or a 2'-O-methyl nucleotide, 2'-deoxy-2'-fluoro nucleotide, or 2'-deoxyribonucleotide;

[N] represents nucleotides that are ribonucleotides;

X1 and X2 are independently integers from 0 to 4;

X3 is an integer from 9 to 30;

X4 is an integer from 11 to 30, provided that the sum of X4 and X5 is 17-36;

X5 is an integer from 1 to 6; and wherein

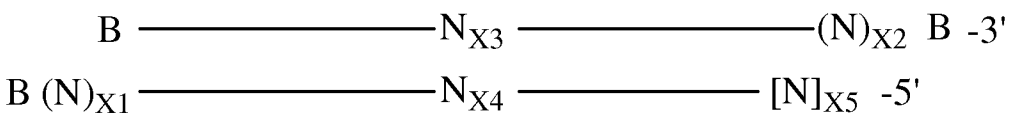
(a) each pyrimidine nucleotide in N_{X4} positions is independently a 2'-deoxy-2'-fluoro nucleotide or a 2'-O-methyl nucleotide;

each purine nucleotide in N_{X4} positions is a 2'-O-methyl nucleotide;

(b) each pyrimidine nucleotide in N_{X3} positions is a ribonucleotide;

each purine nucleotide in N_{X3} positions is a ribonucleotide.

[0207] In another aspect, the invention comprises a double stranded nucleic acid (siNA) molecule comprising structure SXI' having a sense strand and an antisense strand:



SXI'

wherein

the upper strand is the sense strand and the lower strand is the antisense strand of the double stranded nucleic acid molecule; said antisense strand comprises sequence complementary to SEQ ID NO: 10, SEQ ID NO: 13, SEQ ID NO: 16, or SEQ ID NO: 21, and said sense strand comprises a sequence complementary to the antisense strand;

each N is independently a nucleotide which is unmodified or chemically modified;

each B is a terminal cap moiety that is present or absent;

(N) represents overhanging nucleotides, each of which is independently unmodified or a 2'-O-methyl nucleotide, 2'-deoxy-2'-fluoro nucleotide, or 2'-deoxyribonucleotide;

[N] represents nucleotides that are ribonucleotides;

X1 and X2 are independently integers from 0 to 4;

X3 is an integer from 9 to 30;

X4 is an integer from 11 to 30, provided that the sum of X4 and X5 is 17-36;

X5 is an integer from 1 to 6; and wherein

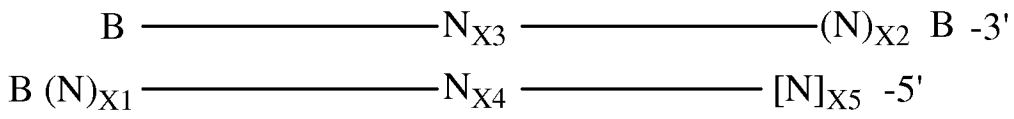
(a) each pyrimidine nucleotide in N_{X4} positions is independently a 2'-deoxy-2'-fluoro nucleotide or a 2'-O-methyl nucleotide;

each purine nucleotide in N_{X4} positions is a 2'-O-methyl nucleotide;

(b) each pyrimidine nucleotide in N_{X3} positions is a 2'-deoxy-2'-fluoro nucleotide;

each purine nucleotide in N_{X3} positions is a ribonucleotide.

[0208] In another aspect, the invention comprises a double stranded nucleic acid (siNA) molecule comprising structure SXII' having a sense strand and an antisense strand:



SXII'

wherein

the upper strand is the sense strand and the lower strand is the antisense strand of the double stranded nucleic acid molecule; said antisense strand comprises sequence complementary to SEQ ID NO: 10, SEQ ID NO: 13, SEQ ID NO: 16, or SEQ ID NO: 21, and said sense strand comprises a sequence complementary to the antisense strand;

each N is independently a nucleotide which is unmodified or chemically modified;

each B is a terminal cap moiety that is present or absent;

(N) represents overhanging nucleotides, each of which is independently unmodified or a 2'-O-methyl nucleotide, 2'-deoxy-2'-fluoro nucleotide, or 2'-deoxyribonucleotide;

[N] represents nucleotides that are ribonucleotides;

X1 and X2 are independently integers from 0 to 4;

X3 is an integer from 9 to 30;

X4 is an integer from 11 to 30, provided that the sum of X4 and X5 is 17-36;

X5 is an integer from 1 to 6; and wherein

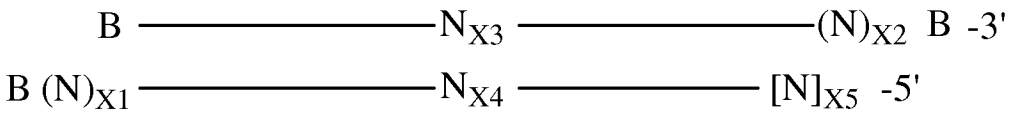
(a) each pyrimidine nucleotide in N_{X4} positions is independently a 2'-deoxy-2'-fluoro nucleotide or a 2'-O-methyl nucleotide;

each purine nucleotide in N_{X4} positions is a 2'-O-methyl nucleotide;

(b) each pyrimidine nucleotide in N_{X3} positions is a 2'-deoxy-2'-fluoro nucleotide;

each purine nucleotide in N_{X3} positions is a 2'-deoxyribonucleotide.

[0209] In another aspect, the invention comprises a double stranded nucleic acid (siNA) molecule comprising structure SXIII' having a sense strand and an antisense strand:



SXIII'

wherein

the upper strand is the sense strand and the lower strand is the antisense strand of the double stranded nucleic acid molecule; said antisense strand comprises sequence complementary to SEQ ID NO: 10, SEQ ID NO: 13, SEQ ID NO: 16, or SEQ ID NO: 21, and said sense strand comprises a sequence complementary to the antisense strand;

each N is independently a nucleotide which is unmodified or chemically modified;

each B is a terminal cap moiety that is present or absent;

(N) represents overhanging nucleotides, each of which is independently unmodified or a 2'-O-methyl nucleotide, 2'-deoxy-2'-fluoro nucleotide, or 2'-deoxyribonucleotide;

[N] represents nucleotides that are ribonucleotides;

X1 and X2 are independently integers from 0 to 4;

X3 is an integer from 9 to 30;

X4 is an integer from 11 to 30, provided that the sum of X4 and X5 is 17-36;

X5 is an integer from 1 to 6; and wherein

(a) each pyrimidine nucleotide in N_{X4} positions is a nucleotide having a ribo-like, Northern or A-form helix configuration;

each purine nucleotide in N_{X4} positions is a 2'-O-methyl nucleotide;

(b) each pyrimidine nucleotide in N_{X3} positions is a nucleotide having a ribo-like, Northern or A-form helix configuration;
each purine nucleotide in N_{X3} positions is a 2'-O-methyl nucleotide.

[0210] In one embodiment of the foregoing aspects, the double-stranded nucleic acid (siNA) molecule comprises structure SIX' wherein X5 is 3. In one embodiment, the double-stranded nucleic acid (siNA) molecule comprises structure SIX' wherein X1 is 2 and X2 is 2. In one embodiment, the double-stranded nucleic acid (siNA) molecule comprises structure SIX' wherein X5 is 3, X1 is 2 and X2 is 2. In one embodiment, the double-stranded nucleic acid (siNA) molecule comprises structure SIX' wherein X5 is 3, X1 is 2, X2 is 2, X3 is 19 and X4 is 16. In one embodiment of the foregoing aspects, including but not limited to the double-stranded nucleic acid (siNA) molecule of structures SIX', SX', SXI', SXII', and SXIII', X5 = 1, 2, or 3; each X1 and X2 = 1 or 2; X3 = 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, or 30, and X4 = 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, or 30.

[0211] In one embodiment of the foregoing aspects, B is present at the 3' and 5' ends of the sense strand and optionally at the 3' end of the antisense strand. In one embodiment B is present at the 3' and 5' ends of the sense strand only.

[0212] The invention also comprises double-stranded nucleic acid (siNA) molecules as otherwise described hereinabove in which the first strand and second strand are complementary to each other and wherein at least one strand has at least 80%, 85%, 90%, 95%, or 99% identity to SEQ ID NO: 10, SEQ ID NO: 13, SEQ ID NO: 16, or SEQ ID NO: 21 over its entire length and wherein any of the nucleotides is unmodified or chemically modified. In one embodiment, the first strand and a second strand are complementary to each other and at least one strand has at least 80%, 85%, 90%, 95%, or 99% identity to the complement of SEQ ID NO: 10, SEQ ID NO: 13, SEQ ID NO: 16, or SEQ ID NO: 21 over its entire length and wherein any of the nucleotides is unmodified or chemically modified. In one embodiment, the first strand and second strand that are complementary to each other and at least one strand has at least 95% identity to SEQ ID NO: 10, SEQ ID NO: 13, SEQ ID NO: 16, or SEQ ID NO: 21 or at least 95% identity to the complement of SEQ ID NO: 10, SEQ ID NO: 13, SEQ ID NO: 16, or SEQ ID NO: 21 over its entire length and wherein each of the nucleotides is unmodified or chemically modified. In one embodiment, the first strand and second strand have 90% complementarity to each other, wherein at least one strand has at

least 95% identity to SEQ ID NO: 10, SEQ ID NO: 13, SEQ ID NO: 16, or SEQ ID NO: 21 or or its complement.

[0213] The invention also comprises double-stranded nucleic acid (siNA) molecules as otherwise described hereinabove in which the first strand and second strand are complementary to each other and wherein at least one strand is hybridisable to the polynucleotide sequence of SEQ ID NO: 10, SEQ ID NO: 13, SEQ ID NO: 16, or SEQ ID NO: 21 or its complement under conditions of high stringency, and wherein any of the nucleotides is unmodified or chemically modified. In one embodiment, the first strand and second strand have 90% complementarity to each other and at least one strand is hybridisable to the polynucleotide sequence of SEQ ID NO: 10, SEQ ID NO: 13, SEQ ID NO: 16, or SEQ ID NO: 21 or its complement under conditions of high stringency, and wherein any of the nucleotides is unmodified or chemically modified.

[0214] For nucleotide acid sequences, the term "identity" indicates the degree of identity between two nucleic acid sequences when optimally aligned and compared with appropriate insertions or deletions. In other words, the percent identity between two sequences is a function of the number of identical positions shared by the sequences (i.e., % identity = # of identical positions/total # of positions times 100), taking into account the number of gaps, and the length of each gap, which need to be introduced for optimal alignment of the two sequences. The comparison of sequences and determination of percent identity between two sequences is accomplished using a mathematical algorithm, as described in the non-limiting examples below.

[0215] The percent identity between two nucleotide sequences is determined using the GAP program in the Accelrys GCG software package (University of Wisconsin), using a NWSgapdna.CMP matrix and a gap weight of 40, 50, 60, 70, or 80 and a length weight of 1, 2, 3, 4, 5, or 6. The percent identity between two nucleotide sequences can also be determined using the algorithm of E. Meyers and W. Miller (Comput. Appl. Biosci., 4:11-17 (1988)) which has been incorporated into the ALIGN program (version 2.0), using a PAM120 weight residue table, a gap length penalty of 12 and a gap penalty of 4.

[0216] Hybridization techniques are well known to the skilled artisan (see for instance, Sambrook *et al.*, Molecular Cloning: A Laboratory Manual, 2nd Ed., Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y. (1989)). Preferred stringent hybridization conditions include overnight incubation at 42°C in a solution comprising: 50% formamide,

5xSSC (150mM NaCl, 15mM trisodium citrate), 50 mM sodium phosphate (pH 7.6), 5x Denhardt's solution, 10 % dextran sulfate, and 20 microgram/ml denatured, sheared salmon sperm DNA; followed by washing the filters in 0.1x SSC at about 65°C.

[0217] Another aspect of the invention comprises a pharmaceutical composition comprising a double stranded nucleic acid (siNA) of the invention in a pharmaceutically acceptable carrier or diluent.

[0218] Another aspect of the invention comprises a method of treating a human subject suffering from a condition which is mediated by the action, or by loss of action, of ENaC which method comprises administering to said subject an effective amount of the double stranded nucleic acid (siNA) molecule of the invention. In one embodiment of this aspect, the condition is or is caused by a respiratory disease. Respiratory disease treatable according to this aspect of the invention include COPD, asthma, cystic fibrosis, eosinophilic cough, bronchitis, sarcoidosis, pulmonary fibrosis, rhinitis, sinusitis (particularly COPD, cystic fibrosis and asthma).

[0219] In an aspect, the invention comprises use of a double stranded nucleic acid according to the invention for use as a medicament. In an embodiment, the medicament is for use in treating a condition that is mediated by the action, or by loss of action, of ENaC. In one embodiment, the medicament is for use for the treatment of a respiratory disease. In an embodiment the medicament is for use for the treatment of a respiratory disease selected from the group consisting of COPD, asthma, cystic fibrosis, eosinophilic cough, bronchitis, sarcoidosis, pulmonary fibrosis, rhinitis, and sinusitis. In a particular embodiment, the use is for the treatment of a respiratory disease selected from the group consisting of COPD, cystic fibrosis and asthma.

[0220] In another aspect, the invention comprises use of a double stranded nucleic acid according to the invention for use in the manufacture of a medicament. In an embodiment, the medicament is for use in treating a condition that is mediated by the action, or by loss of action, of ENaC. In one embodiment, the medicament is for use for the treatment of a respiratory disease. In an embodiment the medicament is for use for the treatment of a respiratory disease selected from the group consisting of COPD, asthma, cystic fibrosis, eosinophilic cough, bronchitis, sarcoidosis, pulmonary fibrosis, rhinitis, and sinusitis. In a particular embodiment, the use is for the treatment of a respiratory disease selected from the group consisting of COPD, cystic fibrosis and asthma.

[0221] It will be appreciated that in the foregoing embodiments, in particular those embodiments described in paragraphs [000197] to [000214], the term "short interfering nucleic acid" (siNA) refers to a nucleic acid molecule that is capable of mediating RNA interference.

[0222] In one embodiment, the invention features a composition comprising a first double stranded nucleic and a second double stranded nucleic acid molecule each having a first strand and a second strand that are complementary to each other, wherein the second strand of the first double stranded nucleic acid molecule comprises sequence complementary to a first ENaC target sequence and the second strand of the second double stranded nucleic acid molecule comprises sequence complementary to a second ENaC target sequence. In one embodiment, the composition further comprises a cationic lipid, a neutral lipid, and a polyethyleneglycol-conjugate. In one embodiment, the composition further comprises a cationic lipid, a neutral lipid, a polyethyleneglycol-conjugate, and a cholesterol. In one embodiment, the composition further comprises a polyethyleneglycol-conjugate, a cholesterol, and a surfactant. In one embodiment, the cationic lipid is selected from the group consisting of CLinDMA, pCLinDMA, eCLinDMA, DMOBA, and DMLBA. In one embodiment, the neutral lipid is selected from the group consisting of DSPC, DOBA, and cholesterol. In one embodiment, the polyethyleneglycol-conjugate is selected from the group consisting of a PEG-dimyristoyl glycerol and PEG-cholesterol. In one embodiment, the PEG is 2KPEG. In one embodiment, the surfactant is selected from the group consisting of palmityl alcohol, stearyl alcohol, oleyl alcohol and linoleyl alcohol. In one embodiment, the cationic lipid is CLinDMA, the neutral lipid is DSPC, the polyethylene glycol conjugate is 2KPEG-DMG, the cholesterol is cholesterol, and the surfactant is linoleyl alcohol. In one embodiment, the CLinDMA, the DSPC, the 2KPEG-DMG, the cholesterol, and the linoleyl alcohol are present in molar ratio of 43:38:10:2:7 respectively.

[0223] In any of the embodiments herein, the siNA molecule of the invention modulates expression of one or more ENaC targets via RNA interference or the inhibition of RNA interference. In one embodiment, the RNA interference is RISC mediated cleavage of the ENaC target (e.g., siRNA mediated RNA interference). In one embodiment, the RNA interference is translational inhibition of the ENaC target (e.g., miRNA mediated RNA interference). In one embodiment, the RNA interference is transcriptional inhibition of the ENaC target (e.g., siRNA mediated transcriptional silencing). In one embodiment, the RNA

interference takes place in the cytoplasm. In one embodiment, the RNA interference takes place in the nucleus.

[0224] In any of the embodiments herein, the siNA molecule of the invention modulates expression of one or more ENaC targets via inhibition of an endogenous ENaC RNA, such as an endogenous ENaC mRNA, ENaC siRNA, ENaC miRNA, or alternately though inhibition of RISC.

[0225] In one embodiment, the invention features one or more RNAi inhibitors that modulate the expression of one or more ENaC gene targets by miRNA inhibition, siRNA inhibition, or RISC inhibition.

[0226] In one embodiment, a RNAi inhibitor of the invention is a siNA molecule as described herein that has one or more strands that are complementary to one or more target miRNA or siRNA molecules.

[0227] In one embodiment, the RNAi inhibitor of the invention is an antisense molecule that is complementary to a target miRNA or siRNA molecule or a portion thereof. An antisense RNAi inhibitor of the invention can be of length of about 10 to about 40 nucleotides in length (e.g., 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36, 37, 38, 39, or 40 nucleotides in length). An antisense RNAi inhibitor of the invention can comprise one or more modified nucleotides or non-nucleotides as described herein (see for example molecules having any of Formulae I-VII herein or any combination thereof). In one embodiment, an antisense RNAi inhibitor of the invention can comprise one or more or all 2'-O-methyl nucleotides. In one embodiment, an antisense RNAi inhibitor of the invention can comprise one or more or all 2'-deoxy-2'-fluoro nucleotides. In one embodiment, an antisense RNAi inhibitor of the invention can comprise one or more or all 2'-O-methoxy-ethyl (also known as 2'-methoxyethoxy or MOE) nucleotides. In one embodiment, an antisense RNAi inhibitor of the invention can comprise one or more or all phosphorothioate internucleotide linkages. In one embodiment, an antisense RNA inhibitor or the invention can comprise a terminal cap moiety at the 3'-end, the 5;'-end, or both the 5' and 3' ends of the the antisense RNA inhibitor.

[0228] In one embodiment, a RNAi inhibitor of the invention is a nucleic acid aptamer having binding affinity for RISC, such as a regulatable aptamer (see for example An *et al.*,

2006, *RNA,* 12:710-716). An aptamer RNAi inhibitor of the invention can be of length of about 10 to about 50 nucleotides in length (e.g., 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36, 37, 38, 39, 40, 41, 42, 43, 44, 45, 46, 47, 48, 49, or 50 nucleotides in length). An aptamer RNAi inhibitor of the invention can comprise one or more modified nucleotides or non-nucleotides as described herein (see for example molecules having any of Formulae I-VII herein or any combination thereof). In one embodiment, an aptamer RNAi inhibitor of the invention can comprise one or more or all 2'-O-methyl nucleotides. In one embodiment, an aptamer RNAi inhibitor of the invention can comprise one or more or all 2'-deoxy-2'-fluoro nucleotides. In one embodiment, an aptamer RNAi inhibitor of the invention can comprise one or more or all 2'-O-methoxy-ethyl (also known as 2'-methoxyethoxy or MOE) nucleotides. In one embodiment, an aptamer RNAi inhibitor of the invention can comprise one or more or all phosphorothioate internucleotide linkages. In one embodiment, an aptamer RNA inhibitor or the invention can comprise a terminal cap moiety at the 3'-end, the 5;'-end, or both the 5' and 3' ends of the the aptamer RNA inhibitor.

[0229] In one embodiment, the invention features a method for modulating the expression of an ENaC target gene within a cell comprising: (a) synthesizing a siNA molecule of the invention, which can be chemically-modified or unmodified, wherein one of the siNA strands comprises a sequence complementary to RNA of the ENaC target gene; and (b) introducing the siNA molecule into a cell under conditions suitable to modulate (*e.g.*, inhibit) the expression of the ENaC target gene in the cell.

[0230] In one embodiment, the invention features a method for modulating the expression of an ENaC target gene within a cell comprising: (a) synthesizing a siNA molecule of the invention, which can be chemically-modified or unmodified, wherein one of the siNA strands comprises a sequence complementary to RNA of the ENaC target gene and wherein the sense strand sequence of the siNA comprises a sequence identical or substantially similar to the sequence of the ENaC target RNA; and (b) introducing the siNA molecule into a cell under conditions suitable to modulate (*e.g.*, inhibit) the expression of the ENaC target gene in the cell.

[0231] In another embodiment, the invention features a method for modulating the expression of more than one ENaC target gene within a cell comprising: (a) synthesizing

siNA molecules of the invention, which can be chemically-modified or unmodified, wherein one of the siNA strands comprises a sequence complementary to RNA of the ENaC target genes; and (b) introducing the siNA molecules into a cell under conditions suitable to modulate (*e.g.*, inhibit) the expression of the ENaC target genes in the cell.

[0232] In another embodiment, the invention features a method for modulating the expression of two or more ENaC target genes within a cell comprising: (a) synthesizing one or more siNA molecules of the invention, which can be chemically-modified or unmodified, wherein the siNA strands comprise sequences complementary to RNA of the ENaC target genes and wherein the sense strand sequences of the siNAs comprise sequences identical or substantially similar to the sequences of the ENaC target RNAs; and (b) introducing the siNA molecules into a cell under conditions suitable to modulate (*e.g.*, inhibit) the expression of the ENaC target genes in the cell.

[0233] In another embodiment, the invention features a method for modulating the expression of more than one ENaC target gene within a cell comprising: (a) synthesizing a siNA molecule of the invention, which can be chemically-modified or unmodified, wherein one of the siNA strands comprises a sequence complementary to RNA of the ENaC target gene and wherein the sense strand sequence of the siNA comprises a sequence identical or substantially similar to the sequences of the ENaC target RNAs; and (b) introducing the siNA molecule into a cell under conditions suitable to modulate (*e.g.*, inhibit) the expression of the ENaC target genes in the cell.

[0234] In another embodiment, the invention features a method for modulating the expression of an ENaC target gene within a cell comprising: (a) synthesizing a siNA molecule of the invention, which can be chemically-modified or unmodified, wherein one of the siNA strands comprises a sequence complementary to RNA of the ENaC target gene, wherein the sense strand sequence of the siNA comprises a sequence identical or substantially similar to the sequences of the ENaC target RNA; and (b) introducing the siNA molecule into a cell under conditions suitable to modulate (*e.g.*, inhibit) the expression of the ENaC target gene in the cell.

[0235] In one embodiment, siNA molecules of the invention are used as reagents in *ex vivo* applications. For example, siNA reagents are introduced into tissue or cells that are

transplanted into a subject for therapeutic effect. The cells and/or tissue can be derived from an organism or subject that later receives the explant, or can be derived from another organism or subject prior to transplantation. The siNA molecules can be used to modulate the expression of one or more genes in the cells or tissue, such that the cells or tissue obtain a desired phenotype or are able to perform a function when transplanted in vivo. In one embodiment, certain target cells from a patient are extracted. These extracted cells are contacted with ENaC siNAs targeting a specific nucleotide sequence within the cells under conditions suitable for uptake of the siNAs by these cells (*e.g.* using delivery reagents such as cationic lipids, liposomes and the like or using techniques such as electroporation to facilitate the delivery of siNAs into cells). The cells are then reintroduced back into the same patient or other patients.

[0236] In one embodiment, the invention features a method of modulating the expression of an ENaC target gene in a tissue explant comprising: (a) synthesizing a siNA molecule of the invention, which can be chemically-modified, wherein one of the siNA strands comprises a sequence complementary to RNA of the ENaC target gene; and (b) introducing the siNA molecule into a cell of the tissue explant derived from a particular organism under conditions suitable to modulate (*e.g.*, inhibit) the expression of the ENaC target gene in the tissue explant. In another embodiment, the method further comprises introducing the tissue explant back into the organism the tissue was derived from or into another organism under conditions suitable to modulate (*e.g.*, inhibit) the expression of the ENaC target gene in that organism.

[0237] In one embodiment, the invention features a method of modulating the expression of an ENaC target gene in a tissue explant comprising: (a) synthesizing a siNA molecule of the invention, which can be chemically-modified, wherein one of the siNA strands comprises a sequence complementary to RNA of the ENaC target gene and wherein the sense strand sequence of the siNA comprises a sequence identical or substantially similar to the sequence of the ENaC target RNA; and (b) introducing the siNA molecule into a cell of the tissue explant derived from a particular organism under conditions suitable to modulate (*e.g.*, inhibit) the expression of the ENaC target gene in the tissue explant. In another embodiment, the method further comprises introducing the tissue explant back into the organism the tissue was derived from or into another organism under conditions suitable to modulate (*e.g.*, inhibit) the expression of the ENaC target gene in that organism.

[0238] In another embodiment, the invention features a method of modulating the expression of more than one ENaC target gene in a tissue explant comprising: (a) synthesizing siNA molecules of the invention, which can be chemically-modified, wherein one of the siNA strands comprises a sequence complementary to RNA of the ENaC target genes; and (b) introducing the siNA molecules into a cell of the tissue explant derived from a particular organism under conditions suitable to modulate (*e.g.*, inhibit) the expression of the ENaC target genes in the tissue explant. In another embodiment, the method further comprises introducing the tissue explant back into the organism the tissue was derived from or into another organism under conditions suitable to modulate (*e.g.*, inhibit) the expression of the ENaC target genes in that organism.

[0239] In one embodiment, the invention features a method of modulating the expression of an ENaC target gene in a subject or organism comprising: (a) synthesizing a siNA molecule of the invention, which can be chemically-modified, wherein one of the siNA strands comprises a sequence complementary to RNA of the ENaC target gene; and (b) introducing the siNA molecule into the subject or organism under conditions suitable to modulate (*e.g.*, inhibit) the expression of the ENaC target gene in the subject or organism. The level of ENaC target protein or RNA can be determined using various methods well-known in the art.

[0240] In another embodiment, the invention features a method of modulating the expression of more than one ENaC target gene in a subject or organism comprising: (a) synthesizing siNA molecules of the invention, which can be chemically-modified, wherein one of the siNA strands comprises a sequence complementary to RNA of the ENaC target genes; and (b) introducing the siNA molecules into the subject or organism under conditions suitable to modulate (*e.g.*, inhibit) the expression of the ENaC target genes in the subject or organism. The level of ENaC target protein or RNA can be determined as is known in the art.

[0241] In one embodiment, the invention features a method for modulating the expression of an ENaC target gene within a cell, (e.g., a lung or lung epithelial 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 ENaC

target gene; and (b) introducing the siNA molecule into a cell under conditions suitable to modulate (*e.g.*, inhibit) the expression of the ENaC target gene in the cell.

[0242] In another embodiment, the invention features a method for modulating the expression of more than one ENaC target gene within a cell, (e.g., a lung or lung epithelial 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 ENaC target gene; and (b) contacting the cell *in vitro* or *in vivo* with the siNA molecule under conditions suitable to modulate (*e.g.*, inhibit) the expression of the ENaC target genes in the cell.

[0243] In one embodiment, the invention features a method of modulating the expression of an ENaC target gene in a tissue explant ((*e.g.*, lung or any other organ, tissue or cell as can be transplanted from one organism to another or back to the same organism from which the organ, tissue or cell is derived) comprising: (a) synthesizing 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 ENaC target gene; and (b) contacting a cell of the tissue explant derived from a particular subject or organism with the siNA molecule under conditions suitable to modulate (*e.g.*, inhibit) the expression of the ENaC target gene in the tissue explant. In another embodiment, the method further comprises introducing the tissue explant back into the subject or organism the tissue was derived from or into another subject or organism under conditions suitable to modulate (*e.g.*, inhibit) the expression of the ENaC target gene in that subject or organism.

[0244] In another embodiment, the invention features a method of modulating the expression of more than one ENaC target gene in a tissue explant (*e.g.*, lung or any other organ, tissue or cell as can be transplanted from one organism to another or back to the same organism from which the organ, tissue or cell is derived) comprising: (a) synthesizing siNA molecules of the invention, which can be chemically-modified, wherein the siNA comprises a single stranded sequence having complementarity to RNA of the ENaC target gene; and (b) introducing the siNA molecules into a cell of the tissue explant derived from a particular subject or organism under conditions suitable to modulate (*e.g.*, inhibit) the expression of the ENaC target genes in the tissue explant. In another embodiment, the method further comprises introducing the tissue explant back into the subject or organism the tissue was

derived from or into another subject or organism under conditions suitable to modulate (*e.g.*, inhibit) the expression of the ENaC target genes in that subject or organism.

[0245] In one embodiment, the invention features a method of modulating the expression of a ENaC target gene in a subject or organism comprising: (a) synthesizing a siNA molecule of the invention, which can be chemically-modified, wherein the siNA comprises a single stranded sequence having complementarity to RNA of the ENaC target gene; and (b) introducing the siNA molecule into the subject or organism under conditions suitable to modulate (*e.g.*, inhibit) the expression of the ENaC target gene in the subject or organism.

[0246] In another embodiment, the invention features a method of modulating the expression of more than one ENaC target gene in a subject or organism comprising: (a) synthesizing siNA molecules of the invention, which can be chemically-modified, wherein the siNA comprises a single stranded sequence having complementarity to RNA of the ENaC target gene; and (b) introducing the siNA molecules into the subject or organism under conditions suitable to modulate (*e.g.*, inhibit) the expression of the ENaC target genes in the subject or organism.

[0247] In one embodiment, the invention features a method of modulating the expression of an ENaC target gene in a subject or organism comprising contacting the subject or organism with a siNA molecule of the invention under conditions suitable to modulate (*e.g.*, inhibit) the expression of the ENaC target gene in the subject or organism.

[0248] In one embodiment, the invention features a method for treating or preventing a disease, disorder, trait or condition related to gene expression or activity in a subject or organism comprising contacting the subject or organism with a siNA molecule of the invention under conditions suitable to modulate the expression of the ENaC target gene in the subject or organism. The reduction of gene expression and thus reduction in the level of the respective protein/RNA relieves, to some extent, the symptoms of the disease, disorder, trait or condition.

[0249] In one embodiment, the invention features a method for treating or preventing one or more respiratory diseases, traits, or conditions in a subject or organism comprising contacting the subject or organism with a siNA molecule of the invention under conditions suitable to modulate the expression of the ENaC target gene in the subject or organism

whereby the treatment or prevention of the respiratory disease(s), trait(s), or condition(s) can be achieved. In one embodiment, the invention features contacting the subject or organism with a siNA molecule of the invention via local administration to relevant tissues or cells, such as lung cells and tissues, such as via pulmonary delivery. In one embodiment, the invention features contacting the subject or organism with a siNA molecule of the invention via systemic administration (such as via intravenous or subcutaneous administration of siNA) to relevant tissues or cells, such as tissues or cells involved in the maintenance or development of the respiratory disease, trait, or condition in a subject or organism. The siNA molecule of the invention can be formulated or conjugated as described herein or otherwise known in the art to target appropriate tisssues or cells in the subject or organism. The siNA molecule can be combined with other therapeutic treatments and modalities as are known in the art for the treatment of or prevention of respiratory diseases, traits, or conditions in a subject or organism.

[0250] In one embodiment, the invention features a method for treating or preventing COPD, asthma, cystic fibrosis, eosinophilic cough, bronchitis, sarcoidosis, pulmonary fibrosis, rhinitis, and/or sinusitis a subject or organism comprising contacting the subject or organism with a siNA molecule of the invention under conditions suitable to modulate the expression of the ENaC target gene in the subject or organism whereby the treatment or prevention of COPD, asthma, cystic fibrosis, eosinophilic cough, bronchitis, sarcoidosis, pulmonary fibrosis, rhinitis, and/or sinusitis can be achieved. In one embodiment, the invention features contacting the subject or organism with a siNA molecule of the invention via local administration to relevant tissues or cells, such as lung or airway cells and tissues involved in COPD, asthma, cystic fibrosis, eosinophilic cough, bronchitis, acute and chronic rejection of lung allograft, sarcoidosis, pulmonary fibrosis, rhinitis, and/or sinusitis. In one embodiment, the invention features contacting the subject or organism with a siNA molecule of the invention via systemic administration (such as via intravenous or subcutaneous administration of siNA) to relevant tissues or cells, such as tissues or cells involved in the maintenance or development of COPD, asthma, cystic fibrosis, eosinophilic cough, bronchitis, acute and chronic rejection of lung allograft, sarcoidosis, pulmonary fibrosis, rhinitis, and/or sinusitis in a subject or organism. The siNA molecule of the invention can be formulated or conjugated as described herein or otherwise known in the art to target appropriate tisssues or cells in the subject or organism. The siNA molecule can be combined with other therapeutic treatments and modalities as are known in the art for the treatment of

or prevention of COPD, asthma, cystic fibrosis, eosinophilic cough, bronchitis, sarcoidosis, pulmonary fibrosis, rhinitis, and/or sinusitis in a subject or organism.

[0251] In one embodiment, the invention features a method for treating or preventing one or more respiratory diseases, traits, or conditions in a subject or organism comprising contacting the subject or organism with a siNA molecule of the invention under conditions suitable to modulate (*e.g.*, inhibit) the expression of an inhibitor of ENaC gene expression in the subject or organism. In one embodiment, the inhibitor of ENaC gene expression is a miRNA.

[0252] In one embodiment, the invention features a method for treating or preventing one or more inflammatory diseases, traits, or conditions in a subject or organism comprising contacting the subject or organism with a siNA molecule of the invention under conditions suitable to modulate the expression of the ENaC target gene in the subject or organism whereby the treatment or prevention of the inflammatory disease(s), trait(s), or condition(s) can be achieved. In one embodiment, the invention features contacting the subject or organism with a siNA molecule of the invention via local administration to relevant tissues or cells, such as lung cells and tissues, such as via pulmonary delivery. In one embodiment, the invention features contacting the subject or organism with a siNA molecule of the invention via systemic administration (such as via intravenous or subcutaneous administration of siNA) to relevant tissues or cells, such as tissues or cells involved in the maintenance or development of the inflammatory disease, trait, or condition in a subject or organism. The siNA molecule of the invention can be formulated or conjugated as described herein or otherwise known in the art to target appropriate tisssues or cells in the subject or organism. The siNA molecule can be combined with other therapeutic treatments and modalities as are known in the art for the treatment of or prevention of inflammatory diseases, traits, or conditions in a subject or organism.

[0253] In one embodiment, the invention features a method for treating or preventing one or more inflammatory diseases, traits, or conditions in a subject or organism comprising contacting the subject or organism with a siNA molecule of the invention under conditions suitable to modulate (*e.g.*, inhibit) the expression of an inhibitor of ENaC gene expression in the subject or organism. In one embodiment, the inhibitor of ENaC gene expression is a miRNA.

[0254] In one embodiment, the siNA molecule or double stranded nucleic acid molecule of the invention is formulated as a composition described in U.S. Provisional patent application No. 60/678,531 and in related U.S. Provisional patent application No. 60/703,946, filed July 29, 2005, and U.S. Provisional patent application No. 60/737,024, filed November 15, 2005 (Vargeese *et al.*).

[0255] In any of the above methods for treating or preventing epithelial sodium channel (ENaC) related diseases, traits, or conditions in a subject, the treatment is combined with administration of a beta-2 agonist composition as is generally recognized in the art, including for example, albuterol or albuterol sulfate.

[0256] In any of the above methods for treating or preventing epithelial sodium channel (ENaC) related diseases, traits, phenotypes or conditions in a subject, the treatment is combined with administration of a PDE4 inhibitor composition as is generally recognized in the art (e.g., sildenafil, motapizone, rolipram, and zaprinast, zardaverine and tolafentrine).

[0257] In one embodiment, the siNA molecule or double stranded nucleic acid molecule of the invention is formulated as a composition described in U.S. Provisional patent application No. 60/678,531 and in related U.S. Provisional patent application No. 60/703,946, filed July 29, 2005, U.S. Provisional patent application No. 60/737,024, filed November 15, 2005, and USSN 11/353,630 , filed February 14, 2006, and USSN 11/586,102, filed October 24, 2006 (Vargeese *et al.*).

[0258] In any of the methods herein for modulating the expression of one or more targets or for treating or preventing diseases, traits, conditions, or phenotypes in a cell, subject, or organism, the siNA molecule of the invention modulates expression of one or more ENaC targets via RNA interference. In one embodiment, the RNA interference is RISC mediated cleavage of the ENaC target (e.g., siRNA mediated RNA interference). In one embodiment, the RNA interference is translational inhibition of the ENaC target (e.g., miRNA mediated RNA interference). In one embodiment, the RNA interference is transcriptional inhibition of the ENaC target (e.g., siRNA mediated transcriptional silencing). In one embodiment, the RNA interference takes place in the cytoplasm. In one embodiment, the RNA interference takes place in the nucleus.

[0259] In any of the methods of treatment of the invention, the siNA can be administered to the subject as a course of treatment, for example administration at various time intervals,

such as once per day over the course of treatment, once every two days over the course of treatment, once every three days over the course of treatment, once every four days over the course of treatment, once every five days over the course of treatment, once every six days over the course of treatment, once per week over the course of treatment, once every other week over the course of treatment, once per month over the course of treatment, etc. In one embodiment, the course of treatment is once every 1, 2, 3, 4, 5, 6, 7, 8, 9, or 10 weeks. In one embodiment, the course of treatment is from about one to about 52 weeks or longer (*e.g.*, indefinitely). In one embodiment, the course of treatment is from about one to about 48 months or longer (*e.g.*, indefinitely).

[0260] In one embodiment, a course of treatment involves an initial course of treatment, such as once every 1, 2, 3, 4, 5, 6, 7, 8, 9, 10 or more weeks for a fixed interval (*e.g.*, 1x, 2x, 3x, 4x, 5x, 6x, 7x, 8x, 9x, 10x or more) followed by a maintenance course of treatment, such as once every 4, 6, 8, 10, 15, 20, 25, 30, 35, 40, or more weeks for an additional fixed interval (*e.g.*, 1x, 2x, 3x, 4x, 5x, 6x, 7x, 8x, 9x, 10x or more).

[0261] In any of the methods of treatment of the invention, the siNA can be administered to the subject systemically as described herein or otherwise known in the art, either alone as a monotherapy or in combination with additional therapies described herein or as are known in the art. Systemic administration can include, for example, pulmonary (inhalation, nebulization etc.) intravenous, subcutaneous, intramuscular, catheterization, nasopharangeal, transdermal, or oral/gastrointestinal administration as is generally known in the art.

[0262] In one embodiment, in any of the methods of treatment or prevention of the invention, the siNA can be administered to the subject locally or to local tissues as described herein or otherwise known in the art, either alone as a monotherapy or in combination with additional therapies as are known in the art. Local administration can include, for example, inhalation, nebulization, catheterization, implantation, direct injection, dermal/transdermal application, stenting, ear/eye drops, or portal vein administration to relevant tissues, or any other local administration technique, method or procedure, as is generally known in the art.

[0263] The compound and pharmaceutical formulations according to the invention can be used in combination with or include one or more other therapeutic agents, for example selected from anti-inflammatory agents, anticholinergic agents (particularly an $M_1/M_2/M_3$ receptor antagonist), β_2-adrenoreceptor agonists, antiinfective agents, such as antibiotics,

antivirals, or antihistamines. The invention thus provides, in a further aspect, a combination comprising a compound of formula (I) or a pharmaceutically acceptable salt, solvate or physiologically functional derivative thereof together with one or more other therapeutically active agents, for example selected from an anti-inflammatory agent, such as a corticosteroid or an NSAID, an anticholinergic agent, a β_2-adrenoreceptor agonist, an antiinfective agent, such as an antibiotic or an antiviral, or an antihistamine. One embodiment of the invention encompasses combinations comprising a compound of formula (I) or a pharmaceutically acceptable salt, solvate or physiologically functional derivative thereof together with a β_2-adrenoreceptor agonist, and/or an anticholinergic, and/or a PDE-4 inhibitor, and/or an antihistamine.

[0264] One embodiment of the invention encompasses combinations comprising one or two other therapeutic agents. It will be clear to a person skilled in the art that, where appropriate, the other therapeutic ingredient(s) can be used in the form of salts, for example as alkali metal or amine salts or as acid addition salts, or prodrugs, or as esters, for example lower alkyl esters, or as solvates, for example hydrates to optimise the activity and/or stability and/or physical characteristics, such as solubility, of the therapeutic ingredient. It will be clear also that, where appropriate, the therapeutic ingredients can be used in optically pure form.

[0265] In one embodiment, the invention encompasses a combination comprising a compound of the invention together with a β2-adrenoreceptor agonist. Non-limiting examples of β2-adrenoreceptor agonists include salmeterol (which can be a racemate or a single enantiomer such as the R-enantiomer), salbutamol (which can be a racemate or a single enantiomer such as the R-enantiomer), formoterol (which can be a racemate or a single diastereomer such as the R,R-diastereomer), salmefamol, fenoterol, carmoterol, etanterol, naminterol, clenbuterol, pirbuterol, flerbuterol, reproterol, bambuterol, indacaterol, terbutaline and salts thereof, for example the xinafoate (1-hydroxy-2-naphthalenecarboxylate) salt of salmeterol, the sulphate salt or free base of salbutamol or the fumarate salt of formoterol. In one embodiment the β2-adrenoreceptor agonists are long-acting β2-adrenoreceptor agonists, for example, compounds which provide effective bronchodilation for about 12 hours or longer.

[0266] Other β2-adrenoreceptor agonists include those described in WO 02/066422, WO 02/070490, WO 02/076933, WO 03/024439, WO 03/072539, WO 03/091204, WO

04/016578, WO 2004/022547, WO 2004/037807, WO 2004/037773, WO 2004/037768, WO 2004/039762, WO 2004/039766, WO01/42193 and WO03/042160.

[0267] Further examples of β2-adrenoreceptor agonists include 3-(4-{[6-({(2R)-2-hydroxy-2-[4-hydroxy-3-(hydroxymethyl)phenyl]ethyl}amino) hexyl] oxy} butyl) benzenesulfonamide; 3-(3-{[7-({(2R)-2-hydroxy-2-[4-hydroxy-3-hydroxymethyl) phenyl] ethyl}-amino) heptyl] oxy} propyl) benzenesulfonamide; 4-{(1R)-2-[(6-{2-[(2, 6-dichlorobenzyl) oxy] ethoxy} hexyl) amino]-1-hydroxyethyl}-2-(hydroxymethyl) phenol; 4-{(1R)-2-[(6-{4-[3-(cyclopentylsulfonyl)phenyl]butoxy}hexyl)amino]-1-hydroxyethyl}-2-(hydroxymethyl)phenol; N-[2-hydroxyl-5-[(1R)-1-hydroxy-2-[[2-4-[[(2R)-2-hydroxy-2 phenylethyl]amino]phenyl]ethyl]amino]ethyl]phenyl]formamide; N-2{2-[4-(3-phenyl-4-methoxyphenyl)aminophenyl]ethyl}-2-hydroxy-2-(8-hydroxy-2(1H)-quinolinon-5-yl)ethylamine; and 5-[(R)-2-(2-{4-[4-(2-amino-2-methyl-propoxy)-phenylamino]-phenyl}-ethylamino)-1-hydroxy-ethyl]-8-hydroxy-1H-quinolin-2-one.

[0268] In one embodiment, the β2-adrenoreceptor agonist can be in the form of a salt formed with a pharmaceutically acceptable acid selected from sulphuric, hydrochloric, fumaric, hydroxynaphthoic (for example 1- or 3-hydroxy-2-naphthoic), cinnamic, substituted cinnamic, triphenylacetic, sulphamic, sulphanilic, naphthaleneacrylic, benzoic, 4-methoxybenzoic, 2- or 4-hydroxybenzoic, 4-chlorobenzoic and 4-phenylbenzoic acid. Suitable anti-inflammatory agents include corticosteroids. Examples of corticosteroids which can be used in combination with the compounds of the invention are those oral and inhaled corticosteroids and their pro-drugs which have anti-inflammatory activity. Non-limiting examples include methyl prednisolone, prednisolone, dexamethasone, fluticasone propionate, 6α,9α-difluoro-11β-hydroxy-16α-methyl-17α-[(4-methyl-1,3-thiazole-5-carbonyl)oxy]-3-oxo-androsta-1,4-diene-17β-carbothioic acid S-fluoromethyl ester, 6α,9α-difluoro-17α-[(2-furanylcarbonyl)oxy]-11β-hydroxy-16α-methyl-3-oxo-androsta-1,4-diene-17β-carbothioic acid S-fluoromethyl ester (fluticasone furoate), 6α,9α-difluoro-11β-hydroxy-16α-methyl-3-oxo-17α-propionyloxy- androsta-1,4-diene-17β-carbothioic acid S-(2-oxo-tetrahydro-furan-3S-yl) ester, 6α,9α-difluoro-11β-hydroxy-16α-methyl-3-oxo-17α-(2,2,3,3-tetramethycyclopropylcarbonyl)oxy-androsta-1,4-diene-17β-carbothioic acid S-cyanomethyl ester and 6α,9α-difluoro-11β-hydroxy-16α-methyl-17α-(1-methycyclopropylcarbonyl)oxy-3-oxo-androsta-1,4-diene-17β-carbothioic acid S-fluoromethyl ester, beclomethasone esters (for example the 17-propionate ester or the 17,21-dipropionate ester), budesonide, flunisolide,

mometasone esters (for example mometasone furoate), triamcinolone acetonide, rofleponide, ciclesonide (16α,17-[[(R)-cyclohexylmethylene]bis(oxy)]-11β,21-dihydroxy-pregna-1,4-diene-3,20-dione), butixocort propionate, RPR-106541, and ST-126. In one embodiment corticosteroids include fluticasone propionate, 6α,9α-difluoro-11β-hydroxy-16α-methyl-17α-[(4-methyl-1,3-thiazole-5-carbonyl)oxy]-3-oxo-androsta-1,4-diene-17β-carbothioic acid S-fluoromethyl ester, 6α,9α-difluoro-17α-[(2-furanylcarbonyl)oxy]-11β-hydroxy-16α-methyl-3-oxo-androsta-1,4-diene-17β-carbothioic acid S-fluoromethyl ester, 6α,9α-difluoro-11β-hydroxy-16α-methyl-3-oxo-17α-(2,2,3,3- tetramethycyclopropylcarbonyl)oxy-androsta-1,4-diene-17β-carbothioic acid S-cyanomethyl ester and 6α,9α-difluoro-11β-hydroxy-16α-methyl-17α-(1-methycyclopropylcarbonyl)oxy-3-oxo-androsta-1,4-diene-17β-carbothioic acid S-fluoromethyl ester. In one embodiment the corticosteroid is 6α,9α-difluoro-17α-[(2-furanylcarbonyl)oxy]-11β-hydroxy-16α-methyl-3-oxo-androsta-1,4-diene-17β-carbothioic acid S-fluoromethyl ester. Non-limiting examples of corticosteroids can include those described in the following published patent applications and patents: WO02/088167, WO02/100879, WO02/12265, WO02/12266, WO05/005451, WO05/005452, WO06/072599 and WO06/072600.

[0269] In one embodiment, non-steroidal compounds having glucocorticoid agonism that can possess selectivity for transrepression over transactivation and that can be useful in combination therapy include those covered in the following published patent applications and patents: WO03/082827, WO98/54159, WO04/005229, WO04/009017, WO04/018429, WO03/104195, WO03/082787, WO03/082280, WO03/059899, WO03/101932, WO02/02565, WO01/16128, WO00/66590, WO03/086294, WO04/026248, WO03/061651, WO03/08277, WO06/000401, WO06/000398 and WO06/015870.

[0270] Non-steroidal compounds having glucocorticoid agonism that can possess selectivity for transrepression over transactivation and that can be useful in combination therapy include those covered in the following patents: WO03/082827, WO98/54159, WO04/005229, WO04/009017, WO04/018429, WO03/104195, WO03/082787, WO03/082280, WO03/059899, WO03/101932, WO02/02565, WO01/16128, WO00/66590, WO03/086294, WO04/026248, WO03/061651 and WO03/08277.

[0271] Non-limiting examples of anti-inflammatory agents include non-steroidal anti-inflammatory drugs (NSAID's).

[0272] Non-limiting examples of NSAID's include sodium cromoglycate, nedocromil sodium, phosphodiesterase (PDE) inhibitors (for example, theophylline, PDE4 inhibitors or mixed PDE3/PDE4 inhibitors), leukotriene antagonists, inhibitors of leukotriene synthesis (for example montelukast), iNOS inhibitors, tryptase and elastase inhibitors, beta-2 integrin antagonists and adenosine receptor agonists or antagonists (e.g. adenosine 2a agonists), cytokine antagonists (for example chemokine antagonists, such as a CCR3 antagonist) or inhibitors of cytokine synthesis, or 5-lipoxygenase inhibitors. In one embodiment, the invention encompasses iNOS (inducible nitric oxide synthase) inhibitors for oral administration. Examples of iNOS inhibitors include those disclosed in the following published international patents and patent applications: WO93/13055, WO98/30537, WO02/50021, WO95/34534 and WO99/62875. Examples of CCR3 inhibitors include those disclosed in WO02/26722.

[0273] In one embodiment the invention provides the use of the compounds of formula (I) in combination with a phosphodiesterase 4 (PDE4) inhibitor, for example in the case of a formulation adapted for inhalation. The PDE4-specific inhibitor useful in this aspect of the invention can be any compound that is known to inhibit the PDE4 enzyme or which is discovered to act as a PDE4 inhibitor, and which are only PDE4 inhibitors, not compounds which inhibit other members of the PDE family, such as PDE3 and PDE5, as well as PDE4.

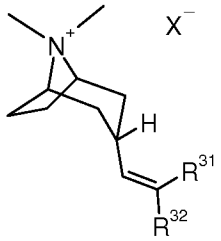
[0274] Compounds include cis-4-cyano-4-(3-cyclopentyloxy-4-methoxyphenyl)cyclohexan-1-carboxylic acid, 2-carbomethoxy-4-cyano-4-(3-cyclopropylmethoxy-4-difluoromethoxyphenyl)cyclohexan-1-one and cis-[4-cyano-4-(3-cyclopropylmethoxy-4-difluoromethoxyphenyl)cyclohexan-1-ol]. Also, cis-4-cyano-4-[3-(cyclopentyloxy)-4-methoxyphenyl]cyclohexane-1-carboxylic acid (also known as cilomilast) and its salts, esters, pro-drugs or physical forms, which is described in U.S. patent 5,552,438 issued 03 September, 1996; this patent and the compounds it discloses are incorporated herein in full by reference.

[0275] Other compounds include AWD-12-281 from Elbion (Hofgen, N. et al. 15th EFMC Int Symp Med Chem (Sept 6-10, Edinburgh) 1998, Abst P.98; CAS reference No. 247584020-9); a 9-benzyladenine derivative nominated NCS-613 (INSERM); D-4418 from Chiroscience and Schering-Plough; a benzodiazepine PDE4 inhibitor identified as CI-1018 (PD-168787) and attributed to Pfizer; a benzodioxole derivative disclosed by Kyowa Hakko in WO99/16766; K-34 from Kyowa Hakko; V-11294A from Napp (Landells, L.J. et al. Eur

Resp J [Annu Cong Eur Resp Soc (Sept 19-23, Geneva) 1998] 1998, 12 (Suppl. 28): Abst P2393); roflumilast (CAS reference No 162401-32-3) and a pthalazinone (WO99/47505, the disclosure of which is hereby incorporated by reference) from Byk-Gulden; Pumafentrine, (-)-p-[(4aR*,10bS*)-9-ethoxy-1,2,3,4,4a,10b-hexahydro-8-methoxy-2-methylbenzo[c][1,6]naphthyridin-6-yl]-N,N-diisopropylbenzamide which is a mixed PDE3/PDE4 inhibitor which has been prepared and published on by Byk-Gulden, now Altana; arofylline under development by Almirall-Prodesfarma; VM554/UM565 from Vernalis; or T-440 (Tanabe Seiyaku; Fuji, K. et al. J Pharmacol Exp Ther,1998, 284(1): 162), and T2585. Further compounds are disclosed in the published international patent applications WO04/024728 (Glaxo Group Ltd), WO04/056823 (Glaxo Group Ltd) and WO04/103998 (Glaxo Group Ltd).

[0276] Examples of anticholinergic agents are those compounds that act as antagonists at the muscarinic receptors, in particular those compounds which are antagonists of the M1 or M3 receptors, dual antagonists of the M1/M3 or M2/M3, receptors or pan-antagonists of the M1/M2/M3 receptors. Exemplary compounds for administration via inhalation include ipratropium (for example, as the bromide, CAS 22254-24-6, sold under the name Atrovent), oxitropium (for example, as the bromide, CAS 30286-75-0) and tiotropium (for example, as the bromide, CAS 136310-93-5, sold under the name Spiriva). Also of interest are revatropate (for example, as the hydrobromide, CAS 262586-79-8) and LAS-34273 which is disclosed in WO01/04118. Exemplary compounds for oral administration include pirenzepine (CAS 28797-61-7), darifenacin (CAS 133099-04-4, or CAS 133099-07-7 for the hydrobromide sold under the name Enablex), oxybutynin (CAS 5633-20-5, sold under the name Ditropan), terodiline (CAS 15793-40-5), tolterodine (CAS 124937-51-5, or CAS 124937-52-6 for the tartrate, sold under the name Detrol), otilonium (for example, as the bromide, CAS 26095-59-0, sold under the name Spasmomen), trospium chloride (CAS 10405-02-4) and solifenacin (CAS 242478-37-1, or CAS 242478-38-2 for the succinate also known as YM-905 and sold under the name Vesicare).

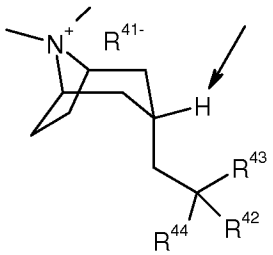
[0277] Other anticholinergic agents include compounds of formula (XXI), which are disclosed in US patent application 60/487981:



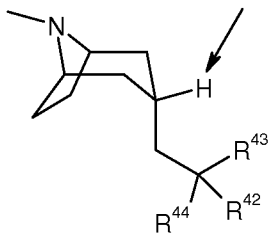
(XXI)

in which the preferred orientation of the alkyl chain attached to the tropane ring is *endo*; R^{31} and R^{32} are, independently, selected from the group consisting of straight or branched chain lower alkyl groups having preferably from 1 to 6 carbon atoms, cycloalkyl groups having from 5 to 6 carbon atoms, cycloalkyl-alkyl having 6 to 10 carbon atoms, 2-thienyl, 2-pyridyl, phenyl, phenyl substituted with an alkyl group having not in excess of 4 carbon atoms and phenyl substituted with an alkoxy group having not in excess of 4 carbon atoms; X^- represents an anion associated with the positive charge of the N atom. X^- can be but is not limited to chloride, bromide, iodide, sulfate, benzene sulfonate, and toluene sulfonate, including, for example: (3-*endo*)-3-(2,2-di-2-thienylethenyl)-8,8-dimethyl-8-azoniabicyclo[3.2.1]octane bromide; (3-*endo*)-3-(2,2-diphenylethenyl)-8,8-dimethyl-8-azoniabicyclo[3.2.1]octane bromide; (3-*endo*)-3-(2,2-diphenylethenyl)-8,8-dimethyl-8-azoniabicyclo[3.2.1]octane 4-methylbenzenesulfonate; (3-*endo*)-8,8-dimethyl-3-[2-phenyl-2-(2-thienyl)ethenyl]-8-azoniabicyclo[3.2.1]octane bromide; and/or (3-*endo*)-8,8-dimethyl-3-[2-phenyl-2-(2-pyridinyl)ethenyl]-8-azoniabicyclo[3.2.1]octane bromide.

[0278] Further anticholinergic agents include compounds of formula (XXII) or (XXIII), which are disclosed in US patent application 60/511009:



(XXII)



(XXIII)

wherein: the H atom indicated is in the exo position; R^{41} represents an anion associated with the positive charge of the N atom. R^{41} can be but is not limited to chloride, bromide, iodide, sulfate, benzene sulfonate and toluene sulfonate; R^{42} and R^{43} are independently selected from the group consisting of straight or branched chain lower alkyl groups (having preferably from 1 to 6 carbon atoms), cycloalkyl groups (having from 5 to 6 carbon atoms), cycloalkyl-alkyl (having 6 to 10 carbon atoms), heterocycloalkyl (having 5 to 6 carbon atoms) and N or O as the heteroatom, heterocycloalkyl-alkyl (having 6 to10 carbon atoms) and N or O as the heteroatom, aryl, optionally substituted aryl, heteroaryl, and optionally substituted heteroaryl; R^{44} is slected from the group consisting of $(C_1\text{-}C_6)$alkyl, $(C_3\text{-}C_{12})$cycloalkyl, $(C_3\text{-}C_7)$heterocycloalkyl, $(C_1\text{-}C_6)$alkyl$(C_3\text{-}C_{12})$cycloalkyl, $(C_1\text{-}C_6)$alkyl$(C_3\text{-}C_7)$heterocycloalkyl, aryl, heteroaryl, $(C_1\text{-}C_6)$alkyl-aryl, $(C_1\text{-}C_6)$alkyl-heteroaryl, $-OR^{45}$, $-CH_2OR^{45}$, $-CH_2OH$, -CN, $-CF_3$, $-CH_2O(CO)R^{46}$, $-CO_2R^{47}$, $-CH_2NH_2$, $-CH_2N(R^{47})SO_2R^{45}$, $-SO_2N(R^{47})(R^{48})$, $-CON(R^{47})(R^{48})$, $-CH_2N(R^{48})CO(R^{46})$, $-CH_2N(R^{48})SO_2(R^{46})$, $-CH_2N(R^{48})CO_2(R^{45})$, $-CH_2N(R^{48})CONH(R^{47})$; R^{45} is selected from the group consisting of $(C_1\text{-}C_6)$alkyl, $(C_1\text{-}C_6)$alkyl$(C_3\text{-}C_{12})$cycloalkyl, $(C_1\text{-}C_6)$alkyl$(C_3\text{-}C_7)$heterocycloalkyl, $(C_1\text{-}C_6)$alkyl-aryl, $(C_1\text{-}C_6)$alkyl-heteroaryl; R^{46} is selected from the group consisting of $(C_1\text{-}C_6)$alkyl, $(C_3\text{-}C_{12})$cycloalkyl, $(C_3\text{-}C_7)$heterocycloalkyl, $(C_1\text{-}C_6)$alkyl$(C_3\text{-}C_{12})$cycloalkyl, $(C_1\text{-}C_6)$alkyl$(C_3\text{-}C_7)$heterocycloalkyl, aryl, heteroaryl, $(C_1\text{-}C_6)$alkyl-aryl, $(C_1\text{-}C_6)$alkyl-heteroaryl; R^{47} and R^{48} are, independently, selected from the group consisting of H, $(C_1\text{-}C_6)$alkyl, $(C_3\text{-}C_{12})$cycloalkyl, $(C_3\text{-}C_7)$heterocycloalkyl, $(C_1\text{-}C_6)$alkyl$(C_3\text{-}C_{12})$cycloalkyl, $(C_1\text{-}C_6)$alkyl$(C_3\text{-}C_7)$heterocycloalkyl, $(C_1\text{-}C_6)$alkyl-aryl, and $(C_1\text{-}C_6)$alkyl-heteroaryl, including, for example: (*endo*)-3-(2-methoxy-2,2-di-thiophen-2-yl-ethyl)-8,8-dimethyl-8-azonia-bicyclo[3.2.1]octane iodide; 3-((*endo*)-8-methyl-8-aza-bicyclo[3.2.1]oct-3-yl)-2,2-diphenyl-propionitrile; (*endo*)-8-methyl-3-(2,2,2-triphenyl-ethyl)-8-aza-bicyclo[3.2.1]octane;

3-((*endo*)-8-methyl-8-aza-bicyclo[3.2.1]oct-3-yl)-2,2-diphenyl-propionamide;

3-((*endo*)-8-methyl-8-aza-bicyclo[3.2.1]oct-3-yl)-2,2-diphenyl-propionic acid;

(*endo*)-3-(2-cyano-2,2-diphenyl-ethyl)-8,8-dimethyl-8-azonia-bicyclo[3.2.1]octane iodide;

(*endo*)-3-(2-cyano-2,2-diphenyl-ethyl)-8,8-dimethyl-8-azonia-bicyclo[3.2.1]octane bromide;

3-((*endo*)-8-methyl-8-aza-bicyclo[3.2.1]oct-3-yl)-2,2-diphenyl-propan-1-ol;

N-benzyl-3-((*endo*)-8-methyl-8-aza-bicyclo[3.2.1]oct-3-yl)-2,2-diphenyl-propionamide;

(*endo*)-3-(2-carbamoyl-2,2-diphenyl-ethyl)-8,8-dimethyl-8-azonia-bicyclo[3.2.1]octane iodide;

1-benzyl-3-[3-((*endo*)-8-methyl-8-aza-bicyclo[3.2.1]oct-3-yl)-2,2-diphenyl-propyl]-urea;

1-ethyl-3-[3-((*endo*)-8-methyl-8-aza-bicyclo[3.2.1]oct-3-yl)-2,2-diphenyl-propyl]-urea;

N-[3-((*endo*)-8-methyl-8-aza-bicyclo[3.2.1]oct-3-yl)-2,2-diphenyl-propyl]-acetamide; *N*-[3-((*endo*)-8-methyl-8-aza-bicyclo[3.2.1]oct-3-yl)-2,2-diphenyl-propyl]-benzamide; 3-((*endo*)-8-methyl-8-aza-bicyclo[3.2.1]oct-3-yl)-2,2-di-thiophen-2-yl-propionitrile; (*endo*)-3-(2-cyano-2,2-di-thiophen-2-yl-ethyl)-8,8-dimethyl-8-azonia-bicyclo[3.2.1]octane iodide; *N*-[3-((*endo*)-8-methyl-8-aza-bicyclo[3.2.1]oct-3-yl)-2,2-diphenyl-propyl]-benzenesulfonamide; [3-((*endo*)-8-methyl-8-aza-bicyclo[3.2.1]oct-3-yl)-2,2-diphenyl-propyl]-urea; *N*-[3-((*endo*)-8-methyl-8-aza-bicyclo[3.2.1]oct-3-yl)-2,2-diphenyl-propyl]-methanesulfonamide; and/or (*endo*)-3-{2,2-diphenyl-3-[(1-phenyl-methanoyl)-amino]-propyl}-8,8-dimethyl-8-azonia-bicyclo[3.2.1]octane bromide.

[0279] Further compounds include: (endo)-3-(2-methoxy-2,2-di-thiophen-2-yl-ethyl)-8,8-dimethyl-8-azonia-bicyclo[3.2.1]octane iodide; (endo)-3-(2-cyano-2,2-diphenyl-ethyl)-8,8-dimethyl-8-azonia-bicyclo[3.2.1]octane iodide; (endo)-3-(2-cyano-2,2-diphenyl-ethyl)-8,8-dimethyl-8-azonia-bicyclo[3.2.1]octane bromide; (endo)-3-(2-carbamoyl-2,2-diphenyl-ethyl)-8,8-dimethyl-8-azonia-bicyclo[3.2.1]octane iodide; (endo)-3-(2-cyano-2,2-di-thiophen-2-yl-ethyl)-8,8-dimethyl-8-azonia-bicyclo[3.2.1]octane iodide; and/or (endo)-3-{2,2-diphenyl-3-[(1-phenyl-methanoyl)-amino]-propyl}-8,8-dimethyl-8-azonia-bicyclo[3.2.1]octane bromide.

[0280] In one embodiment the invention provides a combination comprising a compound of formula (I) or a pharmaceutically acceptable salt thereof together with an H1 antagonist. Examples of H1 antagonists include, without limitation, amelexanox, astemizole, azatadine, azelastine, acrivastine, brompheniramine, cetirizine, levocetirizine, efletirizine, chlorpheniramine, clemastine, cyclizine, carebastine, cyproheptadine, carbinoxamine, descarboethoxyloratadine, doxylamine, dimethindene, ebastine, epinastine, efletirizine, fexofenadine, hydroxyzine, ketotifen, loratadine, levocabastine, mizolastine, mequitazine, mianserin, noberastine, meclizine, norastemizole, olopatadine, picumast, pyrilamine, promethazine, terfenadine, tripelennamine, temelastine, trimeprazine and triprolidine, particularly cetirizine, levocetirizine, efletirizine and fexofenadine. In a further embodiment the invention provides a combination comprising a compound of formula (I), or a pharmaceutically acceptable salt thereof together with an H3 antagonist (and/or inverse agonist). Examples of H3 antagonists include, for example, those compounds disclosed in WO2004/035556 and in WO2006/045416. Other histamine receptor antagonists which can be used in combination with the compounds of the present invention include antagonists (and/or

inverse agonists) of the H4 receptor, for example, the compounds disclosed in Jablonowski et al., J. Med. Chem. 46:3957-3960 (2003).

[0281] The invention thus provides, in a further aspect, a combination comprising a compound of formula (I) and/or a pharmaceutically acceptable salt, solvate or physiologically functional derivative thereof together with a PDE4 inhibitor.

[0282] The invention thus provides, in a further aspect, a combination comprising a compound of formula (I) and/or a pharmaceutically acceptable salt, solvate or physiologically functional derivative thereof together with a β2-adrenoreceptor agonist.

[0283] The invention thus provides, in a further aspect, a combination comprising a compound of formula (I) and/or a pharmaceutically acceptable salt, solvate or physiologically functional derivative thereof together with a corticosteroid.

[0284] The invention thus provides, in a further aspect, a combination comprising a compound of formula (I) and/or a pharmaceutically acceptable salt, solvate or physiologically functional derivative thereof together with an anticholinergic.

[0285] The invention thus provides, in a further aspect, a combination comprising a compound of formula (I) and/or a pharmaceutically acceptable salt, solvate or physiologically functional derivative thereof together with an antihistamine.

[0286] The invention thus provides, in a further aspect, a combination comprising a compound of formula (I) and/or a pharmaceutically acceptable salt, solvate or physiologically functional derivative thereof together with a PDE4 inhibitor and a β2-adrenoreceptor agonist.

[0287] The invention thus provides, in a further aspect, a combination comprising a compound of formula (I) and/or a pharmaceutically acceptable salt, solvate or physiologically functional derivative thereof together with an anticholinergic and a PDE-4 inhibitor.

[0288] The combinations referred to above can conveniently be presented for use in the form of a pharmaceutical formulation and thus pharmaceutical formulations comprising a combination as defined above together with a pharmaceutically acceptable diluent or carrier represent a further aspect of the invention.

[0289] The individual compounds of such combinations can be administered either sequentially or simultaneously in separate or combined pharmaceutical formulations. In one embodiment, the individual compounds will be administered simultaneously in a combined pharmaceutical formulation. Appropriate doses of known therapeutic agents will readily be appreciated by those skilled in the art.

[0290] The invention thus provides, in a further aspect, a pharmaceutical composition comprising a combination of a compound of the invention together with another therapeutically active agent.

[0291] The invention thus provides, in a further aspect, a pharmaceutical composition comprising a combination of a compound of the invention together with a PDE4 inhibitor.

[0292] The invention thus provides, in a further aspect, a pharmaceutical composition comprising a combination of a compound of the invention together with a β2-adrenoreceptor agonist.

[0293] The invention thus provides, in a further aspect, a pharmaceutical composition comprising a combination of a compound of the invention together with a corticosteroid.

[0294] The invention thus provides, in a further aspect, a pharmaceutical composition comprising a combination of a compound of the invention together with an anticholinergic.

[0295] The invention thus provides, in a further aspect, a pharmaceutical composition comprising a combination of a compound of the invention together with an antihistamine.

[0296] The composition of the invention (e.g. siNA and/or LNP formulations thereof) can be formulated for administration in any suitable way, and the invention therefore also includes within its scope pharmaceutical compositions comprising a composition of the invention (e.g. siNA and/or LNP formulations thereof) together, if desirable, in a mixture with one or more physiologically acceptable diluents or carriers.

[0297] In one embodiment, pharmaceutical compositions of the invention (e.g. siNA and/or LNP formulations thereof) are prepared by a process which comprises mixing the ingredients into suitable formulation. Non limiting examples of administration methods of the invention include oral, buccal, sublingual, parenteral, local rectal administration or other local administration. In one embodiment, the composition of the invention can be

administered by insufflation and inhalation. Non limiting examples of various types of formulations for local administration include ointments, lotions, creams, gels, foams, preparations for delivery by transdermal patches, powders, sprays, aerosols, capsules or cartridges for use in an inhaler or insufflator or drops (for example eye or nose drops), solutions/suspensions for nebulisation, suppositories, pessaries, retention enemas and chewable or suckable tablets or pellets (for example for the treatment of aphthous ulcers) or liposome or microencapsulation preparations.

[0298] In one embodiment, a composition of the invention (e.g. siNA and/or LNP formulations thereof and pharmaceutical compositions thereof) are administered topically to the nose for example, for the treatment of rhinitis, including pressurised aerosol formulations and aqueous formulations administered to the nose by pressurised pump. Formulations which are non-pressurised and adapted to be administered topically to the nasal cavity are of particular interest. Suitable formulations contain water as the diluent or carrier for this purpose. In one embodiment, aqueous formulations for administration of the composition of the invention to the lung or nose can be provided with conventional excipients such as buffering agents, tonicity modifying agents and the like. In another embodiment, aqueous formulations can also be administered to the nose by nebulisation.

[0299] The compositions of the invention (e.g. siNA and/or LNP formulations thereof and pharmaceutical compositions thereof) can be formulated as a fluid formulation for delivery from a fluid dispenser, for example a fluid dispenser having a dispensing nozzle or dispensing orifice through which a metered dose of the fluid formulation is dispensed upon the application of a user-applied force to a pump mechanism of the fluid dispenser. In one embodiment, the fluid dispenser of the invention uses reservoir of multiple metered doses of the fluid formulation, the doses being dispensable upon sequential pump actuations. In one embodiment, the dispensing nozzle or orifice of the invention can be configured for insertion into the nostrils of the user for spray dispensing of the fluid formulation comprising the composition of the invention into the nasal cavity. A fluid dispenser of the aforementioned type is described and illustrated in WO05/044354, the entire content of which is hereby incorporated herein by reference. The dispenser has a housing which houses a fluid discharge device having a compression pump mounted on a container for containing a fluid formulation. In one embodiment, the housing has at least one finger-operable side lever which is movable inwardly with respect to the housing to cam the container upwardly in the

housing to cause the pump to compress and pump a metered dose of the formulation out of a pump stem through a nasal nozzle of the housing. In another embodiment, the fluid dispenser is of the general type illustrated in Figures 30-40 of WO05/044354.

[0300] Ointments, creams and gels, can, for example, be formulated with an aqueous or oily base with the addition of suitable thickening and/or gelling agent and/or solvents. Non limiting examples of such bases can thus, for example, include water and/or an oil such as liquid paraffin or a vegetable oil such as arachis oil or castor oil, or a solvent such as polyethylene glycol. Thickening agents and gelling agents which can be used according to the nature of the base. Non limiting examples of such agents include soft paraffin, aluminium stearate, cetostearyl alcohol, polyethylene glycols, woolfat, beeswax, carboxypolymethylene and cellulose derivatives, and/or glyceryl monostearate and/or non-ionic emulsifying agents.

[0301] In one embodiment lotions can be formulated with an aqueous or oily base and will in general also contain one or more emulsifying agents, stabilising agents, dispersing agents, suspending agents or thickening agents.

[0302] In one embodiment powders for external application can be formed with the aid of any suitable powder base, for example, talc, lactose or starch. Drops can be formulated with an aqueous or non-aqueous base also comprising one or more dispersing agents, solubilising agents, suspending agents or preservatives.

[0303] Spray compositions can for example be formulated as aqueous solutions or suspensions or as aerosols delivered from pressurised packs, such as a metered dose inhaler, with the use of a suitable liquefied propellant. In one embodiment, aerosol compositions of the invention suitable for inhalation can be either a suspension or a solution and generally contain a compound of formula (I) and a suitable propellant such as a fluorocarbon or hydrogen-containing chlorofluorocarbon or mixtures thereof, particularly hydrofluoroalkanes, especially 1,1,1,2-tetrafluoroethane, 1,1,1,2,3,3,3-heptafluoro-n-propane or a mixture thereof. The aerosol composition can optionally contain additional formulation excipients well known in the art such as surfactants. Non limiting examples include oleic acid, lecithin or an

oligolactic acid or derivative such as those described in WO94/21229 and WO98/34596 and cosolvents for example ethanol. In one embodiment a pharmaceutical aerosol formulation of the invention comprising a compound of the invention and a fluorocarbon or hydrogen-containing chlorofluorocarbon or mixtures thereof as propellant, optionally in combination with a surfactant and/or a cosolvent.

[0304] Formulations of the composition of the invention can comprise a pharmaceutical aerosol wherein the propellant is selected from 1,1,1,2-tetrafluoroethane, 1,1,1,2,3,3,3-heptafluoro-n-propane and mixtures thereof.

[0305] The formulations of the invention can be buffered by the addition of suitable buffering agents.

[0306] Capsules and cartridges comprising the composition of the invention for use in an inhaler or insufflator, of for example gelatine, can be formulated containing a powder mix for inhalation of a compound of the invention and a suitable powder base such as lactose or starch. In one embodiment, each capsule or cartridge can generally contain from 20μg to 10mg of the compound of formula (I). In another embodiment, the compound of the invention can be presented without excipients such as lactose.

[0307] The proportion of the active compound of formula (I) in the local compositions according to the invention depends on the precise type of formulation to be prepared but will generally be within the range of from 0.001 to 10% by weight. In one embodiment, the proportion of most types of preparations used will be within the range of from 0.005 to 1%, for example from 0.01 to 0.5%. In another embodiment, the composition of the invention comprises powders for inhalation or insufflation wherein the proportion used will normally be within the range of from 0.1 to 5%.

[0308] Aerosol formulations comprising the composition of the invention are preferably arranged so that each metered dose or "puff" of aerosol contains from 20μg to 10mg. In one embodiment, the aerosol formulation is from 20μg to 2000μg. In another embodiment, the aerosol formulation is from 20μg to 500μg of a compound of formula (I). Administration can be once daily or several times daily, for example 2, 3, 4 or 8 times, giving for example 1, 2 or 3 doses each time. In one embodiment, the overall daily dose with an aerosol comprising the

composition of the invention will be within the range from 100μg to 10mg. In another embodiment, the overall daily dose with an aerosol comprising the composition of the invention, will be within the range from 200μg to 2000μg. The overall daily dose and the metered dose delivered by capsules and cartridges in an inhaler or insufflator will generally be double that delivered with aerosol formulations.

[0309] In the case of suspension aerosol formulations, the particle size of the particulate (for example, micronised) drug should be such as to permit inhalation of substantially all the drug into the lungs upon administration of the aerosol formulation. In one embodiment, the particle size of the particulate will be less than 100 microns. In another embodiment, the particle size of the particulate will be less than 20 microns. The range of particulate size can be within the range of from 1 to 10 microns. In one embodiment, the particulate range can be from 1 to 5 microns. In another embodiment, the particulate range can be from 2 to 3 microns.

[0310] The formulations of the invention can be prepared by dispersal or dissolution of the medicament and a compound of the invention in the selected propellant in an appropriate container. In one embodiment, the dispersal or dissolution is with the aid of sonication or a high-shear mixer. The process is desirably carried out under controlled humidity conditions.

[0311] The chemical and physical stability and the pharmaceutical acceptability of the aerosol formulations according to the invention can be determined by techniques well known to those skilled in the art. In one embodiment, the chemical stability of the components can be determined by HPLC assay, for example, after prolonged storage of the product. Physical stability data can be gained from other conventional analytical techniques. In one embodiment, physical stability data can be gained by leak testing, by valve delivery assay (average shot weights per actuation), by dose reproducibility assay (active ingredient per actuation) and spray distribution analysis.

[0312] The stability of the suspension aerosol formulations according to the invention can be measured by conventional techniques. In one embodiment, the stability of the suspension aerosol can be measured by determining flocculation size distribution using a back light scattering instrument or by measuring particle size distribution by cascade impaction or by the "twin impinger" analytical process.

[0313] As used herein reference to the "twin impinger" assay means "Determination of the deposition of the emitted dose in pressurised inhalations using apparatus A" as defined in British Pharmacopaeia 1988, pages A204-207, Appendix XVII C. Such techniques enable the "respirable fraction" of the aerosol formulations to be calculated. In one embodiment, a method used to calculate the "respirable fraction" is by reference to "fine particle fraction" which is the amount of active ingredient collected in the lower impingement chamber per actuation expressed as a percentage of the total amount of active ingredient delivered per actuation using the twin impinger method described above.

[0314] The term "metered dose inhaler" or MDI means a unit comprising a can, a secured cap covering the can and a formulation metering valve situated in the cap. MDI system includes a suitable channelling device. Suitable channelling devices of the invention comprise for example, a valve actuator and a cylindrical or cone-like passage through which medicament can be delivered from the filled canister via the metering valve to the nose or mouth of a patient such as a mouthpiece actuator.

[0315] MDI canisters of the invention typically comprise a container capable of withstanding the vapour pressure of the propellant used such as a plastic or plastic-coated glass bottle or preferably a metal can, for example, aluminium or an alloy thereof which can optionally be anodised, lacquer-coated and/or plastic-coated (for example incorporated herein by reference WO96/32099 wherein part or all of the internal surfaces are coated with one or more fluorocarbon polymers optionally in combination with one or more non-fluorocarbon polymers), which container is closed with a metering valve. In one embodiment the cap can be secured onto the can via ultrasonic welding, screw fitting or crimping. MDIs taught herein can be prepared by methods of the art (for example, see Byron, above and WO96/32099). In one embodiment, the canister of the invention is fitted with a cap assembly, wherein a drug-metering valve is situated in the cap, and said cap is crimped in place.

[0316] In one embodiment of the invention the metallic internal surface of the can is coated with a fluoropolymer, most preferably blended with a non-fluoropolymer. In another embodiment of the invention the metallic internal surface of the can is coated with a polymer blend of polytetrafluoroethylene (PTFE) and polyethersulfone (PES). In a further embodiment of the invention the whole of the metallic internal surface of the can is coated with a polymer blend of polytetrafluoroethylene (PTFE) and polyethersulfone (PES).

[0317] The metering valves are designed to deliver a metered amount of the formulation per actuation and incorporate a gasket to prevent leakage of propellant through the valve. The gasket can comprise any suitable elastomeric material such as, for example, low density polyethylene, chlorobutyl, bromobutyl, EPDM, black and white butadiene-acrylonitrile rubbers, butyl rubber and neoprene. Suitable valves are commercially available from manufacturers well known in the aerosol industry, for example, from Valois, France (e.g. DF10, DF30, DF60), Bespak plc, UK (e.g. BK300, BK357) and 3M-Neotechnic Ltd, UK (e.g. SpraymiserTM).

[0318] In various embodiments, the MDIs can also be used in conjunction with other structures such as, without limitation, overwrap packages for storing and containing the MDIs, including those described in U.S. Patent Nos. 6,119,853; 6,179,118; 6,315,112; 6,352,152; 6,390,291; and 6,679,374, as well as dose counter units such as, but not limited to, those described in U.S. Patent Nos. 6,360,739 and 6,431,168.

[0319] Conventional bulk manufacturing methods and machinery well known to those skilled in the art of pharmaceutical aerosol manufacture can be employed for the preparation of large-scale batches for the commercial production of filled canisters. Thus, for example, in one bulk manufacturing method for preparing suspension aerosol formulations a metering valve is crimped onto an aluminium can to form an empty canister. The particulate medicament is added to a charge vessel and liquefied propellant together with the optional excipients is pressure filled through the charge vessel into a manufacturing vessel. The drug suspension is mixed before recirculation to a filling machine and an aliquot of the drug suspension is then filled through the metering valve into the canister. In one example bulk manufacturing method for preparing solution aerosol formulations, a metering valve is crimped onto an aluminium can to form an empty canister. The liquefied propellant together with the optional excipients and the dissolved medicament is pressure filled through the charge vessel into a manufacturing vessel.

[0320] In another embodiment, an aliquot of the liquefied formulation is added to an open canister under conditions which are sufficiently cold to ensure the formulation does not vaporise, and then a metering valve crimped onto the canister.

[0321] Typically, in batches prepared for pharmaceutical use, each filled canister is check-weighed, coded with a batch number and packed into a tray for storage before release testing.

[0322] Topical preparations can be administered by one or more applications per day to the affected area; over skin areas occlusive dressings can advantageously be used. Continuous or prolonged delivery can be achieved by an adhesive reservoir system.

[0323] For internal administration the compounds according to the invention (e.g. siNA and/or LNP formulations thereof) can, for example, be formulated in conventional manner for oral, nasal, parenteral or rectal administration. In one embodiment, formulations for oral administration include syrups, elixirs, powders, granules, tablets and capsules which typically contain conventional excipients such as binding agents, fillers, lubricants, disintegrants, wetting agents, suspending agents, emulsifying agents, preservatives, buffer salts, flavouring, colouring and/or sweetening agents as appropriate. Dosage unit forms can be preferred as described below.

[0324] The compounds of the invention can in general be given by internal administration in cases wherein systemic glucocorticoid receptor agonist therapy is indicated.

[0325] Slow release or enteric coated formulations can be advantageous, particularly for the treatment of inflammatory bowel disorders.

[0326] In some embodiments, the compounds of the invention (e.g. siNA and/or LNP formulations thereof) will be formulated for oral administration. In other embodiments, the compounds of the invention will be formulated for inhaled administration.

[0327] In another embodiment, the invention features a method of modulating the expression of more than one ENaC target gene in a subject or organism comprising contacting the subject or organism with one or more siNA molecules of the invention under conditions suitable to modulate (*e.g.*, inhibit) the expression of the ENaC target genes in the subject or organism.

[0328] The siNA molecules of the invention can be designed to down regulate or inhibit target gene expression through RNAi targeting of a variety of nucleic acid molecules. In one embodiment, the siNA molecules of the invention are used to target various DNA corresponding to a target gene, for example via heterochromatic silencing or transcriptional inhibition. In one embodiment, the siNA molecules of the invention are used to target various RNAs corresponding to a target gene, for example via RNA target cleavage or translational inhibition. Non-limiting examples of such RNAs include messenger RNA (mRNA), non-

coding RNA (ncRNA) or regulatory elements (see for example Mattick, 2005, *Science*, 309, 1527-1528 and Claverie, 2005, *Science*, 309, 1529-1530) which includes miRNA and other small RNAs, alternate RNA isotypes of target gene(s), post-transcriptionally modified RNA of target gene(s), pre-mRNA of target gene(s), and/or RNA templates. If alternate splicing produces a family of transcripts that are distinguished by usage of appropriate exons, the instant invention can be used to inhibit gene expression through the appropriate exons to specifically inhibit or to distinguish among the functions of gene family members. For example, a protein that contains an alternatively spliced transmembrane domain can be expressed in both membrane bound and secreted forms. Use of the invention to target the exon containing the transmembrane domain can be used to determine the functional consequences of pharmaceutical targeting of the membrane bound as opposed to the secreted form of the protein. Non-limiting examples of applications of the invention relating to targeting these RNA molecules include therapeutic pharmaceutical applications, cosmetic applications, veterinary applications, pharmaceutical discovery applications, molecular diagnostic and gene function applications, and gene mapping, for example using single nucleotide polymorphism mapping with siNA molecules of the invention. Such applications can be implemented using known gene sequences or from partial sequences available from an expressed sequence tag (EST).

[0329] 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 ENaC family genes (*e.g.*, all known ENaC isotypes, or select groupings of ENaC isotypes). As such, siNA molecules targeting multiple ENaC targets can provide increased therapeutic effect. In addition, by avoiding other ENaC isotypes, toxicity can be avoided.

[0330] In one embodiment, siNA molecules can be used to characterize pathways of gene function in a variety of applications. For example, the present invention can be used to inhibit the activity of target gene(s) in a pathway to determine the function of uncharacterized gene(s) in gene function analysis, mRNA function analysis, or translational analysis. The invention can be used to determine potential target gene pathways involved in various diseases and conditions toward pharmaceutical development. The invention can be used to understand pathways of gene expression involved in, for example respiratory, inflammatory, and/or autoimmune diseases, disorders, traits and conditions.

[0331] 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, target genes encoding RNA sequence(s) referred to herein by Genbank Accession number, for example, Genbank Accession Nos. shown herein (*e.g.* in **Table 7**).

[0332] 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 15 to about 30 (*e.g.*, about 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, or 30) nucleotides in length. In one embodiment, the assay can comprise a reconstituted *in vitro* siNA assay as described herein. In another embodiment, the assay can comprise a cell culture system in which target RNA is expressed. In another embodiment, fragments of target RNA are analyzed for detectable levels of cleavage, for example by gel electrophoresis, northern blot analysis, or RNAse protection assays, to determine the most suitable target site(s) within the target RNA sequence. The target RNA sequence can be obtained as is known in the art, for example, by cloning and/or transcription for *in vitro* systems, and by cellular expression in *in vivo* systems.

[0333] In one embodiment, the invention features a method comprising: (a) generating a randomized library of siNA constructs having a predetermined complexity, such as of 4^N, where N represents the number of base paired nucleotides in each of the siNA construct strands (*eg.* for a siNA construct having 21 nucleotide sense and antisense strands with 19 base pairs, the complexity would be 4^{19}); and (b) assaying the siNA constructs of (a) above, under conditions suitable to determine RNAi target sites within the target RNA sequence. In another embodiment, the siNA molecules of (a) have strands of a fixed length, for example about 23 nucleotides in length. In yet another embodiment, the siNA molecules of (a) are of differing length, for example having strands of about 15 to about 30 (*e.g.*, about 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, or 30) nucleotides in length. In one embodiment, the assay can comprise a reconstituted *in vitro* siNA assay. 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 target RNA sequence. The target 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.

[0334] 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 siNA molecules of (b) have strands of a fixed length, for example about 23 nucleotides in length. In another embodiment, the siNA molecules of (b) are of differing length, for example having strands of about 15 to about 30 (*e.g.*, about 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, or 30) nucleotides in length. In one embodiment, the assay can comprise a reconstituted *in vitro* siNA assay as described herein. In another embodiment, the assay can comprise a cell culture system in which target RNA is expressed. Fragments of target RNA are analyzed for detectable levels of cleavage, for example by gel electrophoresis, northern blot analysis, or RNAse protection assays, to determine the most suitable target site(s) within the target RNA sequence. The target RNA sequence can be obtained as is known in the art, for example, by cloning and/or transcription for *in vitro* systems, and by expression in *in vivo* systems.

[0335] By "target site" is meant a sequence within a target RNA that is "targeted" for cleavage mediated by a siNA construct which contains sequences within its antisense region that are complementary to the target sequence.

[0336] 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.

[0337] In one embodiment, the invention features a composition comprising a siNA molecule of the invention, which can be chemically-modified, in a pharmaceutically acceptable carrier or diluent. In another embodiment, the invention features a pharmaceutical

composition comprising siNA molecules of the invention, which can be chemically-modified, targeting one or more genes in a pharmaceutically acceptable carrier or diluent. In another embodiment, the invention features a method for diagnosing a disease, trait, or condition in a subject comprising administering to the subject a composition of the invention under conditions suitable for the diagnosis of the disease, trait, or condition in the subject. In another embodiment, the invention features a method for treating or preventing a disease, trait, or condition, such as respiratory, inflammatory, and/or autoimmune disorders in a subject, comprising administering to the subject a composition of the invention under conditions suitable for the treatment or prevention of the disease, trait, or condition in the subject, alone or in conjunction with one or more other therapeutic compounds.

[0338] In another embodiment, the invention features a method for validating a target gene target, comprising: (a) synthesizing a siNA molecule of the invention, which can be chemically-modified, wherein one of the siNA strands includes a sequence complementary to RNA of a target gene; (b) introducing the siNA molecule into a cell, tissue, subject, or organism under conditions suitable for modulating expression of the target gene in the cell, tissue, subject, or organism; and (c) determining the function of the gene by assaying for any phenotypic change in the cell, tissue, subject, or organism.

[0339] In another embodiment, the invention features a method for validating a target comprising: (a) synthesizing a siNA molecule of the invention, which can be chemically-modified, wherein one of the siNA strands includes a sequence complementary to RNA of a target gene; (b) introducing the siNA molecule into a biological system under conditions suitable for modulating expression of the target gene in the biological system; and (c) determining the function of the gene by assaying for any phenotypic change in the biological system.

[0340] By "biological system" is meant, material, in a purified or unpurified form, from biological sources, including but not limited to human or animal, wherein the system comprises the components required for RNAi activity. The term "biological system" includes, for example, a cell, tissue, subject, or organism, or extract thereof. The term biological system also includes reconstituted RNAi systems that can be used in an *in vitro* setting.

[0341] By "phenotypic change" is meant any detectable change to a cell that occurs in response to contact or treatment with a nucleic acid molecule of the invention (*e.g.*, siNA). Such detectable changes include, but are not limited to, changes in shape, size, proliferation, motility, protein expression or RNA expression or other physical or chemical changes as can be assayed by methods known in the art. The detectable change can also include expression of reporter genes/molecules such as Green Fluorescent Protein (GFP) or various tags that are used to identify an expressed protein or any other cellular component that can be assayed.

[0342] In one embodiment, the invention features a kit containing a siNA molecule of the invention, which can be chemically-modified, that can be used to modulate the expression of a target gene in a biological system, including, for example, in a cell, tissue, subject, or organism. In another embodiment, the invention features a kit containing more than one siNA molecule of the invention, which can be chemically-modified, that can be used to modulate the expression of more than one target gene in a biological system, including, for example, in a cell, tissue, subject, or organism.

[0343] In one embodiment, the invention features a cell containing one or more siNA molecules of the invention, which can be chemically-modified. In another embodiment, the cell containing a siNA molecule of the invention is a mammalian cell. In yet another embodiment, the cell containing a siNA molecule of the invention is a human cell.

[0344] In one embodiment, the synthesis of a siNA molecule of the invention, which can be chemically-modified, comprises: (a) synthesis of two complementary strands of the siNA molecule; (b) annealing the two complementary strands together under conditions suitable to obtain a double-stranded siNA molecule. In another embodiment, synthesis of the two complementary strands of the siNA molecule is by solid phase oligonucleotide synthesis. In yet another embodiment, synthesis of the two complementary strands of the siNA molecule is by solid phase tandem oligonucleotide synthesis.

[0345] In one embodiment, the invention features a method for synthesizing a siNA duplex molecule comprising: (a) synthesizing a first oligonucleotide sequence strand of the siNA molecule, wherein the first oligonucleotide sequence strand comprises a cleavable linker molecule that can be used as a scaffold for the synthesis of the second oligonucleotide sequence strand of the siNA; (b) synthesizing the second oligonucleotide sequence strand of siNA on the scaffold of the first oligonucleotide sequence strand, wherein the second

oligonucleotide sequence strand further comprises a chemical moiety than can be used to purify the siNA duplex; (c) cleaving the linker molecule of (a) under conditions suitable for the two siNA oligonucleotide strands to hybridize and form a stable duplex; and (d) purifying the siNA duplex utilizing the chemical moiety of the second oligonucleotide sequence strand. In one embodiment, cleavage of the linker molecule in (c) above takes place during deprotection of the oligonucleotide, for example, under hydrolysis conditions using an alkylamine base such as methylamine. In one embodiment, the method of synthesis comprises solid phase synthesis on a solid support such as controlled pore glass (CPG) or polystyrene, wherein the first sequence of (a) is synthesized on a cleavable linker, such as a succinyl linker, using the solid support as a scaffold. The cleavable linker in (a) used as a scaffold for synthesizing the second strand can comprise similar reactivity as the solid support derivatized linker, such that cleavage of the solid support derivatized linker and the cleavable linker of (a) takes place concomitantly. In another embodiment, the chemical moiety of (b) that can be used to isolate the attached oligonucleotide sequence comprises a trityl group, for example a dimethoxytrityl group, which can be employed in a trityl-on synthesis strategy as described herein. In yet another embodiment, the chemical moiety, such as a dimethoxytrityl group, is removed during purification, for example, using acidic conditions.

[0346] 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.

[0347] 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.

[0348] 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.

[0349] In another embodiment, the method of synthesis of siNA molecules of the invention comprises the teachings of Scaringe *et al.,* US Patent Nos. 5,889,136; 6,008,400; and 6,111,086, incorporated by reference herein in their entirety.

[0350] In one embodiment, the invention features siNA constructs that mediate RNAi against an ENaC target polynucleotide wherein the siNA construct comprises one or more chemical modifications, for example, one or more chemical modifications having any of

Formulae I-VII or any combination thereof that increases the nuclease resistance of the siNA construct.

[0351] In another embodiment, the invention features a method for generating siNA molecules with increased nuclease resistance comprising (a) introducing nucleotides having any of Formula I-VII or any combination thereof into a siNA molecule, and (b) assaying the siNA molecule of step (a) under conditions suitable for isolating siNA molecules having increased nuclease resistance.

[0352] In another embodiment, the invention features a method for generating siNA molecules with improved toxicologic profiles (*e.g.*, having attenuated or no immunstimulatory properties) comprising (a) introducing nucleotides having any of Formula I-VII (*e.g.*, siNA motifs referred to in **Table 8**) or any combination thereof into a siNA molecule, and (b) assaying the siNA molecule of step (a) under conditions suitable for isolating siNA molecules having improved toxicologic profiles.

[0353] In another embodiment, the invention features a method for generating siNA formulations with improved toxicologic profiles (*e.g.*, having attenuated or no immunstimulatory properties) comprising (a) generating a siNA formulation comprising a siNA molecule of the invention and a delivery vehicle or delivery particle as described herein or as otherwise known in the art, and (b) assaying the siNA formualtion of step (a) under conditions suitable for isolating siNA formulations having improved toxicologic profiles.

[0354] In another embodiment, the invention features a method for generating siNA molecules that do not stimulate an interferon response (*e.g.*, no interferon response or attenuated interferon response) in a cell, subject, or organism, comprising (a) introducing nucleotides having any of Formula I-VII (*e.g.*, siNA motifs referred to in **Table 8**) or any combination thereof into a siNA molecule, and (b) assaying the siNA molecule of step (a) under conditions suitable for isolating siNA molecules that do not stimulate an interferon response.

[0355] In another embodiment, the invention features a method for generating siNA formulations that do not stimulate an interferon response (*e.g.*, no interferon response or attenuated interferon response) in a cell, subject, or organism, comprising (a) generating a siNA formulation comprising a siNA molecule of the invention and a delivery vehicle or delivery particle as described herein or as otherwise known in the art, and (b) assaying the

siNA formualtion of step (a) under conditions suitable for isolating siNA formulations that do not stimulate an interferon response. In one embodiment, the interferon comprises interferon alpha.

[0356] In another embodiment, the invention features a method for generating siNA molecules that do not stimulate an inflammatory or proinflammatory cytokine response (*e.g.*, no cytokine response or attenuated cytokine response) in a cell, subject, or organism, comprising (a) introducing nucleotides having any of Formula I-VII (*e.g.*, siNA motifs referred to in **Table 8**) or any combination thereof into a siNA molecule, and (b) assaying the siNA molecule of step (a) under conditions suitable for isolating siNA molecules that do not stimulate a cytokine response. In one embodiment, the cytokine comprises an interleukin such as interleukin-6 (IL-6) and/or tumor necrosis alpha (TNF-α).

[0357] In another embodiment, the invention features a method for generating siNA formulations that do not stimulate an inflammatory or proinflammatory cytokine response (*e.g.*, no cytokine response or attenuated cytokine response) in a cell, subject, or organism, comprising (a) generating a siNA formulation comprising a siNA molecule of the invention and a delivery vehicle or delivery particle as described herein or as otherwise known in the art, and (b) assaying the siNA formualtion of step (a) under conditions suitable for isolating siNA formulations that do not stimulate a cytokine response. In one embodiment, the cytokine comprises an interleukin such as interleukin-6 (IL-6) and/or tumor necrosis alpha (TNF-α).

[0358] In another embodiment, the invention features a method for generating siNA molecules that do not stimulate Toll-like Receptor (TLR) response (*e.g.*, no TLR response or attenuated TLR response) in a cell, subject, or organism, comprising (a) introducing nucleotides having any of Formula I-VII (*e.g.*, siNA motifs referred to in **Table 8**) or any combination thereof into a siNA molecule, and (b) assaying the siNA molecule of step (a) under conditions suitable for isolating siNA molecules that do not stimulate a TLR response. In one embodiment, the TLR comprises TLR3, TLR7, TLR8 and/or TLR9.

[0359] In another embodiment, the invention features a method for generating siNA formulations that do not stimulate a Toll-like Receptor (TLR) response (*e.g.*, no TLR response or attenuated TLR response) in a cell, subject, or organism, comprising (a) generating a siNA formulation comprising a siNA molecule of the invention and a delivery

vehicle or delivery particle as described herein or as otherwise known in the art, and (b) assaying the siNA formualtion of step (a) under conditions suitable for isolating siNA formulations that do not stimulate a TLR response. In one embodiment, the TLR comprises TLR3, TLR7, TLR8 and/or TLR9.

[0360] In one embodiment, the invention features a chemically synthesized double stranded short interfering nucleic acid (siNA) molecule that directs cleavage of a target RNA via RNA interference (RNAi), wherein: (a) each strand of said siNA molecule is about 18 to about 38 nucleotides in length; (b) one strand of said siNA molecule comprises nucleotide sequence having sufficient complementarity to said target RNA for the siNA molecule to direct cleavage of the target RNA via RNA interference; and (c) wherein the nucleotide positions within said siNA molecule are chemically modified to reduce the immunostimulatory properties of the siNA molecule to a level below that of a corresponding unmodified siRNA molecule. Such siNA molecules are said to have an improved toxicologic profile compared to an unmodified or minimally modified siNA.

[0361] By "improved toxicologic profile", is meant that the chemically modified or formulated siNA construct exhibits decreased toxicity in a cell, subject, or organism compared to an unmodified or unformulated siNA, or siNA molecule having fewer modifications or modifications that are less effective in imparting improved toxicology. Such siNA molecules are also considered to have “improved RNAi activity” In a non-limiting example, siNA molecules and formulations with improved toxicologic profiles are associated with reduced immunostimulatory properties, such as a reduced, decreased or attenuated immunostimulatory response in a cell, subject, or organism compared to an unmodified or unformulated siNA, or siNA molecule having fewer modifications or modifications that are less effective in imparting improved toxicology. Such an improved toxicologic profile is characterized by abrogated or reduced immunostimulation, such as reduction or abrogation of induction of interferons (*e.g.*, interferon alpha), inflammatory cytokines (*e.g.*, interleukins such as IL-6, and/or TNF-alpha), and/or toll like receptors (*e.g.*, TLR3, TLR7, TLR8, and/or TLR9). In one embodiment, a siNA molecule or formulation with an improved toxicological profile comprises no ribonucleotides. In one embodiment, a siNA molecule or formulation with an improved toxicological profile comprises less than 5 ribonucleotides (*e.g.*, 1, 2, 3, or 4 ribonucleotides). In one embodiment, a siNA molecule or formulation with an improved toxicological profile comprises Stab 7, Stab 8, Stab 11, Stab 12, Stab 13, Stab 16, Stab 17,

Stab 18, Stab 19, Stab 20, Stab 23, Stab 24, Stab 25, Stab 26, Stab 27, Stab 28, Stab 29, Stab 30, Stab 31, Stab 32, Stab 33, Stab 34, Stab 35, Stab 36 or any combination thereof (see **Table 8**). Herein, numeric Stab chemistries include both 2'-fluoro and 2'-OCF3 versions of the chemistries shown in **Table 8**. For example, "Stab 7/8" refers to both Stab 7/8 and Stab 7F/8F *etc*. In one embodiment, a siNA molecule or formulation with an improved toxicological profile comprises a siNA molecule of the invention and a formulation as described in United States Patent Application Publication No. 20030077829, incorporated by reference herein in its entirety including the drawings.

[0362] In one embodiment, the level of immunostimulatory response associated with a given siNA molecule can be measured as is described herein or as is otherwise known in the art, for example by determining the level of PKR/interferon response, proliferation, B-cell activation, and/or cytokine production in assays to quantitate the immunostimulatory response of particular siNA molecules (see, for example, Leifer *et al.*, 2003, *J Immunother.* 26, 313-9; and U.S. Patent No. 5,968,909, incorporated in its entirety by reference). In one embodiment, the reduced immunostimulatory response is between about 10% and about 100% compared to an unmodified or minimally modified siRNA molecule, *e.g.*, about 10%, 20%, 30%, 40%, 50%, 60%, 70%, 80%, 90% or 100% reduced immunostimulatory response. In one embodiment, the immunostimulatory response associated with a siNA molecule can be modulated by the degree of chemical modification. For example, a siNA molecule having between about 10% and about 100%, *e.g.*, about 10%, 20%, 30%, 40%, 50%, 60%, 70%, 80%, 90% or 100% or at least about 10%, 20%, 30%, 40%, 50%, 60%, 70%, 80%, 90% or 100% of the nucleotide positions in the siNA molecule modified can be selected to have a corresponding degree of immunostimulatory properties as described herein.

[0363] In one embodiment, the degree of reduced immunostimulatory response is selected for optimized RNAi activity. For example, retaining a certain degree of immunostimulation can be preferred to treat viral infection, where less than 100% reduction in immunostimulation can be preferred for maximal antiviral activity (*e.g.*, about 10%, 20%, 30%, 40%, 50%, 60%, 70%, 80%, or 90% reduction in immunostimulation) whereas the inhibition of expression of an endogenous gene target can be preferred with siNA molecules that posess minimal immunostimulatory properties to prevent non-specific toxicity or off target effects (*e.g.*, about 90% to about 100% reduction in immunostimulation).

[0364] In one embodiment, the invention features a chemically synthesized double stranded siNA molecule that directs cleavage of a target RNA via RNA interference (RNAi), wherein (a) each strand of said siNA molecule is about 18 to about 38 nucleotides in length; (b) one strand of said siNA molecule comprises nucleotide sequence having sufficient complementarity to said target RNA for the siNA molecule to direct cleavage of the target RNA via RNA interference; and (c) wherein one or more nucleotides of said siNA molecule are chemically modified to reduce the immunostimulatory properties of the siNA molecule to a level below that of a corresponding unmodified siNA molecule. In one embodiment, each starnd comprises at least about 18 nucleotides that are complementary to the nucleotides of the other strand.

[0365] In another embodiment, the siNA molecule comprising modified nucleotides to reduce the immunostimulatory properties of the siNA molecule comprises an antisense region having nucleotide sequence that is complemetary to a nucleotide sequence of a target gene or a portion thereof and further comprises a sense region, wherein said sense region comprises a nucleotide sequence substantially similar to the nucleotide sequence of said target gene or protion thereof. In one embodiment thereof, the antisense region and the sense region comprise about 18 to about 38 nucleotides, wherein said antisense region comprises at least about 18 nucleotides that are complementary to nucleotides of the sense region. In one embodiment thereof, the pyrimidine nucleotides in the sense region are 2'-O-methyl pyrimidine nucleotides. In another embodiment thereof, the purine nucleotides in the sense region are 2'-deoxy purine nucleotides. In yet another embodiment thereof, the pyrimidine nucleotides present in the sense region are 2'-deoxy-2'-fluoro pyrimidine nucleotides. In another embodiment thereof, the pyrimidine nucleotides of said antisense region are 2'-deoxy-2'-fluoro pyrimidine nucleotides. In yet another embodiment thereof, the purine nucleotides of said antisense region are 2'-O-methyl purine nucleotides. In still another embodiment thereof, the purine nucleotides present in said antisense region comprise 2'-deoxypurine nucleotides. In another embodiment, the antisense region comprises a phosphorothioate internucleotide linkage at the 3' end of said antisense region. In another embodiment, the antisense region comprises a glyceryl modification at a 3' end of said antisense region.

[0366] In other embodiments, the siNA molecule comprisisng modified nucleotides to reduce the immunostimulatory properties of the siNA molecule can comprise any of the

structural features of siNA molecules described herein. In other embodiments, the siNA molecule comprising modified nucleotides to reduce the immunostimulatory properties of the siNA molecule can comprise any of the chemical modifications of siNA molecules described herein.

[0367] In one embodiment, the invention features a method for generating a chemically synthesized double stranded siNA molecule having chemically modified nucleotides to reduce the immunostimulatory properties of the siNA molecule, comprising (a) introducing one or more modified nucleotides in the siNA molecule, and (b) assaying the siNA molecule of step (a) under conditions suitable for isolating an siNA molecule having reduced immunostimulatory properties compared to a corresponding siNA molecule having unmodified nucleotides. Each strand of the siNA molecule is about 18 to about 38 nucleotides in length. One strand of the siNA molecule comprises nucleotide sequence having sufficient complementarity to the target RNA for the siNA molecule to direct cleavage of the target RNA via RNA interference. In one embodiment, the reduced immunostimulatory properties comprise an abrogated or reduced induction of inflammatory or proinflammatory cytokines, such as interleukin-6 (IL-6) or tumor necrosis alpha (TNF-α), in response to the siNA being introduced in a cell, tissue, or organism. In another embodiment, the reduced immunostimulatory properties comprise an abrogated or reduced induction of Toll Like Receptors (TLRs), such as TLR3, TLR7, TLR8 or TLR9, in response to the siNA being introduced in a cell, tissue, or organism. In another embodiment, the reduced immunostimulatory properties comprise an abrogated or reduced induction of interferons, such as interferon alpha, in response to the siNA being introduced in a cell, tissue, or organism.

[0368] In one embodiment, the invention features siNA constructs that mediate RNAi against a target polynucleotide, wherein the siNA construct comprises one or more chemical modifications described herein that modulates the binding affinity between the sense and antisense strands of the siNA construct.

[0369] 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.

[0370] In one embodiment, the invention features siNA constructs that mediate RNAi against a target polynucleotide, wherein the siNA construct comprises one or more chemical modifications described herein that modulates the binding affinity between the antisense strand of the siNA construct and a complementary target RNA sequence within a cell.

[0371] In one embodiment, the invention features siNA constructs that mediate RNAi against a target polynucleotide, wherein the siNA construct comprises one or more chemical modifications described herein that modulates the binding affinity between the antisense strand of the siNA construct and a complementary target DNA sequence within a cell.

[0372] 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.

[0373] 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.

[0374] In one embodiment, the invention features siNA constructs that mediate RNAi against a target polynucleotide, wherein the siNA construct comprises one or more chemical modifications described herein that modulate the polymerase activity of a cellular polymerase capable of generating additional endogenous siNA molecules having sequence homology to the chemically-modified siNA construct.

[0375] 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.

[0376] In one embodiment, the invention features chemically-modified siNA constructs that mediate RNAi against a target polynucleotide in a cell, wherein the chemical modifications do not significantly effect the interaction of siNA with a target RNA molecule, DNA molecule and/or proteins or other factors that are essential for RNAi in a manner that would decrease the efficacy of RNAi mediated by such siNA constructs.

[0377] In another embodiment, the invention features a method for generating siNA molecules with improved RNAi specificity against polynucleotide targets comprising (a) introducing nucleotides having any of Formula I-VII or any combination thereof into a siNA molecule, and (b) assaying the siNA molecule of step (a) under conditions suitable for isolating siNA molecules having improved RNAi specificity. In one embodiment, improved specificity comprises having reduced off target effects compared to an unmodified siNA molecule. For example, introduction of terminal cap moieties at the 3'-end, 5'-end, or both 3' and 5'-ends of the sense strand or region of a siNA molecule of the invention can direct the siNA to have improved specificity by preventing the sense strand or sense region from acting as a template for RNAi activity against a corresponding target having complementarity to the sense strand or sense region.

[0378] In another embodiment, the invention features a method for generating siNA molecules with improved RNAi activity against a target polynucleotide comprising (a) introducing nucleotides having any of Formula I-VII or any combination thereof into a siNA molecule, and (b) assaying the siNA molecule of step (a) under conditions suitable for isolating siNA molecules having improved RNAi activity.

[0379] In yet another embodiment, the invention features a method for generating siNA molecules with improved RNAi activity against a target RNA comprising (a) introducing nucleotides having any of Formula I-VII or any combination thereof into a siNA molecule, and (b) assaying the siNA molecule of step (a) under conditions suitable for isolating siNA molecules having improved RNAi activity against the target RNA.

[0380] In yet another embodiment, the invention features a method for generating siNA molecules with improved RNAi activity against a target DNA comprising (a) introducing nucleotides having any of Formula I-VII or any combination thereof into a siNA molecule, and (b) assaying the siNA molecule of step (a) under conditions suitable for isolating siNA molecules having improved RNAi activity against the target DNA.

[0381] In one embodiment, the invention features siNA constructs that mediate RNAi against a target polynucleotide, wherein the siNA construct comprises one or more chemical modifications described herein that modulates the cellular uptake of the siNA construct, such as cholesterol conjugation of the siNA.

[0382] In another embodiment, the invention features a method for generating siNA molecules against a target polynucleotide with improved cellular uptake comprising (a) introducing nucleotides having any of Formula I-VII or any combination thereof into a siNA molecule, and (b) assaying the siNA molecule of step (a) under conditions suitable for isolating siNA molecules having improved cellular uptake.

[0383] In one embodiment, the invention features siNA constructs that mediate RNAi against a target polynucleotide, wherein the siNA construct comprises one or more chemical modifications described herein that increases the bioavailability of the siNA construct, for example, by attaching polymeric conjugates such as polyethyleneglycol or equivalent conjugates that improve the pharmacokinetics of the siNA construct, or by attaching conjugates that target specific tissue types or cell types *in vivo*. Non-limiting examples of such conjugates are described in Vargeese *et al.,* U.S. Serial No. 10/201,394 incorporated by reference herein.

[0384] In one embodiment, the invention features a method for generating siNA molecules of the invention with improved bioavailability comprising (a) introducing a conjugate into the structure of a siNA molecule, and (b) assaying the siNA molecule of step (a) under conditions suitable for isolating siNA molecules having improved bioavailability.

Such conjugates can include ligands for cellular receptors, such as peptides derived from naturally occurring protein ligands; protein localization sequences, including cellular ZIP code sequences; antibodies; nucleic acid aptamers; vitamins and other co-factors, such as folate and N-acetylgalactosamine; polymers, such as polyethyleneglycol (PEG); phospholipids; cholesterol; cholesterol derivatives, polyamines, such as spermine or spermidine; and others.

[0385] In one embodiment, the invention features a double stranded short interfering nucleic acid (siNA) molecule that comprises a first nucleotide sequence complementary to a target RNA sequence or a portion thereof, and a second sequence having complementarity to said first sequence, wherein said second sequence is chemically modified in a manner that it can no longer act as a guide sequence for efficiently mediating RNA interference and/or be recognized by cellular proteins that facilitate RNAi. In one embodiment, the first nucleotide sequence of the siNA is chemically modified as described herein. In one embodiment, the first nucleotide sequence of the siNA is not modified (*e.g.*, is all RNA).

[0386] In one embodiment, the invention features a double stranded short interfering nucleic acid (siNA) molecule that comprises a first nucleotide sequence complementary to a target RNA sequence or a portion thereof, and a second sequence having complementarity to said first sequence, wherein the second sequence is designed or modified in a manner that prevents its entry into the RNAi pathway as a guide sequence or as a sequence that is complementary to a target nucleic acid (*e.g.*, RNA) sequence. In one embodiment, the first nucleotide sequence of the siNA is chemically modified as described herein. In one embodiment, the first nucleotide sequence of the siNA is not modified (*e.g.*, is all RNA). Such design or modifications are expected to enhance the activity of siNA and/or improve the specificity of siNA molecules of the invention. These modifications are also expected to minimize any off-target effects and/or associated toxicity.

[0387] In one embodiment, the invention features a double stranded short interfering nucleic acid (siNA) molecule that comprises a first nucleotide sequence complementary to a target RNA sequence or a portion thereof, and a second sequence having complementarity to said first sequence, wherein said second sequence is incapable of acting as a guide sequence for mediating RNA interference. In one embodiment, the first nucleotide sequence of the siNA is chemically modified as described herein. In one embodiment, the first nucleotide sequence of the siNA is not modified (*e.g.*, is all RNA).

[0388] 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.

[0389] In one embodiment, the invention features a double stranded short interfering nucleic acid (siNA) molecule that comprises a first nucleotide sequence complementary to a target RNA sequence or a portion thereof, and a second sequence having complementarity to said first sequence, wherein said second sequence comprises a terminal cap moiety at the 5'-end of said second sequence. In one embodiment, the terminal cap moiety comprises an inverted abasic, inverted deoxy abasic, inverted nucleotide moiety, a group shown in **Figure 7**, 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.

[0390] In one embodiment, the invention features a double stranded short interfering nucleic acid (siNA) molecule that comprises a first nucleotide sequence complementary to a target RNA sequence or a portion thereof, and a second sequence having complementarity to said first sequence, wherein said second sequence comprises a terminal cap moiety at the 5'-end and 3'-end of said second sequence. In one embodiment, each terminal cap moiety individually comprises an inverted abasic, inverted deoxy abasic, inverted nucleotide moiety, a group shown in **Figure 7**, 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.

[0391] In one embodiment, the invention features a method for generating siNA molecules of the invention with improved specificity for down regulating or inhibiting the expression of a target nucleic acid (*e.g.*, a DNA or RNA such as a gene or its corresponding RNA), comprising (a) introducing one or more chemical modifications into the structure of a siNA molecule, and (b) assaying the siNA molecule of step (a) under conditions suitable for isolating siNA molecules having improved specificity. In another embodiment, the chemical modification used to improve specificity comprises terminal cap modifications at the 5'-end, 3'-end, or both 5' and 3'-ends of the siNA molecule. The terminal cap modifications can comprise, for example, structures shown in **Figure 7** (*e.g.* inverted deoxyabasic moieties) or any other chemical modification that renders a portion of the siNA molecule (*e.g.* the sense

strand) incapable of mediating RNA interference against an off target nucleic acid sequence. In a non-limiting example, a siNA molecule is designed such that only the antisense sequence of the siNA molecule can serve as a guide sequence for RISC mediated degradation of a corresponding target RNA sequence. This can be accomplished by rendering the sense sequence of the siNA inactive by introducing chemical modifications to the sense strand that preclude recognition of the sense strand as a guide sequence by RNAi machinery. In one embodiment, such chemical modifications comprise any chemical group at the 5'-end of the sense strand of the siNA, or any other group that serves to render the sense strand inactive as a guide sequence for mediating RNA interference. These modifications, for example, can result in a molecule where the 5'-end of the sense strand no longer has a free 5'-hydroxyl (5'-OH) or a free 5'-phosphate group (*e.g.*, phosphate, diphosphate, triphosphate, cyclic phosphate etc.). Non-limiting examples of such siNA constructs are described herein, such as "Stab 9/10", "Stab 7/8", "Stab 7/19", "Stab 17/22", "Stab 23/24", "Stab 24/25", and "Stab 24/26" (*e.g.*, any siNA having Stab 7, 9, 17, 23, or 24 sense strands) chemistries and variants thereof (see **Table 8**) wherein the 5'-end and 3'-end of the sense strand of the siNA do not comprise a hydroxyl group or phosphate group. Herein, numeric Stab chemistries include both 2'-fluoro and 2'-OCF3 versions of the chemistries shown in **Table 8**. For example, "Stab 7/8" refers to both Stab 7/8 and Stab 7F/8F etc.

[0392] 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 an ENaC target nucleic acid (*e.g.*, a DNA or RNA such as an ENaC gene or its corresponding coding and/or non-coding RNA), comprising introducing one or more chemical modifications into the structure of a siNA molecule that prevent a strand or portion of the siNA molecule from acting as a template or guide sequence for RNAi activity. In one embodiment, the inactive strand or sense region of the siNA molecule is the sense strand or sense region of the siNA molecule, i.e. the strand or region of the siNA that does not have complementarity to the target nucleic acid sequence. In one embodiment, such chemical modifications comprise any chemical group at the 5'-end of the sense strand or region of the siNA that does not comprise a 5'-hydroxyl (5'-OH) or 5'-phosphate group, or any other group that serves to render the sense strand or sense region inactive as a guide sequence for mediating RNA interference. Non-limiting examples of such siNA constructs are described herein, such as "Stab 9/10", "Stab 7/8", "Stab 7/19", "Stab 17/22", "Stab 23/24", "Stab 24/25", and "Stab 24/26" (*e.g.*, any siNA having Stab 7, 9, 17, 23, or 24 sense strands)

chemistries and variants thereof (see **Table 8**) wherein the 5'-end and 3'-end of the sense strand of the siNA do not comprise a hydroxyl group or phosphate group. Herein, numeric Stab chemistries include both 2'-fluoro and 2'-OCF3 versions of the chemistries shown in **Table 8**. For example, "Stab 7/8" refers to both Stab 7/8 and Stab 7F/8F etc.

[0393] 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 molecules that are active in mediating RNA interference against the target nucleic acid sequence, and (c) introducing chemical modifications (*e.g.* chemical modifications as described herein or as otherwise known in the art) into the active siNA molecules of (b). In one embodiment, the method further comprises re-screening the chemically modified siNA molecules of step (c) under conditions suitable for isolating chemically modified siNA molecules that are active in mediating RNA interference against the target nucleic acid sequence.

[0394] In one embodiment, the invention features a method for screening chemically modified siNA molecules that are active in mediating RNA interference against a target nucleic acid sequence comprising (a) generating a plurality of chemically modified siNA molecules (*e.g.* siNA molecules as described herein or as otherwise known in the art), and (b) screening the siNA molecules of step (a) under conditions suitable for isolating chemically modified siNA molecules that are active in mediating RNA interference against the target nucleic acid sequence.

[0395] 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 intracellular and/or intercellular receptor. Interaction of the ligand with the receptor can result in a biochemical reaction, or can simply be a physical interaction or association.

[0396] In another embodiment, the invention features a method for generating siNA molecules of the invention with improved bioavailability comprising (a) introducing an excipient formulation to a siNA molecule, and (b) assaying the siNA molecule of step (a) under conditions suitable for isolating siNA molecules having improved bioavailability.

Such excipients include polymers such as cyclodextrins, lipids, cationic lipids, polyamines, phospholipids, nanoparticles, receptors, ligands, and others.

[0397] In another embodiment, the invention features a method for generating siNA molecules of the invention with improved bioavailability comprising (a) introducing nucleotides having any of Formulae I-VII or any combination thereof into a siNA molecule, and (b) assaying the siNA molecule of step (a) under conditions suitable for isolating siNA molecules having improved bioavailability.

[0398] In another embodiment, polyethylene glycol (PEG) can be covalently attached to siNA compounds of the present invention. The attached PEG can be any molecular weight, preferably from about 100 to about 50,000 daltons (Da).

[0399] The present invention can be used alone or as a component of a kit having at least one of the reagents necessary to carry out the *in vitro* or *in vivo* introduction of RNA to test samples and/or subjects. For example, preferred components of the kit include a siNA molecule of the invention and a vehicle that promotes introduction of the siNA into cells of interest as described herein (*e.g.*, using lipids and other methods of transfection known in the art, see for example Beigelman *et al,* US 6,395,713). The kit can be used for target validation, such as in determining gene function and/or activity, or in drug optimization, and in drug discovery (see for example Usman *et al.*, USSN 60/402,996). Such a kit can also include instructions to allow a user of the kit to practice the invention.

[0400] The term "short interfering nucleic acid", "siNA", "short interfering RNA", "siRNA", "short interfering nucleic acid molecule", "short interfering oligonucleotide molecule", or "chemically-modified short interfering nucleic acid molecule" as used herein refers to any nucleic acid molecule capable of inhibiting or down regulating gene expression or viral replication by mediating RNA interference "RNAi" or gene silencing in a sequence-specific manner. These terms can refer to both individual nucleic acid molecules, a plurality of such nucleic acid molecules, or pools of such nucleic acid molecules. The siNA can be a double-stranded nucleic acid molecule comprising self-complementary sense and antisense regions, wherein the antisense region comprises nucleotide sequence that is complementary to nucleotide sequence in a target nucleic acid molecule or a portion thereof and the sense region having nucleotide sequence corresponding to the target nucleic acid sequence or a portion thereof. The siNA can be assembled from two separate oligonucleotides, where one

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

art, or are alternately non-covalently linked by ionic interactions, hydrogen bonding, van der waals interactions, hydrophobic interactions, and/or stacking interactions. In certain embodiments, the siNA molecules of the invention comprise nucleotide sequence that is complementary to nucleotide sequence of a target gene. In another embodiment, the siNA molecule of the invention interacts with nucleotide sequence of a target gene in a manner that causes inhibition of expression of the target gene. As used herein, siNA molecules need not be limited to those molecules containing only RNA, but further encompasses chemically-modified nucleotides and non-nucleotides. In certain embodiments, the short interfering nucleic acid molecules of the invention lack 2'-hydroxy (2'-OH) containing nucleotides. Applicant describes in certain embodiments short interfering nucleic acids that do not require the presence of nucleotides having a 2'-hydroxy group for mediating RNAi and as such, short interfering nucleic acid molecules of the invention optionally do not include any ribonucleotides (*e.g.*, nucleotides having a 2'-OH group). Such siNA molecules that do not require the presence of ribonucleotides within the siNA molecule to support RNAi can however have an attached linker or linkers or other attached or associated groups, moieties, or chains containing one or more nucleotides with 2'-OH groups. Optionally, siNA molecules can comprise ribonucleotides at about 5, 10, 20, 30, 40, or 50% of the nucleotide positions. The modified short interfering nucleic acid molecules of the invention can also be referred to as short interfering modified oligonucleotides "siMON." As used herein, the term siNA is meant to be equivalent to other terms used to describe nucleic acid molecules that are capable of mediating sequence specific RNAi, for example short interfering RNA (siRNA), double-stranded RNA (dsRNA), micro-RNA (miRNA), short hairpin RNA (shRNA), short interfering oligonucleotide, short interfering nucleic acid, short interfering modified oligonucleotide, chemically-modified siRNA, post-transcriptional gene silencing RNA (ptgsRNA), and others. Non limiting examples of siNA molecules of the invention are shown in **Figures 4-6,** and **Tables 1a and 1b** herein. Such siNA molecules are distinct from other nucleic acid technologies known in the art that mediate inhibition of gene expression, such as ribozymes, antisense, triplex forming, aptamer, 2,5-A chimera, or decoy oligonucleotides.

[0401] By "RNA interference" or "RNAi" is meant a biological process of inhibiting or down regulating gene expression in a cell as is generally known in the art and which is mediated by short interfering nucleic acid molecules, see for example Zamore and Haley, 2005, *Science*, 309, 1519-1524; Vaughn and Martienssen, 2005, *Science*, 309, 1525-1526; Zamore *et al.*, 2000, *Cell*, 101, 25-33; Bass, 2001, *Nature*, 411, 428-429; Elbashir *et al.*,

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

[0402] In one embodiment, a siNA molecule of the invention is a duplex forming oligonucleotide "DFO", (see for example **Figures 11-12** and Vaish *et al.*, USSN 10/727,780 filed December 3, 2003 and International PCT Application No. US04/16390, filed May 24, 2004).

[0403] In one embodiment, a siNA molecule of the invention is a multifunctional siNA, (see for example **Figures 13-25** and Jadhav *et al.,* USSN 60/543,480 filed February 10, 2004

and International PCT Application No. US04/16390, filed May 24, 2004). In one embodiment, the multifunctional siNA of the invention can comprise sequence targeting, for example, two or more regions of ENaC RNA (see for example target sequences in **Tables 1a and 1b**). In one embodiment, the multifunctional siNA of the invention can comprise sequence targeting any of ENaC targets selected from the group consisting of ENaC target sequences in **Tables 1a and 1b** or any of its isotypes or any combination thereof.

[0404] By "asymmetric hairpin" as used herein is meant a linear siNA molecule comprising an antisense region, a loop portion that can comprise nucleotides or non-nucleotides, and a sense region that comprises fewer nucleotides than the antisense region to the extent that the sense region has enough complementary nucleotides to base pair with the antisense region and form a duplex with loop. For example, an asymmetric hairpin siNA molecule of the invention can comprise an antisense region having length sufficient to mediate RNAi in a cell or in vitro system (*e.g.* about 15 to about 30, or about 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, or 30 nucleotides) and a loop region comprising about 4 to about 12 (*e.g.*, about 4, 5, 6, 7, 8, 9, 10, 11, or 12) nucleotides, and a sense region having about 3 to about 25 (*e.g.*, about 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, or 25) nucleotides that are complementary to the antisense region. The asymmetric hairpin siNA molecule can also comprise a 5'-terminal phosphate group that can be chemically modified. The loop portion of the asymmetric hairpin siNA molecule can comprise nucleotides, non-nucleotides, linker molecules, or conjugate molecules as described herein.

[0405] By "asymmetric duplex" as used herein is meant a siNA molecule having two separate strands comprising a sense region and an antisense region, wherein the sense region comprises fewer nucleotides than the antisense region to the extent that the sense region has enough complementary nucleotides to base pair with the antisense region and form a duplex. For example, an asymmetric duplex siNA molecule of the invention can comprise an antisense region having length sufficient to mediate RNAi in a cell or in vitro system (*e.g.*, about 15 to about 30, or about 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, or 30 nucleotides) and a sense region having about 3 to about 25 (*e.g.*, about 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, or 25) nucleotides that are complementary to the antisense region.

[0406] By "RNAi inhibitor" is meant any molecule that can down regulate, reduce or inhibit RNA interference function or activity in a cell or organism. An RNAi inhibitor can down regulate, reduce or inhibit RNAi (e.g., RNAi mediated cleavage of a target polynucleotide, translational inhibition, or transcriptional silencing) by interaction with or interfering the function of any component of the RNAi pathway, including protein components such as RISC, or nucleic acid components such as miRNAs or siRNAs. A RNAi inhibitor can be a siNA molecule, an antisense molecule, an aptamer, or a small molecule that interacts with or interferes with the function of RISC, a miRNA, or a siRNA or any other component of the RNAi pathway in a cell or organism. By inhibiting RNAi (e.g., RNAi mediated cleavage of a target polynucleotide, translational inhibition, or transcriptional silencing), a RNAi inhibitor of the invention can be used to modulate (e.g, up-regulate or down regulate) the expression of a target gene. In one embodiment, a RNA inhibitor of the invention is used to up-regulate gene expression by interfering with (e.g., reducing or preventing) endogenous down-regulation or inhibition of gene expression through translational inhibition, transcriptional silencing, or RISC mediated cleavage of a polynucleotide (e.g., mRNA). By interfering with mechanisms of endogenous repression, silencing, or inhibition of gene expression, RNAi inhibitors of the invention can therefore be used to up-regulate gene expression for the treatment of diseases, traits, or conditions resulting from a loss of function. In one embodiment, the term "RNAi inhibitor" is used in place of the term "siNA" in the various embodiments herein, for example, with the effect of increasing gene expression for the treatment of loss of function diseases, traits, and/or conditions.

[0407] By "aptamer" or "nucleic acid aptamer" as used herein is meant a polynucleotide that binds specifically to a target molecule wherein the nucleic acid molecule has sequence that is distinct from sequence recognized by the target molecule in its natural setting. Alternately, an aptamer can be a nucleic acid molecule that binds to a target molecule where the target molecule does not naturally bind to a nucleic acid. The target molecule can be any molecule of interest. For example, the aptamer can be used to bind to a ligand-binding domain of a protein, thereby preventing interaction of the naturally occurring ligand with the protein. This is a non-limiting example and those in the art will recognize that other embodiments can be readily generated using techniques generally known in the art, see for example Gold et al., 1995, Annu. Rev. Biochem., 64, 763; Brody and Gold, 2000, J. Biotechnol., 74, 5; Sun, 2000, Curr. Opin. Mol. Ther., 2, 100; Kusser, 2000, J. Biotechnol.,

74, 27; Hermann and Patel, 2000, Science, 287, 820; and Jayasena, 1999, Clinical Chemistry, 45, 1628. Aptamer molecules of the invention can be chemically modified as is generally known in the art or as described herein.

[0408] The term "antisense nucleic acid", as used herein, refers to a nucleic acid molecule that binds to target RNA by means of RNA-RNA or RNA-DNA or RNA-PNA (protein nucleic acid; Egholm et al., 1993 Nature 365, 566) interactions and alters the activity of the target RNA (for a review, see Stein and Cheng, 1993 Science 261, 1004 and Woolf et al., US patent No. 5,849,902) by steric interaction or by RNase H mediated target recognition. Typically, antisense molecules are complementary to a target sequence along a single contiguous sequence of the antisense molecule. However, in certain embodiments, an antisense molecule can bind to substrate such that the substrate molecule forms a loop, and/or an antisense molecule can bind such that the antisense molecule forms a loop. Thus, the antisense molecule can be complementary to two (or even more) non-contiguous substrate sequences or two (or even more) non-contiguous sequence portions of an antisense molecule can be complementary to a target sequence or both. For a review of current antisense strategies, see Schmajuk et al., 1999, J. Biol. Chem., 274, 21783-21789, Delihas et al., 1997, Nature, 15, 751-753, Stein et al., 1997, Antisense N. A. Drug Dev., 7, 151, Crooke, 2000, Methods Enzymol., 313, 3-45; Crooke, 1998, Biotech. Genet. Eng. Rev., 15, 121-157, Crooke, 1997, Ad. Pharmacol., 40, 1-49. In addition, antisense DNA or antisense modified with 2'-MOE and other modifictions as are known in the art can be used to target RNA by means of DNA-RNA interactions, thereby activating RNase H, which digests the target RNA in the duplex. The antisense oligonucleotides can comprise one or more RNAse H activating region, which is capable of activating RNAse H cleavage of a target RNA. Antisense DNA can be synthesized chemically or expressed via the use of a single stranded DNA expression vector or equivalent thereof. Antisense molecules of the invention can be chemically modified as is generally known in the art or as described herein.

[0409] By "modulate" is meant that the expression of the gene, or level of a RNA molecule or equivalent RNA molecules encoding one or more proteins or protein subunits, or activity of one or more proteins or protein subunits is up regulated or down regulated, such that expression, level, or activity is greater than or less than that observed in the absence of the modulator. For example, the term "modulate" can mean "inhibit," but the use of the word "modulate" is not limited to this definition.

[0410] By “inhibit”, "down-regulate", or “reduce”, it is meant that the expression of the gene, or level of RNA molecules or equivalent RNA molecules encoding one or more proteins or protein subunits, or activity of one or more proteins or protein subunits, is reduced below that observed in the absence of the nucleic acid molecules (*e.g.*, siNA) of the invention. In one embodiment, inhibition, down-regulation or reduction with an siNA molecule is below that level observed in the presence of an inactive or attenuated molecule. In another embodiment, inhibition, down-regulation, or reduction with siNA molecules is below that level observed in the presence of, for example, an siNA molecule with scrambled sequence or with mismatches. In another embodiment, inhibition, down-regulation, or reduction of gene expression with a nucleic acid molecule of the instant invention is greater in the presence of the nucleic acid molecule than in its absence. In one embodiment, inhibition, down regulation, or reduction of gene expression is associated with post transcriptional silencing, such as RNAi mediated cleavage of a target nucleic acid molecule (*e.g.* RNA) or inhibition of translation. In one embodiment, inhibition, down regulation, or reduction of gene expression is associated with pretranscriptional silencing, such as by alterations in DNA methylation patterns and DNA chromatin structure.

[0411] By "up-regulate", or “promote”, it is meant that the expression of the gene, or level of RNA molecules or equivalent RNA molecules encoding one or more proteins or protein subunits, or activity of one or more proteins or protein subunits, is increased above that observed in the absence of the nucleic acid molecules (*e.g.*, siNA) of the invention. In one embodiment, up-regulation or promotion of gene expression with an siNA molecule is above that level observed in the presence of an inactive or attenuated molecule. In another embodiment, up-regulation or promotion of gene expression with siNA molecules is above that level observed in the presence of, for example, an siNA molecule with scrambled sequence or with mismatches. In another embodiment, up-regulation or promotion of gene expression with a nucleic acid molecule of the instant invention is greater in the presence of the nucleic acid molecule than in its absence. In one embodiment, up-regulation or promotion of gene expression is associated with inhibition of RNA mediated gene silencing, such as RNAi mediated cleavage or silencing of a coding or non-coding RNA target that down regulates, inhibits, or silences the expression of the gene of interest to be up-regulated. The down regulation of gene expression can, for example, be induced by a coding RNA or its encoded protein, such as through negative feedback or antagonistic effects. The down regulation of gene expression can, for example, be induced by a non-coding RNA having

regulatory control over a gene of interest, for example by silencing expression of the gene via translational inhibition, chromatin structure, methylation, RISC mediated RNA cleavage, or translational inhibition. As such, inhibition or down regulation of targets that down regulate, suppress, or silence a gene of interest can be used to up-regulate or promote expression of the gene of interest toward therapeutic use.

[0412] In one embodiment, a RNAi inhibitor of the invention is used to up regulate gene expression by inhibiting RNAi or gene silencing. For example, a RNAi inhibitor of the invention can be used to treat loss of function diseases and conditions by up-regulating gene expression, such as in instances of haploinsufficiency where one allele of a particular gene harbors a mutation (e.g., a frameshift, missense, or nonsense mutation) resulting in a loss of function of the protein encoded by the mutant allele. In such instances, the RNAi inhibitor can be used to up regulate expression of the protein encoded by the wild type or functional allele, thus correcting the haploinsufficiency by compensating for the mutant or null allele. In another embodiment, a siNA molecule of the invention is used to down regulate expression of a toxic gain of function allele while a RNAi inhibitor of the invention is used concomitantly to up regulate expression of the wild type or functional allele, such as in the treatment of diseases, traits, or conditions herein or otherwise known in the art (see for example Rhodes *et al.,* 2004, PNAS USA, 101:11147-11152 and Meisler *et al.* 2005, The Journal of Clinical Investigation, 115:2010-2017).

[0413] By "gene", or "target gene" or “target DNA”, is meant a nucleic acid that encodes an RNA, for example, nucleic acid sequences including, but not limited to, structural genes encoding a polypeptide. A gene or target gene can also encode a functional RNA (fRNA) or non-coding RNA (ncRNA), such as small temporal RNA (stRNA), micro RNA (miRNA), small nuclear RNA (snRNA), short interfering RNA (siRNA), small nucleolar RNA (snRNA), ribosomal RNA (rRNA), transfer RNA (tRNA) and precursor RNAs thereof. Such non-coding RNAs can serve as target nucleic acid molecules for siNA mediated RNA interference in modulating the activity of fRNA or ncRNA involved in functional or regulatory cellular processes. Abberant fRNA or ncRNA activity leading to disease can therefore be modulated by siNA molecules of the invention. siNA molecules targeting fRNA and ncRNA can also be used to manipulate or alter the genotype or phenotype of a subject, organism or cell, by intervening in cellular processes such as genetic imprinting, transcription, translation, or nucleic acid processing (*e.g.*, transamination, methylation etc.).

The target gene can be a gene derived from a cell, an endogenous gene, a transgene, or exogenous genes such as genes of a pathogen, for example a virus, which is present in the cell after infection thereof. The cell containing the target gene can be derived from or contained in any organism, for example a plant, animal, protozoan, virus, bacterium, or fungus. Non-limiting examples of plants include monocots, dicots, or gymnosperms. Non-limiting examples of animals include vertebrates or invertebrates. Non-limiting examples of fungi include molds or yeasts. For a review, see for example Snyder and Gerstein, 2003, *Science,* 300, 258-260.

[0414] By "non-canonical base pair" is meant any non-Watson Crick base pair, such as mismatches and/or wobble base pairs, including flipped mismatches, single hydrogen bond mismatches, trans-type mismatches, triple base interactions, and quadruple base interactions. Non-limiting examples of such non-canonical base pairs include, but are not limited to, AC reverse Hoogsteen, AC wobble, AU reverse Hoogsteen, GU wobble, AA N7 amino, CC 2-carbonyl-amino(H1)-N3-amino(H2), GA sheared, UC 4-carbonyl-amino, UU imino-carbonyl, AC reverse wobble, AU Hoogsteen, AU reverse Watson Crick, CG reverse Watson Crick, GC N3-amino-amino N3, AA N1-amino symmetric, AA N7-amino symmetric, GA N7-N1 amino-carbonyl, GA+ carbonyl-amino N7-N1, GG N1-carbonyl symmetric, GG N3-amino symmetric, CC carbonyl-amino symmetric, CC N3-amino symmetric, UU 2-carbonyl-imino symmetric, UU 4-carbonyl-imino symmetric, AA amino-N3, AA N1-amino, AC amino 2-carbonyl, AC N3-amino, AC N7-amino, AU amino-4-carbonyl, AU N1-imino, AU N3-imino, AU N7-imino, CC carbonyl-amino, GA amino-N1, GA amino-N7, GA carbonyl-amino, GA N3-amino, GC amino-N3, GC carbonyl-amino, GC N3-amino, GC N7-amino, GG amino-N7, GG carbonyl-imino, GG N7-amino, GU amino-2-carbonyl, GU carbonyl-imino, GU imino-2-carbonyl, GU N7-imino, psiU imino-2-carbonyl, UC 4-carbonyl-amino, UC imino-carbonyl, UU imino-4-carbonyl, AC C2-H-N3, GA carbonyl-C2-H, UU imino-4-carbonyl 2 carbonyl-C5-H, AC amino(A) N3(C)-carbonyl, GC imino amino-carbonyl, Gpsi imino-2-carbonyl amino-2- carbonyl, and GU imino amino-2-carbonyl base pairs.

[0415] By "ENaC" as used herein is meant, any epithelial sodium channel or ENaC protein, peptide, or polypeptide such as genes encoding the α (SCNN1A), β (SCNN1B), or γ (SCNN1G) subunit sequences comprising those sequences referred to by GenBank Accession Nos. shown in **Table 7**. References herein to "ENaC" include any or all of the α (SCNN1A), β (SCNN1B), or γ (SCNN1G) subunit sequences. In a preferred embodiment the

invention features one or more siNA molecules and/or RNAi inhibitors and methods that independently or in combination modulate the expression of ENaC gene(s) encoding the α (SCNN1A) subunit. The term ENaC also refers to nucleic acids encoding any ENaC protein, peptide, or polypeptide for example nucleic acids encoding the α (SCNN1A), β (SCNN1B), or γ (SCNN1G) subunit sequences comprising those sequences referred to by GenBank Accession Nos. shown in **Table 7**. The term "ENaC" is also meant to include other ENaC encoding sequences, such as ENaC sequences derived from various subjects or organisms, including other ENaC isoforms, mutant ENaC genes, isotypes of ENaC genes, ENaC gene polymorphisms and ENaC splice variants.

[0416] By "target" as used herein is meant, any ENaC target protein, peptide, or polypeptide, such as encoded by Genbank Accession Nos. shown in **Table 7**. The term "target" also refers to nucleic acid sequences or target polynucleotide sequence encoding any target protein, peptide, or polypeptide, such as proteins, peptides, or polypeptides encoded by sequences having Genbank Accession Nos. shown in **Table 7**. The target of interest can include target polynucleotide sequences, such as target DNA or target RNA. The term "target" is also meant to include other sequences, such as differing isoforms, mutant target genes, isotypes of target polynucleotides, target polymorphisms, and non-coding (*e.g.*, ncRNA, miRNA, stRNA, sRNA) or other regulatory polynucleotide sequences as described herein. Therefore, in various embodiments of the invention, a double stranded nucleic acid molecule of the invention (*e.g.*, siNA) having complementarity to a target RNA can be used to inhibit or down regulate miRNA or other ncRNA activity. In one embodiment, inhibition of miRNA or ncRNA activity can be used to down regulate or inhibit gene expression (*e.g.*, gene targets described herein or otherwise known in the art) that is dependent on miRNA or ncRNA activity. In another embodiment, inhibition of miRNA or ncRNA activity by double stranded nucleic acid molecules of the invention (*e.g.* siNA) having complementarity to the miRNA or ncRNA can be used to up regulate or promote target gene expression (*e.g.*, gene targets described herein or otherwise known in the art) where the expression of such genes is down regulated, suppressed, or silenced by the miRNA or ncRNA. Such up-regulation of gene expression can be used to treat diseases and conditions associated with a loss of function or haploinsufficiency as are generally known in the art.

[0417] By "pathway target" is meant any target involved in pathways of gene expression or activity. For example, any given target can have related pathway targets that can include

upstream, downstream, or modifier genes in a biologic pathway. These pathway target genes can provide additive or synergistic effects in the treatment of diseases, conditions, and traits herein.

[0418] In one embodiment, the target is any of target RNA or a portion thereof.

[0419] In one embodiment, the target is any ENaC RNA or a portion thereof.

[0420] In one embodiment, the target is any ENaC DNA or a portion thereof.

[0421] In one embodiment, the target is any ENaC mRNA or a portion thereof.

[0422] In one embodiment, the target is any ENaC miRNA or a portion thereof.

[0423] In one embodiment, the target is any ENaC siRNA or a portion thereof.

[0424] In one embodiment, the target is an ENaC target or a portion thereof.

[0425] In one embodiment, the target is any ENaC (e.g., one or more) of target sequences described herein and/or shown in **Table 7.** In one embodiment, the target is any (e.g., one or more) of target sequences shown in **Table 1a** or **1b** or a portion thereof. In another embodiment, the target is a siRNA, miRNA, or stRNA corresponding to any (e.g., one or more) target, sequence shown in **Table 1a** or **1b** or its complement or an ENaC target or a portion thereof.

[0426] In one embodiment, the target is any ENaC (e.g., one or more) of target sequences shown in **Table 7**. In one embodiment, the target is any (e.g., one or more) of target sequences shown in **Table 1a** or **1b** (e.g., SEQ ID NOs: 1, 2, 3, and/or 4) or a portion thereof. In another embodiment, the target is a siRNA, miRNA, or stRNA corresponding to any (e.g., one or more) targets shown in **Table 1a** or **1b** (e.g., SEQ ID NOs: 1, 2, 3, and/or 4) or its complement or a portion thereof. In another embodiment, the target is any siRNA, miRNA, or stRNA corresponding any (e.g., one or more) sequence corresponding to a sequence herein or shown in **Table 7.**

[0427] By "homologous sequence" is meant, a nucleotide sequence that is shared by one or more polynucleotide sequences, such as genes, gene transcripts and/or non-coding polynucleotides. For example, a homologous sequence can be a nucleotide sequence that is shared by two or more genes encoding related but different proteins, such as different

members of a gene family, different protein epitopes, different protein isoforms or completely divergent genes, such as a cytokine and its corresponding receptors. A homologous sequence can be a nucleotide sequence that is shared by two or more non-coding polynucleotides, such as noncoding DNA or RNA, regulatory sequences, introns, and sites of transcriptional control or regulation. Homologous sequences can also include conserved sequence regions shared by more than one polynucleotide sequence. Homology does not need to be perfect homology (*e.g.*, 100%), as partially homologous sequences are also contemplated by the instant invention (*e.g.*, 99%, 98%, 97%, 96%, 95%, 94%, 93%, 92%, 91%, 90%, 89%, 88%, 87%, 86%, 85%, 84%, 83%, 82%, 81%, 80% etc.).

[0428] By "conserved sequence region" is meant, a nucleotide sequence of one or more regions in a polynucleotide does not vary significantly between generations or from one biological system, subject, or organism to another biological system, subject, or organism. The polynucleotide can include both coding and non-coding DNA and RNA.

[0429] By "sense region" is meant a nucleotide sequence of a siNA molecule having complementarity to an antisense region of the siNA molecule. In addition, the sense region of a siNA molecule can comprise a nucleic acid sequence having homology with a target nucleic acid sequence. In one embodiment, the sense region of the siNA molecule is referred to as the sense strand or passenger strand.

[0430] By "antisense region" is meant a nucleotide sequence of a siNA molecule having complementarity to a target nucleic acid sequence. In addition, the antisense region of a siNA molecule can optionally comprise a nucleic acid sequence having complementarity to a sense region of the siNA molecule. In one embodiment, the antisense region of the siNA molecule is referred to as the antisense strand or guide strand.

[0431] By "target nucleic acid" or "target polynucleotide" is meant any nucleic acid sequence (e.g, any ENaC sequence) whose expression or activity is to be modulated. The target nucleic acid can be DNA or RNA. In one embodiment, a target nucleic acid of the invention is target RNA or DNA.

[0432] By "complementarity" is meant that a nucleic acid can form hydrogen bond(s) with another nucleic acid sequence by either traditional Watson-Crick or other non-traditional types as described herein. In one embodiment, a double stranded nucleic acid molecule of the invention, such as an siNA molecule, wherein each strand is between 15 and 30

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

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

[0433] In one embodiment, a double stranded nucleic acid molecule of the invention, such as siNA molecule, has perfect complementarity between the sense strand or sense region and the antisense strand or antisense region of the nucleic acid molecule. In one embodiment, double stranded nucleic acid molecule of the invention, such as siNA molecule, is perfectly complementary to a corresponding target nucleic acid molecule.

[0434] In one embodiment, double stranded nucleic acid molecule of the invention, such as siNA molecule, has partial complementarity (i.e., less than 100% complementarity) between the sense strand or sense region and the antisense strand or antisense region of the double stranded nucleic acid molecule or between the antisense strand or antisense region of the nucleic acid molecule and a corresponding target nucleic acid molecule. For example, partial complementarity can include various mismatches or non-base paired nucleotides (*e.g.*, 1, 2, 3, 4, 5 or more mismatches or non-based paired nucleotides, such as nucleotide bulges) within the double stranded nucleic acid molecule, structure which can result in bulges, loops, or overhangs that result between the sense strand or sense region and the antisense strand or antisense region of the double stranded nucleic acid molecule or between the antisense strand or antisense region of the double stranded nucleic acid molecule and a corresponding target nucleic acid molecule. In certain embodiments, partial complementarity can relate to non-base paired nucleotides (*e.g.*, 1, 2, 3, 4, 5, or 6 or more non-base paired nucleotides) located at either the 3'- or 5'-ends of the double stranded nucleic acid molecule. In such

embodiments, the remainder of the double stranded nucleic acid molecule can be perfectly complementary between the strands and/or the target sequence.

[0435] In one embodiment, double stranded nucleic acid molecule of the invention is a microRNA (miRNA). By "microRNA" or "miRNA" is meant, a small double stranded RNA that regulates the expression of target messenger RNAs either by mRNA cleavage, translational repression/inhibition or heterochromatic silencing (see for example Ambros, 2004, Nature, 431, 350-355; Bartel, 2004, Cell, 116, 281-297; Cullen, 2004, Virus Research., 102, 3-9; He *et al.,* 2004, Nat. Rev. Genet., 5, 522-531; Ying *et al.,* 2004, Gene, 342, 25-28; and Sethupathy *et al.,* 2006, RNA, 12:192-197). In one embodiment, the microRNA of the invention, has partial complementarity (i.e., less than 100% complementarity) between the sense strand or sense region and the antisense strand or antisense region of the miRNA molecule or between the antisense strand or antisense region of the miRNA and a corresponding target nucleic acid molecule. For example, partial complementarity can include various mismatches or non-base paired nucleotides (*e.g.*, 1, 2, 3, 4, 5 or more mismatches or non-based paired nucleotides, such as nucleotide bulges) within the double stranded nucleic acid molecule, structure which can result in bulges, loops, or overhangs that result between the sense strand or sense region and the antisense strand or antisense region of the miRNA or between the antisense strand or antisense region of the miRNA and a corresponding target nucleic acid molecule.

[0436] In one embodiment, siNA molecules of the invention that down regulate or reduce target gene expression are used for treating, or preventing respiratory diseases, disorders, traits, or conditions in a subject or organism as described herein or otherwise known in the art.

[0437] In one embodiment of the present invention, each sequence of a siNA molecule of the invention is independently about 15 to about 30 nucleotides in length, in specific embodiments about 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, or 30 nucleotides in length. In another embodiment, the siNA duplexes of the invention independently comprise about 15 to about 30 base pairs (*e.g.*, about 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, or 30). In another embodiment, one or more strands of the siNA molecule of the invention independently comprises about 15 to about 30 nucleotides (*e.g.*, about 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, or 30) that are complementary to a target nucleic acid molecule. In yet another embodiment, siNA molecules of the invention

comprising hairpin or circular structures are about 35 to about 55 (*e.g.*, about 35, 40, 45, 50 or 55) nucleotides in length, or about 38 to about 44 (*e.g.*, about 38, 39, 40, 41, 42, 43, or 44) nucleotides in length and comprising about 15 to about 25 (*e.g.*, about 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, or 25) base pairs. Exemplary siNA molecules of the invention are shown in **Tables II and III** and/or **Figures 4-5**.

[0438] 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.

[0439] The siNA molecules of the invention are added directly, or can be complexed with cationic lipids, packaged within liposomes, or otherwise delivered to target cells or tissues. The nucleic acid or nucleic acid complexes can be locally administered to relevant tissues *ex vivo*, or *in vivo* through local delivery to the lung, with or without their incorporation in biopolymers. In particular embodiments, the nucleic acid molecules of the invention comprise sequences shown in **Tables 1a and 1b** and/or **Figures 4-5**. Examples of such nucleic acid molecules consist essentially of sequences defined in these tables and figures. Furthermore, the chemically modified constructs described in **Table 8** and the lipid nanoparticle (LNP) formulations shown in **Table 10** can be applied to any siNA sequence or group of siNA sequences of the invention.

[0440] 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 within a target polynucleotide of the invention.

[0441] By "RNA" is meant a molecule comprising at least one ribonucleotide residue. By "ribonucleotide" is meant a nucleotide with a hydroxyl group at the 2' position of a β-D-ribofuranose moiety. The terms include double-stranded RNA, single-stranded RNA, isolated RNA such as partially purified RNA, essentially pure RNA, synthetic RNA, recombinantly produced RNA, as well as altered RNA that differs from naturally occurring RNA by the addition, deletion, substitution and/or alteration of one or more nucleotides.

Such alterations can include addition of non-nucleotide material, such as to the end(s) of the siNA or internally, for example at one or more nucleotides of the RNA. Nucleotides in the RNA molecules of the instant invention can also comprise non-standard nucleotides, such as non-naturally occurring nucleotides or chemically synthesized nucleotides or deoxynucleotides. These altered RNAs can be referred to as analogs or analogs of naturally-occurring RNA.

[0442] By "subject" is meant an organism, which is a donor or recipient of explanted cells or the cells themselves. "Subject" also refers to an organism to which the nucleic acid molecules of the invention can be administered. A subject can be a mammal or mammalian cells, including a human or human cells. In one embodiment, the subject is an infant (e.g., subjects that are less than 1 month old, or 1, 2, 3, 4, 5, 6, 7, 8, 9 10, 11, or 12 months old). In one embodiment, the subject is a toddler (e.g., 1, 2, 3, 4, 5 or 6 years old). In one embodiment, the subject is a senior (e.g., anyone over the age of about 65 years of age).

[0443] By "chemical modification" as used herein is meant any modification of chemical structure of the nucleotides that differs from nucleotides of native siRNA or RNA. The term "chemical modification" encompasses the addition, substitution, or modification of native siRNA or RNA nucleosides and nucleotides with modified nucleosides and modified nucleotides as described herein or as is otherwise known in the art. Non-limiting examples of such chemical modifications include without limitation compositions having any of Formulae I, II, III, IV, V, VI, or VII herein, phosphorothioate internucleotide linkages, 2'-deoxyribonucleotides, 2'-O-methyl ribonucleotides, 2'-deoxy-2'-fluoro ribonucleotides, 4'-thio ribonucleotides, 2'-O-trifluoromethyl nucleotides, 2'-O-ethyl-trifluoromethoxy nucleotides, 2'-O-difluoromethoxy-ethoxy nucleotides (see for example USSN 10/981,966 filed November 5, 2004, incorporated by reference herein), FANA, "universal base" nucleotides, "acyclic" nucleotides, 5-C-methyl nucleotides, terminal glyceryl and/or inverted deoxy abasic residue incorporation, or a modification having any of Formulae I-VII herein. In one embodiment, the nucleic acid molecules of the invention (e.g, dsRNA, siNA etc.) are partially modified (e.g., about 5%, 10,%, 15%, 20%, 25%, 30%, 35%, 40%, 45%, 50%, 55%, 60%, 65%, 70%, 75%, 80%, 85%, 90%, or 95% modified) with chemical modifications. In another embodiment, the the nucleic acid molecules of the invention (e.g, dsRNA, siNA etc.) are completely modified (e.g., about 100% modified) with chemical modifications.

[0444] The term "phosphorothioate" as used herein refers to an internucleotide linkage having Formula I, wherein Z and/or W comprise a sulfur atom. Hence, the term phosphorothioate refers to both phosphorothioate and phosphorodithioate internucleotide linkages.

[0445] The term "phosphonoacetate" as used herein refers to an internucleotide linkage having Formula I, wherein Z and/or W comprise an acetyl or protected acetyl group.

[0446] The term "thiophosphonoacetate" as used herein refers to an internucleotide linkage having Formula I, wherein Z comprises an acetyl or protected acetyl group and W comprises a sulfur atom or alternately W comprises an acetyl or protected acetyl group and Z comprises a sulfur atom.

[0447] The term "universal base" as used herein refers to nucleotide base analogs that form base pairs with each of the natural DNA/RNA bases with little discrimination between them. Non-limiting examples of universal bases include C-phenyl, C-naphthyl and other aromatic derivatives, inosine, azole carboxamides, and nitroazole derivatives such as 3-nitropyrrole, 4-nitroindole, 5-nitroindole, and 6-nitroindole as known in the art (see for example Loakes, 2001, *Nucleic Acids Research,* 29, 2437-2447).

[0448] 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.

[0449] The nucleic acid molecules of the instant invention, individually, or in combination or in conjunction with other drugs, can be used to for preventing or treating diseases, disorders, conditions, and traits described herein or otherwise known in the art, in a subject or organism.

[0450] In one embodiment, the siNA molecules of the invention can be administered to a subject or can be administered to other appropriate cells evident to those skilled in the art, individually or in combination with one or more drugs under conditions suitable for the treatment.

[0451] In a further embodiment, the siNA molecules can be used in combination with other known treatments to prevent or treat respiratory diseases, disorders, or conditions in a

subject or organism. For example, the described molecules could be used in combination with one or more known compounds, treatments, or procedures to prevent or treat diseases, disorders, conditions, and traits described herein in a subject or organism as are known in the art, such as PDE inhibitors including 8-methoxymethyl-IBMX (PDE4B 1 inhibitor), rolipram (PDE4B inhibitor), and denbufylline (PDE4B inhibitor).

[0452] In one embodiment, the invention features an expression vector comprising a nucleic acid sequence encoding at least one siNA molecule of the invention, in a manner which allows expression of the siNA molecule. For example, the vector can contain sequence(s) encoding both strands of a siNA molecule comprising a duplex. The vector can also contain sequence(s) encoding a single nucleic acid molecule that is self-complementary and thus forms a siNA molecule. Non-limiting examples of such expression vectors are described in Paul *et al.,* 2002, *Nature Biotechnology,* 19, 505; Miyagishi and Taira, 2002, *Nature Biotechnology,* 19, 497; Lee *et al.,* 2002, *Nature Biotechnology,* 19, 500; and Novina *et al.,* 2002, *Nature Medicine*, advance online publication doi:10.1038/nm725.

[0453] In another embodiment, the invention features a mammalian cell, for example, a human cell, including an expression vector of the invention.

[0454] 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 7** herein.

[0455] 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.

[0456] In another aspect of the invention, siNA molecules that interact with target RNA molecules and down-regulate gene encoding target RNA molecules (for example target RNA molecules referred to by Genbank Accession numbers herein) are expressed from transcription units inserted into DNA or RNA vectors. The recombinant vectors can be DNA plasmids or viral vectors. siNA expressing viral vectors can be constructed based on, but not limited to, adeno-associated virus, retrovirus, adenovirus, or alphavirus. The recombinant vectors capable of expressing the siNA molecules can be delivered as described herein, and persist in target cells. Alternatively, viral vectors can be used that provide for transient expression of siNA molecules. Such vectors can be repeatedly administered as necessary.

Once expressed, the siNA molecules bind and down-regulate gene function or expression via RNA interference (RNAi). Delivery of siNA expressing vectors can be systemic, such as by intravenous or intramuscular administration, by administration to target cells ex-planted from a subject followed by reintroduction into the subject, or by any other means that would allow for introduction into the desired target cell.

[0457] By "vectors" is meant any nucleic acid- and/or viral-based technique used to deliver a desired nucleic acid.

[0458] 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

[0459] **Figure 1** shows a non-limiting example of a scheme for the synthesis of siNA molecules. The complementary siNA sequence strands, strand 1 and strand 2, are synthesized in tandem and are connected by a cleavable linkage, such as a nucleotide succinate or abasic succinate, which can be the same or different from the cleavable linker used for solid phase synthesis on a solid support. The synthesis can be either solid phase or solution phase, in the example shown, the synthesis is a solid phase synthesis. The synthesis is performed such that a protecting group, such as a dimethoxytrityl group, remains intact on the terminal nucleotide of the tandem oligonucleotide. Upon cleavage and deprotection of the oligonucleotide, the two siNA strands spontaneously hybridize to form a siNA duplex, which allows the purification of the duplex by utilizing the properties of the terminal protecting group, for example by applying a trityl on purification method wherein only duplexes/oligonucleotides with the terminal protecting group are isolated.

[0460] **Figure 2** shows a MALDI-TOF mass spectrum of a purified siNA duplex synthesized by a method of the invention. The two peaks shown correspond to the predicted mass of the separate siNA sequence strands. This result demonstrates that the siNA duplex generated from tandem synthesis can be purified as a single entity using a simple trityl-on purification methodology.

[0461] **Figure 3** shows a non-limiting proposed mechanistic representation of target RNA degradation involved in RNAi. Double-stranded RNA (dsRNA), which is generated by RNA-dependent RNA polymerase (RdRP) from foreign single-stranded RNA, for example

viral, transposon, or other exogenous RNA, activates the DICER enzyme that in turn generates siNA duplexes. Alternately, synthetic or expressed siNA can be introduced directly into a cell by appropriate means. An active siNA complex forms which recognizes a target RNA, resulting in degradation of the target RNA by the RISC endonuclease complex or in the synthesis of additional RNA by RNA-dependent RNA polymerase (RdRP), which can activate DICER and result in additional siNA molecules, thereby amplifying the RNAi response.

[0462] **Figure 4A-F** shows non-limiting examples of chemically-modified siNA constructs of the present invention. In the figure, N stands for any nucleotide (adenosine, guanosine, cytosine, uridine, or optionally thymidine, for example thymidine can be substituted in the overhanging regions designated by parenthesis (N N). Various modifications are shown for the sense and antisense strands of the siNA constructs. The (N N) nucleotide positions can be chemically modified as described herein (*e.g.*, 2'-O-methyl, 2'-deoxy-2'-fluoro etc.) and can be either derived from a corresponding target nucleic acid sequence or not (see for example **Figure 6C**). Furthermore, the sequences shown in **Figure 4** can optionally include a ribonucleotide at the 9th position from the 5'-end of the sense strand or the 11th position based on the 5'-end of the guide strand by counting 11 nucleotide positions in from the 5'-terminus of the guide strand (see **Figure 6C**).

[0463] **Figure 4A:** The sense strand comprises 21 nucleotides wherein the two terminal 3'-nucleotides are optionally base paired and wherein all nucleotides present are ribonucleotides except for (N N) nucleotides, which can comprise ribonucleotides, deoxynucleotides, universal bases, or other chemical modifications described herein. The antisense strand comprises 21 nucleotides, optionally having a 3'-terminal glyceryl moiety wherein the two terminal 3'-nucleotides are optionally complementary to the target RNA sequence, and wherein all nucleotides present are ribonucleotides except for (N N) nucleotides, which can comprise ribonucleotides, deoxynucleotides, universal bases, or other chemical modifications described herein. A modified internucleotide linkage, such as a phosphorothioate, phosphorodithioate or other modified internucleotide linkage as described herein, shown as "s", optionally connects the (N N) nucleotides in the antisense strand.

[0464] **Figure 4B:** The sense strand comprises 21 nucleotides wherein the two terminal 3'-nucleotides are optionally base paired and wherein all pyrimidine nucleotides that can be present are 2'deoxy-2'-fluoro modified nucleotides and all purine nucleotides that can 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 can be present are 2'-deoxy-2'-fluoro modified nucleotides and all purine nucleotides that can be present are 2'-O-methyl modified nucleotides except for (N N) nucleotides, which can comprise ribonucleotides, deoxynucleotides, universal bases, or other chemical modifications described herein. A modified internucleotide linkage, such as a phosphorothioate, phosphorodithioate or other modified internucleotide linkage as described herein, shown as "s", optionally connects the (N N) nucleotides in the sense and antisense strand.

[0465] **Figure 4C:** The sense strand comprises 21 nucleotides having 5'- and 3'- terminal cap moieties wherein the two terminal 3'-nucleotides are optionally base paired and wherein all pyrimidine nucleotides that can 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 can be present are 2'-deoxy-2'-fluoro modified nucleotides except for (N N) nucleotides, which can comprise ribonucleotides, deoxynucleotides, universal bases, or other chemical modifications described herein. A modified internucleotide linkage, such as a phosphorothioate, phosphorodithioate or other modified internucleotide linkage as described herein, shown as "s", optionally connects the (N N) nucleotides in the antisense strand.

[0466] **Figure 4D:** The sense strand comprises 21 nucleotides having 5'- and 3'- terminal cap moieties wherein the two terminal 3'-nucleotides are optionally base paired and wherein all pyrimidine nucleotides that can be present are 2'-deoxy-2'-fluoro modified nucleotides except for (N N) nucleotides, which can comprise ribonucleotides, deoxynucleotides, universal bases, or other chemical modifications described herein and wherein and all purine nucleotides that can 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 can be present are 2'-deoxy-2'-fluoro modified nucleotides and all purine nucleotides that can be present are 2'-O-methyl modified nucleotides except for (N N) nucleotides, which can comprise ribonucleotides, deoxynucleotides, universal bases, or other chemical modifications described herein. A modified internucleotide linkage, such as a phosphorothioate, phosphorodithioate or other modified internucleotide linkage as described herein, shown as "s", optionally connects the (N N) nucleotides in the antisense strand.

[0467] **Figure 4E:** The sense strand comprises 21 nucleotides having 5'- and 3'- terminal cap moieties wherein the two terminal 3'-nucleotides are optionally base paired and wherein all pyrimidine nucleotides that can be present are 2'-deoxy-2'-fluoro modified nucleotides except for (N N) nucleotides, which can comprise ribonucleotides, deoxynucleotides, universal bases, or other chemical modifications described herein. The antisense strand comprises 21 nucleotides, optionally having a 3'-terminal glyceryl moiety and wherein the two terminal 3'-nucleotides are optionally complementary to the target RNA sequence, and wherein all pyrimidine nucleotides that can be present are 2'-deoxy-2'-fluoro modified nucleotides and all purine nucleotides that can be present are 2'-O-methyl modified nucleotides except for (N N) nucleotides, which can comprise ribonucleotides, deoxynucleotides, universal bases, or other chemical modifications described herein. A modified internucleotide linkage, such as a phosphorothioate, phosphorodithioate or other modified internucleotide linkage as described herein, shown as "s", optionally connects the (N N) nucleotides in the antisense strand.

[0468] **Figure 4F:** The sense strand comprises 21 nucleotides having 5'- and 3'- terminal cap moieties wherein the two terminal 3'-nucleotides are optionally base paired and wherein all pyrimidine nucleotides that can be present are 2'-deoxy-2'-fluoro modified nucleotides except for (N N) nucleotides, which can comprise ribonucleotides, deoxynucleotides, universal bases, or other chemical modifications described herein and wherein and all purine nucleotides that can be present are 2'-deoxy nucleotides. The antisense strand comprises 21 nucleotides, optionally having a 3'-terminal glyceryl moiety and wherein the two terminal 3'-nucleotides are optionally complementary to the target RNA sequence, and having one 3'-terminal phosphorothioate internucleotide linkage and wherein all pyrimidine nucleotides that can be present are 2'-deoxy-2'-fluoro modified nucleotides and all purine nucleotides that can be present are 2'-deoxy nucleotides except for (N N) nucleotides, which can comprise ribonucleotides, deoxynucleotides, universal bases, or other chemical modifications described

herein. A modified internucleotide linkage, such as a phosphorothioate, phosphorodithioate or other modified internucleotide linkage as described herein, shown as "s", optionally connects the (N N) nucleotides in the antisense strand. The antisense strand of constructs A-F comprise sequence complementary to any target nucleic acid sequence of the invention. Furthermore, when a glyceryl moiety (L) is present at the 3'-end of the antisense strand for any construct shown in **Figure 4 A-F**, the modified internucleotide linkage is optional.

[0469] **Figure 5A-F** shows non-limiting examples of specific chemically-modified siNA sequences of the invention. **A-F** applies the chemical modifications described in **Figure 4A-F** to an exemplary ENaC siNA sequence. Such chemical modifications can be applied to any ENaC sequence. Furthermore, the sequences shown in **Figure 5** can optionally include a ribonucleotide at the 9^{th} position from the 5'-end of the sense strand or the 11^{th} position based on the 5'-end of the guide strand by counting 11 nucleotide positions in from the 5'-terminus of the guide strand (see **Figure 6C**). In addition, the sequences shown in Figure 5 can optionally include terminal ribonucleotides at up to about 4 positions at the 5'-end of the antisense strand (e.g., about 1, 2, 3, or 4 terminal ribonucleotides at the 5'-end of the antisense strand).

[0470] **Figure 6A-C** shows non-limiting examples of different siNA constructs of the invention.

[0471] The examples shown in **Figure 6A** (constructs 1, 2, and 3) have 19 representative base pairs; however, different embodiments of the invention include any number of base pairs described herein. Bracketed regions represent nucleotide overhangs, for example, comprising about 1, 2, 3, or 4 nucleotides in length, preferably about 2 nucleotides. Constructs 1 and 2 can be used independently for RNAi activity. Construct 2 can comprise a polynucleotide or non-nucleotide linker, which can optionally be designed as a biodegradable linker. In one embodiment, the loop structure shown in construct 2 can comprise a biodegradable linker that results in the formation of construct 1 *in vivo* and/or *in vitro*. In another example, construct 3 can be used to generate construct 2 under the same principle wherein a linker is used to generate the active siNA construct 2 *in vivo* and/or *in vitro*, which can optionally utilize another biodegradable linker to generate the active siNA construct 1 *in vivo* and/or *in vitro*. As such, the stability and/or activity of the siNA constructs can be modulated based on the design of the siNA construct for use *in vivo* or *in vitro* and/or *in vitro*.

[0472] The examples shown in **Figure 6B** represent different variations of double stranded nucleic acid molecule of the invention, such as microRNA, that can include overhangs, bulges, loops, and stem-loops resulting from partial complementarity. Such motifs having bulges, loops, and stem-loops are generally characteristics of miRNA. The bulges, loops, and stem-loops can result from any degree of partial complementarity, such as mismatches or bulges of about 1, 2, 3, 4, 5, 6, 7, 8, 9, 10 or more nucleotides in one or both strands of the double stranded nucleic acid molecule of the invention.

[0473] The example shown in **Figure 6C** represents a model double stranded nucleic acid molecule of the invention comprising a 19 base pair duplex of two 21 nucleotide sequences having dinucleotide 3'-overhangs. The top strand (1) represents the sense strand (passenger strand), the middle strand (2) represents the antisense (guide strand), and the lower strand (3) represents a target polynucleotide sequence. The dinucleotide overhangs (NN) can comprise sequence derived from the target polynucleotide. For example, the 3'-(NN) sequence in the guide strand can be complementary to the 5'-[NN] sequence of the target polynucleotide. In addition, the 5'-(NN) sequence of the passenger strand can comprise the same sequence as the 5'-[NN] sequence of the target polynucleotide sequence. In other embodiments, the overhangs (NN) are not derived from the target polynucleotide sequence, for example where the 3'-(NN) sequence in the guide strand are not complementary to the 5'-[NN] sequence of the target polynucleotide and the 5'-(NN) sequence of the passenger strand can comprise different sequence from the 5'-[NN] sequence of the target polynucleotide sequence. In additional embodiments, any (NN) nucleotides are chemically modified, *e.g.*, as 2'-O-methyl, 2'-deoxy-2'-fluoro, and/or other modifications herein. Furthermore, the passenger strand can comprise a ribonucleotide position ***N*** of the passenger strand. For the representative 19 base pair 21 mer duplex shown, position ***N*** can be 9 nucleotides in from the 3' end of the passenger strand. However, in duplexes of differing length, the position ***N*** is determined based on the 5'-end of the guide strand by counting 11 nucleotide positions in from the 5'-terminus of the guide strand and picking the corresponding base paired nucleotide in the passenger strand. Cleavage by Ago2 takes place between positions 10 and 11 as indicated by the arrow. In additional embodiments, there are two ribonucleotides, *NN*, at positions 10 and 11 based on the 5'-end of the guide strand by counting 10 and 11 nucleotide positions in from the 5'-terminus of the guide strand and picking the corresponding base paired nucleotides in the passenger strand.

[0474] **Figure 7** shows non-limiting examples of different stabilization chemistries (1-10) that can be used, for example, to stabilize the 3'-end of siNA sequences of the invention, including **(1)** [3-3']-inverted deoxyribose; **(2)** deoxyribonucleotide; **(3)** [5'-3']-3'-deoxyribonucleotide; **(4)** [5'-3']-ribonucleotide; **(5)** [5'-3']-3'-O-methyl ribonucleotide; **(6)** 3'-glyceryl; **(7)** [3'-5']-3'-deoxyribonucleotide; **(8)** [3'-3']-deoxyribonucleotide; **(9)** [5'-2']-deoxyribonucleotide; and **(10)** [5-3']-dideoxyribonucleotide. In addition to modified and unmodified backbone chemistries indicated in the figure, these chemistries can be combined with different backbone modifications as described herein, for example, backbone modifications having Formula I. In addition, the 2'-deoxy nucleotide shown 5' to the terminal modifications shown can be another modified or unmodified nucleotide or non-nucleotide described herein, for example modifications having any of Formulae I-VII or any combination thereof.

[0475] **Figure 8** shows a non-limiting example of a strategy used to identify chemically modified siNA constructs of the invention that are nuclease resistant while preserving the ability to mediate RNAi activity. Chemical modifications are introduced into the siNA construct based on educated design parameters (*e.g.* introducing 2'-modifications, base modifications, backbone modifications, terminal cap modifications etc). The modified construct in tested in an appropriate system (*e.g.* human serum for nuclease resistance, shown, or an animal model for PK/delivery parameters). In parallel, the siNA construct is tested for RNAi activity, for example in a cell culture system such as a luciferase reporter assay). Lead siNA constructs are then identified which possess a particular characteristic while maintaining RNAi activity, and can be further modified and assayed once again. This same approach can be used to identify siNA-conjugate molecules with improved pharmacokinetic profiles, delivery, and RNAi activity.

[0476] **Figure 9** shows non-limiting examples of phosphorylated siNA molecules of the invention, including linear and duplex constructs and asymmetric derivatives thereof.

[0477] **Figure 10** shows non-limiting examples of chemically modified terminal phosphate groups of the invention.

[0478] **Figure 11A** shows a non-limiting example of methodology used to design self complementary DFO constructs utilizing palindrome and/or repeat nucleic acid sequences that are identified in a target nucleic acid sequence. (i) A palindrome or repeat sequence is

identified in a nucleic acid target sequence. (ii) A sequence is designed that is complementary to the target nucleic acid sequence and the palindrome sequence. (iii) An inverse repeat sequence of the non-palindrome/repeat portion of the complementary sequence is appended to the 3'-end of the complementary sequence to generate a self complementary DFO molecule comprising sequence complementary to the nucleic acid target. (iv) The DFO molecule can self-assemble to form a double stranded oligonucleotide. **Figure 11B** shows a non-limiting representative example of a duplex forming oligonucleotide sequence. **Figure 11C** shows a non-limiting example of the self assembly schematic of a representative duplex forming oligonucleotide sequence. **Figure 11D** 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.

[0479] **Figure 12** shows a non-limiting example of the design of self complementary DFO constructs utilizing palindrome and/or repeat nucleic acid sequences that are incorporated into the DFO constructs that have sequence complementary to any target nucleic acid sequence of interest. Incorporation of these palindrome/repeat sequences allow the design of DFO constructs that form duplexes in which each strand is capable of mediating modulation of target gene expression, for example by RNAi. First, the target sequence is identified. A complementary sequence is then generated in which nucleotide or non-nucleotide modifications (shown as X or Y) are introduced into the complementary sequence that generate an artificial palindrome (shown as XYXYXY in the Figure). An inverse repeat of the non-palindrome/repeat complementary sequence is appended to the 3'-end of the complementary sequence to generate a self complementary DFO comprising sequence complementary to the nucleic acid target. The DFO can self-assemble to form a double stranded oligonucleotide.

[0480] **Figure 13** shows non-limiting examples of multifunctional siNA molecules of the invention comprising two separate polynucleotide sequences that are each capable of mediating RNAi directed cleavage of differing target nucleic acid sequences. **Figure 13A** shows a non-limiting example of a multifunctional siNA molecule having a first region that is complementary to a first target nucleic acid sequence (complementary region 1) and a second region that is complementary to a second target nucleic acid sequence (complementary region 2), wherein the first and second complementary regions are situated at the 3'-ends of each polynucleotide sequence in the multifunctional siNA. The dashed portions of each

polynucleotide sequence of the multifunctional siNA construct have complementarity with regard to corresponding portions of the siNA duplex, but do not have complementarity to the target nucleic acid sequences. **Figure 13B** shows a non-limiting example of a multifunctional siNA molecule having a first region that is complementary to a first target nucleic acid sequence (complementary region 1) and a second region that is complementary to a second target nucleic acid sequence (complementary region 2), wherein the first and second complementary regions are situated at the 5'-ends of each polynucleotide sequence in the multifunctional siNA. 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.

[0481] **Figure 14** shows non-limiting examples of multifunctional siNA molecules of the invention comprising a single polynucleotide sequence comprising distinct regions that are each capable of mediating RNAi directed cleavage of differing target nucleic acid sequences. **Figure 14A** shows a non-limiting example of a multifunctional siNA molecule having a first region that is complementary to a first target nucleic acid sequence (complementary region 1) and a second region that is complementary to a second target nucleic acid sequence (complementary region 2), wherein the second complementary region is situated at the 3'-end of the polynucleotide sequence in the multifunctional siNA. The dashed portions of each polynucleotide sequence of the multifunctional siNA construct have complementarity with regard to corresponding portions of the siNA duplex, but do not have complementarity to the target nucleic acid sequences. **Figure 14B** shows a non-limiting example of a multifunctional siNA molecule having a first region that is complementary to a first target nucleic acid sequence (complementary region 1) and a second region that is complementary to a second target nucleic acid sequence (complementary region 2), wherein the first complementary region is situated at the 5'-end of the polynucleotide sequence in the multifunctional siNA. The dashed portions of each polynucleotide sequence of the multifunctional siNA construct have complementarity with regard to corresponding portions of the siNA duplex, but do not have complementarity to the target nucleic acid sequences. In one embodiment, these multifunctional siNA constructs are processed in vivo or in vitro to generate multifunctional siNA constructs as shown in **Figure 13**.

[0482] **Figure 15** shows non-limiting examples of multifunctional siNA molecules of the invention comprising two separate polynucleotide sequences that are each capable of

mediating RNAi directed cleavage of differing target nucleic acid sequences and wherein the multifunctional siNA construct further comprises a self complementary, palindrome, or repeat region, thus enabling shorter bifuctional siNA constructs that can mediate RNA interference against differing target nucleic acid sequences. **Figure 15A** shows a non-limiting example of a multifunctional siNA molecule having a first region that is complementary to a first target nucleic acid sequence (complementary region 1) and a second region that is complementary to a second target nucleic acid sequence (complementary region 2), wherein the first and second complementary regions are situated at the 3'-ends of each polynucleotide sequence in the multifunctional siNA, and wherein the first and second complementary regions further comprise a self complementary, palindrome, or repeat region. The dashed portions of each polynucleotide sequence of the multifunctional siNA construct have complementarity with regard to corresponding portions of the siNA duplex, but do not have complementarity to the target nucleic acid sequences. **Figure 15B** shows a non-limiting example of a multifunctional siNA molecule having a first region that is complementary to a first target nucleic acid sequence (complementary region 1) and a second region that is complementary to a second target nucleic acid sequence (complementary region 2), wherein the first and second complementary regions are situated at the 5'-ends of each polynucleotide sequence in the multifunctional siNA, and wherein the first and second complementary regions further comprise a self complementary, palindrome, or repeat region. The dashed portions of each polynucleotide sequence of the multifunctional siNA construct have complementarity with regard to corresponding portions of the siNA duplex, but do not have complementarity to the target nucleic acid sequences.

[0483] **Figure 16** shows non-limiting examples of multifunctional siNA molecules of the invention comprising a single polynucleotide sequence comprising distinct regions that are each capable of mediating RNAi directed cleavage of differing target nucleic acid sequences and wherein the multifunctional siNA construct further comprises a self complementary, palindrome, or repeat region, thus enabling shorter bifuctional siNA constructs that can mediate RNA interference against differing target nucleic acid sequences. **Figure 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 second complementary region is situated at the 3'-end of the polynucleotide sequence in the multifunctional siNA, and wherein the first and second complementary

regions further comprise a self complementary, palindrome, or repeat region. The dashed portions of each polynucleotide sequence of the multifunctional siNA construct have complementarity with regard to corresponding portions of the siNA duplex, but do not have complementarity to the target nucleic acid sequences. **Figure 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 complementary region is situated at the 5'-end of the polynucleotide sequence in the multifunctional siNA, and wherein the first and second complementary regions further comprise a self complementary, palindrome, or repeat region. The dashed portions of each polynucleotide sequence of the multifunctional siNA construct have complementarity with regard to corresponding portions of the siNA duplex, but do not have complementarity to the target nucleic acid sequences. In one embodiment, these multifunctional siNA constructs are processed in vivo or in vitro to generate multifunctional siNA constructs as shown in **Figure 15**.

[0484] **Figure 17** shows a non-limiting example of how multifunctional siNA molecules of the invention can target two separate target nucleic acid molecules, such as separate RNA molecules encoding differing proteins (e.g., any of ENaC targets herein), for example, a cytokine and its corresponding receptor, differing viral strains, a virus and a cellular protein involved in viral infection or replication, or differing proteins involved in a common or divergent biologic pathway that is implicated in the maintenance of progression of disease. Each strand of the multifunctional siNA construct comprises a region having complementarity to separate target nucleic acid molecules. The multifunctional siNA molecule is designed such that each strand of the siNA can be utilized by the RISC complex to initiate RNA interference mediated cleavage of its corresponding target. These design parameters can include destabilization of each end of the siNA construct (see for example Schwarz *et al.*, 2003, *Cell*, 115, 199-208). Such destabilization can be accomplished for example by using guanosine-cytidine base pairs, alternate base pairs (*e.g.*, wobbles), or destabilizing chemically modified nucleotides at terminal nucleotide positions as is known in the art.

[0485] **Figure 18** 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 isotype 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.

[0486] **Figure 19(A-H)** shows non-limiting examples of tethered multifunctional siNA constructs of the invention. In the examples shown, a linker (*e.g.*, nucleotide or non-nucleotide linker) connects two siNA regions (*e.g.*, two sense, two antisense, or alternately a sense and an antisense region together. Separate sense (or sense and antisense) sequences corresponding to a first target sequence and second target sequence are hybridized to their corresponding sense and/or antisense sequences in the multifunctional siNA. In addition, various conjugates, ligands, aptamers, polymers or reporter molecules can be attached to the linker region for selective or improved delivery and/or pharmacokinetic properties.

[0487] **Figure 20** shows a non-limiting example of various dendrimer based multifunctional siNA designs.

[0488] **Figure 21** shows a non-limiting example of various supramolecular multifunctional siNA designs.

[0489] **Figure 22** shows a non-limiting example of a dicer enabled multifunctional siNA design using a 30 nucleotide precursor siNA construct. A 30 base pair duplex is cleaved by Dicer into 22 and 8 base pair products from either end (8 b.p. fragments not shown). For ease of presentation the overhangs generated by dicer are not shown – but can be compensated for. Three targeting sequences are shown. The required sequence identity overlapped is indicated by grey boxes. The N's of the parent 30 b.p. siNA are suggested sites of 2'-OH positions to enable Dicer cleavage if this is tested in stabilized chemistries. Note that processing of a 30mer duplex by Dicer RNase III does not give a precise 22+8 cleavage, but rather produces

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

[0490] **Figure 23** shows a non-limiting example of a dicer enabled multifunctional siNA design using a 40 nucleotide precursor siNA construct. A 40 base pair duplex is cleaved by Dicer into 20 base pair products from either end. For ease of presentation the overhangs generated by dicer are not shown – but can be compensated for. Four targeting sequences are shown. The target sequences having homology are enclosed by boxes. This design format can be extended to larger RNAs. If chemically stabilized siNAs are bound by Dicer, then strategically located ribonucleotide linkages can enable designer cleavage products that permit our more extensive repertoire of multiifunctional designs. For example cleavage products not limited to the Dicer standard of approximately 22-nucleotides can allow multifunctional siNA constructs with a target sequence identity overlap ranging from, for example, about 3 to about 15 nucleotides.

[0491] **Figure 24** shows a non-limiting example of additional multifunctional siNA construct designs of the invention. In one example, a conjugate, ligand, aptamer, label, or other moiety is attached to a region of the multifunctional siNA to enable improved delivery or pharmacokinetic profiling.

[0492] **Figure 25** shows a non-limiting example of additional multifunctional siNA construct designs of the invention. In one example, a conjugate, ligand, aptamer, label, or other moiety is attached to a region of the multifunctional siNA to enable improved delivery or pharmacokinetic profiling.

[0493] **Figure 26** shows a non-limiting example of a cholesterol linked phosphoramidite that can be used to synthesize cholesterol conjugated siNA molecules of the invention. An example is shown with the cholesterol moiety linked to the 5'-end of the sense strand of a siNA molecule.

[0494] **Figure 27** depicts an embodiment of 5' and 3' inverted abasic cap moieties linked to a nucleic acid strand.

[0495] **Figure 28** shows the relative IL8 mRNA expression (n=4 with 6 replicates per data point) in TLR7-U2OS cells upon treatment with siRNAs compared to the control Resiquimod (R848) which is an immunostimulatory agonist.

[0496] **Figure 29** shows the relative IL8 mRNA expression (n=4 with 6 replicates per data point) in TLR8-U2OS cells upon treatment with siRNAs compared to the control ssRNA40, which is an immunostimulatory agonist.

[0497] **Figure 30** shows inhibition of the sodium transport in a FLIPR (fluorescence imaging plate reader) assay upon transfection of recombinant HEK cells with the modified ENaC siRNAs for target sites 782 (SEQ ID NOs 51 and 52) and 1181 (SEQ ID NOs: 57 and 58) at 100, 50, 20, and 10nM concentrations.

DETAILED DESCRIPTION OF THE INVENTION

Mechanism of Action of Nucleic Acid Molecules of the Invention

[0498] The discussion that follows discusses the proposed mechanism of RNA interference mediated by short interfering RNA as is presently known, and is not meant to be limiting and is not an admission of prior art. Applicant demonstrates herein that chemically-modified short interfering nucleic acids possess similar or improved capacity to mediate RNAi as do siRNA molecules and are expected to possess improved stability and activity *in vivo*; therefore, this discussion is not meant to be limiting only to siRNA and can be applied to siNA as a whole. By "improved capacity to mediate RNAi" or "improved RNAi activity" is meant to include RNAi activity measured *in vitro* and/or *in vivo* where the RNAi activity is a reflection of both the ability of the siNA to mediate RNAi and the stability of the siNAs of the invention. In this invention, the product of these activities can be increased *in vitro* and/or *in vivo* compared to an all RNA siRNA or a siNA containing a plurality of ribonucleotides. In some cases, the activity or stability of the siNA molecule can be decreased (i.e., less than ten-fold), but the overall activity of the siNA molecule is enhanced *in vitro* and/or *in vivo*.

[0499] 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 can have evolved in response to the production

of double-stranded RNAs (dsRNAs) derived from viral infection or the random integration of transposon elements into a host genome via a cellular response that specifically destroys homologous single-stranded RNA or viral genomic RNA. The presence of dsRNA in cells triggers the RNAi response though a mechanism that has yet to be fully characterized. This mechanism appears to be different from the interferon response that results from dsRNA-mediated activation of protein kinase PKR and 2', 5'-oligoadenylate synthetase resulting in non-specific cleavage of mRNA by ribonuclease L.

[0500] The presence of long dsRNAs in cells stimulates the activity of a ribonuclease III enzyme referred to as Dicer. Dicer is involved in the processing of the dsRNA into short pieces of dsRNA known as short interfering RNAs (siRNAs) (Berstein *et al.,* 2001, *Nature,* 409, 363). Short interfering RNAs derived from Dicer activity are typically about 21 to about 23 nucleotides in length and comprise about 19 base pair duplexes. Dicer has also been implicated in the excision of 21- and 22-nucleotide small temporal RNAs (stRNAs) from precursor RNA of conserved structure that are implicated in translational control (Hutvagner *et al.,* 2001, *Science,* 293, 834). The RNAi response also features an endonuclease complex containing a siRNA, commonly referred to as an RNA-induced silencing complex (RISC), which mediates cleavage of single-stranded RNA having sequence homologous to the siRNA. Cleavage of the target RNA takes place in the middle of the region complementary to the guide sequence of the siRNA duplex (Elbashir *et al.,* 2001, *Genes Dev.,* 15, 188). In addition, RNA interference can also involve small RNA (*e.g.*, micro-RNA or miRNA) mediated gene silencing, presumably though cellular mechanisms that regulate chromatin structure and thereby prevent transcription of target gene sequences (see for example Allshire, 2002, *Science,* 297, 1818-1819; Volpe *et al.,* 2002, *Science,* 297, 1833-1837; Jenuwein, 2002, *Science,* 297, 2215-2218; and Hall *et al.,* 2002, *Science,* 297, 2232-2237). As such, siNA molecules of the invention can be used to mediate gene silencing via interaction with RNA transcripts or alternately by interaction with particular gene sequences, wherein such interaction results in gene silencing either at the transcriptional level or post-transcriptional level.

[0501] RNAi has been studied in a variety of systems. Fire *et al.,* 1998, *Nature,* 391, 806, were the first to observe RNAi in *C. elegans*. Wianny and Goetz, 1999, *Nature Cell Biol.,* 2, 70, describe RNAi mediated by dsRNA in mouse embryos. Hammond *et al.,* 2000, *Nature,* 404, 293, describe RNAi in *Drosophila* cells transfected with dsRNA. Elbashir *et al.,* 2001,

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

Duplex Forming Oligonucleotides (DFO) of the Invention

[0502] In one embodiment, the invention features siNA molecules comprising duplex forming oligonucleotides (DFO) that can self-assemble into double stranded oligonucleotides. The duplex forming oligonucleotides of the invention can be chemically synthesized or expressed from transcription units and/or vectors. The DFO molecules of the instant invention provide useful reagents and methods for a variety of therapeutic, diagnostic, agricultural, veterinary, target validation, genomic discovery, genetic engineering and pharmacogenomic applications.

[0503] Applicant demonstrates herein that certain oligonucleotides, refered to herein for convenience but not limitation as duplex forming oligonucleotides or DFO molecules, are potent mediators of sequence specific regulation of gene expression. The oligonucleotides of the invention are distinct from other nucleic acid sequences known in the art (*e.g.*, siRNA, miRNA, stRNA, shRNA, antisense oligonucleotides etc.) in that they represent a class of linear polynucleotide sequences that are designed to self-assemble into double stranded oligonucleotides, where each strand in the double stranded oligonucleotides comprises a

nucleotide sequence that is complementary to an ENaC target nucleic acid molecule. Nucleic acid molecules of the invention can thus self assemble into functional duplexes in which each strand of the duplex comprises the same polynucleotide sequence and each strand comprises a nucleotide sequence that is complementary to an ENaC target nucleic acid molecule.

[0504] Generally, double stranded oligonucleotides are formed by the assembly of two distinct oligonucleotide sequences where the oligonucleotide sequence of one strand is complementary to the oligonucleotide sequence of the second strand; such double stranded oligonucleotides are assembled from two separate oligonucleotides, or from a single molecule that folds on itself to form a double stranded structure, often referred to in the field as hairpin stem-loop structure (*e.g.*, shRNA or short hairpin RNA). These double stranded oligonucleotides known in the art all have a common feature in that each strand of the duplex has a distict nucleotide sequence.

[0505] Distinct from the double stranded nucleic acid molecules known in the art, the applicants have developed a novel, potentially cost effective and simplified method of forming a double stranded nucleic acid molecule starting from a single stranded or linear oligonucleotide. The two strands of the double stranded oligonucleotide formed according to the instant invention have the same nucleotide sequence and are not covalently linked to each other. Such double-stranded oligonucleotides molecules can be readily linked post-synthetically by methods and reagents known in the art and are within the scope of the invention**.** In one embodiment, the single stranded oligonucleotide of the invention (the duplex forming oligonucleotide) that forms a double stranded oligonucleotide comprises a first region and a second region, where the second region includes a nucleotide sequence that is an inverted repeat of the nucleotide sequence in the first region, or a portion thereof, such that the single stranded oligonucleotide self assembles to form a duplex oligonucleotide in which the nucleotide sequence of one strand of the duplex is the same as the nucleotide sequence of the second strand. Non-limiting examples of such duplex forming oligonucleotides are illustrated in **Figures 11 and 12**. These duplex forming oligonucleotides (DFOs) can optionally include certain palindrome or repeat sequences where such palindrome or repeat sequences are present in between the first region and the second region of the DFO.

[0506] In one embodiment, the invention features a duplex forming oligonucleotide (DFO) molecule, wherein the DFO comprises a duplex forming self complementary nucleic

acid sequence that has nucleotide sequence complementary to an ENaC target nucleic acid sequence. The DFO molecule can comprise a single self complementary sequence or a duplex resulting from assembly of such self complementary sequences.

[0507] In one embodiment, a duplex forming oligonucleotide (DFO) of the invention comprises a first region and a second region, wherein the second region comprises a nucleotide sequence comprising an inverted repeat of nucleotide sequence of the first region such that the DFO molecule can assemble into a double stranded oligonucleotide. Such double stranded oligonucleotides can act as a short interfering nucleic acid (siNA) to modulate gene expression. Each strand of the double stranded oligonucleotide duplex formed by DFO molecules of the invention can comprise a nucleotide sequence region that is complementary to the same nucleotide sequence in an ENaC target nucleic acid molecule (*e.g.*, ENaC target RNA).

[0508] In one embodiment, the invention features a single stranded DFO that can assemble into a double stranded oligonucleotide. The applicant has surprisingly found that a single stranded oligonucleotide with nucleotide regions of self complementarity can readily assemble into duplex oligonucleotide constructs. Such DFOs can assemble into duplexes that can inhibit gene expression in a sequence specific manner. The DFO moleucles of the invention comprise a first region with nucleotide sequence that is complementary to the nucleotide sequence of a second region and where the sequence of the first region is complementary to an ENaC target nucleic acid (*e.g.*, RNA). The DFO can form a double stranded oligonucleotide wherein a portion of each strand of the double stranded oligonucleotide comprises a sequence complementary to an ENaC target nucleic acid sequence.

[0509] In one embodiment, the invention features a double stranded oligonucleotide, wherein the two strands of the double stranded oligonucleotide are not covalently linked to each other, and wherein each strand of the double stranded oligonucleotide comprises a nucleotide sequence that is complementary to the same nucleotide sequence in an ENaC target nucleic acid molecule or a portion thereof (*e.g.*, ENaC RNA target). In another embodiment, the two strands of the double stranded oligonucleotide share an identical nucleotide sequence of at least about 15, preferably at least about 16, 17, 18, 19, 20, or 21 nucleotides.

[0510] In one embodiment, a DFO molecule of the invention comprises a structure having Formula DFO-I:

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

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

[0511] When a sequence is described in this specification as being of "sufficient" length to interact (*i.e.*, base pair) with another sequence, it is meant that the the length is such that the number of bonds (*e.g.*, hydrogen bonds) formed between the two sequences is enough to enable the two sequence to form a duplex under the conditions of interest. Such conditions can be *in vitro* (*e.g.*, for diagnostic or assay purposes) or *in vivo* (*e.g.*, for therapeutic purposes). It is a simple and routine matter to determine such lengths.

[0512] In one embodiment, the invention features a double stranded oligonucleotide construct having Formula DFO-I(a):

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

wherein Z comprises a palindromic or repeat nucleic acid sequence or palindromic or repeat-like nucleic acid sequence with one or more modified nucleotides (*e.g.*, nucleotides with a modified base, such as 2-amino purine, 2-amino-1,6-dihydro purine or a universal base), for example of length about 2 to about 24 nucleotides in even numbers (*e.g.*, about 2, 4, 6, 8, 10, 12, 14, 16, 18, 20, 22 or 24 nucleotides), X represents a nucleic acid sequence, for example of length about 1 to about 21 nucleotides (*e.g.*, about 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, or 21 nucleotides), X' comprises a nucleic acid sequence, for example of length about 1 to about 21 nucleotides (*e.g.*, about 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20 or 21 nucleotides) having nucleotide sequence complementarity to sequence X or a portion thereof, p comprises a terminal phosphate group that can be present or absent, and wherein each X and Z independently comprises a nucleotide sequence that is complementary to an ENaC target nucleic acid sequence or a portion thereof (*e.g.*, ENaC RNA target) and is of length sufficient to interact with the ENaC target nucleic acid sequence of a portion thereof (*e.g.*, ENaC RNA target). For example, sequence X independently can comprise a sequence from about 12 to about 21 or more nucleotides (*e.g.*, about 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, or more) in length that is complementary to a target nucleotide sequence or a portion thereof (*e.g.*, ENaC RNA target). In another non-limiting example, the length of the nucleotide sequence of X and Z together (when X is present) that is complementary to the target sequence or a portion thereof is from about 12 to about 21 or more nucleotides (*e.g.*, about 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, or more). In yet another non-limiting example, when X is absent, the length of the nucleotide sequence of Z that is

complementary to the target sequence or a portion thereof is from about 12 to about 24 or more nucleotides (*e.g.*, about 12, 14, 16, 18, 20, 22, 24 or more). In one embodiment X, Z and X' are independently oligonucleotides, where X and/or Z comprises a nucleotide sequence of length sufficient to interact (*e.g.*, base pair) with nucleotide sequence in the target sequence or a portion thereof (*e.g.*, ENaC RNA target). In one embodiment, the lengths of oligonucleotides X and X' are identical. In another embodiment, the lengths of oligonucleotides X and X' are not identical. In another embodiment, the lengths of oligonucleotides X and Z or Z and X' or X, Z and X' are either identical or different. In one embodiment, the double stranded oligonucleotide construct of Formula I(a) includes one or more, specifically 1, 2, 3 or 4, mismatches, to the extent such mismatches do not significantly diminish the ability of the double stranded oligonucleotide to inhibit ENaC target gene expression.

[0513] In one embodiment, a DFO molecule of the invention comprises structure having Formula DFO-II:

5'-p-X X'-3'

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

[0514] In one embodiment, the invention features a double stranded oligonucleotide construct having Formula DFO-II(a):

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

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

[0515] In one embodiment, the invention features a DFO molecule having Formula DFO-I(b):

5'-p-Z-3'

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

[0516] In one embodiment, a DFO molecule having any of Formula DFO-I, DFO-I(a), DFO-I(b), DFO-II(a) or DFO-II can comprise chemical modifications as described herein without limitation, such as, for example, nucleotides having any of Formulae I-VII, stabilization chemistries as described in **Table 8**, or any other combination of modified nucleotides and non-nucleotides as described in the various embodiments herein.

[0517] In one embodiment, the palidrome or repeat sequence or modified nucleotide (*e.g.*, nucleotide with a modified base, such as 2-amino purine or a universal base) in Z of DFO constructs having Formula DFO-I, DFO-I(a) and DFO-I(b), comprises chemically modified nucleotides that are able to interact with a portion of the ENaC target nucleic acid sequence (*e.g.*, modified base analogs that can form Watson Crick base pairs or non-Watson Crick base pairs).

[0518] In one embodiment, a DFO molecule of the invention, for example a DFO having Formula DFO-I or DFO-II, comprises about 15 to about 40 nucleotides (*e.g.*, about 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36, 37, 38, 39, or 40 nucleotides). In one embodiment, a DFO molecule of the invention comprises one or more chemical modifications. In a non-limiting example, the introduction of chemically modified nucleotides and/or non-nucleotides into nucleic acid molecules of the invention provides a powerful tool in overcoming potential limitations of *in vivo* stability and bioavailability inherent to unmodified RNA molecules that are delivered exogenously. For example, the use of chemically modified nucleic acid molecules can enable a lower dose of a particular nucleic acid molecule for a given therapeutic effect since chemically modified nucleic acid molecules tend to have a longer half-life in serum or in cells or tissues. Furthermore, certain chemical modifications can improve the bioavailability and/or potency of nucleic acid molecules by not only enhancing half-life but also facilitating the targeting of nucleic acid molecules to particular organs, cells or tissues and/or improving cellular uptake of the nucleic acid molecules. Therefore, even if the activity of a chemically modified nucleic acid molecule is reduced in vitro as compared to a native/unmodified nucleic acid molecule, for example when compared to an unmodified RNA molecule, the overall activity of the modified nucleic acid molecule can be greater than the native or unmodified nucleic acid molecule due to improved stability, potency, duration of effect, bioavailability and/or delivery of the molecule.

Multifunctional or Multi-targeted siNA molecules of the Invention

[0519] In one embodiment, the invention features siNA molecules comprising multifunctional short interfering nucleic acid (multifunctional siNA) molecules that modulate the expression of one or more target genes in a biologic system, such as a cell, tissue, or organism. The multifunctional short interfering nucleic acid (multifunctional siNA) molecules of the invention can target more than one region of the target nucleic acid sequence or can target sequences of more than one distinct target nucleic acid molecules (*e.g.*, ENaC RNA targets). The multifunctional siNA molecules of the invention can be chemically synthesized or expressed from transcription units and/or vectors. The multifunctional siNA molecules of the instant invention provide useful reagents and methods for a variety of human applications, therapeutic, diagnostic, agricultural, veterinary, target validation, genomic discovery, genetic engineering and pharmacogenomic applications.

[0520] Applicant demonstrates herein that certain oligonucleotides, refered to herein for convenience but not limitation as multifunctional short interfering nucleic acid or multifunctional siNA molecules, are potent mediators of sequence specific regulation of gene expression. The multifunctional siNA molecules of the invention are distinct from other nucleic acid sequences known in the art (*e.g.*, siRNA, miRNA, stRNA, shRNA, antisense oligonucleotides, *etc.*) in that they represent a class of polynucleotide molecules that are designed such that each strand in the multifunctional siNA construct comprises a nucleotide sequence that is complementary to a distinct nucleic acid sequence in one or more target nucleic acid molecules. A single multifunctional siNA molecule (generally a double-stranded molecule) of the invention can thus target more than one (*e.g.*, 2, 3, 4, 5, or more) differing target nucleic acid target molecules. Nucleic acid molecules of the invention can also target more than one (*e.g.*, 2, 3, 4, 5, or more) region of the same target nucleic acid sequence. As such multifunctional siNA molecules of the invention are useful in down regulating or inhibiting the expression of one or more target nucleic acid molecules. For example, a multifunctional siNA molecule of the invention can target (e.g., have complementarity to) nucleic acid molecules selected from the group consisting of ENaC, isotypes of ENaC or any combination thereof. By reducing or inhibiting expression of more than one target nucleic acid molecule with one multifunctional siNA construct, multifunctional siNA molecules of the invention represent a class of potent therapeutic agents that can provide simultaneous inhibition of multiple targets within a disease (e.g., respiratory) related pathway. Such simultaneous inhibition can provide synergistic therapeutic treatment strategies without the

need for separate preclinical and clinical development efforts or complex regulatory approval process.

[0521] Use of multifunctional siNA molecules that target more then one region of a target nucleic acid molecule (*e.g.*, ENaC target RNA or DNA) is expected to provide potent inhibition of gene expression. For example, a single multifunctional siNA construct of the invention can target both conserved and variable regions of a target nucleic acid molecule (*e.g.*, ENaC RNA or DNA), thereby allowing down regulation or inhibition of, for example, different target ENaC isoforms or variants to optimize therapeutic efficacy and minimize toxicity, or allowing for targeting of both coding and non-coding regions of the ENaC target nucleic acid molecule.

[0522] Generally, double stranded oligonucleotides are formed by the assembly of two distinct oligonucleotides where the oligonucleotide sequence of one strand is complementary to the oligonucleotide sequence of the second strand; such double stranded oligonucleotides are generally assembled from two separate oligonucleotides (*e.g.*, siRNA). Alternately, a duplex can be formed from a single molecule that folds on itself (*e.g.*, shRNA or short hairpin RNA). These double stranded oligonucleotides are known in the art to mediate RNA interference and all have a common feature wherein only one nucleotide sequence region (guide sequence or the antisense sequence) has complementarity to a target nucleic acid sequence, and the other strand (sense sequence) comprises nucleotide sequence that is homologous to the target nucleic acid sequence. Generally, the antisense sequence is retained in the active RISC complex and guides the RISC to the target nucleotide sequence by means of complementary base-pairing of the antisense sequence with the target seqeunce for mediating sequence-specific RNA interference. It is known in the art that in some cell culture systems, certain types of unmodified siRNAs can exhibit "off target" effects. It is hypothesized that this off-target effect involves the participation of the sense sequence instead of the antisense sequence of the siRNA in the RISC complex (see for example Schwarz *et al.*, 2003, Cell, 115, 199-208). In this instance the sense sequence is believed to direct the RISC complex to a sequence (off-target sequence) that is distinct from the intended target sequence, resulting in the inhibition of the off-target sequence. In these double stranded nucleic acid molecules, each strand is complementary to a distinct target nucleic acid sequence. However, the off-targets that are affected by these dsRNAs are not entirely predictable and are non-specific.

[0523] Distinct from the double stranded nucleic acid molecules known in the art, the applicants have developed a novel, potentially cost effective and simplified method of down regulating or inhibiting the expression of more than one target nucleic acid sequence using a single multifunctional siNA construct. The multifunctional siNA molecules of the invention are designed to be double-stranded or partially double stranded, such that a portion of each strand or region of the multifunctional siNA is complementary to a target nucleic acid sequence of choice. As such, the multifunctional siNA molecules of the invention are not limited to targeting sequences that are complementary to each other, but rather to any two differing target nucleic acid sequences. Multifunctional siNA molecules of the invention are designed such that each strand or region of the multifunctional siNA molecule, that is complementary to a given target nucleic acid sequence, is of suitable length (*e.g.*, from about 16 to about 28 nucleotides in length, preferably from about 18 to about 28 nucleotides in length) for mediating RNA interference against the target nucleic acid sequence. The complementarity between the target nucleic acid sequence and a strand or region of the multifunctional siNA must be sufficient (at least about 8 base pairs) for cleavage of the target nucleic acid sequence by RNA interference. Multifunctional siNA of the invention is expected to minimize off-target effects seen with certain siRNA sequences, such as those described in Schwarz *et al.,* supra.

[0524] It has been reported that dsRNAs of length between 29 base pairs and 36 base pairs (Tuschl *et al.,* International PCT Publication No. WO 02/44321) do not mediate RNAi. One reason these dsRNAs are inactive can be the lack of turnover or dissociation of the strand that interacts with the target RNA sequence, such that the RISC complex is not able to efficiently interact with multiple copies of the target RNA resulting in a significant decrease in the potency and efficiency of the RNAi process. Applicant has surprisingly found that the multifunctional siNAs of the invention can overcome this hurdle and are capable of enhancing the efficiency and potency of RNAi process. As such, in certain embodiments of the invention, multifunctional siNAs of length of about 29 to about 36 base pairs can be designed such that, a portion of each strand of the multifunctional siNA molecule comprises a nucleotide sequence region that is complementary to a target nucleic acid of length sufficient to mediate RNAi efficiently (*e.g.*, about 15 to about 23 base pairs) and a nucleotide sequence region that is not complementary to the target nucleic acid. By having both complementary and non-complementary portions in each strand of the multifunctional siNA, the multifunctional siNA can mediate RNA interference against a target nucleic acid sequence

without being prohibitive to turnover or dissociation (*e.g.*, where the length of each strand is too long to mediate RNAi against the respective target nucleic acid sequence). Furthermore, design of multifunctional siNA molecules of the invention with internal overlapping regions allows the multifunctional siNA molecules to be of favorable (decreased) size for mediating RNA interference and of size that is well suited for use as a therapeutic agent (*e.g.*, wherein each strand is independently from about 18 to about 28 nucleotides in length). Non-limiting examples are illustrated in **Figures 13-25 and Table 1b**.

[0525] In one embodiment, a multifunctional siNA molecule of the invention comprises a first region and a second region, where the first region of the multifunctional siNA comprises a nucleotide sequence complementary to a nucleic acid sequence of a first target nucleic acid molecule, and the second region of the multifunctional siNA comprises nucleic acid sequence complementary to a nucleic acid sequence of a second target nucleic acid molecule. In one embodiment, a multifunctional siNA molecule of the invention comprises a first region and a second region, where the first region of the multifunctional siNA comprises nucleotide sequence complementary to a nucleic acid sequence of the first region of a target nucleic acid molecule, and the second region of the multifunctional siNA comprises nucleotide sequence complementary to a nucleic acid sequence of a second region of a the target nucleic acid molecule. In another embodiment, the first region and second region of the multifunctional siNA can comprise separate nucleic acid sequences that share some degree of complementarity (*e.g.*, from about 1 to about 10 complementary nucleotides). In certain embodiments, multifunctional siNA constructs comprising separate nucleic acid seqeunces can be readily linked post-synthetically by methods and reagents known in the art and such linked constructs are within the scope of the invention**.** Alternately, the first region and second region of the multifunctional siNA can comprise a single nucleic acid sequence having some degree of self complementarity, such as in a hairpin or stem-loop structure. Non-limiting examples of such double stranded and hairpin multifunctional short interfering nucleic acids are illustrated in **Figures 13 and 14** respectively. These multifunctional short interfering nucleic acids (multifunctional siNAs) can optionally include certain overlapping nucleotide sequence where such overlapping nucleotide sequence is present in between the first region and the second region of the multifunctional siNA (see for example **Figures 15 and 16**). In one embodiment, the first target nucleic acid molecule and the second nucleic acid target molecule are one or more ENaC target sequences, such as any ENaC nucleic acid sequence or ENaC isotype nucleic acid sequence.

[0526] In one embodiment, the invention features a multifunctional short interfering nucleic acid (multifunctional siNA) molecule, wherein each strand of the the multifunctional siNA independently comprises a first region of nucleic acid sequence that is complementary to a distinct target nucleic acid sequence and the second region of nucleotide sequence that is not complementary to the target sequence. The target nucleic acid sequence of each strand is in the same target nucleic acid molecule or different target nucleic acid molecules. In one embodiment, the nucleic acid target molecule(s) comprises one or more ENaC target sequences, such as any ENaC or ENaC isotype nucleic acid sequence.

[0527] In another embodiment, the multifunctional siNA comprises two strands, where: (a) the first strand comprises a region having sequence complementarity to a target nucleic acid sequence (complementary region 1) and a region having no sequence complementarity to the target nucleotide sequence (non-complementary region 1); (b) the second strand of the multifunction siNA comprises a region having sequence complementarity to a target nucleic acid sequence that is distinct from the target nucleotide sequence complementary to the first strand nucleotide sequence (complementary region 2), and a region having no sequence complementarity to the target nucleotide sequence of complementary region 2 (non-complementary region 2); (c) the complementary region 1 of the first strand comprises a nucleotide sequence that is complementary to a nucleotide sequence in the non-complementary region 2 of the second strand and the complementary region 2 of the second strand comprises a nucleotide sequence that is complementary to a nucleotide sequence in the non-complementary region 1 of the first strand. The target nucleic acid sequence of complementary region 1 and complementary region 2 is in the same target nucleic acid molecule or different target nucleic acid molecules. In one embodiment, the nucleic acid target molecule(s) comprises one or more ENaC target sequences, such as any ENaC or ENaC isotype nucleic acid sequences.

[0528] In another embodiment, the multifunctional siNA comprises two strands, where: (a) the first strand comprises a region having sequence complementarity to a target nucleic acid sequence derived from a gene (*e.g.*, a first ENaC gene) (complementary region 1) and a region having no sequence complementarity to the target nucleotide sequence of complementary region 1 (non-complementary region 1); (b) the second strand of the multifunction siNA comprises a region having sequence complementarity to a target nucleic acid sequence derived from a gene (*e.g.*, a second ENaC gene) that is distinct from the gene

of complementary region 1 (complementary region 2), and a region having no sequence complementarity to the target nucleotide sequence of complementary region 2 (non-complementary region 2); (c) the complementary region 1 of the first strand comprises a nucleotide sequence that is complementary to a nucleotide sequence in the non-complementary region 2 of the second strand and the complementary region 2 of the second strand comprises a nucleotide sequence that is complementary to a nucleotide sequence in the non-complementary region 1 of the first strand. In one embodiment, the nucleic acid target sequence comprises one or more ENaC target sequences, such as any ENaC or ENaC isotype nucleic acid sequences.

[0529] In another embodiment, the multifunctional siNA comprises two strands, where: (a) the first strand comprises a region having sequence complementarity to a target nucleic acid sequence derived from a first gene (*e.g.*, ENaC gene) (complementary region 1) and a region having no sequence complementarity to the target nucleotide sequence of complementary region 1 (non-complementary region 1); (b) the second strand of the multifunction siNA comprises a region having sequence complementarity to a second target nucleic acid sequence distinct from the first target nucleic acid sequence of complementary region 1 (complementary region 2), provided, however, that the target nucleic acid sequence for complementary region 1 and target nucleic acid sequence for complementary region 2 are both derived from the same gene, and a region having no sequence complementarity to the target nucleotide sequence of complementary region 2 (non-complementary region 2); (c) the complementary region 1 of the first strand comprises a nucleotide sequence that is complementary to a nucleotide sequence in the non-complementary region 2 of the second strand and the complementary region 2 of the second strand comprises a nucleotide sequence that is complementary to nucleotide sequence in the non-complementary region 1 of the first strand. In one embodiment, the nucleic acid target sequence comprises one or more ENaC target sequences, such as any ENaC or ENaC isotype nucleic acid sequences.

[0530] In one embodiment, the invention features a multifunctional short interfering nucleic acid (multifunctional siNA) molecule, wherein the multifunctional siNA comprises two complementary nucleic acid sequences in which the first sequence comprises a first region having nucleotide sequence complementary to nucleotide sequence within a first target nucleic acid molecule, and in which the second seqeunce comprises a first region having nucleotide sequence complementary to a distinct nucleotide sequence within the same target

nucleic acid molecule. Preferably, the first region of the first sequence is also complementary to the nucleotide sequence of the second region of the second sequence, and where the first region of the second sequence is complementary to the nucleotide sequence of the second region of the first sequence. In one embodiment, the nucleic acid target sequence comprises one or more ENaC target sequences, such as any ENaC or ENaC isotype nucleic acid sequences.

[0531] In one embodiment, the invention features a multifunctional short interfering nucleic acid (multifunctional siNA) molecule, wherein the multifunctional siNA comprises two complementary nucleic acid sequences in which the first sequence comprises a first region having a nucleotide sequence complementary to a nucleotide sequence within a first target nucleic acid molecule, and in which the second seqeunce comprises a first region having a nucleotide sequence complementary to a distinct nucleotide sequence within a second target nucleic acid molecule. Preferably, the first region of the first sequence is also complementary to the nucleotide sequence of the second region of the second sequence, and where the first region of the second sequence is complementary to the nucleotide sequence of the second region of the first sequence. In one embodiment, the nucleic acid target sequence comprises one or more ENaC target sequences, such as any ENaC or ENaC isotype nucleic acid sequences.

[0532] In one embodiment, the invention features a multifunctional siNA molecule comprising a first region and a second region, where the first region comprises a nucleic acid sequence having about 18 to about 28 nucleotides complementary to a nucleic acid sequence within a first target nucleic acid molecule, and the second region comprises nucleotide sequence having about 18 to about 28 nucleotides complementary to a distinct nucleic acid sequence within a second target nucleic acid molecule. In one embodiment, the first nucleic acid target molecule and the second target nucleic acid molecule are selected from the group consisting of any of the ENaC target sequences, such as any ENaC or ENaC isotype nucleic acid sequences.

[0533] In one embodiment, the invention features a multifunctional siNA molecule comprising a first region and a second region, where the first region comprises nucleic acid sequence having about 18 to about 28 nucleotides complementary to a nucleic acid sequence within a target nucleic acid molecule, and the second region comprises nucleotide sequence having about 18 to about 28 nucleotides complementary to a distinct nucleic acid sequence

within the same target nucleic acid molecule. In one embodiment, the nucleic acid target molecule is selected from the group consisting of any ENaC target sequences, such as ENaC or any ENaC isotype nucleic acid sequences.

[0534] In one embodiment, the invention features a double stranded multifunctional short interfering nucleic acid (multifunctional siNA) molecule, wherein one strand of the multifunctional siNA comprises a first region having nucleotide sequence complementary to a first target nucleic acid sequence, and the second strand comprises a first region having a nucleotide sequence complementary to a second target nucleic acid sequence. The first and second target nucleic acid sequences can be present in separate target nucleic acid molecules or can be different regions within the same target nucleic acid molecule. As such, multifunctional siNA molecules of the invention can be used to target the expression of different genes, isotypes of the same gene, both mutant and conserved regions of one or more gene transcripts, or both coding and non-coding sequences of the same or differeing genes or gene transcripts. In one embodiment, the first nucleic acid target sequence and the second target nucleic acid sequence are selected from the group consisting of any of the ENaC target sequences, such as any ENaC or ENaC isotype nucleic acid sequences.

[0535] In one embodiment, a target nucleic acid molecule of the invention encodes a single protein. In another embodiment, a target nucleic acid molecule encodes more than one protein (*e.g.*, 1, 2, 3, 4, 5 or more proteins). As such, a multifunctional siNA construct of the invention can be used to down regulate or inhibit the expression of several proteins (e.g., any of ENaC, any ENaC isotype protein or any combination thereof). For example, a multifunctional siNA molecule comprising a region in one strand having nucleotide sequence complementarity to a first target nucleic acid sequence derived from an ENaC target, such as any of ENaC or a isotype of ENaC, and the second strand comprising a region with nucleotide sequence complementarity to a second ENaC target, such as any of ENaC or a isotype of ENaC, which can be used to down regulate, inhibit, or shut down a particular biologic pathway by targeting multiple ENaC genes.

[0536] In one embodiment the invention takes advantage of conserved nucleotide sequences present in different ENaC isoforms, such as any of ENaC or isotypes thereof. By designing multifunctional siNAs in a manner where one strand includes a sequence that is complementary to a target nucleic acid sequence conserved among various ENaC family members and the other strand optionally includes sequence that is complementary to ENaC

pathway target nucleic acid sequences, it is possible to selectively and effectively modulate or inhibit an ENaC disease related biological pathway using a single multifunctional siNA.

[0537] In one embodiment, a multifunctional short interfering nucleic acid (multifunctional siNA) of the invention comprises a first region and a second region, wherein the first region comprises nucleotide sequence complementary to a first ENaC RNA of a first ENaC target and the second region comprises nucleotide sequence complementary to a second ENaC RNA of a second ENaC target. In one embodiment, the first and second regions can comprise nucleotide sequence complementary to shared or conserved RNA sequences of differing ENaC target sites within the same ENaC isoform or shared amongst different classes of ENaC isoforms.

[0538] In one embodiment, a double stranded multifunctional siNA molecule of the invention comprises a structure having Formula MF-I:

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

wherein each 5'-p-XZX'-3' and 5'-p-YZY'-3' are independently an oligonucleotide of length about 20 nucleotides to about 300 nucleotides, preferably about 20 to about 200 nucleotides, about 20 to about 100 nucleotides, about 20 to about 40 nucleotides, about 20 to about 40 nucleotides, about 24 to about 38 nucleotides, or about 26 to about 38 nucleotides; XZ comprises a nucleic acid sequence that is complementary to a first ENaC target nucleic acid sequence; YZ is an oligonucleotide comprising nucleic acid sequence that is complementary to a second ENaC target nucleic acid sequence; Z comprises nucleotide sequence of length about 1 to about 24 nucleotides (*e.g.*, about 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, or 24 nucleotides) that is self complementary; X comprises nucleotide sequence of length about 1 to about 100 nucleotides, preferably about 1 to about 21 nucleotides (*e.g.*, about 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, or 21 nucleotides) that is complementary to nucleotide sequence present in region Y'; Y comprises nucleotide sequence of length about 1 to about 100 nucleotides, preferably about 1 to about 21 nucleotides (*e.g.*, about 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20 or 21 nucleotides) that is complementary to nucleotide sequence present in region X'; each p comprises a terminal phosphate group that is independently present or absent; each XZ and YZ is independently of length sufficient to stably interact (i.e., base pair) with the first

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

[0539] In one embodiment, a multifunctional siNA molecule of the invention comprises a structure having Formula MF-II:

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

wherein each 5'-p-XX'-3' and 5'-p-YY'-3' are independently an oligonucleotide of length of about 20 nucleotides to about 300 nucleotides, preferably about 20 to about 200 nucleotides, about 20 to about 100 nucleotides, about 20 to about 40 nucleotides, about 20 to about 40 nucleotides, about 24 to about 38 nucleotides, or about 26 to about 38 nucleotides; X

comprises a nucleic acid sequence that is complementary to a first target nucleic acid sequence; Y is an oligonucleotide comprising nucleic acid sequence that is complementary to a second target nucleic acid sequence; X comprises a nucleotide sequence of length about 1 to about 100 nucleotides, preferably about 1 to about 21 nucleotides (*e.g.*, about 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, or 21 nucleotides) that is complementary to nucleotide sequence present in region Y'; Y comprises nucleotide sequence of length about 1 to about 100 nucleotides, preferably about 1 to about 21 nucleotides (*e.g.*, about 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20 or 21 nucleotides) that is complementary to nucleotide sequence present in region X'; each p comprises a terminal phosphate group that is independently present or absent; each X and Y independently is of length sufficient to stably interact (i.e., base pair) with the first and second target nucleic acid sequence, respectively, or a portion thereof. For example, each sequence X and Y can independently comprise sequence from about 12 to about 21 or more nucleotides in length (*e.g.*, about 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, or more) that is complementary to a target nucleotide sequence in different target nucleic acid molecules, such as ENaC target RNAs or a portion thereof. In one embodiment, the first ENaC target nucleic acid sequence and the second ENaC target nucleic acid sequence are present in the same target nucleic acid molecule (*e.g.*, ENaC target RNA or ENaC pathway target RNA). In another embodiment, the first ENaC target nucleic acid sequence and the second ENaC target nucleic acid sequence are present in different target nucleic acid molecules (*e.g.*, ENaC target RNA and ENaC pathway target RNA). In one embodiment, Z comprises a palindrome or a repeat sequence. In one embodiment, the lengths of oligonucleotides X and X' are identical. In another embodiment, the lengths of oligonucleotides X and X' are not identical. In one embodiment, the lengths of oligonucleotides Y and Y' are identical. In another embodiment, the lengths of oligonucleotides Y and Y' are not identical. In one embodiment, the double stranded oligonucleotide construct of Formula I(a) includes one or more, specifically 1, 2, 3 or 4, mismatches, to the extent such mismatches do not significantly diminish the ability of the double stranded oligonucleotide to inhibit target gene expression.

[0540] In one embodiment, a multifunctional siNA molecule of the invention comprises a structure having Formula MF-III:

X X'
Y'-W-Y

wherein each X, X', Y, and Y' is independently an oligonucleotide of length about 15 nucleotides to about 50 nucleotides, preferably about 18 to about 40 nucleotides, or about 19 to about 23 nucleotides; X comprises nucleotide sequence that is complementary to nucleotide sequence present in region Y'; X' comprises nucleotide sequence that is complementary to nucleotide sequence present in region Y; each X and X' is independently of length sufficient to stably interact (i.e., base pair) with a first and a second ENaC target nucleic acid sequence, respectively, or a portion thereof; W represents a nucleotide or non-nucleotide linker that connects sequences Y' and Y; and the multifunctional siNA directs cleavage of the first and second ENaC target sequence via RNA interference. In one embodiment, the first ENaC target nucleic acid sequence and the second ENaC target nucleic acid sequence are present in the same target nucleic acid molecule (*e.g.*, ENaC target RNA or ENaC pathway target RNA). In another embodiment, the first ENaC target nucleic acid sequence and the second ENaC target nucleic acid sequence are present in different target nucleic acid molecules or a portion thereof. (*e.g.*, ENaC target RNA and ENaC pathway target RNA). In one embodiment, region W connects the 3'-end of sequence Y' with the 3'-end of sequence Y. In one embodiment, region W connects the 3'-end of sequence Y' with the 5'-end of sequence Y. In one embodiment, region W connects the 5'-end of sequence Y' with the 5'-end of sequence Y. In one embodiment, region W connects the 5'-end of sequence Y' with the 3'-end of sequence Y. In one embodiment, a terminal phosphate group is present at the 5'-end of sequence X. In one embodiment, a terminal phosphate group is present at the 5'-end of sequence X'. In one embodiment, a terminal phosphate group is present at the 5'-end of sequence Y. In one embodiment, a terminal phosphate group is present at the 5'-end of sequence Y'. In one embodiment, W connects sequences Y and Y' via a biodegradable linker. In one embodiment, W further comprises a conjugate, label, aptamer, ligand, lipid, or polymer.

[0541] In one embodiment, a multifunctional siNA molecule of the invention comprises a structure having Formula MF-IV:

X X'
Y'-W-Y

wherein each X, X', Y, and Y' is independently an oligonucleotide of length of about 15 nucleotides to about 50 nucleotides, preferably about 18 to about 40 nucleotides, or about 19 to about 23 nucleotides; X comprises nucleotide sequence that is complementary to

nucleotide sequence present in region Y'; X' comprises nucleotide sequence that is complementary to nucleotide sequence present in region Y; each Y and Y' is independently of length sufficient to stably interact (i.e., base pair) with a first and a second ENaC target nucleic acid sequence, respectively, or a portion thereof; W represents a nucleotide or non-nucleotide linker that connects sequences Y' and Y; and the multifunctional siNA directs cleavage of the first and second ENaC target sequence via RNA interference. In one embodiment, the first ENaC target nucleic acid sequence and the second ENaC target nucleic acid sequence are present in the same target nucleic acid molecule (*e.g.*, ENaC target RNA or ENaC pathway target RNA). In another embodiment, the first ENaC target nucleic acid sequence and the second ENaC target nucleic acid sequence are present in different target nucleic acid molecules or a portion thereof (*e.g.*, ENaC target RNA and ENaC pathway target RNA). In one embodiment, region W connects the 3'-end of sequence Y' with the 3'-end of sequence Y. In one embodiment, region W connects the 3'-end of sequence Y' with the 5'-end of sequence Y. In one embodiment, region W connects the 5'-end of sequence Y' with the 5'-end of sequence Y. In one embodiment, region W connects the 5'-end of sequence Y' with the 3'-end of sequence Y. In one embodiment, a terminal phosphate group is present at the 5'-end of sequence X. In one embodiment, a terminal phosphate group is present at the 5'-end of sequence X'. In one embodiment, a terminal phosphate group is present at the 5'-end of sequence Y. In one embodiment, a terminal phosphate group is present at the 5'-end of sequence Y'. In one embodiment, W connects sequences Y and Y' via a biodegradable linker. In one embodiment, W further comprises a conjugate, label, aptamer, ligand, lipid, or polymer.

[0542] In one embodiment, a multifunctional siNA molecule of the invention comprises a structure having Formula MF-V:

X X'
Y'-W-Y

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

or fourth ENaC target nucleic acid sequence, respectively, or a portion thereof; W represents a nucleotide or non-nucleotide linker that connects sequences Y' and Y; and the multifunctional siNA directs cleavage of the first, second, third, and/or fourth target sequence via RNA interference. In one embodiment, the first, second, third and fourth ENaC target nucleic acid sequence are all present in the same target nucleic acid molecule (*e.g.*, ENaC target RNA or ENaC pathway target RNA). In another embodiment, the first, second, third and fourth ENaC target nucleic acid sequence are independently present in different target nucleic acid molecules or a portion thereof (*e.g.*, ENaC target RNA and ENaC pathway target RNA). In one embodiment, region W connects the 3'-end of sequence Y' with the 3'-end of sequence Y. In one embodiment, region W connects the 3'-end of sequence Y' with the 5'-end of sequence Y. In one embodiment, region W connects the 5'-end of sequence Y' with the 5'-end of sequence Y. In one embodiment, region W connects the 5'-end of sequence Y' with the 3'-end of sequence Y. In one embodiment, a terminal phosphate group is present at the 5'-end of sequence X. In one embodiment, a terminal phosphate group is present at the 5'-end of sequence X'. In one embodiment, a terminal phosphate group is present at the 5'-end of sequence Y. In one embodiment, a terminal phosphate group is present at the 5'-end of sequence Y'. In one embodiment, W connects sequences Y and Y' via a biodegradable linker. In one embodiment, W further comprises a conjugate, label, aptamer, ligand, lipid, or polymer.

[0543] In one embodiment, regions X and Y of multifunctional siNA molecule of the invention (*e.g.*, having any of Formula MF-I - MF-V), are complementary to different target nucleic acid sequences that are portions of the same target nucleic acid molecule. In one embodiment, such target nucleic acid sequences are at different locations within the coding region of a RNA transcript. In one embodiment, such target nucleic acid sequences comprise coding and non-coding regions of the same RNA transcript. In one embodiment, such target nucleic acid sequences comprise regions of alternately spliced transcripts or precursors of such alternately spliced transcripts.

[0544] In one embodiment, a multifunctional siNA molecule having any of Formula MF-I - MF-V can comprise chemical modifications as described herein without limitation, such as, for example, nucleotides having any of Formulae I-VII described herein, stabilization chemistries as described in **Table 8**, or any other combination of modified nucleotides and non-nucleotides as described in the various embodiments herein.

[0545] In one embodiment, the palidrome or repeat sequence or modified nucleotide (*e.g.*, nucleotide with a modified base, such as 2-amino purine or a universal base) in Z of multifunctional siNA constructs having Formula MF-I or MF-II comprises chemically modified nucleotides that are able to interact with a portion of the target nucleic acid sequence (*e.g.*, modified base analogs that can form Watson Crick base pairs or non-Watson Crick base pairs).

[0546] In one embodiment, a multifunctional siNA molecule of the invention, for example each strand of a multifunctional siNA having MF-I – MF-V, independently comprises about 15 to about 40 nucleotides (*e.g.*, about 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36, 37, 38, 39, or 40 nucleotides). In one embodiment, a multifunctional siNA molecule of the invention comprises one or more chemical modifications. In a non-limiting example, the introduction of chemically modified nucleotides and/or non-nucleotides into nucleic acid molecules of the invention provides a powerful tool in overcoming potential limitations of *in vivo* stability and bioavailability inherent to unmodified RNA molecules that are delivered exogenously. For example, the use of chemically modified nucleic acid molecules can enable a lower dose of a particular nucleic acid molecule for a given therapeutic effect since chemically modified nucleic acid molecules tend to have a longer half-life in serum or in cells or tissues. Furthermore, certain chemical modifications can improve the bioavailability and/or potency of nucleic acid molecules by not only enhancing half-life but also facilitating the targeting of nucleic acid molecules to particular organs, cells or tissues and/or improving cellular uptake of the nucleic acid molecules. Therefore, even if the activity of a chemically modified nucleic acid molecule is reduced *in vitro* as compared to a native/unmodified nucleic acid molecule, for example when compared to an unmodified RNA molecule, the overall activity of the modified nucleic acid molecule can be greater than the native or unmodified nucleic acid molecule due to improved stability, potency, duration of effect, bioavailability and/or delivery of the molecule.

[0547] In another embodiment, the invention features multifunctional siNAs, wherein the multifunctional siNAs are assembled from two separate double-stranded siNAs, with one of the ends of each sense strand is tethered to the end of the sense strand of the other siNA molecule, such that the two antisense siNA strands are annealed to their corresponding sense strand that are tethered to each other at one end (see **Figure 19**). The tethers or linkers can

be nucleotide-based linkers or non-nucleotide based linkers as generally known in the art and as described herein.

[0548] In one embodiment, the invention features a multifunctional siNA, wherein the multifunctional siNA is assembled from two separate double-stranded siNAs, with the 5'-end of one sense strand of the siNA is tethered to the 5'- end of the sense strand of the other siNA molecule, such that the 5'-ends of the two antisense siNA strands, annealed to their corresponding sense strand that are tethered to each other at one end, point away (in the opposite direction) from each other (see **Figure 19 (A)**). The tethers or linkers can be nucleotide-based linkers or non-nucleotide based linkers as generally known in the art and as described herein.

[0549] In one embodiment, the invention features a multifunctional siNA, wherein the multifunctional siNA is assembled from two separate double-stranded siNAs, with the 3'-end of one sense strand of the siNA is tethered to the 3'- end of the sense strand of the other siNA molecule, such that the 5'-ends of the two antisense siNA strands, annealed to their corresponding sense strand that are tethered to each other at one end, face each other (see **Figure 19 (B)**). The tethers or linkers can be nucleotide-based linkers or non-nucleotide based linkers as generally known in the art and as described herein.

[0550] In one embodiment, the invention features a multifunctional siNA, wherein the multifunctional siNA is assembled from two separate double-stranded siNAs, with the 5'-end of one sense strand of the siNA is tethered to the 3'- end of the sense strand of the other siNA molecule, such that the 5'-end of the one of the antisense siNA strands annealed to their corresponding sense strand that are tethered to each other at one end, faces the 3'-end of the other antisense strand (see **Figure 19 (C-D)**). The tethers or linkers can be nucleotide-based linkers or non-nucleotide based linkers as generally known in the art and as described herein.

[0551] In one embodiment, the invention features a multifunctional siNA, wherein the multifunctional siNA is assembled from two separate double-stranded siNAs, with the 5'-end of one antisense strand of the siNA is tethered to the 3'- end of the antisense strand of the other siNA molecule, such that the 5'-end of the one of the sense siNA strands annealed to their corresponding antisense sense strand that are tethered to each other at one end, faces the 3'-end of the other sense strand (see **Figure 19 (G-H)**). In one embodiment, the linkage between the 5'-end of the first antisense strand and the 3'-end of the second antisense strand

is designed in such a way as to be readily cleavable (*e.g.*, biodegradable linker) such that the 5'end of each antisense strand of the multifunctional siNA has a free 5'-end suitable to mediate RNA interefence-based cleavage of the target RNA. The tethers or linkers can be nucleotide-based linkers or non-nucleotide based linkers as generally known in the art and as described herein.

[0552] In one embodiment, the invention features a multifunctional siNA, wherein the multifunctional siNA is assembled from two separate double-stranded siNAs, with the 5'-end of one antisense strand of the siNA is tethered to the 5'- end of the antisense strand of the other siNA molecule, such that the 3'-end of the one of the sense siNA strands annealed to their corresponding antisense sense strand that are tethered to each other at one end, faces the 3'-end of the other sense strand (see **Figure 19 (E)**). In one embodiment, the linkage between the 5'-end of the first antisense strand and the 5'-end of the second antisense strand is designed in such a way as to be readily cleavable (*e.g.*, biodegradable linker) such that the 5'end of each antisense strand of the multifunctional siNA has a free 5'-end suitable to mediate RNA interefence-based cleavage of the target RNA. The tethers or linkers can be nucleotide-based linkers or non-nucleotide based linkers as generally known in the art and as described herein.

[0553] In one embodiment, the invention features a multifunctional siNA, wherein the multifunctional siNA is assembled from two separate double-stranded siNAs, with the 3'-end of one antisense strand of the siNA is tethered to the 3'- end of the antisense strand of the other siNA molecule, such that the 5'-end of the one of the sense siNA strands annealed to their corresponding antisense sense strand that are tethered to each other at one end, faces the 3'-end of the other sense strand (see **Figure 19 (F)**). In one embodiment, the linkage between the 5'-end of the first antisense strand and the 5'-end of the second antisense strand is designed in such a way as to be readily cleavable (*e.g.*, biodegradable linker) such that the 5'end of each antisense strand of the multifunctional siNA has a free 5'-end suitable to mediate RNA interefence-based cleavage of the target RNA. The tethers or linkers can be nucleotide-based linkers or non-nucleotide based linkers as generally known in the art and as described herein.

[0554] In any of the above embodiments, a first target nucleic acid sequence or second target nucleic acid sequence can independently comprise ENaC and/or a isotype of ENaC. In any of the above embodiments, a first target nucleic acid sequence or second target nucleic

acid sequence can independently comprise ENaC or a isotype of ENaC RNA. In one embodiment, the first ENaC target nucleic acid sequence is an ENaC target RNA, or a portion thereof and the second ENaC target nucleic acid sequence is an ENaC pathway target RNA or DNA. In one embodiment, the first target nucleic acid sequence is a target RNA, DNA or a portion thereof and the second target nucleic acid sequence is a another RNA, DNA of a portion thereof.

[0555] In one embodiment, in any of the embodiments herein the first target sequence is an ENaC target sequence or a portion thereof and the second target sequence is an ENaC target sequence or a portion thereof. In one embodiment, in any of the embodiments herein the first target sequence is an ENaC (e.g., any of ENaC or ENaC isotypes) target sequence or a portion thereof and the second target sequence is an ENaC (e.g any of ENaC or ENaC isotypes) target sequence or a portion thereof.

[0556] Synthesis of Nucleic Acid Molecules

[0557] Synthesis of nucleic acids greater than 100 nucleotides in length is difficult using automated methods, and the therapeutic cost of such molecules is prohibitive. In this invention, small nucleic acid motifs ("small" refers to nucleic acid motifs no more than 100 nucleotides in length, preferably no more than 80 nucleotides in length, and most preferably no more than 50 nucleotides in length; *e.g.*, individual siNA oligonucleotide sequences or siNA sequences synthesized in tandem) are preferably used for exogenous delivery. The simple structure of these molecules increases the ability of the nucleic acid to invade targeted regions of protein and/or RNA structure. Exemplary molecules of the instant invention are chemically synthesized, and others can similarly be synthesized.

[0558] 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 9** outlines the amounts and the contact times of the reagents used in the synthesis cycle. Alternatively, syntheses at the 0.2 µmol scale can be performed on a 96-well plate synthesizer, such as the instrument produced by Protogene (Palo Alto, CA) with minimal modification to the cycle. A 33-fold excess (60 µL of 0.11 M = 6.6 µmol) of 2'-O-methyl phosphoramidite and a 105-fold excess of S-ethyl tetrazole (60 µL of 0.25 M = 15 µmol) can be used in each coupling cycle of 2'-O-methyl residues relative to polymer-bound 5'-hydroxyl. A 22-fold excess (40 µL of 0.11 M = 4.4 µmol) of deoxy phosphoramidite and a 70-fold excess of S-ethyl tetrazole (40 µL of 0.25 M = 10 µmol) can be used in each coupling cycle of deoxy residues relative to polymer-bound 5'-hydroxyl. Average coupling yields on the 394 Applied Biosystems, Inc. synthesizer, determined by colorimetric quantitation of the trityl fractions, are typically 97.5-99%. Other oligonucleotide synthesis reagents for the 394 Applied Biosystems, Inc. synthesizer include the following: detritylation solution is 3% TCA in methylene chloride (ABI); capping is performed with 16% *N*-methyl imidazole in THF (ABI) and 10% acetic anhydride/10% 2,6-lutidine in THF (ABI); and oxidation solution is 16.9 mM I_2, 49 mM pyridine, 9% water in THF (PerSeptive Biosystems, Inc.). Burdick & Jackson Synthesis Grade acetonitrile is used directly from the reagent bottle. S-Ethyltetrazole solution (0.25 M in acetonitrile) is made up from the solid obtained from American International Chemical, Inc. Alternately, for the introduction of phosphorothioate linkages, Beaucage reagent (3H-1,2-Benzodithiol-3-one 1,1-dioxide, 0.05 M in acetonitrile) is used.

[0559] Deprotection of the DNA-based oligonucleotides is performed as follows: the polymer-bound trityl-on oligoribonucleotide is transferred to a 4 mL glass screw top vial and suspended in a solution of 40% aqueous methylamine (1 mL) at 65 °C for 10 minutes. After cooling to –20 °C, the supernatant is removed from the polymer support. The support is washed three times with 1.0 mL of EtOH:MeCN:H2O/3:1:1, vortexed and the supernatant is then added to the first supernatant. The combined supernatants, containing the oligoribonucleotide, are dried to a white powder. In one embodiment, the nucleic acid molecules of the invention are synthesized, deprotected, and analyzed according to methods described in US 6,995,259, US 6,686,463, US 6,673,918, US 6,649,751, US 6,989,442, and USSN 10/190,359, all incorporated by reference herein in their entirety.

[0560] The method of synthesis used for RNA including certain siNA molecules of the invention follows the procedure as described in Usman *et al.,* 1987, *J. Am. Chem. Soc.*, 109, 7845; Scaringe *et al.,* 1990, *Nucleic Acids Res.*, 18, 5433; and Wincott *et al.,* 1995, *Nucleic Acids Res.* 23, 2677-2684 Wincott *et al.,* 1997, *Methods Mol. Bio.*, 74, 59, and makes use of common nucleic acid protecting and coupling groups, such as dimethoxytrityl at the 5'-end, and phosphoramidites at the 3'-end. In a non-limiting example, small scale syntheses are conducted on a 394 Applied Biosystems, Inc. synthesizer using a 0.2 µmol scale protocol with a 7.5 min coupling step for alkylsilyl protected nucleotides and a 2.5 min coupling step for 2'-O-methylated nucleotides. **Table 9** outlines the amounts and the contact times of the reagents used in the synthesis cycle. Alternatively, syntheses at the 0.2 µmol scale can be done on a 96-well plate synthesizer, such as the instrument produced by Protogene (Palo Alto, CA) with minimal modification to the cycle. A 33-fold excess (60 µL of 0.11 M = 6.6 µmol) of 2'-O-methyl phosphoramidite and a 75-fold excess of S-ethyl tetrazole (60 µL of 0.25 M = 15 µmol) can be used in each coupling cycle of 2'-O-methyl residues relative to polymer-bound 5'-hydroxyl. A 66-fold excess (120 µL of 0.11 M = 13.2 µmol) of alkylsilyl (ribo) protected phosphoramidite and a 150-fold excess of S-ethyl tetrazole (120 µL of 0.25 M = 30 µmol) can be used in each coupling cycle of ribo residues relative to polymer-bound 5'-hydroxyl. Average coupling yields on the 394 Applied Biosystems, Inc. synthesizer, determined by colorimetric quantitation of the trityl fractions, are typically 97.5-99%. Other oligonucleotide synthesis reagents for the 394 Applied Biosystems, Inc. synthesizer include the following: detritylation solution is 3% TCA in methylene chloride (ABI); capping is performed with 16% *N*-methyl imidazole in THF (ABI) and 10% acetic anhydride/10% 2,6-lutidine in THF (ABI); oxidation solution is 16.9 mM I_2, 49 mM pyridine, 9% water in THF (PerSeptive Biosystems, Inc.). Burdick & Jackson Synthesis Grade acetonitrile is used directly from the reagent bottle. S-Ethyltetrazole solution (0.25 M in acetonitrile) is made up from the solid obtained from American International Chemical, Inc. Alternately, for the introduction of phosphorothioate linkages, Beaucage reagent (3H-1,2-Benzodithiol-3-one 1,1-dioxide0.05 M in acetonitrile) is used.

[0561] Deprotection of the RNA is performed using either a two-pot or one-pot protocol. For the two-pot protocol, the polymer-bound trityl-on oligoribonucleotide is transferred to a 4 mL glass screw top vial and suspended in a solution of 40% aq. methylamine (1 mL) at 65 °C for 10 min. After cooling to –20 °C, the supernatant is removed from the polymer support. The support is washed three times with 1.0 mL of EtOH:MeCN:H2O/3:1:1, vortexed and the

supernatant is then added to the first supernatant. The combined supernatants, containing the oligoribonucleotide, are dried to a white powder. The base deprotected oligoribonucleotide is resuspended in anhydrous TEA/HF/NMP solution (300 µL of a solution of 1.5 mL N-methylpyrrolidinone, 750 µL TEA and 1 mL TEA•3HF to provide a 1.4 M HF concentration) and heated to 65 °C. After 1.5 h, the oligomer is quenched with 1.5 M NH_4HCO_3. In one embodiment, the nucleic acid molecules of the invention are synthesized, deprotected, and analyzed according to methods described in US 6,995,259, US 6,686,463, US 6,673,918, US 6,649,751, US 6,989,442, and USSN 10/190,359, all incorporated by reference herein in their entirety.

[0562] Alternatively, for the one-pot protocol, the polymer-bound trityl-on oligoribonucleotide is transferred to a 4 mL glass screw top vial and suspended in a solution of 33% ethanolic methylamine/DMSO: 1/1 (0.8 mL) at 65 °C for 15 minutes. The vial is brought to room temperature TEA•3HF (0.1 mL) is added and the vial is heated at 65 °C for 15 minutes. The sample is cooled at –20 °C and then quenched with 1.5 M NH_4HCO_3.

[0563] For purification of the trityl-on oligomers, the quenched NH_4HCO_3 solution is loaded onto a C-18 containing cartridge that had been prewashed with acetonitrile followed by 50 mM TEAA. After washing the loaded cartridge with water, the RNA is detritylated with 0.5% TFA for 13 minutes. The cartridge is then washed again with water, salt exchanged with 1 M NaCl and washed with water again. The oligonucleotide is then eluted with 30% acetonitrile.

[0564] The average stepwise coupling yields are typically >98% (Wincott *et al.,* 1995 *Nucleic Acids Res.* 23, 2677-2684). Those of ordinary skill in the art will recognize that the scale of synthesis can be adapted to be larger or smaller than the example described above including but not limited to 96-well format.

[0565] Alternatively, the nucleic acid molecules of the present invention can be synthesized separately and joined together post-synthetically, for example, by ligation (Moore *et al.,* 1992, *Science* 256, 9923; Draper *et al.,* International PCT publication No. WO 93/23569; Shabarova *et al.,* 1991, *Nucleic Acids Research* 19, 4247; Bellon *et al.,* 1997, *Nucleosides & Nucleotides*, 16, 951; Bellon *et al.,* 1997, *Bioconjugate Chem.* 8, 204), or by hybridization following synthesis and/or deprotection.

[0566] The siNA molecules of the invention can also be synthesized via a tandem synthesis methodology as described in Example 1 herein, wherein both siNA strands are synthesized as a single contiguous oligonucleotide fragment or strand separated by a cleavable linker which is subsequently cleaved to provide separate siNA fragments or strands that hybridize and permit purification of the siNA duplex. The linker can be a polynucleotide linker or a non-nucleotide linker. The tandem synthesis of siNA as described herein can be readily adapted to both multiwell/multiplate synthesis platforms such as 96 well or similarly larger multi-well platforms. The tandem synthesis of siNA as described herein can also be readily adapted to large scale synthesis platforms employing batch reactors, synthesis columns and the like.

[0567] 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.

[0568] The nucleic acid molecules of the present invention can be modified extensively to enhance stability by modification with nuclease resistant groups, for example, 2'-amino, 2'-*C*-allyl, 2'-fluoro, 2'-*O*-methyl, 2'-H (for a review see Usman and Cedergren, 1992, *TIBS* 17, 34; Usman *et al.,* 1994, *Nucleic Acids Symp. Ser.* 31, 163). siNA constructs can be purified by gel electrophoresis using general methods or can be purified by high pressure liquid chromatography (HPLC; see Wincott *et al., supra*, the totality of which is hereby incorporated herein by reference) and re-suspended in water.

[0569] In another aspect of the invention, siNA molecules of the invention are expressed from transcription units inserted into DNA or RNA vectors. The recombinant vectors can be DNA plasmids or viral vectors. siNA expressing viral vectors can be constructed based on, but not limited to, adeno-associated virus, retrovirus, adenovirus, or alphavirus. The recombinant vectors capable of expressing the siNA molecules can be delivered as described herein, and persist in target cells. Alternatively, viral vectors can be used that provide for transient expression of siNA molecules.

Optimizing Activity of the nucleic acid molecule of the invention.

[0570] Chemically synthesizing nucleic acid molecules with modifications (base, sugar and/or phosphate) can prevent their degradation by serum ribonucleases, which can increase their potency (see *e.g.,* Eckstein *et al.,* International Publication No. WO 92/07065; Perrault

et al., 1990 *Nature* 344, 565; Pieken *et al.,* 1991, *Science* 253, 314; Usman and Cedergren, 1992, *Trends in Biochem. Sci.* 17, 334; Usman *et al.,* International Publication No. WO 93/15187; and Rossi *et al.,* International Publication No. WO 91/03162; Sproat, U.S. Pat. No. 5,334,711; Gold *et al.,* U.S. Pat. No. 6,300,074; and Burgin *et al., supra;* all of which are incorporated by reference herein). All of the above references describe various chemical modifications that can be made to the base, phosphate and/or sugar moieties of the nucleic acid molecules described herein. Modifications that enhance their efficacy in cells, and removal of bases from nucleic acid molecules to shorten oligonucleotide synthesis times and reduce chemical requirements are desired.

[0571] There are several examples in the art describing sugar, base and phosphate modifications that can be introduced into nucleic acid molecules with significant enhancement in their nuclease stability and efficacy. For example, oligonucleotides are modified to enhance stability and/or enhance biological activity by modification with nuclease resistant groups, for example, 2'-amino, 2'-*C*-allyl, 2'-fluoro, 2'-*O*-methyl, 2'-O-allyl, 2'-H, nucleotide base modifications (for a review see Usman and Cedergren, 1992, *TIBS.* 17, 34; Usman *et al.,* 1994, *Nucleic Acids Symp. Ser.* 31, 163; Burgin *et al.,* 1996, *Biochemistry,* 35, 14090). Sugar modification of nucleic acid molecules have been extensively described in the art (see Eckstein *et al., International Publication* PCT No. WO 92/07065; Perrault *et al. Nature*, 1990, *344*, 565-568; Pieken *et al. Science,* 1991, *253*, 314-317; Usman and Cedergren, *Trends in Biochem. Sci.*, 1992, *17*, 334-339; Usman *et al. International Publication* PCT No. WO 93/15187; Sproat, *U.S. Pat.* No. 5,334,711 and Beigelman *et al.,* 1995, *J. Biol. Chem.,* 270, 25702; Beigelman *et al.,* International PCT publication No. WO 97/26270; Beigelman *et al.,* U.S. Pat. No. 5,716,824; Usman *et al.,* U.S. Pat. No. 5,627,053; Woolf *et al.,* International PCT Publication No. WO 98/13526; Thompson *et al.,* USSN 60/082,404 which was filed on April 20, 1998; Karpeisky *et al.,* 1998, *Tetrahedron Lett.*, 39, 1131; Earnshaw and Gait, 1998, *Biopolymers (Nucleic Acid Sciences),* 48, 39-55; Verma and Eckstein, 1998, *Annu. Rev. Biochem.,* 67, 99-134; and Burlina *et al.,* 1997, *Bioorg. Med. Chem.,* 5, 1999-2010; all of the references are hereby incorporated in their totality by reference herein). Such publications describe general methods and strategies to determine the location of incorporation of sugar, base and/or phosphate modifications and the like into nucleic acid molecules without modulating catalysis, and are incorporated by reference herein. In view of such teachings, similar modifications can be used as described herein to

modify the siNA nucleic acid molecules of the instant invention so long as the ability of siNA to promote RNAi is cells is not significantly inhibited.

[0572] In one embodiment, a nucleic acid molecule of the invention is chemically modified as described in US 20050020521, incorporated by reference herein in its entirety.

[0573] While chemical modification of oligonucleotide internucleotide linkages with phosphorothioate, phosphorodithioate, and/or 5'-methylphosphonate linkages improves stability, excessive modifications can cause some toxicity or decreased activity. Therefore, when designing nucleic acid molecules, the amount of these internucleotide linkages should be minimized. The reduction in the concentration of these linkages should lower toxicity, resulting in increased efficacy and higher specificity of these molecules.

[0574] Short interfering nucleic acid (siNA) molecules having chemical modifications that maintain or enhance activity are provided. Such a nucleic acid is also generally more resistant to nucleases than an unmodified nucleic acid. Accordingly, the *in vitro* and/or *in vivo* activity should not be significantly lowered. In cases in which modulation is the goal, therapeutic nucleic acid molecules delivered exogenously should optimally be stable within cells until translation of the target RNA has been modulated long enough to reduce the levels of the undesirable protein. This period of time varies between hours to days depending upon the disease state. Improvements in the chemical synthesis of RNA and DNA (Wincott *et al.*, 1995, *Nucleic Acids Res.* 23, 2677; Caruthers *et al.*, 1992, *Methods in Enzymology* 211, 3-19 (incorporated by reference herein)) have expanded the ability to modify nucleic acid molecules by introducing nucleotide modifications to enhance their nuclease stability, as described above.

[0575] In one embodiment, nucleic acid molecules of the invention include one or more (*e.g.*, about 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, or more) G-clamp nucleotides. A G-clamp nucleotide is a modified cytosine analog wherein the modifications confer the ability to hydrogen bond both Watson-Crick and Hoogsteen faces of a complementary guanine within a duplex, see for example Lin and Matteucci, 1998, *J. Am. Chem. Soc.*, 120, 8531-8532. A single G-clamp analog substitution within an oligonucleotide can result in substantially enhanced helical thermal stability and mismatch discrimination when hybridized to complementary oligonucleotides. The inclusion of such nucleotides in nucleic acid molecules of the invention results in both enhanced affinity and specificity to nucleic acid targets,

complementary sequences, or template strands. In another embodiment, nucleic acid molecules of the invention include one or more (*e.g.*, about 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, or more) LNA "locked nucleic acid" nucleotides such as a 2', 4'-C methylene bicyclo nucleotide (see for example Wengel *et al.,* International PCT Publication No. WO 00/66604 and WO 99/14226).

[0576] In another embodiment, the invention features conjugates and/or complexes of siNA molecules of the invention. Such conjugates and/or complexes can be used to facilitate delivery of siNA molecules into a biological system, such as a cell. The conjugates and complexes provided by the instant invention can impart therapeutic activity by transferring therapeutic compounds across cellular membranes, altering the pharmacokinetics, and/or modulating the localization of nucleic acid molecules of the invention. The present invention encompasses the design and synthesis of novel conjugates and complexes for the delivery of molecules, including, but not limited to, small molecules, lipids, cholesterol, phospholipids, nucleosides, nucleotides, nucleic acids, antibodies, toxins, negatively charged polymers and other polymers, for example proteins, peptides, hormones, carbohydrates, polyethylene glycols, or polyamines, across cellular membranes. In general, the transporters described are designed to be used either individually or as part of a multi-component system, with or without degradable linkers. These compounds are expected to improve delivery and/or localization of nucleic acid molecules of the invention into a number of cell types originating from different tissues, in the presence or absence of serum (see Sullenger and Cech, U.S. Pat. No. 5,854,038). Conjugates of the molecules described herein can be attached to biologically active molecules via linkers that are biodegradable, such as biodegradable nucleic acid linker molecules.

[0577] The term "biodegradable linker" as used herein, refers to a nucleic acid or non-nucleic acid linker molecule that is designed as a biodegradable linker to connect one molecule to another molecule, for example, a biologically active molecule to a siNA molecule of the invention or the sense and antisense strands of a siNA molecule of the invention. The biodegradable linker is designed such that its stability can be modulated for a particular purpose, such as delivery to a particular tissue or cell type. The stability of a nucleic acid-based biodegradable linker molecule can be modulated by using various chemistries, for example combinations of ribonucleotides, deoxyribonucleotides, and chemically-modified nucleotides, such as 2'-O-methyl, 2'-fluoro, 2'-amino, 2'-O-amino, 2'-C-

allyl, 2'-O-allyl, and other 2'-modified or base modified nucleotides. The biodegradable nucleic acid linker molecule can be a dimer, trimer, tetramer or longer nucleic acid molecule, for example, an oligonucleotide of about 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, or 20 nucleotides in length, or can comprise a single nucleotide with a phosphorus-based linkage, for example, a phosphoramidate or phosphodiester linkage. The biodegradable nucleic acid linker molecule can also comprise nucleic acid backbone, nucleic acid sugar, or nucleic acid base modifications.

[0578] The term "biodegradable" as used herein, refers to degradation in a biological system, for example, enzymatic degradation or chemical degradation.

[0579] The term "biologically active molecule" as used herein refers to compounds or molecules that are capable of eliciting or modifying a biological response in a system. Non-limiting examples of biologically active siNA molecules either alone or in combination with other molecules contemplated by the instant invention include therapeutically active molecules such as antibodies, cholesterol, hormones, antivirals, peptides, proteins, chemotherapeutics, small molecules, vitamins, co-factors, nucleosides, nucleotides, oligonucleotides, enzymatic nucleic acids, antisense nucleic acids, triplex forming oligonucleotides, 2,5-A chimeras, siNA, dsRNA, allozymes, aptamers, decoys and analogs thereof. Biologically active molecules of the invention also include molecules capable of modulating the pharmacokinetics and/or pharmacodynamics of other biologically active molecules, for example, lipids and polymers such as polyamines, polyamides, polyethylene glycol and other polyethers.

[0580] 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.

[0581] Therapeutic nucleic acid molecules (*e.g.,* siNA molecules) delivered exogenously optimally are stable within cells until reverse transcription of the RNA has been modulated long enough to reduce the levels of the RNA transcript. The nucleic acid molecules are resistant to nucleases in order to function as effective intracellular therapeutic agents. Improvements in the chemical synthesis of nucleic acid molecules described in the instant

invention and in the art have expanded the ability to modify nucleic acid molecules by introducing nucleotide modifications to enhance their nuclease stability as described above.

[0582] In yet another embodiment, siNA molecules having chemical modifications that maintain or enhance enzymatic activity of proteins involved in RNAi are provided. Such nucleic acids are also generally more resistant to nucleases than unmodified nucleic acids. Thus, *in vitro* and/or *in vivo* the activity should not be significantly lowered.

[0583] Use of the nucleic acid-based molecules of the invention will lead to better treatments by affording the possibility of combination therapies (*e.g.,* multiple siNA molecules targeted to different genes; nucleic acid molecules coupled with known small molecule modulators; or intermittent treatment with combinations of molecules, including different motifs and/or other chemical or biological molecules). The treatment of subjects with siNA molecules can also include combinations of different types of nucleic acid molecules, such as enzymatic nucleic acid molecules (ribozymes), allozymes, antisense, 2,5-A oligoadenylate, decoys, and aptamers.

[0584] In another aspect a siNA molecule of the invention comprises one or more 5' and/or a 3'- cap structure, for example, on only the sense siNA strand, the antisense siNA strand, or both siNA strands.

[0585] By "cap structure" is meant chemical modifications, which have been incorporated at either terminus of the oligonucleotide (see, for example, Adamic *et al.,* U.S. Pat. No. 5,998,203, incorporated by reference herein). These terminal modifications protect the nucleic acid molecule from exonuclease degradation, and can help in delivery and/or localization within a cell. The cap can be present at the 5'-terminus (5'-cap) or at the 3'-terminal (3'-cap) or can be present on both termini. In non-limiting examples, the 5'-cap includes, but is not limited to, glyceryl, inverted deoxy abasic residue (moiety); 4',5'-methylene nucleotide; 1-(beta-D-erythrofuranosyl) nucleotide, 4'-thio nucleotide; carbocyclic nucleotide; 1,5-anhydrohexitol nucleotide; L-nucleotides; alpha-nucleotides; modified base nucleotide; phosphorodithioate linkage; *threo*-pentofuranosyl nucleotide; acyclic 3',4'-seco nucleotide; acyclic 3,4-dihydroxybutyl nucleotide; acyclic 3,5-dihydroxypentyl nucleotide, 3'-3'-inverted nucleotide moiety; 3'-3'-inverted abasic moiety; 3'-2'-inverted nucleotide moiety; 3'-2'-inverted abasic moiety; 1,4-butanediol phosphate; 3'-phosphoramidate; hexylphosphate; aminohexyl phosphate; 3'-phosphate; 3'-phosphorothioate;

phosphorodithioate; or bridging or non-bridging methylphosphonate moiety. Non-limiting examples of cap moieties are shown in **Figure 10.**

[0586] Non-limiting examples of the 3'-cap include, but are not limited to, glyceryl, inverted deoxy abasic residue (moiety), 4', 5'-methylene nucleotide; 1-(beta-D-erythrofuranosyl) nucleotide; 4'-thio nucleotide, carbocyclic nucleotide; 5'-amino-alkyl phosphate; 1,3-diamino-2-propyl phosphate; 3-aminopropyl phosphate; 6-aminohexyl phosphate; 1,2-aminododecyl phosphate; hydroxypropyl phosphate; 1,5-anhydrohexitol nucleotide; L-nucleotide; alpha-nucleotide; modified base nucleotide; phosphorodithioate; *threo*-pentofuranosyl nucleotide; acyclic 3',4'-seco nucleotide; 3,4-dihydroxybutyl nucleotide; 3,5-dihydroxypentyl nucleotide, 5'-5'-inverted nucleotide moiety; 5'-5'-inverted abasic moiety; 5'-phosphoramidate; 5'-phosphorothioate; 1,4-butanediol phosphate; 5'-amino; bridging and/or non-bridging 5'-phosphoramidate, phosphorothioate and/or phosphorodithioate, bridging or non bridging methylphosphonate and 5'-mercapto moieties (for more details see Beaucage and Iyer, 1993, *Tetrahedron* 49, 1925; incorporated by reference herein).

[0587] 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.

[0588] An "alkyl" group refers to a saturated aliphatic hydrocarbon, including straight-chain, branched-chain, and cyclic alkyl groups. Preferably, the alkyl group has 1 to 12 carbons. More preferably, it is a lower alkyl of from 1 to 7 carbons, more preferably 1 to 4 carbons. The alkyl group can be substituted or unsubstituted. When substituted the substituted group(s) is preferably, hydroxyl, cyano, alkoxy, =O, =S, NO_2 or $N(CH_3)_2$, amino, or SH. The term also includes alkenyl groups that are unsaturated hydrocarbon groups containing at least one carbon-carbon double bond, including straight-chain, branched-chain, and cyclic groups. Preferably, the alkenyl group has 1 to 12 carbons. More preferably, it is a lower alkenyl of from 1 to 7 carbons, more preferably 1 to 4 carbons. The alkenyl group can be substituted or unsubstituted. When substituted the substituted group(s) is preferably, hydroxyl, cyano, alkoxy, =O, =S, NO_2, halogen, $N(CH_3)_2$, amino, or SH. The

term "alkyl" also includes alkynyl groups that have an unsaturated hydrocarbon group containing at least one carbon-carbon triple bond, including straight-chain, branched-chain, and cyclic groups. Preferably, the alkynyl group has 1 to 12 carbons. More preferably, it is a lower alkynyl of from 1 to 7 carbons, more preferably 1 to 4 carbons. The alkynyl group can be substituted or unsubstituted. When substituted the substituted group(s) is preferably, hydroxyl, cyano, alkoxy, =O, =S, NO_2 or $N(CH_3)_2$, amino or SH.

[0589] 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 can be optionally substituted. The preferred substituent(s) of aryl groups are halogen, trihalomethyl, hydroxyl, SH, OH, cyano, alkoxy, alkyl, alkenyl, alkynyl, and amino groups. An "alkylaryl" group refers to an alkyl group (as described above) covalently joined to an aryl group (as described above). Carbocyclic aryl groups are groups wherein the ring atoms on the aromatic ring are all carbon atoms. The carbon atoms are optionally substituted. Heterocyclic aryl groups are groups having from 1 to 3 heteroatoms as ring atoms in the aromatic ring and the remainder of the ring atoms are carbon atoms. Suitable heteroatoms include oxygen, sulfur, and nitrogen, and include furanyl, thienyl, pyridyl, pyrrolyl, N-lower alkyl pyrrolo, pyrimidyl, pyrazinyl, imidazolyl and the like, all optionally substituted. An "amide" refers to an -C(O)-NH-R, where R is either alkyl, aryl, alkylaryl or hydrogen. An "ester" refers to an -C(O)-OR', where R is either alkyl, aryl, alkylaryl or hydrogen.

[0590] By "nucleotide" as used herein is as recognized in the art to include natural bases (standard), and modified bases well known in the art. Such bases are generally located at the 1' position of a nucleotide sugar moiety. Nucleotides generally comprise a base, sugar and a phosphate group. The nucleotides can be unmodified or modified at the sugar, phosphate and/or base moiety, (also referred to interchangeably as nucleotide analogs, modified nucleotides, non-natural nucleotides, non-standard nucleotides and other; see, for example, Usman and McSwiggen, supra; Eckstein et al., International PCT Publication No. WO 92/07065; Usman et al., International PCT Publication No. WO 93/15187; Uhlman & Peyman, supra, all are hereby incorporated by reference herein). There are several examples of modified nucleic acid bases known in the art as summarized by Limbach et al., 1994, Nucleic Acids Res. 22, 2183. Some of the non-limiting examples of base modifications that

can be introduced into nucleic acid molecules include, inosine, purine, pyridin-4-one, pyridin-2-one, phenyl, pseudouracil, 2, 4, 6-trimethoxy benzene, 3-methyl uracil, dihydrouridine, naphthyl, aminophenyl, 5-alkylcytidines (e.g., 5-methylcytidine), 5-alkyluridines (e.g., ribothymidine), 5-halouridine (e.g., 5-bromouridine) or 6-azapyrimidines or 6-alkylpyrimidines (e.g. 6-methyluridine), propyne, and others (Burgin *et al.,* 1996, *Biochemistry*, 35, 14090; Uhlman & Peyman, *supra*). By "modified bases" in this aspect is meant nucleotide bases other than adenine, guanine, cytosine and uracil at 1' position or their equivalents.

[0591] In one embodiment, the invention features modified siNA molecules, with phosphate backbone modifications comprising one or more phosphorothioate, phosphorodithioate, methylphosphonate, phosphotriester, morpholino, amidate carbamate, carboxymethyl, acetamidate, polyamide, sulfonate, sulfonamide, sulfamate, formacetal, thioformacetal, and/or alkylsilyl, substitutions. For a review of oligonucleotide backbone modifications, see Hunziker and Leumann, 1995, *Nucleic Acid Analogues: Synthesis and Properties,* in *Modern Synthetic Methods,* VCH, 331-417, and Mesmaeker *et al.,* 1994, *Novel Backbone Replacements for Oligonucleotides,* in *Carbohydrate Modifications in Antisense Research,* ACS, 24-39.

[0592] By "abasic" is meant sugar moieties lacking a nucleobase or having a hydrogen atom (H) or other other non-nucleobase chemical groups in place of a nucleobase at the 1' position of the sugar moiety, see for example Adamic *et al.,* U.S. Pat. No. 5,998,203. In one embodiment, an abasic moiety of the invention is a ribose, deoxyribose, or dideoxyribose sugar. .

[0593] 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.

[0594] By "modified nucleoside" is meant any nucleotide base which contains a modification in the chemical structure of an unmodified nucleotide base, sugar and/or phosphate. Non-limiting examples of modified nucleotides are shown by Formulae I-VII and/or other modifications described herein.

[0595] In connection with 2'-modified nucleotides as described for the present invention, by "amino" is meant 2'-NH_2 or 2'-*O*- NH_2, which can be modified or unmodified. Such modified groups are described, for example, in Eckstein *et al.,* U.S. Pat. No. 5,672,695 and

Matulic-Adamic *et al.,* U.S. Pat. No. 6,248,878, which are both incorporated by reference in their entireties.

[0596] Various modifications to nucleic acid siNA structure can be made to enhance the utility of these molecules. Such modifications will enhance shelf-life, half-life *in vitro,* stability, and ease of introduction of such oligonucleotides to the target site, *e.g.,* to enhance penetration of cellular membranes, and confer the ability to recognize and bind to targeted cells.

Administration of Nucleic Acid Molecules

[0597] A siNA molecule of the invention can be adapted for use to treat, prevent, inhibit, or reduce respiratory, inflammatory, autoimmune diseases, traits, conditions, and phenotypes and/or any other trait, disease, condition, or phenotype that is related to or will respond to the levels of ENaC targets or ENaC pathway targets in a cell or tissue, alone or in combination with other therapies. In one embodiment, the siNA molecules of the invention and formulations or compositions thereof are administered to the lung as is described herein and as is generally known in the art. In one embodiment, the siNA molecules of the invention and formulations or compositions thereof are administered to a cell, subject, or organism as is described herein and as is generally known in the art.

[0598] In one embodiment, a siNA composition of the invention can comprise a delivery vehicle, including liposomes, for administration to a subject, carriers and diluents and their salts, and/or can be present in pharmaceutically acceptable formulations. Methods for the delivery of nucleic acid molecules are described in Akhtar *et al.,* 1992, *Trends Cell Bio.*, 2, 139; *Delivery Strategies for Antisense Oligonucleotide Therapeutics*, ed. Akhtar, 1995, Maurer *et al.,* 1999, *Mol. Membr. Biol.,* 16, 129-140; Hofland and Huang, 1999, *Handb. Exp. Pharmacol.,* 137, 165-192; and Lee *et al.,* 2000, *ACS Symp. Ser.,* 752, 184-192, all of which are incorporated herein by reference. Beigelman *et al.,* U.S. Pat. No. 6,395,713 and Sullivan *et al.,* PCT WO 94/02595 further describe the general methods for delivery of nucleic acid molecules. These protocols can be utilized for the delivery of virtually any nucleic acid molecule. Nucleic acid molecules can be administered to cells by a variety of methods known to those of skill in the art, including, but not restricted to, encapsulation in liposomes, by iontophoresis, or by incorporation into other vehicles, such as biodegradable polymers, hydrogels, cyclodextrins (see for example Gonzalez *et al.,* 1999, *Bioconjugate Chem.,* 10,

1068-1074; Wang *et al.,* International PCT publication Nos. WO 03/47518 and WO 03/46185), poly(lactic-co-glycolic)acid (PLGA) and PLCA microspheres (see for example US Patent 6,447,796 and US Patent Application Publication No. US 2002130430), biodegradable nanocapsules, and bioadhesive microspheres, or by proteinaceous vectors (O'Hare and Normand, International PCT Publication No. WO 00/53722). In another embodiment, the nucleic acid molecules of the invention can also be formulated or complexed with polyethyleneimine and derivatives thereof, such as polyethyleneimine-polyethyleneglycol-N-acetylgalactosamine (PEI-PEG-GAL) or polyethyleneimine-polyethyleneglycol-tri-N-acetylgalactosamine (PEI-PEG-triGAL) derivatives. In one embodiment, the nucleic acid molecules of the invention are formulated as described in United States Patent Application Publication No. 20030077829, incorporated by reference herein in its entirety.

[0599] In one embodiment, a siNA molecule of the invention is formulated as a composition described in U.S. Provisional patent application No. 60/678,531 and in related U.S. Provisional patent application No. 60/703,946, filed July 29, 2005, U.S. Provisional patent application No. 60/737,024, filed November 15, 2005, USSN 11/353,630, filed February 14, 2006, and USSN 11/586,102, filed October 24, 2006 (Vargeese *et al.*), all of which are incorporated by reference herein in their entirety. Such siNA formuations are generally referred to as "lipid nucleic acid particles" (LNP). In one embodiment, a siNA molecule of the invention is formulated with one or more LNP compositions described herein in **Table 10** (see USSN 11/353,630 *supra*).

[0600] In one embodiment, the siNA molecules of the invention and formulations or compositions thereof are administered to lung tissues and cells as is described in US 2006/0062758; US 2006/0014289; and US 2004/0077540.

[0601] In one embodiment, a siNA molecule of the invention is complexed with membrane disruptive agents such as those described in U.S. Patent Application Publication No. 20010007666, incorporated by reference herein in its entirety including the drawings. In another embodiment, the membrane disruptive agent or agents and the siNA molecule are also complexed with a cationic lipid or helper lipid molecule, such as those lipids described in U.S. Patent No. 6,235,310, incorporated by reference herein in its entirety including the drawings.

[0602] In one embodiment, a siNA molecule of the invention is complexed with delivery systems as described in U.S. Patent Application Publication No. 2003077829 and International PCT Publication Nos. WO 00/03683 and WO 02/087541, all incorporated by reference herein in their entirety including the drawings.

[0603] In one embodiment, a siNA molecule of the invention is complexed with delivery systems as is generally described in U.S. Patent Application Publication Nos. US-20050287551; US-20050164220; US-20050191627; US-20050118594; US-20050153919; US-20050085486; and US-20030158133; all incorporated by reference herein in their entirety including the drawings.

[0604] In one embodiment, the nucleic acid molecules of the invention are administered to skeletal tissues (e.g., bone, cartilage, tendon, ligament) or bone metastatic tumors via atelocollagen complexation or conjugation (see for example Takeshita *et al.,* 2005, PNAS, 102, 12177-12182). Therefore, in one embodiment, the instant invention features one or more dsiNA molecules as a composition complexed with atelocollagen. In another embodiment, the instant invention features one or more siNA molecules conjugated to atelocollagen via a linker as described herein or otherwise known in the art.

[0605] In one embodiment, the nucleic acid molecules of the invention and formulations thereof (e.g., LNP formulations of double stranded nucleic acid molecules of the invention) are administered via pulmonary delivery, such as by inhalation of an aerosol or spray dried formulation administered by an inhalation device or nebulizer, providing rapid local uptake of the nucleic acid molecules into relevant pulmonary tissues. Solid particulate compositions containing respirable dry particles of micronized nucleic acid compositions can be prepared by grinding dried or lyophilized nucleic acid compositions, and then passing the micronized composition through, for example, a 400 mesh screen to break up or separate out large agglomerates. A solid particulate composition comprising the nucleic acid compositions of the invention can optionally contain a dispersant which serves to facilitate the formation of an aerosol as well as other therapeutic compounds. A suitable dispersant is lactose, which can be blended with the nucleic acid compound in any suitable ratio, such as a 1 to 1 ratio by weight.

[0606] Aerosols of liquid or non-liquid particles comprising a nucleic acid composition of the invention (e.g., siNA and/or LNP formulations thereof) can be produced by any suitable

means, such as with a device comprising a nebulizer (see for example US 4,501,729, incorporated by reference herein). In one embodiment, nebulizer devices of the invention are used in applications for conscious, spontaneously breathing subjects, and for controlled ventilated subjects of all ages. Nebulizer devices of the invention can be used for targeted topical and systemic drug delivery to the lung. In one embodiment, a device comprising a nebulizer is used to deliver a composition of the invention (e.g., siNA and/or LNP formulations thereof) locally to lung or pulmonary tissues. In one embodiment, a device comprising a nebulizer is used to deliver a composition of the invention (e.g., siNA and/or LNP formulations thereof) systemically. Non-limiting examples of diseases and conditions that can be treated or managed using a device comprising a nebulizer of the invention include asthma, bronchitis, COPD, cystic fibrosis, emphysema, respiratory syncytial virus, influenza virus, and other respiratory tract or pulmonary diseases and infections. Nebulizer devices of the invention can be used to deliver various classes of drugs and combinations thereof; including, for example but not limited to siNA composition and/or LNP formulations thereof, anti-histamines, anti-infective agents, anti-viral agents, anti-bacterial agents, blood modifiers, cardiovascular agents, decongestants, diagnostics, immunosuppressives, mast cell stabilizers, anti-inflammatories, respiratory agents, skin and mucous membrane agents and other classes. In one embodiment, a nebulizer device of the invention is used for the effective delivery of proteins, peptides, oligonucleotides, plasmids, and small molecules (i.e.,. interleukins, DNase, antisense RNA, streptococcus B polypeptides and HIV integrases). In another embodiment, nebulizer devices of the invention are used to deliver respiratory dispersions comprising emulsions, microemulsions, or submicron and nanoparticulate suspensions of at least one active agent. See for example U.S. Pat. No. 7128,897 and 7,090,830 B2, both incorporated by reference herein).

[0607] Delivery of liquid or non-liquid aerosols comprising the compostion of the invention (e.g., siNA and/or LNP formulations thereof) can be accomplished using any suitable device such as an ultrasonic or air jet nebulizer. In one embodiment, the device comprising a nebulizer relies on oscillation signals to drive a piezoelectric ceramic oscillator for producing high energy ultrasonic waves which mechanically agitate a composition of the invention (e.g., siNA and/or LNP formulations thereof) generating a medicament aerosol cloud. (see for example U.S. Pat. Nos. 7,129, 619 B2 and 7,131,439 B2, incorporated by reference herein). In another embodiment, the device comprising a nebulizer relies on air jet

mixing of compressed air with a composition of the invention (e.g., siNA and/or LNP formulations thereof) to form droplets in an aerosol cloud.

[0608] Nebulizer devices can be used to administer aerosols comprising a composition of the invention (e.g., siNA and/or LNP formulations thereof) continuously or periodically and can be regulated manually, automatically, or in coordination with a patient's breathing. (See U.S. Pat. No. 3,812,854, WO 92/11050). In one embodiment, a device comprising a nebulizer can periodically administer a composition of the invention (e.g., siNA and/or LNP formulations thereof) via a microchannel extrusion chamber or cyclic pressurization single-bolus. In another embodiment, devices comprising a nebulizer can be used to continuously administer suspension aerosols comprising the composition of the invention (e.g., siNA and/or LNP formulations thereof).

[0609] Nebularizer devices of the invention can use carriers, typically water or a dilute aqueous or non-aqueous solutions comprising compositions of the invention (e.g., siNA and/or LNP formulations thereof) . In one embodiment, a device comprising a nebulizer uses an alcoholic solution, preferably made isotonic with body fluids by the addition of, for example, sodium chloride or other suitable salts comprising the composition of the invention (e.g., siNA and/or LNP formulations thereof). In another embodiment, nebulizer devices of the invention use non-aqueous fluorochemical carriers comprising the composition of the invention (e.g., siNA and/or LNP formulations thereof). A device comprising a nebulizer can deliver compositions of the invention in amounts of about 0.001% to 90% w/w of carrier formulation. In one embodiment, a device comprising a nebulizer uses suitable formulations comprising the composition of the invention (e.g., siNA and/or LNP formulations thereof) in a liquid carrier in an amount of up to 40% w/w preferably less than 20% w/w of the formulation. In another embodiment, a device comprising a nebulizer uses stabilized non-liquid particulate, sub-micron, nanoparticle suspensions comprising as little as 0.001% up to 90% w/w of composition of the invention (e.g., siNA and/or LNP formulations thereof) relative to the non-liquid particulate, sub-micron, and/or nanoparticle weight (U.S. Pat. No.6,946,117 B1).

[0610] Aerosol formulations can include optional additives including preservatives if the formulation is not prepared sterile. Non-limiting examples include, methyl hydroxybenzoate, anti-oxidants, flavorings, volatile oils, buffering agents and emulsifiers and other formulation surfactants. In one embodiment, fluorocarbon or perfluorocarbon carriers are used to reduce

degradation and provide safer biocompatible non-liquid particulate suspension compositions of the invention (e.g., siNA and/or LNP formulations thereof). In another embodiment, a device comprising a nebulizer delivers a composition of the invention (e.g., siNA and/or LNP formulations thereof) comprising flurochemicals that are bacteriostatic thereby decreasing the potential for microbial growth in compabitable devices.

[0611] The aerosols of solid particles comprising the active composition and surfactant can likewise be produced with any solid particulate aerosol generator. In one embodiment, aerosol generators for administering solid particulate agents to a subject produce particles which are respirable, as explained above, and generate a volume of aerosol containing a predetermined metered dose of a composition. In another embodiment, the aerosol comprises a combination of particulates comprising at least one composition of the invention (e.g., siNA and/or LNP formulations thereof) with a pretermined volume of suspension medium or surfactant to provide a respiratory blend.

[0612] In one embodiment, a solid particulate aerosol generator of the invention is an insufflator. Suitable formulations for administration by insufflation include finely comminuted powders which can be delivered by means of an insufflator. In the insufflator, the powder, *e.g.*, a metered dose thereof effective to carry out the treatments described herein, is contained in capsules or cartridges, typically made of gelatin or plastic, which are either pierced or opened in situ and the powder delivered by air drawn through the device upon inhalation or by means of a manually-operated pump. The powder employed in the insufflator consists either solely of the active ingredient or of a powder blend comprising the active ingredient, a suitable powder diluent, such as lactose, and an optional surfactant. The active ingredient typically comprises from 0.1 to 100 w/w of the formulation. A second type of illustrative aerosol generator comprises a metered dose inhaler. Metered dose inhalers are pressurized aerosol dispensers, typically containing a suspension or solution formulation of the active ingredient in a liquified propellant. During use these devices discharge the formulation through a valve adapted to deliver a metered volume to produce a fine particle spray containing the active ingredient. Suitable propellants include certain chlorofluorocarbon compounds, for example, dichlorodifluoromethane, trichlorofluoromethane, dichlorotetrafluoroethane and mixtures thereof. The formulation can additionally contain one or more co-solvents, for example, ethanol, emulsifiers and other formulation surfactants, such as oleic acid or sorbitan trioleate, anti-oxidants and suitable

flavoring agents. Other methods for pulmonary delivery are described in, for example US Patent Application No. 20040037780, and US Patent Nos. 6,592,904; 6,582,728; 6,565,885, all incorporated by reference herein.

[0613] In one embodiment, the siNA and LNP compositions and formulations provided herein for use in pulmonary delivery further comprise one or more surfactants. Suitable surfactants or surfactant components for enhancing the uptake of the compositions of the invention include synthetic and natural as well as full and truncated forms of surfactant protein A, surfactant protein B, surfactant protein C, surfactant protein D and surfactant Protein E, di-saturated phosphatidylcholine (other than dipalmitoyl), dipalmitoylphosphatidylchol- ine, phosphatidylcholine, phosphatidylglycerol, phosphatidylinositol, phosphatidylethanolamine, phosphatidylserine; phosphatidic acid, ubiquinones, lysophosphatidylethanolamine, lysophosphatidylcholine, palmitoyl-lysophosphatidylcholine, dehydroepiandrosterone, dolichols, sulfatidic acid, glycerol-3-phosphate, dihydroxyacetone phosphate, glycerol, glycero-3-phosphocholine, dihydroxyacetone, palmitate, cytidine diphosphate (CDP) diacylglycerol, CDP choline, choline, choline phosphate; as well as natural and artificial lamelar bodies which are the natural carrier vehicles for the components of surfactant, omega-3 fatty acids, polyenic acid, polyenoic acid, lecithin, palmitinic acid, non-ionic block copolymers of ethylene or propylene oxides, polyoxypropylene, monomeric and polymeric, polyoxyethylene, monomeric and polymeric, poly (vinyl amine) with dextran and/or alkanoyl side chains, Brij 35, Triton X-100 and synthetic surfactants ALEC, Exosurf, Survan and Atovaquone, among others. These surfactants can be useed either as single or part of a multiple component surfactant in a formulation, or as covalently bound additions to the 5' and/or 3' ends of the nucleic acid component of a pharmaceutical composition herein.

[0614] The composition of the present invention can be administered into the respiratory system as a formulation including particles of respirable size, e.g. particles of a size sufficiently small to pass through the nose, mouth and larynx upon inhalation and through the bronchi and alveoli of the lungs. In general, respirable particles range from about 0.5 to 10 microns in size. Particles of non-respirable size which are included in the aerosol tend to deposit in the throat and be swallowed, and the quantity of non-respirable particles in the aerosol is thus minimized. For nasal administration, a particle size in the range of 10-500 um is preferred to ensure retention in the nasal cavity.

[0615] In one embodiment, the siNA molecules of the invention and formulations or compositions thereof are administered to the liver as is generally known in the art (see for example Wen *et al.,* 2004, *World J Gastroenterol.,* 10, 244-9; Murao *et al.,* 2002, *Pharm Res.,* 19, 1808-14; Liu *et al.,* 2003, *gene Ther.,* 10, 180-7; Hong *et al.,* 2003, *J Pharm Pharmacol.,* 54, 51-8; Herrmann *et al.,* 2004, *Arch Virol.,* 149, 1611-7; and Matsuno *et al.,* 2003, *gene Ther.,* 10, 1559-66).

[0616] In one embodiment, the invention features the use of methods to deliver the nucleic acid molecules of the instant invention to hematopoietic cells, including monocytes and lymphocytes. These methods are described in detail by Hartmann *et al.,* 1998, *J. Phamacol. Exp. Ther.,* 285(2), 920-928; Kronenwett *et al.,* 1998, *Blood,* 91(3), 852-862; Filion and Phillips, 1997, *Biochim. Biophys. Acta.,* 1329(2), 345-356; Ma and Wei, 1996, *Leuk. Res.,* 20(11/12), 925-930; and Bongartz *et al.,* 1994, *Nucleic Acids Research,* 22(22), 4681-8. Such methods, as described above, include the use of free oligonucleitide, cationic lipid formulations, liposome formulations including pH sensitive liposomes and immunoliposomes, and bioconjugates including oligonucleotides conjugated to fusogenic peptides, for the transfection of hematopoietic cells with oligonucleotides.

[0617] . In one embodiment, the siNA molecules of the invention and formulations or compositions thereof are administered directly or topically (e.g., locally) to the dermis or follicles as is generally known in the art (see for example Brand, 2001, *Curr. Opin. Mol. Ther.,* 3, 244-8; Regnier *et al.,* 1998, *J. Drug Target,* 5, 275-89; Kanikkannan, 2002, *BioDrugs,* 16, 339-47; Wraight *et al.,* 2001, *Pharmacol. Ther.,* 90, 89-104; and Preat and Dujardin, 2001, STP PharmaSciences, 11, 57-68). In one embodiment, the siNA molecules of the invention and formulations or compositions thereof are administered directly or topically using a hydroalcoholic gel formulation comprising an alcohol (e.g., ethanol or isopropanol), water, and optionally including additional agents such isopropyl myristate and carbomer 980.

[0618] In one embodiment, a siNA molecule of the invention is administered iontophoretically, for example to a particular organ or compartment (e.g., the eye, back of the eye, heart, liver, kidney, bladder, prostate, tumor, CNS etc.). Non-limiting examples of iontophoretic delivery are described in, for example, WO 03/043689 and WO 03/030989, which are incorporated by reference in their entireties herein.

[0619] In one embodiment, siNA compounds and compositions of the invention are administered either systemically or locally about every 1-50 weeks (*e.g.*, about every 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36, 37, 38, 39, 40, 41, 42, 43, 44, 45, 46, 47, 48, 49, or 50 weeks), alone or in combination with other comounds and/or therapeis herein. In one embodiment, siNA compounds and compositions of the invention are administered systemically (*e.g.*, via intravenous, subcutaneous, intramuscular, infusion, pump, implant etc.) about every 1-50 weeks (*e.g.*, about every 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36, 37, 38, 39, 40, 41, 42, 43, 44, 45, 46, 47, 48, 49, or 50 weeks), alone or in combination with other comounds and/or therapies described herein and/or otherwise known in the art.

[0620] In one embodiment, delivery systems of the invention include, for example, aqueous and nonaqueous gels, creams, multiple emulsions, microemulsions, liposomes, ointments, aqueous and nonaqueous solutions, lotions, aerosols, hydrocarbon bases and powders, and can contain excipients such as solubilizers, permeation enhancers (*e.g.*, fatty acids, fatty acid esters, fatty alcohols and amino acids), and hydrophilic polymers (*e.g.*, polycarbophil and polyvinylpyrolidone). In one embodiment, the pharmaceutically acceptable carrier is a liposome or a transdermal enhancer. Examples of liposomes which can be used in this invention include the following: (1) CellFectin, 1:1.5 (M/M) liposome formulation of the cationic lipid N,NI,NII,NIII-tetramethyl-N,NI,NII,NIII-tetrapalmit-y-spermine and dioleoyl phosphatidylethanolamine (DOPE) (GIBCO BRL); (2) Cytofectin GSV, 2:1 (M/M) liposome formulation of a cationic lipid and DOPE (Glen Research); (3) DOTAP (N-[1-(2,3-dioleoyloxy)-N,N,N-tri-methyl-ammoniummethylsulfate) (Boehringer Manheim); and (4) Lipofectamine, 3:1 (M/M) liposome formulation of the polycationic lipid DOSPA and the neutral lipid DOPE (GIBCO BRL).

[0621] In one embodiment, delivery systems of the invention include patches, tablets, suppositories, pessaries, gels and creams, and can contain excipients such as solubilizers and enhancers (*e.g.*, propylene glycol, bile salts and amino acids), and other vehicles (*e.g.*, polyethylene glycol, fatty acid esters and derivatives, and hydrophilic polymers such as hydroxypropylmethylcellulose and hyaluronic acid).

[0622] In one embodiment, siNA molecules of the invention are formulated or complexed with polyethylenimine (*e.g.*, linear or branched PEI) and/or polyethylenimine derivatives,

including for example grafted PEIs such as galactose PEI, cholesterol PEI, antibody derivatized PEI, and polyethylene glycol PEI (PEG-PEI) derivatives thereof (see for example Ogris *et al.,* 2001, *AAPA PharmSci,* 3, 1-11; Furgeson *et al.*, 2003, Bioconjugate Chem., 14, 840-847; Kunath *et al.*, 2002, Phramaceutical Research, 19, 810-817; Choi *et al.*, 2001, Bull. Korean Chem. Soc., 22, 46-52; Bettinger *et al.*, 1999, Bioconjugate Chem., 10, 558-561; Peterson *et al.*, 2002, Bioconjugate Chem., 13, 845-854; Erbacher *et al.*, 1999, Journal of Gene Medicine Preprint, 1, 1-18; Godbey *et al.*, 1999., PNAS USA, 96, 5177-5181; Godbey *et al.*, 1999, Journal of Controlled Release, 60, 149-160; Diebold *et al.*, 1999, Journal of Biological Chemistry, 274, 19087-19094; Thomas and Klibanov, 2002, PNAS USA, 99, 14640-14645; and Sagara, US 6,586,524, incorporated by reference herein.

[0623] In one embodiment, a siNA molecule of the invention comprises a bioconjugate, for example a nucleic acid conjugate as described in Vargeese *et al.*, USSN 10/427,160, filed April 30, 2003; US 6,528,631; US 6,335,434; US 6, 235,886; US 6,153,737; US 5,214,136; US 5,138,045, all incorporated by reference herein.

[0624] Thus, the invention features a pharmaceutical composition comprising one or more nucleic acid(s) of the invention in an acceptable carrier, such as a stabilizer, buffer, and the like. The polynucleotides of the invention can be administered (*e.g.*, RNA, DNA or protein) and introduced to a subject by any standard means, with or without stabilizers, buffers, and the like, to form a pharmaceutical composition. When it is desired to use a liposome delivery mechanism, standard protocols for formation of liposomes can be followed. The compositions of the present invention can also be formulated and used as creams, gels, sprays, oils and other suitable compositions for topical, dermal, or transdermal administration as is known in the art.

[0625] 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.

[0626] A pharmacological composition or formulation refers to a composition or formulation in a form suitable for administration, *e.g.,* systemic or local administration, into a cell or subject, including for example a human. Suitable forms, in part, depend upon the use or the route of entry, for example oral, transdermal, or by injection. Such forms should not

prevent the composition or formulation from reaching a target cell (*i.e.*, a cell to which the negatively charged nucleic acid is desirable for delivery). For example, pharmacological compositions injected into the blood stream should be soluble. Other factors are known in the art, and include considerations such as toxicity and forms that prevent the composition or formulation from exerting its effect.

[0627] In one embodiment, siNA molecules of the invention are administered to a subject by systemic administration in a pharmaceutically acceptable composition or formulation. By "systemic administration" is meant *in vivo* systemic absorption or accumulation of drugs in the blood stream followed by distribution throughout the entire body. Administration routes that lead to systemic absorption include, without limitation: intravenous, subcutaneous, portal vein, intraperitoneal, inhalation, oral, intrapulmonary and intramuscular. Each of these administration routes exposes the siNA molecules of the invention to an accessible diseased tissue (e.g., lung). The rate of entry of a drug into the circulation has been shown to be a function of molecular weight or size. The use of a liposome or other drug carrier comprising the compounds of the instant invention can potentially localize the drug, for example, in certain tissue types, such as the tissues of the reticular endothelial system (RES). A liposome formulation that can facilitate the association of drug with the surface of cells, such as, lymphocytes and macrophages is also useful. This approach can provide enhanced delivery of the drug to target cells by taking advantage of the specificity of macrophage and lymphocyte immune recognition of abnormal cells.

[0628] By "pharmaceutically acceptable formulation" or "pharmaceutically acceptable composition" is meant, a composition or formulation that allows for the effective distribution of the nucleic acid molecules of the instant invention in the physical location most suitable for their desired activity. Non-limiting examples of agents suitable for formulation with the nucleic acid molecules of the instant invention include: P-glycoprotein inhibitors (such as Pluronic P85),; biodegradable polymers, such as poly (DL-lactide-coglycolide) microspheres for sustained release delivery (Emerich, DF *et al*, 1999, *Cell Transplant*, 8, 47-58); and loaded nanoparticles, such as those made of polybutylcyanoacrylate. Other non-limiting examples of delivery strategies for the nucleic acid molecules of the instant invention include material described in Boado *et al.,* 1998, *J. Pharm. Sci.*, 87, 1308-1315; Tyler *et al.,* 1999, *FEBS Lett.,* 421, 280-284; Pardridge *et al.,* 1995, *PNAS USA.,* 92, 5592-5596; Boado, 1995,

Adv. Drug Delivery Rev., 15, 73-107; Aldrian-Herrada *et al.,* 1998, *Nucleic Acids Res.,* 26, 4910-4916; and Tyler *et al.,* 1999, *PNAS USA.,* 96, 7053-7058.

[0629] The invention also features the use of a composition comprising surface-modified liposomes containing poly (ethylene glycol) lipids (PEG-modified, or long-circulating liposomes or stealth liposomes) and nucleic acid molecules of the invention. These formulations offer a method for increasing the accumulation of drugs (*e.g.*, siNA) in target tissues. This class of drug carriers resists opsonization and elimination by the mononuclear phagocytic system (MPS or RES), thereby enabling longer blood circulation times and enhanced tissue exposure for the encapsulated drug (Lasic *et al. Chem. Rev.* 1995, 95, 2601-2627; Ishiwata *et al., Chem. Pharm. Bull.* 1995, 43, 1005-1011). Such liposomes have been shown to accumulate selectively in tumors, presumably by extravasation and capture in the neovascularized target tissues (Lasic *et al., Science* 1995, 267, 1275-1276; Oku *et al.,*1995, *Biochim. Biophys. Acta*, 1238, 86-90). The long-circulating liposomes enhance the pharmacokinetics and pharmacodynamics of DNA and RNA, particularly compared to conventional cationic liposomes which are known to accumulate in tissues of the MPS (Liu *et al., J. Biol. Chem.* 1995, 42, 24864-24870; Choi *et al.,* International PCT Publication No. WO 96/10391; Ansell *et al.,* International PCT Publication No. WO 96/10390; Holland *et al.,* International PCT Publication No. WO 96/10392). Long-circulating liposomes are also likely to protect drugs from nuclease degradation to a greater extent compared to cationic liposomes, based on their ability to avoid accumulation in metabolically aggressive MPS tissues such as the liver and spleen.

[0630] In one embodiment, a liposomal formulation of the invention comprises a double stranded nucleic acid molecule of the invention (e.g, siNA) formulated or complexed with compounds and compositions described in US 6,858,224; 6,534,484; 6,287,591; 6,835,395; 6,586,410; 6,858,225; 6,815,432; US 6,586,001; 6,120,798; US 6,977,223; US 6,998,115; 5,981,501; 5,976,567; 5,705,385; US 2006/0019912; US 2006/0019258; US 2006/0008909; US 2005/0255153; US 2005/0079212; US 2005/0008689; US 2003/0077829, US 2005/0064595, US 2005/0175682, US 2005/0118253; US 2004/0071654; US 2005/0244504; US 2005/0265961 and US 2003/0077829, all of which are incorporated by reference herein in their entirety.

[0631] The present invention also includes compositions prepared for storage or administration that include a pharmaceutically effective amount of the desired compounds in

a pharmaceutically acceptable carrier or diluent. Acceptable carriers or diluents for therapeutic use are well known in the pharmaceutical art, and are described, for example, in *Remington's Pharmaceutical Sciences*, Mack Publishing Co. (A.R. Gennaro edit. 1985), hereby incorporated by reference herein. For example, preservatives, stabilizers, dyes and flavoring agents can be provided. These include sodium benzoate, sorbic acid and esters of *p*-hydroxybenzoic acid. In addition, antioxidants and suspending agents can be used.

[0632] 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.

[0633] The nucleic acid molecules of the invention and formulations thereof can be administered orally, topically, parenterally, by inhalation or spray, or rectally in dosage unit formulations containing conventional non-toxic pharmaceutically acceptable carriers, adjuvants and/or vehicles. The term parenteral as used herein includes percutaneous, subcutaneous, intravascular (*e.g.*, intravenous), intramuscular, or intrathecal injection or infusion techniques and the like. In addition, there is provided a pharmaceutical formulation comprising a nucleic acid molecule of the invention and a pharmaceutically acceptable carrier. One or more nucleic acid molecules of the invention can be present in association with one or more non-toxic pharmaceutically acceptable carriers and/or diluents and/or adjuvants, and if desired other active ingredients. The pharmaceutical compositions containing nucleic acid molecules of the invention can be in a form suitable for oral use, for example, as tablets, troches, lozenges, aqueous or oily suspensions, dispersible powders or granules, emulsion, hard or soft capsules, or syrups or elixirs.

[0634] Compositions intended for oral use can be prepared according to any method known to the art for the manufacture of pharmaceutical compositions and such compositions can contain one or more such sweetening agents, flavoring agents, coloring agents or preservative agents in order to provide pharmaceutically elegant and palatable preparations. Tablets contain the active ingredient in admixture with non-toxic pharmaceutically acceptable

excipients that are suitable for the manufacture of tablets. These excipients can be, for example, inert diluents; such as calcium carbonate, sodium carbonate, lactose, calcium phosphate or sodium phosphate; granulating and disintegrating agents, for example, corn starch, or alginic acid; binding agents, for example starch, gelatin or acacia; and lubricating agents, for example magnesium stearate, stearic acid or talc. The tablets can be uncoated or they can be coated by known techniques. In some cases such coatings can be prepared by known techniques to delay disintegration and absorption in the gastrointestinal tract and thereby provide a sustained action over a longer period. For example, a time delay material such as glyceryl monosterate or glyceryl distearate can be employed.

[0635] 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.

[0636] Aqueous suspensions contain the active materials in a mixture with excipients suitable for the manufacture of aqueous suspensions. Such excipients are suspending agents, for example sodium carboxymethylcellulose, methylcellulose, hydropropyl-methylcellulose, sodium alginate, polyvinylpyrrolidone, gum tragacanth and gum acacia; dispersing or wetting agents can be a naturally-occurring phosphatide, for example, lecithin, or condensation products of an alkylene oxide with fatty acids, for example polyoxyethylene stearate, or condensation products of ethylene oxide with long chain aliphatic alcohols, for example heptadecaethyleneoxycetanol, or condensation products of ethylene oxide with partial esters derived from fatty acids and a hexitol such as polyoxyethylene sorbitol monooleate, or condensation products of ethylene oxide with partial esters derived from fatty acids and hexitol anhydrides, for example polyethylene sorbitan monooleate. The aqueous suspensions can also contain one or more preservatives, for example ethyl, or n-propyl p-hydroxybenzoate, one or more coloring agents, one or more flavoring agents, and one or more sweetening agents, such as sucrose or saccharin.

[0637] 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

[0638] 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.

[0639] 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.

[0640] Syrups and elixirs can be formulated with sweetening agents, for example glycerol, propylene glycol, sorbitol, glucose or sucrose. Such formulations can also contain a demulcent, a preservative and flavoring and coloring agents. The pharmaceutical compositions can be in the form of a sterile injectable aqueous or oleaginous suspension. This suspension can be formulated according to the known art using those suitable dispersing or wetting agents and suspending agents that have been mentioned above. The sterile injectable preparation can also be a sterile injectable solution or suspension in a non-toxic parentally acceptable diluent or solvent, for example as a solution in 1,3-butanediol. Among the acceptable vehicles and solvents that can be employed are water, Ringer's solution and isotonic sodium chloride solution. In addition, sterile, fixed oils are conventionally employed as a solvent or suspending medium. For this purpose, any bland fixed oil can be employed including synthetic mono-or diglycerides. In addition, fatty acids such as oleic acid find use in the preparation of injectables.

[0641] 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.

[0642] 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.

[0643] 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.

[0644] 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.

[0645] 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.

[0646] 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.

[0647] In one embodiment, the invention comprises compositions suitable for administering nucleic acid molecules of the invention to specific cell types. For example, the asialoglycoprotein receptor (ASGPr) (Wu and Wu, 1987, *J. Biol. Chem.* 262, 4429-4432) is unique to hepatocytes and binds branched galactose-terminal glycoproteins, such as

asialoorosomucoid (ASOR). In another example, the folate receptor is overexpressed in many cancer cells. Binding of such glycoproteins, synthetic glycoconjugates, or folates to the receptor takes place with an affinity that strongly depends on the degree of branching of the oligosaccharide chain, for example, triatennary structures are bound with greater affinity than biatenarry or monoatennary chains (Baenziger and Fiete, 1980, *Cell,* 22, 611-620; Connolly *et al.,* 1982, *J. Biol. Chem.,* 257, 939-945). Lee and Lee, 1987, *Glycoconjugate J.*, 4, 317-328, obtained this high specificity through the use of N-acetyl-D-galactosamine as the carbohydrate moiety, which has higher affinity for the receptor, compared to galactose. This "clustering effect" has also been described for the binding and uptake of mannosyl-terminating glycoproteins or glycoconjugates (Ponpipom *et al.,* 1981, *J. Med. Chem.,* 24, 1388-1395). The use of galactose, galactosamine, or folate based conjugates to transport exogenous compounds across cell membranes can provide a targeted delivery approach to, for example, the treatment of liver disease, cancers of the liver, or other cancers. The use of bioconjugates can also provide a reduction in the required dose of therapeutic compounds required for treatment. Furthermore, therapeutic bioavailability, pharmacodynamics, and pharmacokinetic parameters can be modulated through the use of nucleic acid bioconjugates of the invention. Non-limiting examples of such bioconjugates are described in Vargeese *et al.,* USSN 10/201,394, filed August 13, 2001; and Matulic-Adamic *et al.,* USSN 60/362,016, filed March 6, 2002.

[0648] Alternatively, certain siNA molecules of the instant invention can be expressed within cells from eukaryotic promoters (*e.g.,* Izant and Weintraub, 1985, *Science,* 229, 345; McGarry and Lindquist, 1986, *Proc. Natl. Acad. Sci.*, USA 83, 399; Scanlon *et al.,* 1991, *Proc. Natl. Acad. Sci. USA*, 88, 10591-5; Kashani-Sabet *et al.,* 1992, *Antisense Res. Dev.,* 2, 3-15; Dropulic *et al.,* 1992, *J. Virol.,* 66, 1432-41; Weerasinghe *et al.,* 1991, *J. Virol.,* 65, 5531-4; Ojwang *et al.,* 1992, *Proc. Natl. Acad. Sci. USA*, 89, 10802-6; Chen *et al.,* 1992, *Nucleic Acids Res.,* 20, 4581-9; Sarver *et al.,* 1990 *Science,* 247, 1222-1225; Thompson *et al.,* 1995, *Nucleic Acids Res.,* 23, 2259; Good *et al.,* 1997, *Gene Therapy*, 4, 45. Those skilled in the art realize that any nucleic acid can be expressed in eukaryotic cells from the appropriate DNA/RNA vector. The activity of such nucleic acids can be augmented by their release from the primary transcript by a enzymatic nucleic acid (Draper *et al.,* PCT WO 93/23569, and Sullivan *et al.,* PCT WO 94/02595; Ohkawa *et al.,* 1992, *Nucleic Acids Symp. Ser.,* 27, 15-6; Taira *et al.,* 1991, *Nucleic Acids Res.,* 19, 5125-30; Ventura *et al.,* 1993, *Nucleic Acids Res.,* 21, 3249-55; Chowrira *et al.,* 1994, *J. Biol. Chem.,* 269, 25856.

[0649] In another aspect of the invention, RNA molecules of the present invention can be expressed from transcription units (see for example Couture *et al.,* 1996, *TIG.,* 12, 510) inserted into DNA or RNA vectors. The recombinant vectors can be DNA plasmids or viral vectors. siNA expressing viral vectors can be constructed based on, but not limited to, adeno-associated virus, retrovirus, adenovirus, or alphavirus. In another embodiment, pol III based constructs are used to express nucleic acid molecules of the invention (see for example Thompson, U.S. Pats. Nos. 5,902,880 and 6,146,886). The recombinant vectors capable of expressing the siNA molecules can be delivered as described above, and persist in target cells. Alternatively, viral vectors can be used that provide for transient expression of nucleic acid molecules. Such vectors can be repeatedly administered as necessary. Once expressed, the siNA molecule interacts with the target mRNA and generates an RNAi response. Delivery of siNA molecule expressing vectors can be systemic, such as by intravenous or intra-muscular administration, by administration to target cells ex-planted from a subject followed by reintroduction into the subject, or by any other means that would allow for introduction into the desired target cell (for a review see Couture *et al.,* 1996, *TIG.,* 12, 510).

[0650] In one aspect the invention features an expression vector comprising a nucleic acid sequence encoding at least one siNA molecule of the instant invention. The expression vector can encode one or both strands of a siNA duplex, or a single self-complementary strand that self hybridizes into a siNA duplex. The nucleic acid sequences encoding the siNA molecules of the instant invention can be operably linked in a manner that allows expression of the siNA molecule (see for example Paul *et al.,* 2002, *Nature Biotechnology,* 19, 505; Miyagishi and Taira, 2002, *Nature Biotechnology,* 19, 497; Lee *et al.,* 2002, *Nature Biotechnology,* 19, 500; and Novina *et al.,* 2002, *Nature Medicine*, advance online publication doi:10.1038/nm725).

[0651] In another aspect, the invention features an expression vector comprising: a) a transcription initiation region (*e.g.,* eukaryotic pol I, II or III initiation region); b) a transcription termination region (*e.g.,* eukaryotic pol I, II or III termination region); and c) a nucleic acid sequence encoding at least one of the siNA molecules of the instant invention, wherein said sequence is operably linked to said initiation region and said termination region in a manner that allows expression and/or delivery of the siNA molecule. The vector can optionally include an open reading frame (ORF) for a protein operably linked on the 5' side or

the 3'-side of the sequence encoding the siNA of the invention; and/or an intron (intervening sequences).

[0652] Transcription of the siNA molecule sequences can be driven from a promoter for eukaryotic RNA polymerase I (pol I), RNA polymerase II (pol II), or RNA polymerase III (pol III). Transcripts from pol II or pol III promoters are expressed at high levels in all cells; the levels of a given pol II promoter in a given cell type depends on the nature of the gene regulatory sequences (enhancers, silencers, etc.) present nearby. Prokaryotic RNA polymerase promoters are also used, providing that the prokaryotic RNA polymerase enzyme is expressed in the appropriate cells (Elroy-Stein and Moss, 1990, *Proc. Natl. Acad. Sci. U S A*, 87, 6743-7; Gao and Huang 1993, *Nucleic Acids Res.*, 21, 2867-72; Lieber *et al.,* 1993, *Methods Enzymol.,* 217, 47-66; Zhou *et al.,* 1990, *Mol. Cell. Biol.*, 10, 4529-37). Several investigators have demonstrated that nucleic acid molecules expressed from such promoters can function in mammalian cells (*e.g.* Kashani-Sabet *et al.,* 1992, *Antisense Res. Dev.,* 2, 3-15; Ojwang *et al.,* 1992, *Proc. Natl. Acad. Sci. U S A*, 89, 10802-6; Chen *et al.,* 1992, *Nucleic Acids Res.*, 20, 4581-9; Yu *et al.,* 1993, *Proc. Natl. Acad. Sci. U S A*, 90, 6340-4; L'Huillier *et al.,* 1992, *EMBO J.,* 11, 4411-8; Lisziewicz *et al.,* 1993, *Proc. Natl. Acad. Sci. U. S. A*, 90, 8000-4; Thompson *et al.,* 1995, *Nucleic Acids Res.,* 23, 2259; Sullenger & Cech, 1993, *Science,* 262, 1566). More specifically, transcription units such as the ones derived from genes encoding U6 small nuclear (snRNA), transfer RNA (tRNA) and adenovirus VA RNA are useful in generating high concentrations of desired RNA molecules such as siNA in cells (Thompson *et al., supra*; Couture and Stinchcomb, *1996, supra*; Noonberg *et al.,* 1994, *Nucleic Acid Res.*, 22, 2830; Noonberg *et al.,* U.S. Pat. No. 5,624,803; Good *et al.,* 1997, *Gene Ther.,* 4, 45; Beigelman *et al.,* International PCT Publication No. WO 96/18736. The above siNA transcription units can be incorporated into a variety of vectors for introduction into mammalian cells, including but not restricted to, plasmid DNA vectors, viral DNA vectors (such as adenovirus or adeno-associated virus vectors), or viral RNA vectors (such as retroviral or alphavirus vectors) (for a review see Couture and Stinchcomb, *1996, supra).*

[0653] In another aspect the invention features an expression vector comprising a nucleic acid sequence encoding at least one of the siNA molecules of the invention in a manner that allows expression of that siNA molecule. The expression vector comprises in one embodiment; a) a transcription initiation region; b) a transcription termination region; and c) a nucleic acid sequence encoding at least one strand of the siNA molecule, wherein the

sequence is operably linked to the initiation region and the termination region in a manner that allows expression and/or delivery of the siNA molecule.

[0654] In another embodiment the expression vector comprises: a) a transcription initiation region; b) a transcription termination region; c) an open reading frame; and d) a nucleic acid sequence encoding at least one strand of a siNA molecule, wherein the sequence is operably linked to the 3'-end of the open reading frame and wherein the sequence is operably linked to the initiation region, the open reading frame and the termination region in a manner that allows expression and/or delivery of the siNA molecule. In yet another embodiment, the expression vector comprises: a) a transcription initiation region; b) a transcription termination region; c) an intron; and d) a nucleic acid sequence encoding at least one siNA molecule, wherein the sequence is operably linked to the initiation region, the intron and the termination region in a manner which allows expression and/or delivery of the nucleic acid molecule.

[0655] In another embodiment, the expression vector comprises: a) a transcription initiation region; b) a transcription termination region; c) an intron; d) an open reading frame; and e) a nucleic acid sequence encoding at least one strand of a siNA molecule, wherein the sequence is operably linked to the 3'-end of the open reading frame and wherein the sequence is operably linked to the initiation region, the intron, the open reading frame and the termination region in a manner which allows expression and/or delivery of the siNA molecule.

ENaC biology and biochemistry

[0656] The epithelial sodium channel (ENaC, or sodium channel non-neuronal 1 (SCNN1) or amiloride sensitive sodium channel (ASSC)) is a membrane-bound ion-channel that is permeable for Li^+, protons and especially Na^+. It is a 'constitutively active' channel, i.e. does not require a gating stimulus and is open at rest. ENaC is a heteromeric protein comprised of three different subunits - α (SCNN1A), β (SCNN1B), and γ (SCNN1G). The exact stoichiometry was until recently unclear, but based on homology to ASIC channels, is almost certainly a heterotrimer (Jasti et al. *supra*). Each subunit consists of two transmembrane helices and an extracellular loop. The amino- and carboxy-termini of all polypeptides are located in the cytosol. In addition there is a fourth, so-called δ-subunit, that shares significant homology with the α-subunit and can form a functional ion-channel together with

the β- and γ-subunits. Such δ2, β, γ-tetramers appear in pancreas, testes and ovaries although their function is yet unknown.

[0657] ENaC is located in the apical membrane of polarized epithelial cells particularly in the kidney, the lung and the colon. It is involved in the transepithelial Na^+-ion transport which it accomplishes together with the Na^+/K^+-ATPase. It plays a major role in the Na^+- and K^+-ion homeostasis of blood, epithelia and extraepithelial fluids by resorption of Na^+-ions.

[0658] The airways are lined with a film of liquid about 10 micrometres deep that is in two layers. Around the cilia is the watery periciliary sol. Over this is a mucous blanket that traps inhaled particles. The mucus layer itself traps inhaled pathogens/particles, allowing their removal via ongoing mucociliary clearance, without the need to trigger a potentially injurious inflammatory response. The low viscosity of the periciliary sol allows the cilia to beat and propel the mucous blanket along airways to the mouth. In large airways, mucus comes predominantly from the mucous glands but also from goblet cells in the surface epithelium. Water is added to the airway surface by gland secretion that is driven by active Cl^- secretion by serous cells. Water is removed by Na^+ transport via ENaC across the surface epithelium. In airway diseases, the balance is shifted from water secretion to mucus secretion (Widdicombe, J.H. (2002) J. Anat. 201 pp313 to 318). Thus ENaC represents the rate-limiting step of sodium absorption across airway epithelia, and therefore controls water absorption from the surface of the airway epithelium. Both COPD (Melton, L. (2002) Lancet 359 p 1924; Hogg, J.C. et al (2004) N. Engl. J. Med. 350 pp2645 to 2653; deMarco, R. et al (2007) Am. J. Respir. Crit. Care Med. 175 pp32 to 39) and cystic fibrosis are characterized by relative dehydration of the airways causing adhesion of mucus. The result is mucus stasis. Adherent mucus obstructs the airways and can become the nidus for the onset of first intermittent, and then chronic airway infection/disease exacerbation.

[0659] Mucus dehydration in COPD is likely multifactorial. It is important to note that relative dehydration can be manifest as either less airway surface liquid or an increase in the % solids of materials present in the lumen. While goblet cell hyperplasia, a key feature of COPD (Hogg et al. *supra*) and the resulting mucin hypersecretion *per se* may increase the % solids content of airway surface liquid, causing relative dehydration in COPD it is also likely that defects in ion and water transport contribute (Boucher, R.C. (2004) Proc. Am. Thorac. Soc. 1 pp66-70). Perhaps the most compelling data that mucus dehydration is a problem in

COPD are those of Hogg (Hogg et al. *supra*) that describe mucus adhesion to airway surfaces and mucus obstruction in the small airways. In this study, the progressive pathological effects of airway obstruction in patients with COPD was assessed in surgically resected lung tissue. The progression of COPD was strongly associated with an increase in the volume of tissue in the wall ($P<0.001$) and the accumulation of inflammatory mucous exudates in the lumen ($P<0.001$) of the small airways.

[0660] With regard cystic fibrosis Knowles et al., (Knowles, M, et al. (1981) N. Engl. J. Med. 305 pp1489 to 1495) measured the transepithelial electrical potential difference across the respiratory mucosa in patients with cystic fibrosis and control subjects. Transepithelial potential differences in cystric fibrosis airways were significantly greater than in controls. This was due to excessive ENaC-mediated Na^+ transport as superfusion of the luminal surface with amiloride induced greater reductions in transepithelial potential difference in cystic fibrosis than in controls. These seminal observations changed the view of the underling cause of the respiratory pathology of cystic fibrosis from a defect due solely to a lack of Cl^- ion secretion to one associated with excessive Na^+ absorption.

[0661] The greater reduction in potential difference in response to amiloride indicated excessive salt absorption and therefore liquid absorption from respiratory epithelial surfaces. This is strongly supported by a variety of in-vitro and in-vivo studies (Boucher, R.C (2007) Annu. Rev. Med. 58 pp157 to 170; Boucher, R.C. (2007) Trends Mol. Med. 13 pp231 to 240; Boucher, R.C. (2007) J. Int. Med. 261 pp5 to 16; Donaldson, S.H. and Boucher, R.C. (2007) Chest 132 pp1631-1636). The result of airway surface dehydration is mucus stasis. Adherent mucus obstructs the airways and can become the nidus for the onset of first intermittent, and then chronic airway infection/disease exacerbation. The development of treatment strategies that address this defect is a logical and promising means of slowing, delaying or potentially preventing these lung diseases.

Examples:

[0662] The following are non-limiting examples showing the selection, isolation, synthesis and activity of nucleic acids of the instant invention.

Example 1: Design, Synthesis, and Identification of siRNAs Active Against ENaCα

ENaCα siNA Synthesis

[0663] A series of 64 siNA strands were designed, synthesized and evaluated for efficacy against ENaC. The primary criteria for design of ENaC siNAs were (i) conservation of ENaC across all human, mouse, and rat isoforms and (ii) high efficacy scores as determined by a proprietary algorithm. The effects of the siNAs on ENaC protein production and RNA levels were also examined. The sequences of the siNAs that were designed, synthesized, and evaluated for efficacy against ENaC are described in **Table 1a** (target sequences) and **Table 1b** (modified sequences).

Table 1a: ENaCα Target Sequences, noting target site.

Duplex #	Antisense Comp #	Sense Comp #	Target Site	Target Sequence	SEQ ID NO:
15580-DC	51062	51061	310	GCCAUCCGCCUGGUGUGCU	1
15581-DC	51064	51063	311	CCAUCCGCCUGGUGUGCUC	2
15582-DC	51066	51065	334	CACAACCGCAUGAAGACGG	3
15583-DC	51068	51067	337	AACCGCAUGAAGACGGCCU	4
15584-DC	51070	51069	338	ACCGCAUGAAGACGGCCUU	5
15585-DC	51072	51071	339	CCGCAUGAAGACGGCCUUC	6
15586-DC	51074	51073	340	CGCAUGAAGACGGCCUUCU	7
15587-DC	51076	51075	341	GCAUGAAGACGGCCUUCUG	8
15588-DC	51078	51077	781	CUGUGCAACCAGAACAAAU	9
15589-DC	51080	51079	782	UGUGCAACCAGAACAAAUC	10
15590-DC	51082	51081	1100	CAGAGCAGAAUGACUUCAU	11
15591-DC	51084	51083	1121	CCCUGCUGUCCACAGUGAC	12
15592-DC	51086	51085	1181	UUAUGGAUGAUGGUGGCUU	13
15593-DC	51088	51087	1351	CACUCCUGCUUCCAGGAGA	14
15594-DC	51090	51089	1382	AGUGUGGCUGUGCCUACAU	15
15595-DC	51092	51091	1383	GUGUGGCUGUGCCUACAUC	16
15596-DC	51094	51093	1384	UGUGGCUGUGCCUACAUCU	17
15597-DC	51096	51095	1385	GUGGCUGUGCCUACAUCUU	18
15598-DC	51098	51097	1386	UGGCUGUGCCUACAUCUUC	19
15599-DC	51100	51099	1387	GGCUGUGCCUACAUCUUCU	20
15600-DC	51102	51101	1388	GCUGUGCCUACAUCUUCUA	21
15601-DC	51104	51103	1738	CUCCUGUCCAACCUGGGCA	22
15602-DC	51106	51105	1739	UCCUGUCCAACCUGGGCAG	23
15603-DC	51108	51107	1742	UGUCCAACCUGGGCAGCCA	24
15604-DC	51110	51109	1743	GUCCAACCUGGGCAGCCAG	25
15605-DC	51112	51111	1747	AACCUGGGCAGCCAGUGGA	26
15606-DC	51114	51113	1748	ACCUGGGCAGCCAGUGGAG	27
15607-DC	51116	51115	1751	UGGGCAGCCAGUGGAGCCU	28
15608-DC	51118	51117	1752	GGGCAGCCAGUGGAGCCUG	29
15609-DC	51120	51119	1753	GGCAGCCAGUGGAGCCUGU	30
15610-DC	51122	51121	1756	AGCCAGUGGAGCCUGUGGU	31
15611-DC	51124	51123	1757	GCCAGUGGAGCCUGUGGUU	32

Example 2: Tandem synthesis of siNA constructs

[0664] 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.

[0665] 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.

[0666] Standard phosphoramidite synthesis chemistry is used up to the point of introducing a tandem linker, such as an inverted deoxy abasic succinate or glyceryl succinate linker (see **Figure 1**) or an equivalent cleavable linker. A non-limiting example of linker coupling conditions that can be used includes a hindered base such as diisopropylethylamine (DIPA) and/or DMAP in the presence of an activator reagent such as Bromotripyrrolidinophosphoniumhexaflurorophosphate (PyBrOP). After the linker is coupled, standard synthesis chemistry is utilized to complete synthesis of the second sequence leaving the terminal the 5'-O-DMT intact. Following synthesis, the resulting oligonucleotide is deprotected according to the procedures described herein and quenched with a suitable buffer, for example with 50mM NaOAc or 1.5M $NH_4H_2CO_3$.

[0667] Purification of the siNA duplex can be readily accomplished using solid phase extraction, for example, using a Waters C18 SepPak 1g cartridge conditioned with 1 column volume (CV) of acetonitrile, 2 CV H2O, and 2 CV 50mM NaOAc. The sample is loaded and then washed with 1 CV H2O or 50mM NaOAc. Failure sequences are eluted with 1 CV 14%

ACN (Aqueous with 50mM NaOAc and 50mM NaCl). The column is then washed, for example with 1 CV H2O followed by on-column detritylation, for example by passing 1 CV of 1% aqueous trifluoroacetic acid (TFA) over the column, then adding a second CV of 1% aqueous TFA to the column and allowing to stand for approximately 10 minutes. The remaining TFA solution is removed and the column washed with H20 followed by 1 CV 1M NaCl and additional H2O. The siNA duplex product is then eluted, for example, using 1 CV 20% aqueous CAN.

[0668] **Figure 2** provides an example of MALDI-TOF mass spectrometry analysis of a purified siNA construct in which each peak corresponds to the calculated mass of an individual siNA strand of the siNA duplex. The same purified siNA provides three peaks when analyzed by capillary gel electrophoresis (CGE), one peak presumably corresponding to the duplex siNA, and two peaks presumably corresponding to the separate siNA sequence strands. Ion exchange HPLC analysis of the same siNA contract 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 3: Chemical Synthesis and Purification of siNA

[0669] siNA molecules can be designed to interact with various sites in the RNA message, for example, target sequences within the RNA sequences described herein. The sequence of one strand of the siNA molecule(s) is complementary to the target site sequences described above. The siNA molecules can be chemically synthesized using methods described herein. Inactive siNA molecules that are used as control sequences can be synthesized by scrambling the sequence of the siNA molecules such that it is not complementary to the target sequence. Generally, siNA constructs can by synthesized using solid phase oligonucleotide synthesis methods as described herein (see for example Usman *et al.,* US Patent Nos. 5,804,683; 5,831,071; 5,998,203; 6,117,657; 6,353,098; 6,362,323; 6,437,117; 6,469,158; Scaringe *et al.,* US Patent Nos. 6,111,086; 6,008,400; 6,111,086 all incorporated by reference herein in their entirety).

[0670] In a non-limiting example, RNA oligonucleotides are synthesized in a stepwise fashion using the phosphoramidite chemistry as is known in the art. Standard phosphoramidite chemistry involves the use of nucleosides comprising any of 5'-O-

dimethoxytrityl, 2'-O-tert-butyldimethylsilyl, 3'-O-2-Cyanoethyl N,N-diisopropylphosphoroamidite groups, and exocyclic amine protecting groups (*e.g.* N6-benzoyl adenosine, N4 acetyl cytidine, and N2-isobutyryl guanosine). Alternately, 2´-O-Silyl Ethers can be used in conjunction with acid-labile 2´-O-orthoester protecting groups in the synthesis of RNA as described by Scaringe *supra*. Differing 2' chemistries can require different protecting groups, for example 2'-deoxy-2'-amino nucleosides can utilize N-phthaloyl protection as described by Usman *et al.,* US Patent 5,631,360, incorporated by reference herein in its entirety).

[0671] During solid phase synthesis, each nucleotide is added sequentially (3´- to 5´-direction) to the solid support-bound oligonucleotide. The first nucleoside at the 3´-end of the chain is covalently attached to a solid support (*e.g.*, controlled pore glass or polystyrene) using various linkers. The nucleotide precursor, a ribonucleoside phosphoramidite, and activator are combined resulting in the coupling of the second nucleoside phosphoramidite onto the 5´-end of the first nucleoside. The support is then washed and any unreacted 5´-hydroxyl groups are capped with a capping reagent such as acetic anhydride to yield inactive 5´-acetyl moieties. The trivalent phosphorus linkage is then oxidized to a more stable phosphate linkage. At the end of the nucleotide addition cycle, the 5´-O-protecting group is cleaved under suitable conditions (*e.g.*, acidic conditions for trityl-based groups and fluoride for silyl-based groups). The cycle is repeated for each subsequent nucleotide.

[0672] Modification of synthesis conditions can be used to optimize coupling efficiency, for example by using differing coupling times, differing reagent/phosphoramidite concentrations, differing contact times, differing solid supports and solid support linker chemistries depending on the particular chemical composition of the siNA to be synthesized. Deprotection and purification of the siNA can be performed as is generally described in Usman *et al.,* US 5,831,071, US 6,353,098, US 6,437,117, and Bellon *et al.,* US 6,054,576, US 6,162,909, US 6,303,773, or Scaringe *supra,* incorporated by reference herein in their entireties. Additionally, deprotection conditions can be modified to provide the best possible yield and purity of siNA constructs. For example, applicant has observed that oligonucleotides comprising 2'-deoxy-2'-fluoro nucleotides can degrade under inappropriate deprotection conditions. Such oligonucleotides are deprotected using aqueous methylamine at about 35°C for 30 minutes. If the 2'-deoxy-2'-fluoro containing oligonucleotide also comprises ribonucleotides, after deprotection with aqueous methylamine at about 35°C for 30

minutes, TEA-HF is added and the reaction maintained at about 65°C for an additional 15 minutes. The deprotected single strands of siNA are purified by anion exchange to achieve a high purity while maintaining high yields. To form the siNA duplex molecule the single strands are combined in equal molar ratios in a saline solution to form the duplex. The duplex siNA is concentrated and desalted by tangential filtration prior to lyophilization.

[0673] Below is a table showing various synthesized siNAs.

Table 1b: ENaCα siNAs Strands Synthesized.

Target Site	SEQ ID NO:	Target Sequence	Modified Sequence	SEQ ID NO:
310	1	GCCAUCCGCCUGGUGUGCU	B *GccAuccGccuGGuGuGcu TT*B	33
310	1	GCCAUCCGCCUGGUGUGCU	AGCAcAccAGGcGGAuGGcUU	34
311	2	CCAUCCGCCUGGUGUGCUC	B cc*AuccGccuGGuGuGcuc TT*B	35
311	2	CCAUCCGCCUGGUGUGCUC	GAGcAcAccAGGcGGAuGGUU	36
334	3	CACAACCGCAUGAAGACGG	B c*AcAAccGcAuGAAGAcGG TT*B	37
334	3	CACAACCGCAUGAAGACGG	CCGucuucAuGcGGuuGuGUU	38
337	4	AACCGCAUGAAGACGGCCU	B *AAccGCAuGAAGAcGGccu TT*B	39
337	4	AACCGCAUGAAGACGGCCU	AGGccGucuucAuGcGGuuUU	40
338	5	ACCGCAUGAAGACGGCCUU	B *AccGcAuGAAGAcGGccuu TT*B	41
338	5	ACCGCAUGAAGACGGCCUU	AAGGccGucuucAuGcGGuUU	42
339	6	CCGCAUGAAGACGGCCUUC	B cc*GcAuGAAGAcGGccuuc TT*B	43
339	6	CCGCAUGAAGACGGCCUUC	GAAGGccGucuucAuGcGGUU	44
340	7	CGCAUGAAGACGGCCUUCU	B c*GcAuGAAGAcGGccuucu TT*B	45
340	7	CGCAUGAAGACGGCCUUCU	AGAAGGccGucuucAuGcGUU	46
341	8	GCAUGAAGACGGCCUUCUG	B *GcAuGAAGAcGGccuucuG TT*B	47
341	8	GCAUGAAGACGGCCUUCUG	CAGAAGGccGuCuucAuGcUU	48
781	9	CUGUGCAACCAGAACAAAU	B cu*GuGcAAccAGAAcAAAu TT*B	49
781	9	CUGUGCAACCAGAACAAAU	AUUuGuucuGGuuGcAcAGUU	50
782	10	UGUGCAACCAGAACAAAUC	B u*GuGcAAccAGAAcAAAuc TT*B	51
782	10	UGUGCAACCAGAACAAAUC	GAUuuGuucuGGuuGcAcAUU	52
1100	11	CAGAGCAGAAUGACUUCAU	B c*AGAGcAGAAuGAcuucAu TT*B	53
1100	11	CAGAGCAGAAUGACUUCAU	AUGAAGucAuucuGcucuGUU	54
1121	12	CCCUGCUGUCCACAGUGAC	B cccu*GcuGuccAcAGuGAc TT*B	55
1121	12	CCCUGCUGUCCACAGUGAC	GUCAcuGuGGAcAGcAGGGUU	56
1181	13	UUAUGGAUGAUGGUGGCUU	B uu*AuGGAuGAuGGuGGcuu TT*B	57
1181	13	UUAUGGAUGAUGGUGGCUU	AAGccAccAucAuccAuAAUU	58
1351	14	CACUCCUGCUUCCAGGAGA	B c*AcuccuGcuucc AGGAGA TT*B	59
1351	14	CACUCCUGCUUCCAGGAGA	UCUccuGGAAGcAGGAGuGUU	60
1382	15	AGUGUGGCUGUGCCUACAU	B *AGuGuGGcuGuGccuAcAu TT*B	61
1382	15	AGUGUGGCUGUGCCUACAU	AUGuAGGcAcAGccAcAcuUU	62
1383	16	GUGUGGCUGUGCCUACAUC	B *GuGuGGcuGuGccuAcAuc TT*B	63
1383	16	GUGUGGCUGUGCCUACAUC	GAUGuAGGcAcAGccAcAcUU	64
1384	17	UGUGGCUGUGCCUACAUCU	B u*GuGGcuGuGccuAcAucu TT*B	65
1384	17	UGUGGCUGUGCCUACAUCU	AGAuGuAGGcAcAGccAcAUU	66
1385	18	GUGGCUGUGCCUACAUCUU	B *GuGGcuGuGccuAcAucuu TT*B	67

1385	18	GUGGCUGUGCCUACAUCUU	AAGAuGuAGGcAcAGccAcUU	68
1386	19	UGGCUGUGCCUACAUCUUC	B u*GG*cu*G*u*G*ccu*A*cAucuuc *TT*B	69
1386	19	UGGCUGUGCCUACAUCUUC	GAAGAuGuAGGcAcAGccAUU	70
1387	20	GGCUGUGCCUACAUCUUCU	B *GG*cu*G*u*G*ccu*A*cAucuucu *TT*B	71
1387	20	GGCUGUGCCUACAUCUUCU	AGAAGAuGucGGcAcAGccUU	72
1388	21	GCUGUGCCUACAUCUUCUA	B *G*cu*G*u*G*ccu*A*cAucuucu*A TT*B	73
1388	21	GCUGUGCCUACAUCUUCUA	UAGAAGAuGuAGGcAcAGcUU	74
1738	22	CUCCUGUCCAACCUGGGCA	B cuccu*G*ucc*AA*ccu*GGGcA TT*B	75
1738	22	CUCCUGUCCAACCUGGGCA	UGCccAGGuuGGAcAGGAGUU	76
1739	23	UCCUGUCCAACCUGGGCAG	B uccu*G*ucc*AA*ccu*GGGcAG TT*B	77
1739	23	UCCUGUCCAACCUGGGCAG	CUGcccAGGuuGGAcAGGAUU	78
1742	24	UGUCCAACCUGGGCAGCCA	B u*G*ucc*AA*ccu*GGGcAG*cc*A TT*B	79
1742	24	UGUCCAACCUGGGCAGCCA	UGGcuGcccAGGuuGGAcAUU	80
1743	25	GUCCAACCUGGGCAGCCAG	B *G*ucc*AA*ccu*GGGcAG*cc*AG TT*B	81
1743	25	GUCCAACCUGGGCAGCCAG	CUGGcuGcccAGGuuGGAcUU	82
1747	26	AACCUGGGCAGCCAGUGGA	B *AA*ccu*GGGcAG*cc*AG*u*GG*A *TT*B	83
1747	26	AACCUGGGCAGCCAGUGGA	UCCAcuGGcuGcccAGGuuUU	84
1748	27	ACCUGGGCAGCCAGUGGAG	B *A*ccu*GGGcAG*cc*AG*u*GGAG TT*B	85
1748	27	ACCUGGGCAGCCAGUGGAG	CUCcAcuGGcuGcccAGGuUU	86
1751	28	UGGGCAGCCAGUGGAGCCU	B u*GGGcAG*cc*AG*u*GGAG*ccu *TT*B	87
1751	28	UGGGCAGCCAGUGGAGCCU	AGGcuccAcuGGcuGcccAUU	88
1752	29	GGGCAGCCAGUGGAGCCUG	B *GGGcAG*cc*AG*u*GGAG*ccu*G TT*B	89
1752	29	GGGCAGCCAGUGGAGCCUG	CAGGcuccAcuGGcuGcccUU	90
1753	30	GGCAGCCAGUGGAGCCUGU	B *GGcAG*cc*AG*u*GGAG*ccu*G*u *TT*B	91
1753	30	GGCAGCCAGUGGAGCCUGU	ACAGGcuccAcuGGcuGccUU	92
1756	31	AGCCAGUGGAGCCUGUGGU	B *AG*cc*AG*u*GGAG*ccu*G*u*GG*u *TT*B	93
1756	31	AGCCAGUGGAGCCUGUGGU	ACCAcAGGcuccAcuGGcuUU	94
1757	32	GCCAGUGGAGCCUGUGGUU	B *G*cc*AG*u*GGAG*ccu*G*u*GG*uu *TT*B	95
1757	32	GCCAGUGGAGCCUGUGGUU	AACcAcAGGcuccAcuGGcUU	96

wherein:
A, C, G, and U = ribose A, C, G or U
c and u = 2'-deoxy-2'-fluoro C or U
A, U and G = 2'-O-methyl (2'-OMe) A, U or G
A and *G* = deoxy A or G
B = inverted abasic
T = thymidine

Manufacture of siNA compositions

[0674] In a non-limiting example, for each siNA composition, the two individual, complementary strands of the siNA are synthesized separately using solid phase synthesis, then purified separately by ion exchange chromatography. The complementary strands are annealed to form the double strand (duplex). The duplex is then ultrafiltered and lyophilized to form the solid siNA composition (e.g., pharmaceutical composition). A non-limiting example of the manufacturing process is shown in the flow diagram in **Table 12**.

Solid Phase Synthesis

[0675] The single strand oligonucleotides are synthesized using phosphoramidite chemistry on an automated solid-phase synthesizer, such as an Amersham Pharmacia AKTA Oligopilot (e.g., Oligopilot or Oligopilot 100 plus). An adjustable synthesis column is packed with solid support derivatized with the first nucleoside residue. Synthesis is initiated by detritylation of the acid labile 5'-O-dimethoxytrityl group to release the 5'-hydroxyl. Phosphoramidite and a suitable activator in acetonitrile are delivered simultaneously to the synthesis column resulting in coupling of the amidite to the 5'-hydroxyl. The column is then washed with acetonitrile. Iodine is pumped through the column to oxidize the phosphite triester linkage P(III) to its phosphotriester P(V) analog. Unreacted 5'-hydroxyl groups are capped using reagents such as acetic anhydride in the presence of 2,6-lutidine and N-methylimidazole. The elongation cycle resumes with the detritylation step for the next phosphoramidite incorporation. This process is repeated until the desired sequence has been synthesized. The synthesis concludes with the removal of the terminal dimethoxytrityl group.

Cleavage and Deprotection

[0676] On completion of the synthesis, the solid-support and associated oligonucleotide are transferred to a filter funnel, dried under vacuum, and transferred to a reaction vessel. Aqueous base is added and the mixture is heated to effect cleavage of the succinyl linkage, removal of the cyanoethyl phosphate protecting group, and deprotection of the exocyclic amine protection.

[0677] The following process is performed on single strands that do not contain ribonucleotides: After treating the solid support with the aqueous base, the mixture is filtered under vacuum to separate the solid support from the deprotected crude synthesis material. The solid support is then rinsed with water which is combined with the filtrate. The resultant basic solution is neutralized with acid to provide a solution of the crude single strand.

[0678] The following process is performed on single strands that contain ribonucleotides: After treating the solid support with the aqueous base, the mixture is filtered under vacuum to separate the solid support from the deprotected crude synthesis material. The solid support is then rinsed with dimethylsulfoxide (DMSO) which is combined with the filtrate. The mixture is cooled, fluoride reagent such as triethylamine trihydrofluoride is added, and the

solution is heated. The reaction is quenched with suitable buffer to provide a solution of crude single strand.

Anion Exchange Purification

[0679] The solution of each crude single strand is purified using chromatographic purification. The product is eluted using a suitable buffer gradient. Fractions are collected in closed sanitized containers, analyzed by HPLC, and the appropriate fractions are combined to provide a pool of product which is analyzed for purity (HPLC), identity (HPLC), and concentration (UV A260).

Annealing

[0680] Based on the analysis of the pools of product, equal molar amounts (calculated using the theoretical extinction coefficient) of the sense and antisense oligonucleotide strands are transferred to a reaction vessel. The solution is mixed and analyzed for purity of duplex by chromatographic methods. If the analysis indicates an excess of either strand, then additional non-excess strand is titrated until duplexing is complete. When analysis indicates that the target product purity has been achieved, the material is transferred to the tangential flow filtration (TFF) system for concentration and desalting.

Ultrafiltration

[0681] The annealed product solution is concentrated using a TFF system containing an appropriate molecular weight cut-off membrane. Following concentration, the product solution is desalted via diafiltration using WFI quality water until the conductivity of the filtrate is that of water.

Lyophilization

[0682] The concentrated solution is transferred to sanitized trays in a shelf lyophilizer. The product is then freeze-dried to a powder. The trays are removed from the lyophilizer and transferred to a class 100 Laminar Air Flow (LAF) hood for packaging.

Packaging Drug Substance

[0683] The lyophilizer trays containing the freeze-dried product are opened in a class 100 LAF hood. The product is transferred to sanitized containers of appropriate size, which are then sealed and labeled.

Drug Substance Container Closure System

[0684] Lyophilized drug substance is bulk packaged in sanitized Nalgene containers with sanitized caps. The bottle size used is dependent upon the quantity of material to be placed within it. After filling, each bottle is additionally sealed at the closure with polyethylene tape.

Analytical Methods and Specifications

Raw Material and In-Process Methods

[0685] Raw materials are tested for identity prior to introduction into the drug substance manufacturing process. Critical raw materials, those incorporated into the drug substance molecule, are tested additionally using a purity test or an assay test as appropriate. In-process samples are tested at key control points in the manufacturing process to monitor and assure the quality of the final drug substance.

Drug Substance Analytical Methods and Specifications

[0686] Controls incorporating analytical methods and acceptance criteria for oligonucleotides are established prior to clinical testing of bulk siNA compositions. The following test methods and acceptance criteria reflect examples of these controls.

Summary of Analytical Methods

Identification (ID) Tests

[0687] ID Oligonucleotide Main Peak: The identity of the drug substance is established using a chromatographic method. The data used for this determination is generated by one of the HPLC test methods (see Purity Tests). The peak retention times of the drug substance sample and the standard injections are compared. Drug substance identity is supported by a favorable comparison of the main peak retention times.

[0688] Molecular Weight: The identity of the drug substance is established using a spectroscopic method. A sample of drug substance is prepared for analysis by precipitation with aqueous ammonium acetate. The molecular weight of the drug substance is determined by mass spectrometry. The test is controlled to within a set number of atomic mass units from the theoretical molecular weight.

[0689] Melting Temperature: This method supports the identity of the drug substance by measurement of the melting temperature (Tm) of the double stranded drug substance. A sample in solution is heated while monitoring the ultraviolet (UV) absorbance of the solution. The Tm is marked by the inflection point of the absorbance curve as the absorbance increases due to the dissociation of the duplex into single strands.

Assay Tests

[0690] Oligonucleotide Content: This assay determines the total oligonucleotide content in the drug substance. The oligonucleotide absorbs UV light with a local maximum at 260 nm. The oligonucleotide species present consist of the double stranded siRNA product and other minor related oligonucleotide substances from the manufacturing process, including residual single strands. A sample of the drug substance is accurately weighed, dissolved, and diluted volumetrically in water. The absorbance is measured in a quartz cell using a UV spectrophotometer. The total oligonucleotide assay value is calculated using the experimentally determined molar absorptivity of the working standard and reported in micrograms of sodium oligonucleotide per milligram of solid drug substance.

[0691] Purity Tests: Purity will be measured using one or more chromatographic methods. Depending on the separation and the number of nucleic acid analogs of the drug substance present, orthogonal separation methods may be employed to monitor purity of the API. Separation may be achieved by the following means:

[0692] SAX-HPLC: an ion exchange interaction between the oligonucleotide phosphodiesters and a strong anion exchange HPLC column using a buffered salt gradient to perform the separation.

[0693] RP-HPLC: a partitioning interaction between the oligonucleotide and a hydrophobic reversed-phase HPLC column using an aqueous buffer versus organic solvent gradient to perform the separation.

[0694] Capillary Gel Electrophoresis (CGE): an electrophoretic separation by molecular sieving in a buffer solution within a gel filled capillary. Separation occurs as an electrical field is applied, causing anionic oligonucleotides to separate by molecular size as they migrate through the gel matrix. In all separation methods, peaks elute generally in order of oligonucleotide length and are detected by UV at 260 nm.

Other Tests

[0695] Physical Appearance: The drug substance sample is visually examined. This test determines that the material has the character of a lyophilized solid, identifies the color of the solid, and determines whether any visible contaminants are present.

[0696] Bacterial Endotoxins Test: Bacterial endotoxin testing is performed by the Limulus Amebocyte Lysate (LAL) assay using the kinetic turbidimetric method in a 96-well plate. Endotoxin limits for the drug substance will be set appropriately such that when combined with the excipients, daily allowable limits for endotoxin in the administered drug product are not exceeded.

[0697] Aerobic Bioburden: Aerobic bioburden is performed by a contract laboratory using a method based on USP chapter <61>.

[0698] Acetonitrile content: Residual acetonitrile analysis is performed by a contract laboratory using gas chromatography (GC). Acetonitrile is the major organic solvent used in the upstream synthesis step although several other organic reagents are employed in synthesis. Subsequent purification process steps typically remove solvents in the drug substances. Other solvents may be monitored depending on the outcome of process development work. Solvents will be limited within ICH limits.

[0699] Water content: Water content is determined by volumetric Karl Fischer (KF) titration using a solid evaporator unit (oven). Water is typically present in nucleic acid drug substances as several percent of the composition by weight, and therefore, will be monitored.

[0700] pH: The pH of reconstituted drug substance will be monitored to ensure suitability for human injection.

[0701] Ion Content: Testing for sodium, chloride, and phosphate will be performed by a contract laboratory using standard atomic absorption and ion chromatographic methods.

General monitoring of ions will be performed to ensure that the osmolality of the drug product incorporating the drug substances will be within an acceptable physiological range.

[0702] Metals Content: Testing for pertinent metals is performed by a contract laboratory using a standard method of analysis, Inductively Coupled Plasma (ICP) spectroscopy.

Example 4: RNAi *in vitro* assay to assess siNA activity

[0703] An *in vitro* assay that recapitulates RNAi in a cell-free system is used to evaluate siNA constructs targeting RNA targets. The assay comprises the system described by Tuschl *et al.,* 1999, *Genes and Development*, 13, 3191-3197 and Zamore *et al.,* 2000, *Cell*, 101, 25-33 adapted for use with a target RNA. A Drosophila extract derived from syncytial blastoderm is used to reconstitute RNAi activity *in vitro*. Target RNA is generated via *in vitro* transcription from an appropriate target expressing plasmid using T7 RNA polymerase or via chemical synthesis as described herein. Sense and antisense siNA strands (for example 20 uM each) are annealed by incubation in buffer (such as 100 mM potassium acetate, 30 mM HEPES-KOH, pH 7.4, 2 mM magnesium acetate) for 1 minute at 90°C followed by 1 hour at 37°C , then diluted in lysis buffer (for example 100 mM potassium acetate, 30 mM HEPES-KOH at pH 7.4, 2mM magnesium acetate). Annealing can be monitored by gel electrophoresis on an agarose gel in TBE buffer and stained with ethidium bromide. The Drosophila lysate is prepared using zero to two-hour-old embryos from Oregon R flies collected on yeasted molasses agar that are dechorionated and lysed. The lysate is centrifuged and the supernatant isolated. The assay comprises a reaction mixture containing 50% lysate [vol/vol], RNA (10-50 pM final concentration), and 10% [vol/vol] lysis buffer containing siNA (10 nM final concentration). The reaction mixture also contains 10 mM creatine phosphate, 10 ug/ml creatine phosphokinase, 100 um GTP, 100 uM UTP, 100 uM CTP, 500 uM ATP, 5 mM DTT, 0.1 U/uL RNasin (Promega), and 100 uM 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.

[0704] Alternately, internally-labeled target RNA for the assay is prepared by *in vitro* transcription in the presence of [alpha-^{32}P] CTP, passed over a G50 Sephadex column by spin chromatography and used as target RNA without further purification. Optionally, target RNA is 5'-^{32}P-end labeled using T4 polynucleotide kinase enzyme. Assays are performed as described above and target RNA and the specific RNA cleavage products generated by RNAi are visualized on an autoradiograph of a gel. The percentage of cleavage is determined by PHOSPHOR IMAGER® (autoradiography) quantitation of bands representing intact control RNA or RNA from control reactions without siNA and the cleavage products generated by the assay.

[0705] In one embodiment, this assay is used to determine target sites in the target RNA target for siNA mediated RNAi cleavage, wherein a plurality of siNA constructs are screened for RNAi mediated cleavage of the target RNA target, for example, by analyzing the assay reaction by electrophoresis of labeled target RNA, or by northern blotting, as well as by other methodology well known in the art.

Example 5: Animal Models useful to evaluate the down-regulation of ENaC gene expression

[0706] Following identification of active siNA constructs in vitro, a rodent model of airway ENaC function can be used to assess the effectiveness of siNAs targeting ENaC in reducing ion transport. A suitable model is the guinea pig model described by Coote, K.J. et al (2008) Br. J. Pharmacol. (online submission 22 September 2008 pp1-9; doi: 10.1038/bjp.2008.363). This model uses tracheal potential difference to measure airway epithelial ion transport in the guinea pig.

Example 6: RNAi mediated inhibition of ENaC gene expression

[0707] siNA constructs (**Table 1b**) may be tested for efficacy in reducing ENaC RNA expression in, for example, A549 human lung carcinoma cells. A549 (human; ATCC cat# CCL-185) cells were cultured at 37°C in the presence of 5% CO_2 and grown in Ham's F12K medium with 2 mM L-glutamine adjusted to contain 1.5 g/L sodium bicarbonate and supplemented with fetal bovine serum at a final concentration of 10% and 100U/mL penicillin. The A549 cells were plated approximately 24 hours before transfection in 96-well plates at 7,500 cells/well, 100 µl/well. After 24 hours complexes containing siNA and Lipofectamine 2000 (Invitrogen) were created as follows: a solution of Lipofectamine 2000

in OPTI-MEM was prepared containing Lipofectamine 2000 at a final concentration of 14μg/ml. In parallel, solutions of the siNAs were prepared in OPTI-MEM at a final concentration of 150nM. After both solutions were incubated at 20°C for 20 minutes an equal volume of the siNA solutions and the Lipofectamine 2000 solution were added together for each of the siNAs. The resultant solution has the siNAs at a final concentration of 75nM and Lipofectamine 2000 at a final concentration of 7μg/ml. This solution was incubated at 20°C for 20 minutes. After incubation 50μl of the solution were added to each of the wells (in triplicate). The final concentration of siNA in each well was 25nM and the final concentration of Lipofectamine 2000 in each well was 2.33μg/ml. The plates were incubated for 48 hours with no change of media before harvesting. Dose response curves were determined in a similar manner with each concentration being done in triplicate and maintaining a constant amount of Lipofectamine 2000 in each transfection.

[0708] RNA was extracted from 96-well plates using the Invitek Invisorb RNA Cell HTS 96 Kit/C (Cat# 70619000) with a slightly modified protocol. The details of the protocol for isolating RNA from each plate are described as follows:

1) Aspirate media with rake
2) Add 150 ul/well Lysis buffer
3) Place on plate shaker for 1 minute
4) Place DNA binding plate on top of .5 ml collection plate
5) Pipette 190 ul up and down twice and add to DNA binding plate
6) Cover plate
7) Centrifuge plates 4 minutes at 4000 RPM
8) Discard DNA binding plate
9) Place the RNA binding plate on top of 2 ml collection plate
10) Add 150 ul/well of Binding Buffer R to 0.5 ml collection plate
11) Move contents of 0.5 ml collection plate to RNA binding plate
12) Let incubate for 1 minute
13) Centrifuge plates 4 minutes at 4000 RPM
14) Add 600 ul/well of Wash Buffer R1 to RNA binding plate
15) Centrifuge plates 4 minutes at 4000 RPM
16) Dump wash buffer from 2 ml collection plate
17) Add 400 ul/ well of Wash Buffer R2 to RNA binding plate
18) Centrifuge plates 4 minutes at 4000 RPM
19) Add 400 ul/well of Wash Buffer R2 to RNA binding plate
20) Centrifuge plates 10 minutes at 4000 RPM
21) Dump wash buffer from 2 ml collection plate
22) Centrifuge plates 3 minutes at 4000 RPM

23) Discard 2 ml collection plate and place RNA binding plate on to microtiter plate
24) Add 100 ul/well of elution buffer to RNA binding plate
25) Incubate for 2 minutes
26) Seal plate
27) Centrifuge plates 3 minutes at 4000 RPM
28) Discard RNA binding plate
29) Cover microtiter plate for storage in –80 freezer

6-Well Plate Transfection Protocol

[0709] On the day of transfection, about 150,000 cells were seeded in 6-well plate in 2 ml of growth medium. After about 3 hours, the cells were transfected with siNA at 12.5, 25, 50 or 100 nM final concentration using Lipofectamine 2000 reagent (2.5 µl per well). The siNA -Lipofectamine 2000 complex (at 25 nM C_f) was prepared as follows: (a) 1 µM siNA stock solution was diluted in 250 µl of Opti-MEM Reduced Serum Medium (resulting concentration of siNA was 250 nM); (b) the working solution of Lipofectamine 2000 reagent was prepared by diluting the stock in Opti-MEM at 1:100 ratio, it was then mixed and incubated for 5 min at room temperature; (c) 250 µl of diluted siNA was combined with an equal volume of the working solution of Lipofectamine 2000 (resulting concentration of siNA was 125 nM); and (d) 500 µl of the mixture was added to the well (resulting concentration of siNA is 25 nM). Cells ere then incubated at 37°C for 48 hours.

Quantitative RT-PCR (Taqman)

[0710] A series of probes and primers were used to detect the various mRNA transcripts of the genes of ENaCα and GAPDH in mouse, rat, and human cell lines. The assays were performed on an ABI 7500 instrument, according to the manufacturer's instructions. Within each experiment, the baseline was set in the exponential phase of the amplification curve, and based on the intersection point of the baselines with the amplification curve, a Ct value was assigned by the instrument. This Ct value was then assigned a QTY value based on the Standard Curve. The standard curve in the various experiments herein used 1 ng to 300 ng of RNA extracted from the same cell line used for the experimental transfections.

ENaCα Western Blot

[0711] The protein source for the Western Blot experiments were from transfection of A549 cells in a 6 well plate. Cells were seeded (~150,000/well) in 2 mL complete media

(Ham's F12, Cellgro Cat# 10-080-CV + 10% FBS). Cells were transfected 3 to 4 hours after seeding, with siNA molecules at a final concentration of 25 nM (in triplicate) using Lipofectamine 2000. The siNAs were allowed to incubate for 48 hours. Protein samples were prepared using the "PARIS" Protein and RNA Isolation System (Ambion, Cat# 1921) according to manufacturer's instructions. The amount of total protein in cell lysates were measured using a Bradford Dye Reagent (Bio-Rad, Cat# 500-0205).

[0712] Western blot assays were run on 4-12% NuPAGE precast gels (Invitrogen Cat# NPO0322BOX) by diluting protein samples 1:1 in 2X Laemmli buffer with 5% 2-mercaptoethanol, and incubated for 5 minutes at 95°C. Each lane was loaded with 20µg protein (except for marker lanes which were added according to manufacturer's recommended protocol). The gels were run at 100V for about 2 hours, or until the 20kD standard marker reached the bottom of the gel. After the resolution by electrophoresis, the proteins were transferred to a PVDF membrane (1 hr at 100V). Using Casein (1%) in PBS, the membrane was blocked (60 min. at room temperature; on plate shaker). Primary rabbit polyclonal antibody (Abcam # ab3464) was diluted 1:500 in 1% Casein/PBS, and incubated with the membrane at 4°C on a rocker overnight. The blot was washed for three time for 5 minutes each with 0.1%Twen/PBS solution. The blot was incubated with a secondary antibody solution (goat anti-rabbit antibody, Jactson Immunoresearch Cat. # 111-035-144) at a 1:25,000 dilution for 30 minutes at room temperature on a rocker. Following this incubation, the blot was quickly rinsed three time for five minutes each with 0.1% Tween/PBS solution.. The blot was the incubated for 1-2 minutes with ECL+reagents (Amersham # RPN2133) according to the amnaufacturer's insurcions. The blot was then imaged.

[0713] The Western Blots assays as described above, were used to confirm that the siNA molecules of the invention reduced the protein level of ENaCα.

RACE Analysis

[0714] Using 96-well plates, A549 cells were treated with either 25nM active or 25nM control siNA. To obtain sufficient RNA (5µg), one 96-well plate was used for each treatment (96 replicates). Following 24hr transfection, total RNA was isolated using standard Trizol (Invitrogen) isolation, using 2mls Trizol per 96-well plate. The isolated RNA was used for RACE protocol.

[0715] The GeneRacer Oligo was ligated to total RNA by adding 5µg total RNA, in 7µl of H_2O, to lyophilised GeneRacer Oligo and incubating at 65C for 5 minutes. The mixture was placed on ice for 2 minutes, centrifuged briefly, then to it was added 1µl 10x ligase buffer, 1µl 10mM ATP, 1µl RNAse Out (40U/µl), and 1µl T4 RNA ligase (5U/µl). The mixture was mixed gently, and then incubated at 37C for 1 hour. Following 1 hour incubation, 90µl DEPC H_2O and 100µl phenol:cholorform was added. The mixture was vortexed on high for 30 seconds then centrifuged 5 minutes on high. The aqueous layer was transferred to a new eppendorf tube. To this layer was added 2µl glycogen, 10µl 3M sodium acetate and then mixed. 220µl of ethanol was then added. The mixture was inverted several times to mix then placed on dry ice for 10 minutes. It was centrifuge on high at 4C for 20 minutes. The supernatant was aspirated then wash with 70% ethanol. It was centrifuged for 5 minutes on high and the supernatant was removed. The pellet was allowed to dry for 2-3 minutes. The pellet was suspended in 10µl of DEPC H_2O.

[0716] To 10µl of the ligated RNA was added 1µl 10µM RT-Primer (ENaCα primer 1), 1µl 10mM dNTP mix, and 1µl DEPC H_2O then the mixture was incubated at 65C for 5 minutes, followed by placing on ice for 2 minutes, and centrifuging briefly To this was added 4µl 5x first strand buffer, 1µl 0.1M DTT, 1µl RNAse Out (40U/µl), and 1µl Superscript III RT (200U/µl). The mixture was mixed gently then incubated at 50C for 45 minutes followed by 65C for 7 minutes. The RT reaction was inactivated by incubating at 70C for 15 minute and then placing on ice. The mixture was centrifuged briefly. To it was then added 1µl RNAse H (2U) followed by mixing gently and then incubating at 37C for 20 minutes. This mixture was then either stored at –20C or used immediately for PCR.

[0717] Using 1µl of the RT reaction above as template, a standard PCR amplification was performed for 34 cycles using 5' GeneRacer primer and 3' Gene Specific primer (ENaCα primer 1). The specificity was increased by use of a 60C annealing temperature. Using 1µl of the PCR reaction above as template, a Nested PCR was performed reaction for 34 cycles using the GeneRacer Nested 5' primer and Nested 3' Gene Specific primers (ENaCα primer 2). The specificity was increased by use of a 60C annealing temperature. The samples were analyzed on native 6% PAGE. The bands of expected size were cut out and eluted from gel using SNAP columns provided with the GeneRacer kit. The eluted gel bands were cloned

using a TOPO TA Cloning kit provided with the GeneRacer kit. LB-Amp agar plates containing colonies were PCR screened and sequenced.

Table 2: Primer sequences used in RACE method

PRIMER	SEQUENCE	SEQ ID NO:
GeneRacer 5' Primer	5'- CGACTGGAGCACGAGGACACTGA	97
GeneRacer 5' Nested Primer	5'- GGACACTGACATGGACTGAAGGAGTA	98
Site 782: ENaCα primer 1	5'- GGAAGACATCCAGAGGTTGG	99
Site 782: ENaCα primer 2	5'- GGTTGCAGGAGACCTGGTT	100
Site 1181: ENaCα primer 1	5'- GCCGCGGATAGAAGATGTAG	101
Site 1181: ENaCα primer 2	5'- TCCTGGAAGCAGGAGTGAAT	102
Site 1383: ENaCα primer 1	5'- TTCTGTCGCGATAGCATCTG	103
Site 1383: ENaCα primer 2	5'- CCAGGTGGTCTGAGGAGAAG	104
Site 1388: ENaCα primer 1	5'- TTCTGTCGCGATAGCATCTG	105
Site 1388: ENaCα primer 2	5'- GCAGAGAGCTGGTAGCTGGT	106

Calculations

[0718] All IC_{50} values were calculated from the data using GraphPad Prizm software, specifically a sigmoidal, variable slope curve for simple ligand binding. Also, unless otherwise indicated, all calculations of the efficacy and potency (*e.g.*, % knockdown) of the siNAs were done relative to a non-targeting control siNA. If reported, P-values were computed using The Students t-test, including the Welch Correction for unequal variances.

[0719] In all of the calculations of the % knock-down of mRNA, the calculation was made relative to the normalized level of expression of the gene of interest in the samples treated with the non-targeting control (NTC) unless otherwise indicated. The gene of interest expression level was divided by the level of expression of GAPDH or 36B4 (depending on the species) in each sample. The three replicates for each condition in each experiment were averaged and the standard deviation of those samples was calculated. The following formula was then used to calculate the % of knock-down of the gene of interest:

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

[0720] Normalized data are graphed and the percent reduction of target mRNA by an active siNA in comparison to its respective inverted control siNA is determined.

Results:

[0721] The ENaCα siNAs were designed and synthesized as described previously. The siNAs were screened in three cell lines. Human A549 cells, mouse NIH 3T3 and Rat H-4-II-E cells. The data from the screen of ENaCα siNAs in all three species is shown in **Tables 3a, 3b, 4a, 4b, 5a, and 5b** and a summary of the data for certain siNA molecules is presented in **Table 6**. Each screen was performed at 24 hrs. The decision to use this time point was based upon the degree of knockdown of the mRNA seen at that time point.

Table 3a. Screening of ENaCα siNAs in human A549 cells. Expression of ENaCα in the transfected cells, the levels of expression of GAPDH, the level of ENaCα expression normalized to the level of GAPDH and the % reduction of the ENaCα mRNA relative to a non-targeting control siNA (NTC) in the transfected cells For each value, n = 3 and cells were harvested 24 hours post-transfection. The % reduction of the ENaCα mRNA relative to a non-targeting control siNA (NTC) in the transfected cells. UNT is untreated control and LF2K is Lipofectamine 2K alone.

Treatment	Avg ENaCα Expression	Avg GAPDH Expression	Mean	Stdev	Percent Reduction	%sd
UNT	74.97	64.07	1.19	0.17	23.1%	11.2%
LF2K	70.75	71.93	1.07	0.39	31.1%	24.8%
Site 310	68.10	45.78	1.57	0.42	-1.0%	27.1%
Site 311	65.43	53.29	1.29	0.39	17.0%	25.0%
Site 334	58.30	42.86	1.49	0.50	4.5%	32.2%
Site 337	67.27	49.84	1.48	0.44	5.0%	28.4%
Site 338	65.83	76.32	0.90	0.25	42.2%	16.0%
Site 339	69.05	62.02	1.13	0.17	27.1%	10.7%
Site 340	84.94	63.09	1.40	0.29	9.9%	18.8%
Site 341	62.42	48.12	1.62	0.33	-4.3%	21.3%
Site 781	61.44	66.47	0.97	0.31	37.7%	20.1%
Site 782	39.99	66.25	0.61	0.07	60.9%	4.7%
Site 1100	66.11	64.16	1.14	0.38	26.8%	24.2%
Site 1121	72.95	46.89	1.65	0.41	-6.0%	26.1%
Site 1181	36.75	70.22	0.55	0.13	64.7%	8.5%
Site 1351	68.14	64.22	1.12	0.28	28.2%	18.1%
Site 1382	72.49	57.29	1.30	0.21	16.6%	13.8%
Site 1383	40.44	52.36	0.78	0.07	49.8%	4.7%
MAPK14 1033	62.51	40.83	1.55	0.33	0.0%	21.5%

Table 3b. Screening of ENaCα siNAs in human A549 cells. Expression of ENaCα in the transfected cells, the levels of expression of GAPDH, the level of ENaCα expression normalized to the level of GAPDH and the % reduction of the ENaCα mRNA relative to a non-targeting control siNA (NTC) in the transfected cells. For each value, n = 3 and cells were harvested 24 hours post-transfection. The % reduction of the ENaCα mRNA relative to a non-targeting control siNA (NTC) in the transfected cells. UNT is untreated control and LF2K is Lipofectamine 2K alone.

Treatment	Avg ENaCα Expression	Avg GAPDH Expression	Mean	Stdev	Percent Reduction	%sd
UNT	84.50	128.02	0.66	0.03	0.1%	4.4%
LF2K	69.96	126.21	0.56	0.04	15.7%	6.0%
Site 1384	82.65	103.38	0.80	0.05	-21.6%	7.9%
Site 1385	90.81	119.04	0.81	0.01	-23.6%	1.4%
Site 1386	72.05	112.63	0.64	0.03	2.8%	4.3%
Site 1387	46.10	106.19	0.43	0.02	34.2%	3.2%
Site 1388	36.49	120.24	0.30	0.01	54.1%	1.8%
Site 1738	77.23	108.96	0.71	0.01	-7.4%	1.7%
Site 1739	75.77	100.60	0.75	0.05	-13.8%	7.2%
Site 1742	58.12	109.65	0.53	0.01	19.7%	2.2%
Site 1743	68.82	121.24	0.57	0.02	13.9%	3.1%
Site 1747	75.36	130.33	0.58	0.02	12.3%	3.4%
Site 1748	80.25	131.14	0.61	0.00	7.2%	0.6%
Site 1751	63.35	128.83	0.49	0.01	25.4%	1.0%
Site 1752	82.06	137.51	0.60	0.01	9.4%	1.2%
Site 1753	84.05	128.19	0.66	0.01	0.6%	2.1%
Site 1756	73.20	102.64	0.71	0.02	-8.2%	3.0%
Site 1757	66.53	101.11	0.66	0.04	0.0%	6.5%
MAPK14 1033	72.88	110.51	0.66	0.03	0.0%	4.8%
MAPK14 control2	67.76	105.69	0.64	0.04	3.1%	5.6%

Table 4a: Screening of ENaCα siNAs in mouse NIH 3T3 cells. Expression of mSCNN1A, m36B4, and the levels of mSCNN1A expression are normalized to the level of m36B4 and the % reduction of the mSCNN1A mRNA relative to a non-targeting control siNA For each point, n = 3 and cells were harvested 24 hours post-transfection.

Treatment	Avg ENaCα Expression	Avg 36B4 Expression	Mean	Stdev	Percent Reduction	%sd
UNT	2.31	17.14	0.15	0.04	35.6%	18.1%
LF2K	3.42	17.25	0.20	0.01	16.7%	3.9%

Site 310	3.19	16.02	0.20	0.04	16.6%	16.4%
Site 311	3.83	16.80	0.23	0.02	4.6%	10.0%
Site 334	3.57	16.98	0.21	0.03	12.3%	11.9%
Site 337	3.42	14.44	0.24	0.02	0.8%	7.2%
Site 338	3.24	12.29	0.26	0.02	-9.5%	8.7%
Site 339	5.05	14.61	0.35	0.01	-44.9%	2.7%
Site 340	3.35	14.93	0.23	0.01	5.4%	5.0%
Site 341	3.17	13.56	0.23	0.04	3.9%	17.0%
Site 781	3.50	16.49	0.22	0.06	9.8%	25.5%
Site 782	0.85	16.72	0.05	0.04	78.2%	14.9%
Site 1100	3.43	14.25	0.24	0.04	-1.5%	18.4%
Site 1121	3.39	11.45	0.27	0.04	-14.5%	17.2%
Site 1181	0.54	15.48	0.04	0.04	84.4%	16.2%
Site 1351	2.56	11.91	0.22	0.01	9.6%	4.8%
Site 1382	2.94	13.77	0.21	0.02	10.7%	6.8%
Site 1383	0.91	14.23	0.04	0.04	82.4%	15.6%
MAPK14 1033	3.04	15.69	0.20	0.03	17.2%	14.1%
MAPK14 control2	3.20	13.51	0.24	0.03	0.0%	14.0%

Table 4b: Screening of ENaCα siNAs in mouse NIH 3T3 cells. Expression of mSCNN1A, m36B4, and the levels of mSCNN1A expression are normalized to the level of m36B4 and the % reduction of the mSCNN1A mRNA relative to a non-targeting control siNA For each point, n = 3 and cells were harvested 24 hours post-transfection.

Treatment	Avg ENaC Expression	Avg 36B4 Expression	Mean	Stdev	Percent Reduction	%sd
UNT	3.65	32.64	0.11	0.01	19.1%	5.2%
LF2K	2.78	26.75	0.10	0.01	24.0%	5.4%
Site 1384	3.11	20.56	0.14	0.02	-0.1%	12.5%
Site 1385	2.95	22.75	0.13	0.01	4.4%	10.1%
Site 1386	2.35	25.00	0.09	0.01	31.9%	9.8%
Site 1387	1.37	24.37	0.06	0.01	59.1%	4.4%
Site 1388	0.90	24.18	0.04	0.01	73.4%	7.1%
Site 1738	2.63	20.59	0.13	0.02	5.1%	11.1%
Site 1739	2.87	22.65	0.13	0.01	7.3%	7.9%
Site 1742	2.91	21.48	0.14	0.01	1.0%	6.1%
Site 1743	2.87	22.47	0.13	0.01	6.7%	4.8%
Site 1747	3.67	25.19	0.15	0.01	-6.1%	4.7%
Site 1748	4.06	26.15	0.15	0.02	-12.7%	16.1%
Site 1751	2.85	28.97	0.10	0.01	27.6%	4.6%
Site 1752	3.21	29.27	0.11	0.00	19.8%	3.2%
Site 1753	3.13	25.27	0.12	0.01	9.6%	3.8%
Site 1756	2.58	21.66	0.12	0.02	13.5%	11.2%
Site 1757	2.58	24.24	0.11	0.01	22.2%	4.0%
MAPK14 1033	2.86	22.44	0.13	0.01	7.3%	5.9%
MAPK14	3.20	23.23	0.14	0.02	0.0%	12.7%

control2						

Table 5a: Screening of ENaCα siNAs in Rat H-4-II-E. Expression of rSCNN1A, rGAPDH, the level of rSCNN1A expression normalized to the level of rGAPDH and the % reduction of the r ENaCα mRNA relative to a non-targeting control siNA (NTCs) are shown. For each point, n = 3 and cells were harvested 24 hours post-transfection.

Treatment	Avg ENaCα Expression	Avg GAPDH Expression	Mean	Stdev	Percent Reduction	%sd
UNT	21.38	23.18	0.92	0.04	28.2%	2.8%
LF2K	19.41	22.45	0.87	0.04	32.3%	3.5%
Site 310	18.39	20.12	0.92	0.10	28.3%	7.5%
Site 311	16.42	19.71	0.83	0.04	35.2%	3.0%
Site 334	16.10	19.11	0.85	0.15	33.7%	11.7%
Site 337	16.40	17.17	0.96	0.06	25.4%	4.7%
Site 338	14.22	18.42	0.79	0.15	38.7%	11.6%
Site 339	15.81	19.92	0.79	0.05	38.3%	4.2%
Site 340	22.68	21.20	1.07	0.15	16.7%	12.0%
Site 341	19.10	18.30	1.05	0.09	18.4%	6.9%
Site 781	23.09	26.57	0.86	0.06	32.8%	4.9%
Site 782	13.71	29.23	0.47	0.06	63.7%	4.7%
Site 1100	21.68	26.15	0.83	0.09	35.4%	7.3%
Site 1121	19.44	23.01	0.84	0.03	34.6%	2.6%
Site 1181	10.34	24.78	0.41	0.07	68.1%	5.7%
Site 1351	18.06	24.87	0.72	0.08	43.7%	6.0%
Site 1382	22.02	26.11	0.84	0.05	34.5%	4.0%
Site 1383	15.01	23.52	0.63	0.07	50.7%	5.4%
MAPK14 control2	22.87	17.91	1.28	0.06	0.0%	4.9%

Table 5b: Screening of ENaCα siNAs in Rat H-4-II-E. Expression of rSCNN1A, rGAPDH, the level of rSCNN1A expression normalized to the level of rGAPDH and the % reduction of the r ENaCα mRNA relative to a non-targeting control siNA (NTCs) are shown. For each point, n = 3 and cells were harvested 24 hours post-transfection.

Treatment	Avg ENaCα Expression	Avg GAPDH Expression	Mean	Stdev	Percent Reduction	%sd
UNT	30.74	38.15	0.77	0.03	16.5%	3.1%
LF2K	26.78	37.32	0.72	0.05	21.8%	5.7%
Site 1384	23.39	34.69	0.66	0.10	27.8%	11.0%
Site 1385	19.09	30.04	0.58	0.14	36.7%	14.9%
Site 1386	17.21	36.31	0.48	0.04	48.3%	4.8%
Site 1387	24.12	34.37	0.71	0.06	23.3%	6.8%
Site 1388	11.65	37.10	0.32	0.02	65.6%	2.7%
Site 1738	21.90	35.43	0.63	0.05	31.1%	5.5%

Site 1739	32.26	35.80	1.00	0.07	-8.7%	7.1%
Site 1742	28.30	35.68	0.79	0.04	14.3%	4.7%
Site 1743	22.13	42.82	0.52	0.04	43.9%	4.3%
Site 1747	23.39	41.88	0.56	0.02	39.5%	2.6%
Site 1748	26.11	43.45	0.60	0.05	34.7%	5.3%
Site 1751	26.87	45.19	0.59	0.16	36.0%	17.5%
Site 1752	19.23	43.83	0.44	0.03	52.1%	3.3%
Site 1753	24.59	41.36	0.59	0.01	35.4%	0.7%
Site 1756	23.08	40.27	0.57	0.06	38.3%	6.6%
Site 1757	29.36	40.49	0.72	0.08	21.5%	8.4%
MAPK14 1033	29.93	38.22	0.77	0.07	16.1%	7.2%
MAPK14 control2	28.96	31.54	0.92	0.07	0.0%	8.1%

Table 6 Summary of efficacy (%KD) and potency (IC_{50}) of ENaCα mRNA knockdown for certain siNA in human, rat and mouse cell lines.

Site	**Human**		**Mouse**		**Rat**	
	%KD	IC_{50}	%KD	IC_{50}	%KD	IC_{50}
782	60-85%	0.36 nM	78%	3.9 nM	63%	0.80 nM
1181	65-80%	0.40 nM	84%	4.1 nM	68%	1.0 nM
1383	50-70%	0.41 nM	82%	3.0 nM	50%	1.0 nM
1388	55-80%	0.19 nM	73%	1.9 nM	65%	0.90 nM

[0722] RACE experiments confirmed that the siNA constructs that correspond to the sites in **Table 6** showed RISC mediated cleavage of target RNA. Thus, verifying that the RNA knockdown was the direct result of RNAi activity.

Example 7: Maximum ENaC mRNA Knockdown and Potency of ENaC siNAs in Human Bronchial Epithelial Cells.

Cell Culture Preparation

[0723] Human Bronchial Epithelial cells (NHBE cells) obtained from Lonza (Cat. No. CC-2540) were grown at 37deg in the presence of 5% CO2 and cultured in BEBM basal medium (Lonza,Cat.No. CC-3171) on Biocoat Collagen1 coated flasks (Becton Dickinson).

[0724] Human U2OS-TLR7 and Human U2OS-TLR8 cells were grown at 37deg in the presence of 5% CO2 and were cultured in Dulbeco's modified Eagle's Medium (DMEM) , 1% non essential amino acids, supplemented with foetal bovine serum at 10% and 100ug/ml streptomycin and 100u/ml penicillin. Stable expression of TLR7 and TLR8 was maintained by the addition of 300ug/ml Gentamycin.

[0725] Recombinant HEK293 cell line (expressing ENaC beta & gamma) SCNN1B (P618AY620L) + SCNN1G (P624stop) #24.were grown at 37deg in the presence of CO2 and were cultured in M1 medium M1 + 0.5mg/ml geneticin and 0.2mg/ml hygromycin.

Transfection

[0726] mRNA Knockdown and EC50 in NHBEs:Cells were plated in collagen 1 coated plates and cultured in appropriate culture media. The cells were cultured for 24hours after plating at 37deg in the presence of 5% CO2. siNAs were diluted in OptiMEM 1 to 1uM and the delivery lipid GSK212357A to 25ug/ml. For formulation of the siNAs equal volumes of the diluted siNA and delivery lipid were combined and incubated for 20 minutes at room temperature. Cells were meanwhile trypsinised and resuspended in antibiotic free BEBM media at 150,000 cells/ml. 20ul of the formulated siNA and 80ul of BEBM media was added per well of a 96 well plate (6 replicates/data point/siRNA concentration) so as to give a nine point dose range of the siRNAs (100nM, 30nM, 10nM, 3nM, 1nM, 0.3nM, 0.1nM, 0.03nM, 0.01nM). The time of incubation with the GSK212357A-siNA complexes were 48 hours with one change of media at 24 hours.

[0727] TLR3 Mediated Immunostimulation: NHBE cell were treated as above for the measurement of endosomal TLR3 mediated immunostimulation, with the inclusion of polyI:C as a positive control for OAS1 mRNA upregulation. For the measurement of membrane bound TLR3 mediated immunostimulation the NHBE cells were cultured at 1200 cells/ per 96 well and siRNAs administered in PBS the absence of a delivery vehicle at (100nM, 30nM, 10nM, 3nM, 1nM, 0.3nM, 0.1nM, 0.03nM, 0.01nM).

[0728] TLR7 and TLR8 Mediated Immunostimulation: TLR7 and TLR8 expressing U2OS osteosarcoma cells were seeded in 96-well plate format at a concentration of $2x10^4$cells/well 24 hours prior to transfection. Cells were transfected with the siRNAs using DharmaFECT1 lipid transfection reagent using Resiquimod (R848) and the LyoVec-complexed, GU-rich oligonucleotide ssRNA40 respectively as controls (100µl/well). The

treatment media were replaced after 6 hours with antibiotic containing DMEM. Cells were harvested 24 hours following transfection. R848 and ssRNA40 are characterised agonists of the two receptors upon stimulation; the transformed osteosarcoma cells exhibited an increased IL8 expression in a dose response manner. An agonist concentration range of 4-10μg/ml was established since it caused optimal levels of IL8 mRNA expression for the assay. No significant IL8 expression was observed in the native U2OS cell line lacking TLRs following treatment with the two agonists, R848 and ssRNA40.

[0729] DharmaFECT1 was used as the delivery agent for the siRNAs as it combines low immunostimulatory effects with high delivery efficiency.

[0730] Functional Effects on ENaC via FLIPR in Cells Over Expressing ENaCα: A reverse transfection methodology was used to transfect the HEK recombinant cell line. 5ul of siRNA (diluted in Optimem to the appropriate concentration) was mixed with 5ul of the Gemini lipid transfection reagent diluted in Optimem in 384 well non-coated FLIPR plate. Plates were incubated at room temperature for 20 minutes to allow complex formation. Cells were harvested, spun down, diluted to $3x10^5$/ml and plated out at 40ul/well (12K/well) in 384 well plate format containing the transfection complex. The plates were incubated overnight for 20 hours. The cells were then transduced with BacMam virus expressing ENaC alpha (BacMam viruses expressing SCNN1A, GRITS 29703, titre $0.356x10^8$/ml). 30ul of virus was added (1.7%) 20K/well, MOI was approximately 1. Cells were incubated for 24 hours. On day of the FLIPR assay, media was aspirated, leaving 10ul volume. 20ul of dye per well was loaded (FMP2 Blue dye, R8181, MDS. For each pot, 133ml Tyrodes buffer was diluted to make 1X stock prior to assay). The plate was incubated for 0.5 hours at room temperature and 10ul of the compound added on-line prior to FLIPR read (FLIPR settings: protocol Na MP.fcf, with Exposure length 0.4 sec and Filter #2). Compound plate: 1ul volume, column 1, 2 and 3 are 16, 4 and 0 uM amiloride final (1/200 dilution) (IC80, IC50 and ctrl). Added FMP2 dye/quencher measure Na+ in FLIPR assay, added inhibitor Amiloride at IC80 (16uM) or IC50 (4uM).

[0731] RNA Isolation 96 well Plate: Total RNA was isolated from the cells in the 96-well plate format using the Automated SV96 Total RNA Isolation System (Promega) according to the manufacturer's instructions. The Biomek 2000 Laboratory Automation Workstation (Beckman Coulter) was used to apply the transfected cell lysates to a silica membrane. RNase-Free DNase I was then applied directly to the silica membrane to digest contaminating

genomic DNA. The bound total RNA was further purified from contaminating salts, proteins and cellular impurities by simple washing steps. Finally, total RNA was eluted from the membrane by the addition of Nuclease-Free Water.

[0732] RNA isolation 384 well Plate: Extract mRNA using Qiagen Turbo Capture RNA preparation kit, following manufacturer's instructions.

[0733] Quantitative RT-PCR (TaqMan)**:** A series of probes and primers were used for the detection of mRNA transcripts of ENaCα, OAS1, IL8 and GAPDH (as control/normalisation) in the human cell lines. The assays were performed on an ABI 7900HT instrument according to the manufacturer's instructions. Primer Probe sets used **GAPDH** Forward 5'-CAAGGTCATCCATGACAACTTTG-3 (SEQ ID NO: 128), 'Reverse 5'-GGGCCATCCACAGTCTTCT-3' (SEQ ID NO: 129), Probe 5'd FAM-ACCACAGTCCATGCCATCACTGCCA-TAMRA 3' (SEQ ID NO: 130), **ENaCα** Forward 5'-ACATCCCAGGAATGGGTCTTC-3' (SEQ ID NO: 131), Reverse 5'-ACTTTGGCCACTCCATTTCTCT-3' (SEQ ID NO: 132), Probe 5'd FAM-TGCTATCGCGACAGAACAATTACACCGTC-TAMRA 3' (SEQ ID NO: 133), **OAS1** Forward 5'-ACCTAACCCCCAAATCTATGTCAA-3' (SEQ ID NO: 134), Reverse 5'-TGGAGAACTCGCCCTCTTTC-3' (SEQ ID NO: 135), Probe 5'd FAM-CTCATCGAGGAGTGCACCGACCTG-TAMRA 3' (SEQ ID NO: 136),**IL8** Forward 5'-CTGGCCGTGGCTCTCTTG-3' (SEQ ID NO: 137), Reverse 5'-CCTTGGCAAAACTGCACCTT-3' (SEQ ID NO: 138), Probe 5'd FAM-CAGCCTTCCTGATTTCTGCAGTCTGTG-TAMRA 3' (SEQ ID NO: 139).

Calculations:

[0734] **TaqMan**: Critical threshold values (Ct) were converted to copy numbers corresponding to the particular gene analysed in each well of the 384 well plate. Six identical wells were prepared in each plate for a given treatment. Hence, an average gene copy number and standard deviation were calculated. Determination of the percentage coefficient of variation (CV) (%C.V.=[standard deviation/average]*100) allowed the omitting of wells whose value was an outlier (so that %C.V.<25). Relative abundance (aka relative expression) of a gene was determined by dividing the mean copy number of that gene with its GAPDH counterpart in that particular sample.

Statistical Analysis of Data

[0735] **EC50 values** were calculated from the data using sigma plot. All calculations of the efficacy and potency of the siNAs were done relative to the non-targeting control siNA.

[0736] Percentage knockdown was compared between the four chemically modified siNAs (target site 1181, (SEQ ID NOs: 57 and 58), target site 782 (SEQ ID NOs: 51 and 52), target site 1383 (SEQ ID NOs: 63 and 64), and target site 1388 (SEQ ID NOs 73 and 74). The data was analysed using an ANOVA test and then the p-values were corrected for multiple comparisons using the False Discovery Rate correction (FDR). A 95% confidence interval plot was also produced to show graphically where leads were significantly different from each other.

Results

[0737] Highly efficacious and potent siNAs to target sites 782 (SEQ ID NOs: 51 and 52) , 1181 (SEQ ID NOs: 57 and 58), 1383 (SEQ ID NOs: 63 and 64), and 1388 (SEQ ID NOs 73 and 74)) have been used to demonstrate a maximum as well as a dose dependent knock-down (KD) of ENaCα mRNA in human normal bronchial epithelial cells (NHBEs)

Table 14: Efficacy (%KD) and potency (EC_{50}) of siNAs targeting human ENaCα. at 48hrs post transfection

Target Site	**Maximum mRNA knockdown NHBEs n=3 donors**	**EC50 mRNA knockdown NHBEs n=3 donors**
1181	78%	1.59nM
782	79%	0.47nM
1383	64%	4.9nM
1388	79%	4.2nM

[0738] Additionally, the immunostimulation TLR3 mediated % increase in OAS1 (immunostimulatory biomarker) mRNA levels of 4 siNAs at a maximum dose of 100nM was assessed. A nine point dose response was performed 0.01-100nM siNA.

[0739] The endosomal TLR3 mediated immunostimulation was measured by recording the % increase in OAS1 mRNA levels when the NHBE cells were transfected with the siNAs

that had been formulated with the delivery vehicle (the Gemini surfactant GSC170 Dab – example 37 in WO03/82809) (% increase in OAS1 mRNA levels relative to the Gemini delivery vehicle control). The TLR3 agonist Poly I: C is used as a positive control for OAS1 mRNA induction.

[0740] The TLR7 and TLR8 mediated immunostimulation was measured by the increase in IL8 mRNA levels when the siNAs were formulated with DharmaFect1 (Gibco BRL) and delivered to U2OS cells that were engineered to stably express TLR7 or TLR8**.** The cells were treated with the TLR7 and TLR8 agonists Resiquimod (R848) and ssRNA40/LyoVec respectively to act as positive controls for IL8 mRNA induction. IL8 mRNA levels were used as a biomarker of TLR7 and TLR8 mediated immunostimulation.

[0741] The results of the immunostimulation testing described above are shown in **Figures 28** and **29** and **Table 15.**

Table 15: Summary of TLR3, TLR7 and TLR8 immunostimulatory activity of siNAs

Target Site	**Immunostimulation TLR3 mediated NHBEs OAS1 mRNA increase n=3donors**	**Immunostimulation TLR7 mediated in Human U2OS- TLR7 cells TLR8 mediated in Human U2OS-TLR8 cells IL8 mRNA increase n=4 individual expts**
1181	No significant effect Up to 100nM	No significant effect Up to 100nM
782	No significant effect Up to 100nM	No significant effect Up to 100nM
1383	No significant effect Up to 100nM	No significant effect Up to 100nM
1388	No significant effect Up to 100nM	No significant effect Up to 100nM

[0742] The ability of the ENaC siNAs to inhibit functional activity of ENaC was also tested. **Figure 30** shows that transfection of the recombinant HEK cells with the ENaC siNAs to target sites 782 and 1181, specifically siNAs corresponding to SEQ ID NOs: 51 and 52 and SEQ ID NOs: 57 and 58 respectively.

[0743] A dose dependent inhibition of response was observed with both ENaC-alpha siNAs. An inhibition of up to 90% of response was observed at the highest concentration of siNA. The degree of inhibition in the FLIPR assay correlates with the level of ENaC mRNA knockdown. ENaC-alpha mRNA knockdown of up to 85% was observed at the highest concentration of siNA. The results are summarized in **Table 16**. Transfection with the control (UC-3) siNA (Ctrl) resulted in a background inhibition of 10-30% compared to untransfected cells in the FLIPR assay .

Table 16: Summary of Inhibition of FLIPR assay response and ENaC mRNA knockdown (n=6, mean data from 3 independent experiments

ENaC-alpha siNA Motif	Final siNA Concentration (nM)	% ENaC-alpha mRNA knockdown	%Inhibition of Maximal Response (4uM amiloride)	%Inhibition of Maximal Response (16uM amiloride)
782	10	61.7	37.1	40.8
782	20	67.7	41.4	47.9
782	50	75.1	52.5	59.7
782	100	73.1	81.5	85.2
1181	10	52.6	43.8	45.8
1181	20	65.6	48.9	51.4
1181	50	76.1	62.2	65.5
1181	100	76.9	77.5	77.5

Example 8: Indications

[0744] The present body of knowledge in ENaC research indicates the need for methods to assay ENaC activity and for compounds that can regulate ENaC expression for research, diagnostic, and therapeutic use. As described herein, the nucleic acid molecules of the present invention can be used in assays to diagnose disease state related of ENaC levels. In addition, the nucleic acid molecules can be used to treat disease state related to ENaC levels. Particular disease states that can be associated with ENaC expression modulation include, but are not limited to, respiratory, inflammatory, and autoimmune disease, traits, conditions, and phenotypes. Non-limiting examples of such indiations are discussed below.

[0745] Chronic Obstructive Pulmonary Disease (COPD) is one example of an inflammatory airway and alveolar disease where persistent upregulation of inflammation is thought to play a role. Inflammation in COPD is characterized by increased infiltration of neutrophils, CD8 positive lymphocytes, and macrophages into the airways. Neutrophils and

macrophages play an important role in the pathogenesis of airway inflammation in COPD because of their ability to release a number of mediators including elastase, metalloproteases, and oxygen radicals that promote tissue inflammation and damage. It has been suggested that inflammatory cell accumulation in the airways of patients with COPD is driven by increased release of pro-inflammatory cytokines and of chemokines that attract the inflammatory cells into the airways, activate them and maintain their presence. The cells that are present also release enzymes (like metalloproteases) and oxygen radicals which have a negative effect on tissue and perpetuate the disease. A vast array of pro-inflammatory cytokines and chemokines have been shown to be increased within the lungs of patients with COPD. Among them, an important role is played by tumor necrosis factor alpha (TNF-alpha), granulocyte-macrophage colony stimulating factor (GM-CSF) and interleukin 8 (IL-8), which are increased in the airways of patients with COPD.

[0746] Other examples of respiratory diseases where inflammation seems to play a role include: asthma, cystic fibrosis, eosinophilic cough, bronchitis, acute and chronic rejection of lung allograft, sarcoidosis, pulmonary fibrosis, rhinitis, bronchiectasis, and sinusitis. Asthma is defined by airway inflammation, reversible obstruction and airway hyperresponsiveness. In this disease the inflammatory cells that are involved are predominantly eosinophils, T lymphocytes and mast cells, although neutrophils and macrophages can also be important. A vast array of cytokines and chemokines have been shown to be increased in the airways and play a role in the pathophysiology of this disease by promoting inflammation, obstruction and hyperresponsiveness.

[0747] Eosinophilic cough is characterized by chronic cough and the presence of inflammatory cells, mostly eosinophils, within the airways of patients in the absence of airway obstruction or hyperresponsiveness. Several cytokines and chemokines are increased in this disease, although they are mostly eosinophil directed. Eosinophils are recruited and activated within the airways and potentially release enzymes and oxygen radicals that play a role in the perpetuation of inflammation and cough.

[0748] Acute bronchitis is an acute disease that occurs during an infection or irritating event for example by pollution, dust, gas or chemicals, of the lower airways. Chronic bronchitis is defined by the presence of cough and phlegm production on most days for at least three months of the year, for two years. One can also find during acute or chronic bronchitis within the airways inflammatory cells, mostly neutrophils, with a broad array of

chemokines and cytokines. These mediators are thought to play a role in the inflammation, symptoms and mucus production that occur during these diseases.

[0749] Sarcoidosis is a disease of unknown cause where chronic non-caseating granulomas occur within tissue. The lung is the organ most commonly affected. Lung bronchoalveolar lavage shows an increase in mostly lymphocytes, macrophages and sometimes neutrophils and eosinophils. These cells are also recruited and activated by cytokines and chemokines and are thought to be involved in the pathogenesis of the disease.

[0750] Pulmonary fibrosis is a disease of lung tissue characterized by progressive and chronic fibrosis (scarring) which will lead to chronic respiratory insufficiency. Different types and causes of pulmonary fibrosis exist but all are characterized by inflammatory cell influx and persistence, activation and proliferation of fibroblasts with collagen deposition in lung tissue. These events seem related to the release of cytokines and chemokines within lung tissue.

[0751] Acute rhinitis is an acute disease that occurs during an infection or irritating event, for example, by pollution, dust, gas or chemicals, of the nose or upper airways. Chronic rhinitis is defined by the presence of a constant chronic runny nose, nasal congestion, sneezing and pruritis. One can also find within the upper airways during acute or chronic rhinitis inflammatory cells with a broad array of Chemokines and cytokines. These mediators are thought to play a role in the inflammation, symptoms and mucus production that occur during these diseases.

[0752] Acute sinusitis is an acute, usually infectious disease of the sinuses characterized by nasal congestion, runny, purulent phlegm, headache or sinus pain, with or without fever. Chronic sinusitis is defined by the persistence for more than 6 months of the symptoms of acute sinusitis. One can also find during acute or chronic sinusitis within the upper airways and sinuses inflammatory cells with a broad array of chemokines and cytokines. These mediators are thought to play a role in the inflammation, symptoms and phlegm production that occur during these diseases.

[0753] Bronchiectasis is a respiratory disease that is characterized by inflamed thick walled and dilated airways. The damage from this disease is the end result of a vicious cycle of inflammation and infections arising from a number of causes, such as cystic fibrosis, non-

cystic fibrosis causes, postinfection damage, etc.. The sysmptoms can include chronic cough, sputum production, and malaise, as the airwasy become chronically infected with bacteria.

[0754] As described above, these inflammatory respiratory diseases are all characterized by the presence of mediators that recruit and activate different inflammatory cells which release enzymes or oxygen radicals causing symptoms, the persistence of inflammation and when chronic, destruction or disruption of normal tissue.

Example 9: Multifunctional siNA Inhibition of target RNA expression

Multifunctional siNA design

[0755] Once target sites have been identified for multifunctional siNA constructs, each strand of the siNA is designed with a complementary region of length, for example, of about 18 to about 28 nucleotides, that is complementary to a different target nucleic acid sequence. Each complementary region is designed with an adjacent flanking region of about 4 to about 22 nucleotides that is not complementary to the target sequence, but which comprises complementarity to the complementary region of the other sequence (see for example **Figure 13**). Hairpin constructs can likewise be designed (see for example **Figure 14**). Identification of complementary, palindrome or repeat sequences that are shared between the different target nucleic acid sequences can be used to shorten the overall length of the multifunctional siNA constructs (see for example **Figures 15 and 16**).

[0756] In a non-limiting example, three additional categories of additional multifunctional siNA designs are presented that allow a single siNA molecule to silence multiple targets. The first method utilizes linkers to join siNAs (or multiunctional siNAs) in a direct manner. This can allow the most potent siNAs to be joined without creating a long, continuous stretch of RNA that has potential to trigger an interferon response. The second method is a dendrimeric extension of the overlapping or the linked multifunctional design; or alternatively the organization of siNA in a supramolecular format. The third method uses helix lengths greater than 30 base pairs. Processing of these siNAs by Dicer will reveal new, active 5' antisense ends. Therefore, the long siNAs can target the sites defined by the original 5' ends and those defined by the new ends that are created by Dicer processing. When used in combination with traditional multifunctional siNAs (where the sense and antisense strands each define a target) the approach can be used for example to target 4 or more sites.

I. Tethered Bifunctional siNAs

[0757] The basic idea is a novel approach to the design of multifunctional siNAs in which two antisense siNA strands are annealed to a single sense strand. The sense strand oligonucleotide contains a linker (*e.g.*, non-nulcoetide linker as described herein) and two segments that anneal to the antisense siNA strands (see **Figure 19**). The linkers can also optionally comprise nucleotide-based linkers. Several potential advantages and variations to this approach include, but are not limited to:

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

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

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

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

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

6. The linker (or modifications attached to it, such as conjugates described herein) can improve the pharmacokinetic properties of the complex or improve its incorporation into liposomes. Modifications introduced to the linker should not impact siNA activity to the same extent that they would if directly attached to the siNA (see for example **Figures 24 and 25**).

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

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

Dendrimer and supramolecular siNAs

[0758] In the dendrimer siNA approach, the synthesis of siNA is initiated by first synthesizing the dendrimer template followed by attaching various functional siNAs. Various constructs are depicted in **Figure 20**. The number of functional siNAs that can be attached is only limited by the dimensions of the dendrimer used.

Supramolecular approach to multifunctional siNA

[0759] The supramolecular format simplifies the challenges of dendrimer synthesis. In this format, the siNA strands are synthesized by standard RNA chemistry, followed by annealing of various complementary strands. The individual strand synthesis contains an antisense sense sequence of one siNA at the 5'-end followed by a nucleic acid or synthetic linker, such as hexaethyleneglyol, which in turn is followed by sense strand of another siNA in 5' to 3' direction. Thus, the synthesis of siNA strands can be carried out in a standard 3' to 5' direction. Representative examples of trifunctional and tetrafunctional siNAs are depicted in **Figure 21**. Based on a similar principle, higher functionality siNA constucts can be designed as long as efficient annealing of various strands is achieved.

Dicer enabled multifunctional siNA

[0760] Using bioinformatic analysis of multiple targets, stretches of identical sequences shared between differeing target sequences can be identified ranging from about two to about fourteen nucleotides in length. These identical regions can be designed into extended siNA helixes (*e.g.*, >30 base pairs) such that the processing by Dicer reveals a secondary functional 5'-antisense site (see for example **Figure 22**). For example, when the first 17 nucleotides of a siNA antisense strand (*e.g.*, 21 nucleotide strands in a duplex with 3'-TT overhangs) are complementary to a target RNA, robust silencing was observed at 25 nM. 80% silencing was observed with only 16 nucleotide complementarity in the same format.

[0761] Incorporation of this property into the designs of siNAs of about 30 to 40 or more base pairs results in additional multifunctional siNA constructs. The example in **Figure 22**

illustrates how a 30 base-pair duplex can target three distinct sequences after processing by Dicer-RNaseIII; these sequences can be on the same mRNA or separate RNAs, such as viral and host factor messages, or multiple points along a given pathway (*e.g.*, inflammatory cascades). Furthermore, a 40 base-pair duplex can combine a bifunctional design in tandem, to provide a single duplex targeting four target sequences. An even more extensive approach can include use of homologous sequences to enable five or six targets silenced for one multifunctional duplex. The example in **Figure 22** demonstrates how this can be achieved. A 30 base pair duplex is cleaved by Dicer into 22 and 8 base pair products from either end (8 b.p. fragments not shown). For ease of presentation the overhangs generated by dicer are not shown – but can be compensated for. Three targeting sequences are shown. The required sequence identity overlapped is indicated by grey boxes. The N's of the parent 30 b.p. siNA are suggested sites of 2'-OH positions to enable Dicer cleavage if this is tested in stabilized chemistries. Note that processing of a 30mer duplex by Dicer RNase III does not give a precise 22+8 cleavage, but rather produces a series of closely related products (with 22+8 being the primary site). Therefore, processing by Dicer will yield a series of active siNAs. Another non-limiting example is shown in **Figure 23**. A 40 base pair duplex is cleaved by Dicer into 20 base pair products from either end. For ease of presentation the overhangs generated by dicer are not shown – but can be compensated for. Four targeting sequences are shown in four colors, blue, light-blue and red and orange. The required sequence identity overlapped is indicated by grey boxes. This design format can be extended to larger RNAs. If chemically stabilized siNAs are bound by Dicer, then strategically located ribonucleotide linkages can enable designer cleavage products that permit our more extensive repertoire of multiifunctional designs. For example cleavage products not limited to the Dicer standard of approximately 22-nucleotides can allow multifunctional siNA constructs with a target sequence identity overlap ranging from, for example, about 3 to about 15 nucleotides.

Example 10: Diagnostic uses

[0762] The siNA molecules of the invention can be used in a variety of diagnostic applications, such as in the identification of molecular targets (*e.g.*, RNA) in a variety of applications, for example, in clinical, industrial, environmental, agricultural and/or research settings. Such diagnostic use of siNA molecules involves utilizing reconstituted RNAi systems, for example, using cellular lysates or partially purified cellular lysates. siNA molecules of this invention can be used as diagnostic tools to examine genetic drift and

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

[0763] In a specific example, siNA molecules that cleave only wild-type or mutant forms of the target RNA are used for the assay. The first siNA molecules (*i.e.*, those that cleave only wild-type forms of target RNA) are used to identify wild-type RNA present in the sample and the second siNA molecules (*i.e.*, those that cleave only mutant forms of target RNA) are used to identify mutant RNA in the sample. As reaction controls, synthetic substrates of both wild-type and mutant RNA are cleaved by both siNA molecules to demonstrate the relative siNA efficiencies in the reactions and the absence of cleavage of the "non-targeted" RNA species. The cleavage products from the synthetic substrates also serve to generate size markers for the analysis of wild-type and mutant RNAs in the sample population. Thus, each analysis requires two siNA molecules, two substrates and one unknown sample, which is combined into six reactions. The presence of cleavage products is determined using an RNase protection assay so that full-length and cleavage fragments of each RNA can be analyzed in one lane of a polyacrylamide gel. It is not absolutely required to quantify the results to gain insight into the expression of mutant RNAs and putative risk of the desired phenotypic changes in target cells. The expression of mRNA whose protein product is implicated in the development of the phenotype (*i.e.*, disease related or infection

related) is adequate to establish risk. If probes of comparable specific activity are used for both transcripts, then a qualitative comparison of RNA levels is adequate and decreases the cost of the initial diagnosis. Higher mutant form to wild-type ratios are correlated with higher risk whether RNA levels are compared qualitatively or quantitatively.

[0764] 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.

[0765] 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.

[0766] It will be readily apparent to one skilled in the art that varying substitutions and modifications can be made to the invention disclosed herein without departing from the scope and spirit of the invention. Thus, such additional embodiments are within the scope of the present invention and the following claims. The present invention teaches one skilled in the art to test various combinations and/or substitutions of chemical modifications described herein toward generating nucleic acid constructs with improved activity for mediating RNAi activity. Such improved activity can comprise improved stability, improved bioavailability, and/or improved activation of cellular responses mediating RNAi. Therefore, the specific embodiments described herein are not limiting and one skilled in the art can readily appreciate that specific combinations of the modifications described herein can be tested without undue experimentation toward identifying siNA molecules with improved RNAi activity.

[0767] 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" can 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 can 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.

[0768] 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.

Example 11: Preparation of Nanoparticle encapsulated siNA/carrier formulations

General LNP preparation

[0769] siNA nanoparticle solutions were prepared by dissolving siNAs and/or carrier molecules in 25 mM citrate buffer (pH 4.0) at a concentration of 0.9 mg/mL. Lipid solutions were prepared by dissolving a mixture of cationic lipid (e.g., CLinDMA or DOBMA, see structures and ratios for Formulations in **Table 10**), DSPC, Cholesterol, and PEG-DMG (ratios shown in **Table 10**) in absolute ethanol at a concentration of about 15 mg/mL. The nitrogen to phosphate ratio was approximate to 3:1.

[0770] Equal volume of siNA/carrier and lipid solutions was delivered with two FPLC pumps at the same flow rates to a mixing T connector. A back pressure valve was used to adjust to the desired particle size. The resulting milky mixture was collected in a sterile glass bottle. This mixture was then diluted slowly with an equal volume of citrate buffer, and filtered through an ion-exchange membrane to remove any free siNA/carrier in the mixture. Ultra filtration against citrate buffer (pH 4.0) was employed to remove ethanol (test stick from ALCO screen), and against PBS (pH 7.4) to exchange buffer. The final LNP was obtained by concentrating to a desired volume and sterile filtered through a 0.2 μm filter.

The obtained LNPs were characterized in term of particle size, Zeta potential, alcohol content, total lipid content, nucleic acid encapsulated, and total nucleic acid concentration.

LNP Manufacture Process

[0771] In a non-limiting example, a LNP-086 siNA/carrier formulation is prepared in bulk as follows. A process flow diagram for the process is shown in **Table 13** which can be adapted for siNA/carrier cocktails (2 siNA/carrier duplexes are shown) or for a single siNA/carrier duplex. The process consists of (1) preparing a lipid solution; (2) preparing a siNA/carrier solution; (3) mixing/particle formation; (4) Incubation; (5) Dilution; (6) Ultrafiltration and Concentration.

1. Preparation of Lipid Solution

Summary: To a 3-necked round bottom flask fitted with a condenser was added a mixture of CLinDMA, DSPC, Cholesterol, PEG-DMG, and Linoeyl alcohol. Ethanol was then added. The suspension was stirred with a stir bar under Argon, and was heated at 30 °C using a heating mantle controlled with a process controller. After the suspension became clear, the solution was allowed to cool to room temperature.

Detailed Procedure for formulating 8L batch of LNP

1. Depyrogenate a 3-necked 2L round bottom flask, a condenser, measuring cylinders, and two 10L conical glass vessels.
2. Warm the lipids to room temperature. Tare the weight of the round bottom flask. Transfer the **CLinDMA (50.44g)** with a pipette using a pipette aid into the 3-necked round bottom flask.
3. Weigh **DSPC (43.32g), Cholesterol (5.32g) and PEG-DMG (6.96g)** with a weighing paper sequentially into the round bottom flask.
4. **Linoleyl alcohol (2.64g)** was weighed in a separate glass vial (depyrogenated). Tare the vial first, and then transfer the compound with a pipette into the vial.
5. Take the total weight of the round bottom flask with the lipids in, subtract the tare weight. The error was usually much less than ± 1.0%.
6. Transfer one-eighth of the ethanol **(1L)** needed for the lipid solution into the round bottom flask.
7. The round bottom flask placed in a heating mantle was connected to a J-CHEM process controller. The lipid suspension was stirred under Argon with a stir bar and a condenser on top. A thermocouple probe was put into the suspension through one neck of the round bottom flask with a sealed adapter.
8. The suspension was heated at 30 °C until it became clear. The solution was allowed to cool to room temperature and transferred to a conical glass vessel and sealed with a cap.

2. Preparation of siNA/carrier Solution

Summary: The siNA/carrier solution can comprise a single siNA duplex and or carrier or can alternately comprise a cocktail of two or more siNA duplexes and/or carriers. In the case of a single siNA/carrier duplex, the siNA/carrier is dissolved in 25 mM citrate buffer (pH 4.0, 100 mM of NaCl) to give a final concentration of 0.9 mg/mL. In the case of a cocktail of two siNA/carrier molecules, the siNA/carrier solutions are prepared by dissolving each siNA/carrier molecule in 50% of the total expected volume of a 25 mM citrate buffer (pH 4.0, 100 mM of NaCl) to give a final concentration of 0.9 mg/mL. This procedure is repeated for the other siNA/carrier molecule. The two 0.9 mg/ mL siNA/carrier solutions are combined to give a 0.9 mg/mL solution at the total volume containing two siNA molecules.

Detailed Procedure for formulating 8L batch of LNP with siNA cocktail

1. Weigh 3.6 g times the water correction factor (Approximately 1.2) of siNA-1 powder into a sterile container such as the Corning storage bottle.
2. Transfer the siNA to a depyrogenated 5 L glass vessel. Rinse the weighing container 3x with of citrate buffer (25mM, pH 4.0, and 100mM NaCl) placing the rinses into the 5 L vessel, QS with citrate buffer to 4 L.
3. Determine the concentration of the siNA solution with UV spectrometer. Generally, take 20 µL from the solution, dilute 50 times to 1000 µL, and record the UV reading at A260 nm after blanking with citrate buffer. Make a parallel sample and measure. If the readings for the two samples are consistent, take an average and calculate the concentration based on the extinction coefficients of the siNAs. If the final concentration is out of the range of 0.90 ± 0.01 mg/mL, adjust the concentration by adding more siNA/carrier powder, or adding more citrate buffer.
4. Repeat for siNA-2.
5. In a 10 l depyrogenated 10L glass vessel transfer 4 L of each 0.9 mg/mL siNA solution

Sterile Filtration.

The process describes the procedure to sterile filter the Lipid/Ethanol solution. The purpose is to provide a sterile starting material for the encapsulation process. The filtration process was run at an 80 mL scale with a membrane area of 20 cm^2. The flow rate is 280 mL/min. This process is scaleable by increasing the tubing diameter and the filtration area.

1. **Material**s
   a. Nalgene 50 Silicone Tubing PN 8060-0040 Autoclaved
   b. Master Flex Peristaltic Pump Model 7520-40
      i. Master flex Pump Head Model 7518-00
   c. Pall Acropak 20 0.8/0.2 µm sterile filter. PN 12203
   d. Depyrogenated 10 L glass vessel
   e. Autoclaved lid for glass vessel.

2. **Procedure.**
   a. Place tubing into pump head. Set pump to 50% total pump speed and measure flow for 1 minute with a graduated cylinder
   b. Adjust pump setting and measure flow to 280 mL/min.
   c. Set up Tubing with filter attach securely with a clamp.
   d. Set up pump and place tubing into pump head.
   e. Place the feed end of the tubing into the material to be filtered.
   f. Place the filtrate side of filter with filling bell into depyrogenated glass vessel.
   g. Pump material through filter until all material is filtered.

AKTA Pump Setup

1. **Materials**
   a. AKTA P900 Pump
   b. Teflon tubing 2 mm ID x 3 mm OD 2 each x 20.5 cm Upchurch PN 1677
   c. Teflon tubing 1 mm ID x 3 mm OD 6.5 cm Upchurch PN 1675
   d. Peek Tee 1 mm ID 1 each Upchurch PN P-714
   e. ¼ - 28F to 10-32M 2 each Upchurch PN P-652
   f. ETFE Ferrule for 3.0 mm OD tubing 6 each Upchurch PN P-343x
   g. Flangless Nut 6 each Upchurch PN P-345x
   h. ETFE cap for ¼ - 28 flat bottom fitting 1 each Upchurch PN P-755
   i. Argon Compressed gas
   j. Regulator 0-60 psi
   k. Teflon tubing
   l. Peek Y fitting
   m. Depyrogenated glassware conical base.2/pump
   n. Autoclaved lids.
   o. Pressure lids

2. **Pump Setup**
   a. Turn pump on
   b. Allow pump to perform self test
   c. Make certain that there are no caps or pressure regulators attached to tubing (This will cause the pumps to over pressure.)
   d. Press "OK" to synchronize pumps
   e. Turn knob 4 clicks clockwise to "Setup" – press "OK"
   f. Turn knob 5 clicks clockwise to "Setup Gradient Mode" – press "OK"
   g. Turn knob 1 click clockwise to "D" – press "OK"
   h. Press "Esc" twice

3. **Pump Sanitization.**
   a. Place 1000 mL of 1 N NaOH into a 1 L glass vessel
   b. Attach to pump with a pressure lid
   c. Place 1000 mL of 70 % Ethanol into a 1 L glass vessel
   d. Attach to pump with a pressure lid.
   e. Place a 2000 mL glass vessel below pump outlet.
   f. Turn knob 1 click clockwise to "Set Flow Rate" – press "OK"

g. Turn knob clockwise to increase Flow Rate to 40 mL/min; counter clockwise to decrease; press "OK" when desired Flow Rate is set.
h. Set time for 40 minute.
i. Turn on argon gas at 10 psi.
j. Turn knob 2 clicks counter clockwise to "Run" – press "OK", and start timer.
k. Turn knob 1 click counter clockwise to "End Hold Pause"
l. When timer sounds Press "OK" on pump
m. Turn off gas
n. Store pump in sanitizing solutions until ready for use (overnight?)

4. Pump Flow Check

a. Place 200 mL of Ethanol into a depyrogenated 500 mL glass bottle.
b. Attach to pump with a pressure cap.
c. Place 200 mL of Sterile Citrate buffer into a 500 mL depyrogenated glass bottle.
d. Attach to pump with a pressure cap.
e. Place a 100 mL graduated cylinder below pump outlet.
f. Turn knob 1 click clockwise to "Set Flow Rate" – press "OK"
g. Turn knob clockwise to increase Flow Rate to 40 mL/min; counter clockwise to decrease; press "OK" when desired Flow Rate is set.
h. Set time for 1 minute.
i. Turn on argon gas at 10 psi.

j. Turn knob 2 clicks counter clockwise to "Run" – press "OK", and start timer.
k. Turn knob 1 click counter clockwise to "End Hold Pause"
l. When timer sounds Press "OK" on pump
m. Turn off gas
n. Verify that 40 mL of the ethanol/citrate solution was delivered.

3. Particle formation – Mixing step

o. Attach the sterile Lipid / Ethanol solution to the AKTA pump.
p. Attach the sterile siNA/carrier or siNA/carrier cocktail / Citrate buffer solution to the AKTA pump.
q. Attach depyrogenated received vessel (2x batch size) with lid
r. Set time for calculated mixing time.
s. Turn on Argon gas and maintain pressure between 5 to10 psi.
t. Turn knob 2 clicks counter clockwise to "Run" – press "OK", and start timer.
u. Turn knob 1 click counter clockwise to "End Hold Pause"
v. When timer sounds Press "OK" on pump
w. Turn off gas

4. Incubation

The solution is held after mixing for a 22 ± 2 hour incubation. The incubation is at room temperature (20 – 25°C) and the in-process solution is protected from light.

5. Dilution.

The lipid siNA solution is diluted with an equal volume of Citrate buffer. The solution is diluted with a dual head peristaltic pump, set up with equal lengths of tubing and a Tee connection. The flow rate is 360 mL/minute.

1. Materials

h. Nalgene 50 Silicone Tubing PN 8060-0040 Autoclaved
i. Tee ¼ ' ID
j. Master Flex Peristaltic Pump Model 7520-40
    i. Master flex Pump Head Model 7518-00
    ii. Master flex Pump Head Model 7518-00
k. Depyrogenated 2 x 20 L glass vessel
l. Autoclaved lids for glass vessels.

2. Procedure.

a. Attach two equal lengths of tubing to the Tee connector. The tubing should be approximately 1 meter in length. Attach a third piece of tubing approximately 50 cm to the outlet end of the Tee connector.
b. Place the tubing apparatus into the dual pump heads.
c. Place one feed end of the tubing apparatus into an Ethanol solution. Place the other feed end into an equal volume of Citrate buffer.
d. Set the pump speed control 50%. Set a time for 1 minute.
e. Place the outlet end of the tubing apparatus into a 500 mL graduated cylinder.
f. Turn on the pump and start the timer.
g. When the timer sounds stop the pump and determine the delivered volume.
h. Adjust the pump flow rate to 360 mL/minute.
i. Drain the tubing when the flow rate is set.
j. Place one feed end of the tubing apparatus into the Lipid/siNA solution. Place the other feed end into an equal volume of Citrate buffer (16 L).
k. Place the outlet end of the tubing apparatus into the first of 2 x 20 L depyrogenated glass vessels.
l. Set a timer for 90 minutes and start the pump. Visually monitor the dilution progress to ensure that the flow rates are equal.
m. When the receiver vessel is at 16 liters change to the next vessel and collect 16L.
n. Stop the pump when all the material has been transferred.

6. Ultrafiltration and Concentration

Summary: The ultrafiltration process is a timed process and the flow rates must be monitored carefully. The membrane area has been determined based on the volume of the batch. This is a two step process; the first is a concentration step taking the diluted material from 32 liters to 3600 mLs and a concentration of approximately 2 mg/mL. The concentration step is 4 hours ± 15 minutes. The second step is a diafiltration step exchanging the ethanol citrate buffer to

Phosphate buffered saline. The diafiltration step is 3 hours and again the flow rates must be carefully monitored. During this step the ethanol concentration is monitored by head space GC. After 3 hours (20 diafiltration volumes) a second concentration is undertaken to concentrate the solution to approximately 6 mg/mL or a volume of 1.2 liters. This material is collected into a depyrogenated glass vessel. The system is rinsed with 400 mL of PBS at high flow rate and the permeate line closed. This material is collected and added to the first collection. The expected concentration at this point is 4.5 mg/mL. The concentration and volume are determined.

1. **Materials**
   - x. Quatroflow pump
   - y. Flexstand system with autoclaved 5 L reservoir.
   - z. Ultrafiltration membrane GE PN UFP-100-C-35A
   - aa. PBS 0.05µm filtered 100 L
   - bb. 0.5 N Sodium Hydroxide.
   - cc. WFI
   - dd. Nalgene 50 Silicone Tubing PN 8060-0040 Autoclaved
   - ee. Master Flex Peristaltic Pump Model 7520-40
     - i. Master flex Pump Head Model 7518-00
   - ff. Permeate collection vessels 100 L capacity
   - gg. Graduated cylinders depyrogenated 2 L, 11, 500 mL.

2. **Procedure**
   - a. **System preparation.**
     - i. Install the membrane in the Flexstand holder, using the appropriate size sanitary fittings for the membrane. Attach the Flexstand to the quatroflow pump. Attach tubing to the retentate and permeate connections and place these in a suitable waste container.
     - ii. Determine the system hold up volume.
       1. Place 1 liter of WFI in the reservoir.
       2. Clamp the permeate line.
       3. Start the Quatroflow pump and recirculate until no bubbles are present in the retentate line. Stop pump
       4. Mark the reservoir and record the reading for 1 liter.
       5. Add 200 mL of WFI to the reservoir and mark the 1200 mL level.
     - iii. Add 3 liters of 0.5 N sodium hydroxide to the reservoir and flush through the retentate to waste. Add 3 L of 0.5 N sodium hydroxide to the reservoir recirculate the retentate line and flush through the permeate to waste. Add a third 3 L of 0.5 N sodium hydroxide to the reservoir and recirculate through the permeate line to the reservoir for 30 minutes. Store the system in 0.5 N sodium hydroxide overnight prior to use.
     - iv. Flush the sodium hydroxide to waste.

v. Add 3 L WFI to the reservoir and flush the retentate to waste until the pH is neutral, replace the WFI as necessary. Return the retentate line to the reservoir.
vi. Add 3 Liters of WFI and flush the permeate line to waste until the pH is neutral, replacing the WFI as necessary. Drain system.
vii. Add 3 Liters of Citrate buffer to the reservoir. Flush through the permeate line until pH is < 5. Add citrate buffer as necessary.
viii. Drain system.

b. LNP Concentration

i. Place a suitable length on tubing into the peristaltic pump head.
ii. Place the feed end into the diluted LNP solution; place the other end into the reservoir.
iii. Pump the diluted LNP solution into the reservoir to the 4 liter mark.
iv. Place the permeate line into a clean waste container.
v. Start the quatroflow pump and adjust the pump speed so the permeate flow rate is 300 mL/min.
vi. Adjust the peristaltic pump to 300 mL/min so the liquid level is constant at 4L in the reservoir.
vii. When all the diluted LNP solution has been transferred to the reservoir stop the peristaltic pump.
viii. Concentrate the diluted LNP solution to 3600 mL in 240 minutes by adjusting the pump speed as necessary.
ix. Monitor the permeate flow rate, pump setting and feed and retentate pressures.

c. LNP Diafiltration

i. Place the feed tubing of the peristaltic pump into a container containing 72 L of PBS (0.05 μm filtered).
ii. Start the peristaltic pump and adjust the flow rate to maintain a constant volume of 3600 mL in the reservoir.
iii. Increase the Quatroflow pump flow rate to 400 mL/min.
iv. Monitor the permeate flow rate, pump setting and feed and retentate pressures.
v. Monitor the ethanol concentration by GC
vi. The LNP solution is diafiltred with PBS (20 volumes) for 180 minutes.
vii. Stop the peristaltic pump. Remove tubing from reservoir.

d. Final concentration

i. Concentrate the LNP solution to the 1.2 Liter mark.
ii. Collect the LNP solution into a depyrogenated 2 L graduated cylinder.
iii. Add 400 mL of PBS to the reservoir.
iv. Start the pump and recirculate for 2 minutes.
v. Collect the rinse and add to the collected LNP solution in the graduated cylinder.
vi. Record the volume of the LNP solution.
vii. Transfer to a 2 L depyrogenated glass vessel.
viii. Label and refrigerate.

e. Clean system

i. Add 1 L WFI to the reservoir

ii. Recirculate for 5 minutes with permeate closed.
iii. Drain system
iv. Add 2 L 0.5 N sodium hydroxide to the reservoir
v. Recirculate for 5 minutes.
vi. Drain system
vii. Add 2 L of 0.5 N sodium hydroxide to the reservoir.
viii. Recirculate for 5 minutes and stop pump.
ix. Neutralize system with WFI.
x. Drain system and discard membrane.

[0772] The obtained LNPs were characterized in term of particle size, Zeta potential, alcohol content, total lipid content, nucleic acid encapsulated, and total nucleic acid concentration.

[0773] 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.

[0774] 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.

[0775] It will be readily apparent to one skilled in the art that varying substitutions and modifications can be made to the invention disclosed herein without departing from the scope and spirit of the invention. Thus, such additional embodiments are within the scope of the present invention and the following claims. The present invention teaches one skilled in the art to test various combinations and/or substitutions of chemical modifications described herein toward generating nucleic acid constructs with improved activity for mediating RNAi activity. Such improved activity can comprise improved stability, improved bioavailability, and/or improved activation of cellular responses mediating RNAi. Therefore, the specific embodiments described herein are not limiting and one skilled in the art can readily appreciate that specific combinations of the modifications described herein can be tested

without undue experimentation toward identifying siNA molecules with improved RNAi activity.

Table 7: ENaC Accession Numbers

NM_001038– SEQ ID NO: 127
Homo sapiens sodium channel, nonvoltage-gated 1 alpha (SCNN1A), mRNA
gi|47834319|ref|NM_001038.4|[47834319] – Version 4, updated on May 14, 2006

NM_000336
Homo sapiens sodium channel, nonvoltage-gated 1, beta (SCNN1B), mRNA
gi|124301195|ref|NM_000336.2|[124301195]

NM_001130413
Homo sapiens sodium channel, nonvoltage-gated 1, delta (SCNN1D), transcript variant 1, mRNA
gi|194353988|ref|NM_001130413.1|[194353988]

NM_002978
Homo sapiens sodium channel, nonvoltage-gated 1, delta (SCNN1D), transcript variant 2, mRNA
gi|34101281|ref|NM_002978.2|[34101281]

NM_001039
Homo sapiens sodium channel, nonvoltage-gated 1, gamma (SCNN1G), mRNA
gi|148839327|ref|NM_001039.3|[148839327]

DQ898177
Homo sapiens sodium channel nonvoltage-gated 1 delta (SCNN1D) mRNA, 5' UTR
gi|114325733|gb|DQ898177.1|[114325733]

DQ898176
Homo sapiens clone 16HBE140L sodium channel nonvoltage-gated 1 delta (SCNN1D) mRNA, complete cds
gi|114325731|gb|DQ898176.1|[114325731]

DQ898175
Homo sapiens clone 16HBE140S sodium channel nonvoltage-gated 1 delta (SCNN1D) mRNA, complete cds
gi|114325729|gb|DQ898175.1|[114325729]

NM_031548
Rattus norvegicus sodium channel, nonvoltage-gated, type I, alpha (Scnn1a), mRNA
gi|47575865|ref|NM_031548.2|[47575865]

NM_011324
Mus musculus sodium channel, nonvoltage-gated 1 alpha (Scnn1a), mRNA
gi|33859617|ref|NM_011324.1|[33859617]

Table 8

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

Chemistry	pyrimidine	Purine	cap	p=S	Strand
"Stab 00"	Ribo	Ribo	TT at 3'-ends		S/AS
"Stab 1"	Ribo	Ribo	-	5 at 5'-end 1 at 3'-end	S/AS
"Stab 2"	Ribo	Ribo	-	All linkages	Usually AS
"Stab 3"	2'-fluoro	Ribo	-	4 at 5'-end 4 at 3'-end	Usually S
"Stab 4"	2'-fluoro	Ribo	5' and 3'-ends	-	Usually S
"Stab 5"	2'-fluoro	Ribo	-	1 at 3'-end	Usually AS
"Stab 6"	2'-O-Methyl	Ribo	5' and 3'-ends	-	Usually S
"Stab 7"	2'-fluoro	2'-deoxy	5' and 3'-ends	-	Usually S
"Stab 8"	2'-fluoro	2'-O-Methyl	-	1 at 3'-end	S/AS
"Stab 9"	Ribo	Ribo	5' and 3'-ends	-	Usually S
"Stab 10"	Ribo	Ribo	-	1 at 3'-end	Usually AS
"Stab 11"	2'-fluoro	2'-deoxy	-	1 at 3'-end	Usually AS
"Stab 12"	2'-fluoro	LNA	5' and 3'-ends		Usually S
"Stab 13"	2'-fluoro	LNA		1 at 3'-end	Usually AS
"Stab 14"	2'-fluoro	2'-deoxy		2 at 5'-end 1 at 3'-end	Usually AS
"Stab 15"	2'-deoxy	2'-deoxy		2 at 5'-end 1 at 3'-end	Usually AS
"Stab 16"	Ribo	2'-O-Methyl	5' and 3'-ends		Usually S
"Stab 17"	2'-O-Methyl	2'-O-Methyl	5' and 3'-ends		Usually S
"Stab 18"	2'-fluoro	2'-O-Methyl	5' and 3'-ends		Usually S
"Stab 19"	2'-fluoro	2'-O-Methyl	3'-end		S/AS
"Stab 20"	2'-fluoro	2'-deoxy	3'-end		Usually AS
"Stab 21"	2'-fluoro	Ribo	3'-end		Usually AS
"Stab 22"	Ribo	Ribo	3'-end		Usually AS
"Stab 23"	2'-fluoro*	2'-deoxy*	5' and 3'-ends		Usually S

Chemistry	pyrimidine	Purine	cap	p=S	Strand
"Stab 24"	2'-fluoro*	2'-O-Methyl*	-	1 at 3'-end	S/AS
"Stab 25"	2'-fluoro*	2'-O-Methyl*	-	1 at 3'-end	S/AS
"Stab 26"	2'-fluoro*	2'-O-Methyl*	-		S/AS
"Stab 27"	2'-fluoro*	2'-O-Methyl*	3'-end		S/AS
"Stab 28"	2'-fluoro*	2'-O-Methyl*	3'-end		S/AS
"Stab 29"	2'-fluoro*	2'-O-Methyl*		1 at 3'-end	S/AS
"Stab 30"	2'-fluoro*	2'-O-Methyl*			S/AS
"Stab 31"	2'-fluoro*	2'-O-Methyl*	3'-end		S/AS
"Stab 32"	2'-fluoro	2'-O-Methyl			S/AS
"Stab 33"	2'-fluoro	2'-deoxy*	5' and 3'-ends	-	Usually S
"Stab 34"	2'-fluoro	2'-O-Methyl*	5' and 3'-ends		Usually S
"Stab 35"	2'-fluoro*†	2'-O-Methyl*†			Usually AS
"Stab 36"	2'-fluoro*†	2'-O-Methyl*†			Usually AS
"Stab 3F"	2'-OCF3	Ribo	-	4 at 5'-end 4 at 3'-end	Usually S
"Stab 4F"	2'-OCF3	Ribo	5' and 3'-ends	-	Usually S
"Stab 5F"	2'-OCF3	Ribo	-	1 at 3'-end	Usually AS
"Stab 7F"	2'-OCF3	2'-deoxy	5' and 3'-ends	-	Usually S
"Stab 8F"	2'-OCF3	2'-O-Methyl	-	1 at 3'-end	S/AS
"Stab 11F"	2'-OCF3	2'-deoxy	-	1 at 3'-end	Usually AS
"Stab 12F"	2'-OCF3	LNA	5' and 3'-ends		Usually S
"Stab 13F"	2'-OCF3	LNA		1 at 3'-end	Usually AS
"Stab 14F"	2'-OCF3	2'-deoxy		2 at 5'-end 1 at 3'-end	Usually AS
"Stab 15F"	2'-OCF3	2'-deoxy		2 at 5'-end 1 at 3'-end	Usually AS
"Stab 18F"	2'-OCF3	2'-O-Methyl	5' and 3'-ends		Usually S
"Stab 19F"	2'-OCF3	2'-O-Methyl	3'-end		S/AS

Chemistry	pyrimidine	Purine	cap	p=S	Strand
"Stab 20F"	2'-OCF3	2'-deoxy	3'-end		Usually AS
"Stab 21F"	2'-OCF3	Ribo	3'-end		Usually AS
"Stab 23F"	2'-OCF3*	2'-deoxy*	5' and 3'-ends		Usually S
"Stab 24F"	2'-OCF3*	2'-O-Methyl*	-	1 at 3'-end	S/AS
"Stab 25F"	2'-OCF3*	2'-O-Methyl*	-	1 at 3'-end	S/AS
"Stab 26F"	2'-OCF3*	2'-O-Methyl*	-		S/AS
"Stab 27F"	2'-OCF3*	2'-O-Methyl*	3'-end		S/AS
"Stab 28F"	2'-OCF3*	2'-O-Methyl*	3'-end		S/AS
"Stab 29F"	2'-OCF3*	2'-O-Methyl*		1 at 3'-end	S/AS
"Stab 30F"	2'-OCF3*	2'-O-Methyl*			S/AS
"Stab 31F"	2'-OCF3*	2'-O-Methyl*	3'-end		S/AS
"Stab 32F"	2'-OCF3	2'-O-Methyl			S/AS
"Stab 33F"	2'-OCF3	2'-deoxy*	5' and 3'-ends	-	Usually S
"Stab 34F"	2'-OCF3	2'-O-Methyl*	5' and 3'-ends		Usually S
"Stab 35F"	2'-OCF3*†	2'-O-Methyl*†			Usually AS
"Stab 36F"	2'-OCF3*†	2'-O-Methyl*†			Usually AS

CAP = any terminal cap, see for example **Figure 7**.
All Stab 00-34 chemistries can comprise 3'-terminal thymidine (TT) residues
All Stab 00-34 chemistries typically comprise about 21 nucleotides, but can vary as described herein.
All Stab 00-36 chemistries can also include a single ribonucleotide in the sense or passenger strand at the 11th base paired position of the double stranded nucleic acid duplex as determined from the 5'-end of the antisense or guide strand (see **Figure 6C**)
S = sense strand
AS = antisense strand
*Stab 23 has a single ribonucleotide adjacent to 3'-CAP
*Stab 24 and Stab 28 have a single ribonucleotide at 5'-terminus
*Stab 25, Stab 26, Stab 27, Stab 35 and Stab 36 have three ribonucleotides at 5'-terminus
*Stab 29, Stab 30, Stab 31, Stab 33, and Stab 34 any purine at first three nucleotide positions from 5'-terminus are ribonucleotides
p = phosphorothioate linkage
†Stab 35 has 2'-O-methyl U at 3'-overhangs and three ribonucleotides at 5'-terminus

†Stab 36 has 2'-O-methyl overhangs that are complementary to the target sequence (naturally occurring overhangs) and three ribonucleotides at 5'-terminus

Table 9

A. 2.5 µmol Synthesis Cycle ABI 394 Instrument

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

B. 0.2 µmol Synthesis Cycle ABI 394 Instrument

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

C. 0.2 µmol Synthesis Cycle 96 well Instrument

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

- Wait time does not include contact time during delivery.
- Tandem synthesis utilizes double coupling of linker molecule

Table 10

Lipid Nanoparticle (LNP) Formulations

Formulation #	Composition	Molar Ratio
L051	CLinDMA / DSPC / Chol / PEG-n-DMG	48 / 40 / 10 / 2
L053	DMOBA / DSPC / Chol / PEG-n-DMG	30 / 20 / 48 / 2
L054	DMOBA / DSPC / Chol / PEG-n-DMG	50 / 20 / 28 / 2
L069	CLinDMA / DSPC / Cholesterol / PEG-Cholesterol	48 / 40 / 10 / 2
L073	pCLinDMA or CLin DMA/ DMOBA / DSPC / Chol / PEG-n-DMG	25 / 25 / 20 / 28 / 2
L077	eCLinDMA / DSPC / Cholesterol / 2KPEG-Chol	48 / 40 / 10 / 2
L080	eCLinDMA / DSPC / Cholesterol / 2KPEG-DMG	48 / 40 / 10 / 2
L082	pCLinDMA / DSPC / Cholesterol / 2KPEG-DMG	48 / 40 / 10 / 2
L083	pCLinDMA / DSPC / Cholesterol / 2KPEG-Chol	48 / 40 / 10 / 2
L086	CLinDMA/DSPC/Cholesterol/2KPEG-DMG/Linoleyl alcohol	43 / 38 / 10 / 2 / 7
L061	DMLBA/Cholesterol/2KPEG-DMG	52 / 45 / 3
L060	DMOBA/Cholesterol/2KPEG-DMG N/P ratio of 5	52 / 45 / 3
L097	DMLBA/DSPC/Cholesterol/2KPEG-DMG	50 / 20 / 28
L098	DMOBA/Cholesterol/2KPEG-DMG, N/P ratio of 3	52 / 45 / 3
L099	DMOBA/Cholesterol/2KPEG-DMG, N/P ratio of 4	52 / 45 / 3
L100	DMOBA/DOBA/3% PEG-DMG, N/P ratio of 3	52 / 45 / 3
L101	DMOBA/Cholesterol/2KPEG-Cholesterol	52 / 45 / 3
L102	DMOBA/Cholesterol/2KPEG-Cholesterol, N/P ratio of 5	52 / 45 / 3
L103	DMLBA/Cholesterol/2KPEG-Cholesterol	52 / 45 / 3
L104	CLinDMA/DSPC/Cholesterol/2KPEG-cholesterol/Linoleyl alcohol	43 / 38 / 10 / 2 / 7
L105	DMOBA/Cholesterol/2KPEG-Chol, N/P ratio of 2	52 / 45 / 3
L106	DMOBA/Cholesterol/2KPEG-Chol, N/P ratio of 3	67 / 30 / 3
L107	DMOBA/Cholesterol/2KPEG-Chol, N/P ratio of 1.5	52 / 45 / 3
L108	DMOBA/Cholesterol/2KPEG-Chol, N/P ratio of 2	67 / 30 / 3
L109	DMOBA/DSPC/Cholesterol/2KPEG-Chol,	50 / 20/28 / 2

	N/P ratio of 2	
L110	DMOBA/Cholesterol/2KPEG-DMG, N/P ratio of 1.5	52 / 45 / 3
L111	DMOBA/Cholesterol/2KPEG-DMG, N/P ratio of 1.5	67 / 30 / 3
L112	DMLBA/Cholesterol/2KPEG-DMG, N/P ratio of 1.5	52 / 45 / 3
L113	DMLBA/Cholesterol/2KPEG-DMG, N/P ratio of 1.5	67 /30 / 3
L114	DMOBA/Cholesterol/2KPEG-DMG, N/P ratio of 2	52 / 45 / 3
L115	DMOBA/Cholesterol/2KPEG-DMG, N/P ratio of 2	67 / 30 / 3
L116	DMLBA/Cholesterol/2KPEG-DMG, N/Pratio of 2	52 / 45 / 3
L117	DMLBA/Cholesterol/2KPEG-DMG, N/P ratio of 2	52 / 45 / 3
L118	LinCDMA/DSPC/Cholesterol/2KPEG-DMG/Linoleyl alcohol, N/P ratio of 2.85	43 / 38 / 10 / 2 / 7
L121	2-CLIM/DSPC/Cholesterol/2KPEG-DMG/, N/P ratio of 3	48 / 40 / 10 / 2
L122	2-CLIM/ Cholesterol/2KPEG-DMG/, N/P ratio of 3	68 / 30 / 2
L123	CLinDMA/DSPC/Cholesterol/2KPEG-DMG/Linoleyl alcohol, N/P ratio of 2.85	43 / 38 / 10 / 3 / 7
L124	CLinDMA/DSPC/Cholesterol/2KPEG-DMG/Linoleyl alcohol, N/P ratio of 2.85	43 / 36 / 10 / 4 / 7
L130	CLinDMA / DOPC / Chol / PEG-n-DMG, N/P ratio of 3	48 / 39 / 10 / 3
L131	DMLBA/Cholesterol/2KPEG-DMG, N/Pratio of 3	52 / 43 / 5
L132	DMOBA/Cholesterol/2KPEG-DMG, N/Pratio of 3	52 / 43 / 5
L133	CLinDMA / DOPC / Chol / PEG-n-DMG, N/P ratio of 3	48 / 40 / 10 / 2
L134	CLinDMA / DOPC / Chol / PEG-n-DMG, N/P ratio of 3	48 / 37 / 10 / 5
L149	COIM/DSPC/Cholesterol/2KPEG-DMG/, N/P ratio of 3	48 / 40 / 10 / 2
L155	CLinDMA/DOPC/Cholesterol/2KPEG-DMG/Linoleyl alcohol, N/P ratio of 2.85	43 / 38 / 10 / 2 / 7
L156	CLinDMA/DOPC/Cholesterol/2KPEG-DMG, N/P ratio of 2.85	45 / 43 / 10 / 2
L162	CLinDMA/DOPC/Cholesterol/2KPEG-DMG, N/P ratio of 2.5	45 / 43 / 10 / 2
L163	CLinDMA/DOPC/Cholesterol/2KPEG-DMG, N/P ratio of 2	45 / 43 / 10 / 2
L164	CLinDMA/DOPC/Cholesterol/2KPEG-DMG, N/P ratio of 2.25	45 / 43 / 10 / 2

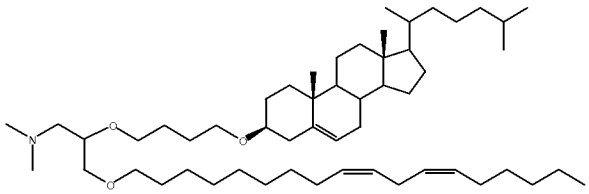
L165	CLinDMA/DOPC/Cholesterol/2KPEG-DMG, N/P ratio of 2.25	40 / 43 / 15 / 2
L166	CLinDMA/DOPC/Cholesterol/2KPEG-DMG, N/P ratio of 2.5	40 / 43 / 15 / 2
L167	CLinDMA/DOPC/Cholesterol/2KPEG-DMG, N/P ratio of 2	40 / 43 / 15 / 2
L174	CLinDMA/DSPC/DOPC/Cholesterol/2KPEG-DMG/Linoleyl alcohol, N/P ratio of 2.85	43 / 9 / 27 / 10 / 4/ 7
L175	CLinDMA/DSPC/DOPC/Cholesterol/2KPEG-DMG/Linoleyl alcohol, N/P ratio of 2.85	43 / 27 / 9 / 10 / 4/ 7
L176	CLinDMA/DOPC/Cholesterol/2KPEG-DMG/Linoleyl alcohol, N/P ratio of 2.85	43 / 38 / 10 / 4 / 7
L180	CLinDMA/DOPC/Cholesterol/2KPEG-DMG/Linoleyl alcohol, N/P ratio of 2.25	43 / 38 / 10 / 4 / 7
L181	CLinDMA/DOPC/Cholesterol/2KPEG-DMG/Linoleyl alcohol, N/P ratio of 2	43 / 38 / 10 / 4 / 7
L182	CLinDMA/DOPC/Cholesterol/2KPEG-DMG, N/P ratio of 2.25	45 / 41 / 10 / 4
L197	CODMA/DOPC/Cholesterol/2KPEG-DMG, N/P ratio of 2.85	43 / 36 / 10 / 4 / 7
L198	CLinDMA/DOPC/Cholesterol/2KPEG-DMG/2KPEG-DSG/Linoleyl alcohol, N/P ratio of 2.85	43 / 34 / 10 / 4 / 2 / 7
L199	CLinDMA/DOPC/Cholesterol/2KPEG-DMG/Linoleyl alcohol, N/P ratio of 2.85	43 / 34 / 10 / 6 / 7
L200	CLinDMA/Cholesterol/2KPEG-DMG, N/P ratio of 3.0	50 / 46 / 4
L201	CLinDMA/Cholesterol/2KPEG-DMG, N/P ratio of 3.0	50 / 44 / 6
L206	CLinDMA/Cholesterol/2KPEG-DMG, N/P ratio of 3.0	40 / 56 / 4
L207	CLinDMA/Cholesterol/2KPEG-DMG, N/P ratio of 3.0	60 / 36 / 4
L208	CLinDMA/DOPC/Cholesterol/2KPEG-DMG, N/P ratio of 3.0	40 / 10 / 46 / 4
L209	CLinDMA/DOPC/Cholesterol/2KPEG-DMG, N/P ratio of 3.0	60 / 10 / 26 / 4

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

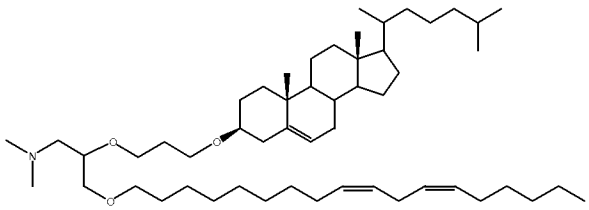
The 2KPEG utilized is PEG2000, a polydispersion which can typically vary from ~1500 to ~3000 Da (i.e., where PEG(n) is about 33 to about 67, or on average ~45).

Table 11

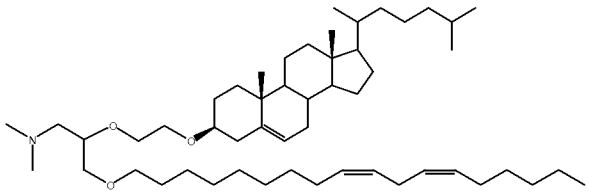
CLinDMA structure



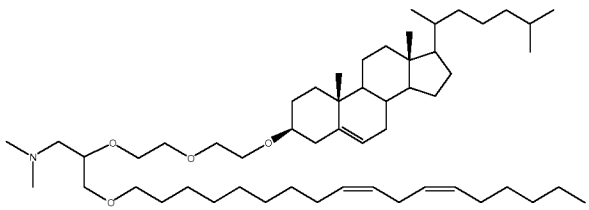
pCLinDMA structure



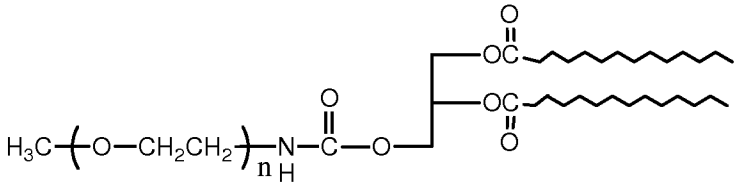
eCLinDMA structure



DEGCLinDMA structure

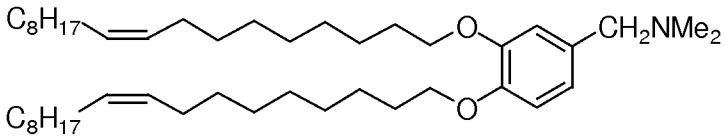


PEG-n-DMG structure

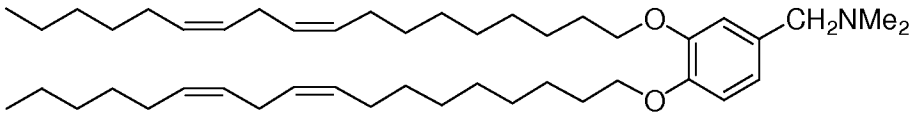


n = about 33 to 67, average = 45 for 2KPEG/PEG2000

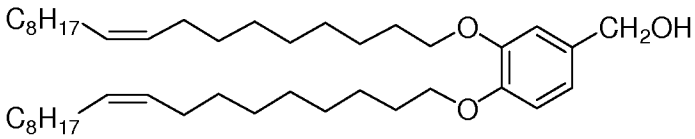
DMOBA structure



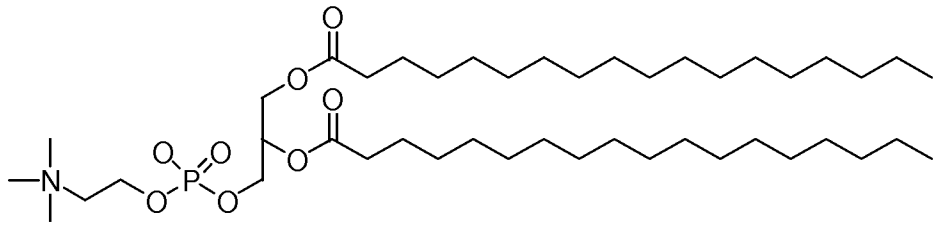
DMLBA structure



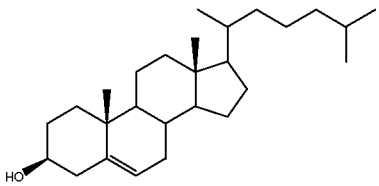
DOBA structure



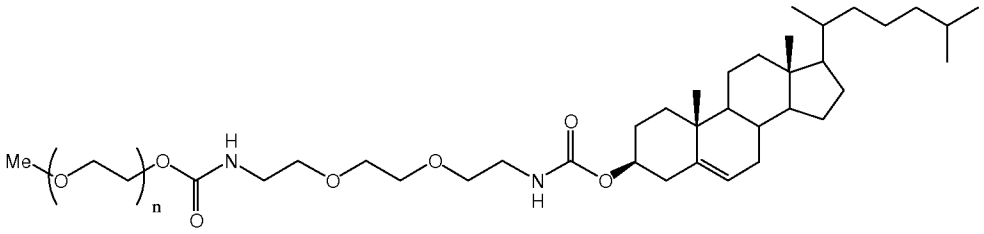
DSPC structure



Cholesterol structure

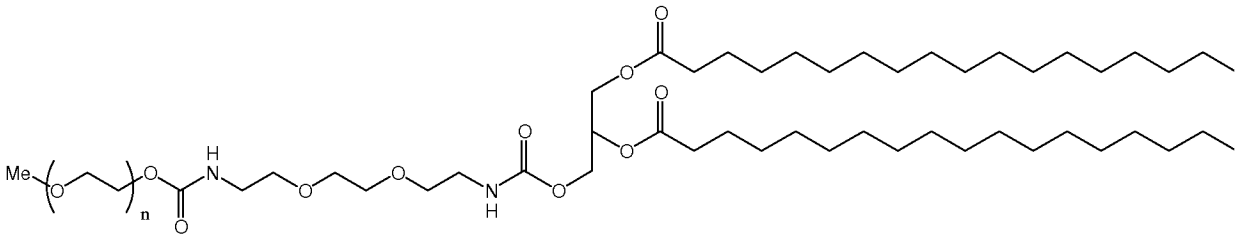


2KPEG-***Cholesterol structure***



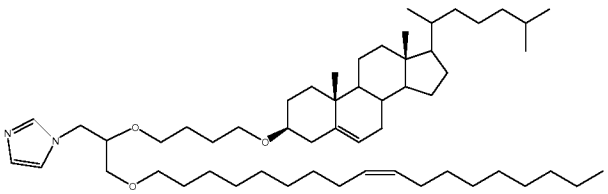
n = about 33 to 67, average = 45 for 2KPEG/PEG2000

2KPEG-***DMG structure***

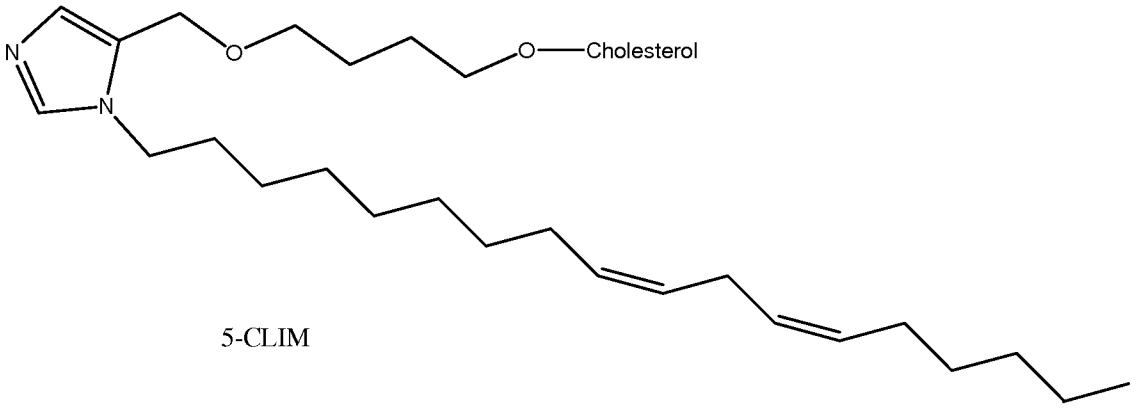


n = about 33 to 67, average = 45 for 2KPEG/PEG2000

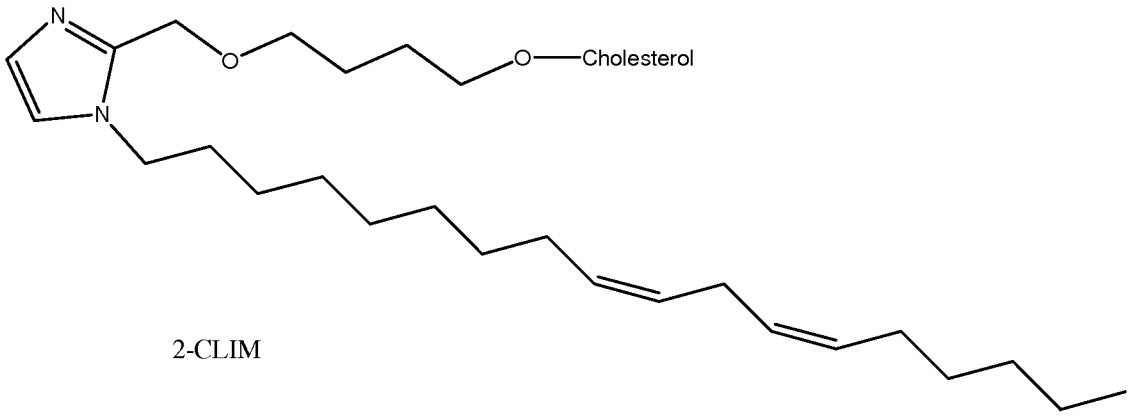
COIM STRUCTURE



5-CLIM AND 2-CLIM STRUCTURE



5-CLIM



2-CLIM

Table 12: Manufacturing Flow Diagram

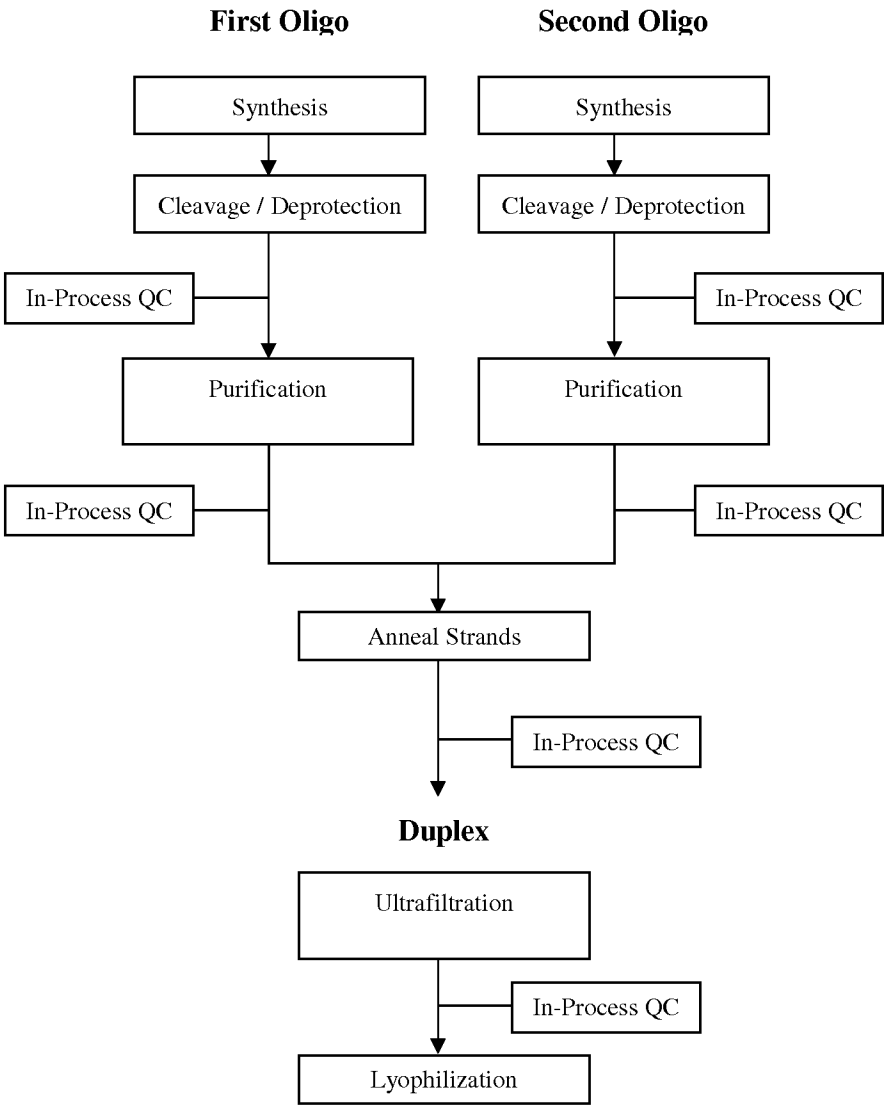
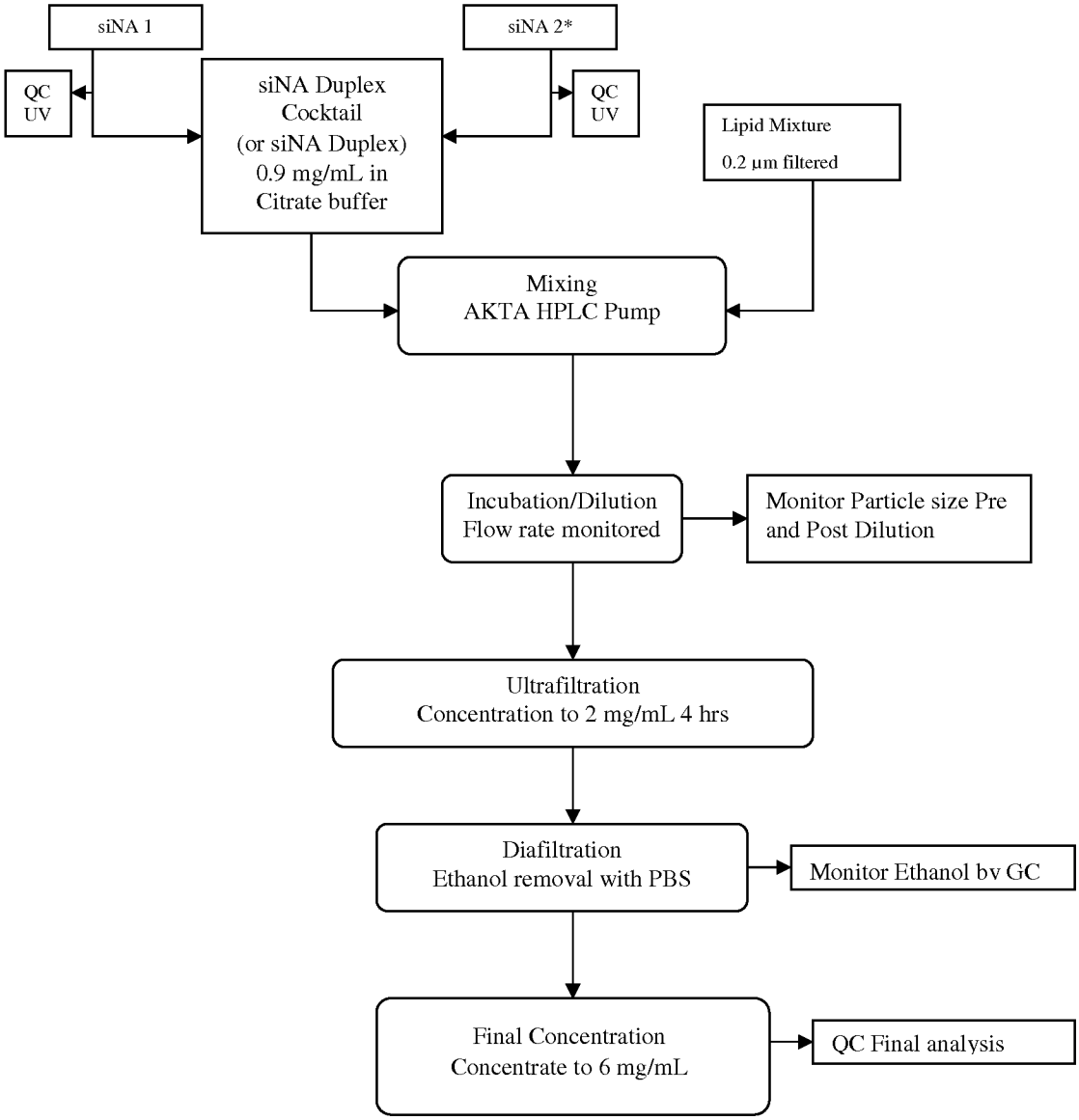


TABLE 13: LNP PROCESS FLOW CHART

(*siNA 2 is optional, shown for input into LNP siNA cocktail formulation, additional siNA duplexes, e.g., siNA 3, siNA 4, siNA 5 etc. can be used for siNA cocktails)



CLAIMS

What we claim is:

1. A double stranded nucleic acid (siNA) molecule having a first strand and a second strand that are complementary to each other, wherein at least one strand comprises:

5'- UGUGCAACCAGAACAAAUC -3' (SEQ ID NO: 10);

5'- GAUUUGUUCUGGUUGCACA -3' (SEQ ID NO: 107);

5'- UUAUGGAUGAUGGUGGCUU -3' (SEQ ID NO: 13);

5'- AAGCCACCAUCAUCCAUAA -3' (SEQ ID NO: 124);

5'- GUGUGGCUGUGCCUACAUC -3' (SEQ ID NO: 16);

5'- GAUGUAGGCACAGCCACAC -3' (SEQ ID NO: 125)

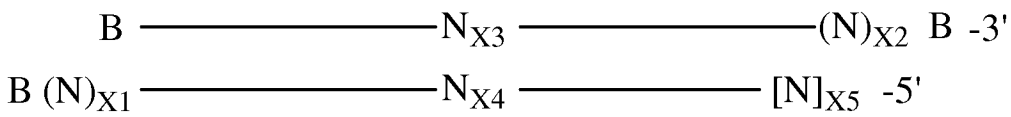
5'- GCUGUGCCUACAUCUUCUA -3' (SEQ ID NO: 21); or

5'- UAGAAGAUGUAGGCACAGC -3' (SEQ ID NO: 126); and

wherein one or more of the nucleotides are optionally chemically modified.

2. A double-stranded nucleic acid (siNA) molecule of claim 1 wherein all the nucleotides are unmodified.

3. A double stranded nucleic acid (siNA) molecule, comprising structure SIX' having a sense strand and an antisense strand:



SIX'

wherein

the upper strand is the sense strand and the lower strand is the antisense strand of the double stranded nucleic acid molecule; said antisense strand comprises a sequence having complementarity to SEQ ID NO: 10, SEQ ID NO: 13, SEQ ID NO: 16, or SEQ ID NO: 21, and said sense strand comprises a sequence having complementarity to the antisense strand;

each N is independently a nucleotide which is unmodified or chemically modified;

each B is a terminal cap moiety that is present or absent;

(N) represents overhanging nucleotides, each of which is independently unmodified or a 2'-O-methyl nucleotide, 2'-deoxy-2'-fluoro nucleotide, or 2'-deoxyribonucleotide;

[N] represents nucleotides that are ribonucleotides;

X1 and X2 are independently integers from 0 to 4;

X3 is an integer from 17 to 36;

X4 is an integer from 11 to 35, provided that the sum of X4 and X5 is 17-36;

X5 is an integer from 1 to 6; and wherein

(a) each pyridmidine nucleotide in N_{X4} positions is independently a 2'-deoxy-2'-fluoro nucleotide or a 2'-O-methyl nucleotide;

each purine nucleotide in N_{X4} positions is independently a 2'-O-methyl nucleotide or a 2'-deoxyribonucleotide;

(b) each pyrimidine nucleotide in N_{X3} positions is a 2'-deoxy-2'-fluoro nucleotide;

each purine nucleotide in N_{X3} positions is independently a 2'-deoxyribonucleotide or a 2'-O-methyl nucleotide.

4. A double-stranded nucleic acid (siNA) molecule according to claim 3 wherein X5 is 3.

5. A double-stranded nucleic acid (siNA) molecule according to claim 3 wherein X1 is 2 and X2 is 2.

6. A double-stranded nucleic acid (siNA) molecule according to claim 3 wherein X5 is 3, X1 is 2 and X2 is 2.

7. A double-stranded nucleic acid (siNA) molecule according to claim 3 wherein X5 is 3, X1 is 2, X2 is 2, X3 is 19 and X4 is 16.

8. A double-stranded short interfering nucleic acid (siNA) molecule wherein the siNA is:

5'- BuGuGcAAccAGAAcAAAucTTB -3' (Sense) (SEQ ID NO:51)
||||||||||||||||||||
3'- UUAcAcGuuGGucuuGuuUAG -5' (Antisense) (SEQ ID NO:52)

wherein:

each B is an inverted abasic cap moiety;

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

u is 2'-deoxy-2'fluorouridine;

A is a 2'-deoxyadenosine;

G is a 2'deoxyguanosine;

T is a thymidine;

A is adenosine;

G is guanosine;

U is uridine

A is a 2'-O-methyl-adenosine;

G is a 2'-O-methyl-guanosine;

U is a 2'-O-methyl-uridine; and

the internucleotide linkages are chemically modified or unmodified.

9. The double-stranded short interfering nucleic acid (siNA) molecule according to claim 8, wherein the internucleotide linkages are unmodified.

10. A double-stranded short interfering nucleic acid (siNA) molecule wherein the siNA is:

5'- BuuAuGGAuGAuGGuGGcuuTTB -3' (Sense) (SEQ ID NO:57)
||||||||||||||||||||
3' UUAAuAccuAcuAccAccGAA -5' (Antisense) (SEQ ID NO:58)

wherein:

each B is an inverted abasic cap;

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

u is 2'-deoxy-2'fluorouridine;

A is a 2'-deoxyadenosine;

G is a 2'deoxyguanosine;

T is a thymidine;

A is adenosine;

G is guanosine;

A is a 2'-O-methyl-adenosine;

U is a 2'-O-methyl-uridine; and

the internucleotide linkages are chemically modified or unmodified.

11. The double-stranded short interfering nucleic acid (siNA) molecule according to claim 10, wherein the internucleotide linkages are unmodified.

12. A double-stranded short interfering nucleic acid (siNA) molecule wherein the siNA is:

```
5'  BGuGuGGcuGuGccuAcAucTTB 3'(Sense) (SEQ ID NO:63)
     ||||||||||||||||||||
3' UUcAcAccGAcAcGGAuGUAG    5'(Antisense) (SEQ ID NO:64)
```

wherein:

each B is an inverted abasic cap moiety;

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

u is 2'-deoxy-2'fluorouridine;

A is a 2'-deoxyadenosine;

G is a 2'deoxyguanosine;

T is a thymidine;

A is adenosine;

G is guanosine;

U is uridine;

A is a 2'-O-methyl-adenosine;

G is a 2'-O-methyl-guanosine;

U is a 2'-O-methyl-uridine; and

the internucleotide linkages are chemically modified or unmodified.

13. The double-stranded short interfering nucleic acid (siNA) molecule according to claim 12, wherein the internucleotide linkages are unmodified.

14. A double-stranded short interfering nucleic acid (siNA) molecule wherein the siNA is:

```
5'- BGcuGuGccuAcAucuucuATTB -3'     (Sense)     (SEQ ID NO:73)
     ||||||||||||||||||||
3' UUcGAcAcGGAuGuAGAAGAU    -5'     (Antisense) (SEQ ID NO:74)
```

wherein:

each B is an inverted abasic cap moiety;

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

u is 2'-deoxy-2'fluorouridine;

A is a 2'-deoxyadenosine;

G is a 2'deoxyguanosine;

T is a thymidine;

A is adenosine;

G is guanosine;

U is uridine;

A is a 2'-O-methyl-adenosine;

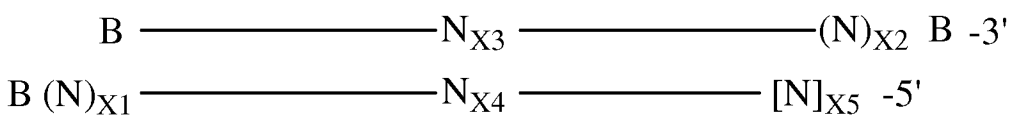
G is a 2'-O-methyl-guanosine;

U is a 2'-O-methyl-uridine; and

the internucleotide linkages are chemically modified or unmodified.

15. The double-stranded short interfering nucleic acid (siNA) molecule according to claim 14, wherein the internucleotide linkages are unmodified.

16. A double stranded nucleic acid (siNA) molecule comprising structure SX' having a sense strand and an antisense strand:



SX'

wherein

the upper strand is the sense strand and the lower strand is the antisense strand of the double stranded nucleic acid molecule; said antisense strand comprises a sequence having complementarity to SEQ ID NO: 10, SEQ ID NO: 13, SEQ ID NO: 16, or SEQ ID NO: 21, and said sense strand comprises a sequence having complementarity to the antisense strand;

each N is independently a nucleotide which is unmodified or chemically modified;

each B is a terminal cap moiety that is present or absent;

(N) represents overhanging nucleotides, each of which is independently unmodified or a 2'-O-methyl nucleotide, 2'-deoxy-2'-fluoro nucleotide, or 2'-deoxyribonucleotide;

[N] represents nucleotides that are ribonucleotides;

X1 and X2 are independently integers from 0 to 4;

X3 is an integer from 17 to 36;

X4 is an integer from 11 to 35, provided that the sum of X4 and X5 is 17-36;

X5 is an integer from 1 to 6; and wherein

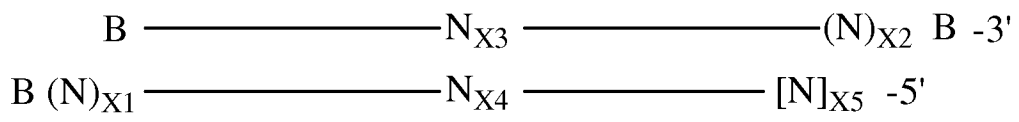
(a) each pyridmidine nucleotide in N_{X4} positions is independently a 2'-deoxy-2'-fluoro nucleotide or a 2'-O-methyl nucleotide;

each purine nucleotide in N_{X4} positions is a 2'-O-methyl nucleotide;

(b) each pyrimidine nucleotide in N_{X3} positions is a ribonucleotide;

each purine nucleotide in N_{X3} positions is a ribonucleotide.

17. A double stranded nucleic acid (siNA) molecule comprising structure SXI' having a sense strand and an antisense strand:



SXI'

wherein

the upper strand is the sense strand and the lower strand is the antisense strand of the double stranded nucleic acid molecule; said antisense strand comprises a sequence having complementarity to SEQ ID NO: 10, SEQ ID NO: 13, SEQ ID NO: 16, or SEQ ID NO: 21, and said sense strand comprises a sequence having complementarity to the antisense strand;

each N is independently a nucleotide which is unmodified or chemically modified;

each B is a terminal cap moiety that is present or absent;

(N) represents overhanging nucleotides, each of which is independently unmodified or a 2'-O-methyl nucleotide, 2'-deoxy-2'-fluoro nucleotide, or 2'-deoxyribonucleotide;

[N] represents nucleotides that are ribonucleotides;

X1 and X2 are independently integers from 0 to 4;

X3 is an integer from 17 to 36;

X4 is an integer from 11 to 35, provided that the sum of X4 and X5 is 17-36;

X5 is an integer from 1 to 6; and wherein

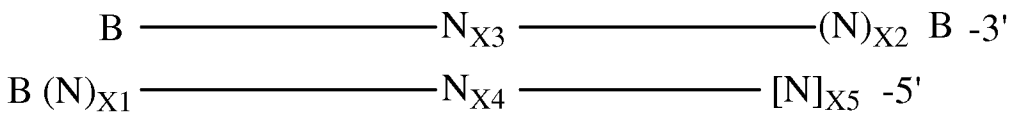
(a) each pyridmidine nucleotide in N_{X4} positions is independently a 2'-deoxy-2'-fluoro nucleotide or a 2'-O-methyl nucleotide;

each purine nucleotide in N_{X4} positions is a 2'-O-methyl nucleotide;

(b) each pyrimidine nucleotide in N_{X3} positions is a 2'-deoxy-2'-fluoro nucleotide;

each purine nucleotide in N_{X3} positions is a ribonucleotide.

18. A double stranded nucleic acid (siNA) molecule comprising structure SXII' having a sense strand and an antisense strand:



SXII'

wherein

the upper strand is the sense strand and the lower strand is the antisense strand of the double stranded nucleic acid molecule; said antisense strand comprises a sequence having complementarity to SEQ ID NO: 10, SEQ ID NO: 13, SEQ ID NO: 16, or SEQ ID NO: 21, and said sense strand comprises a sequence having complementarity to the antisense strand;

each N is independently a nucleotide which is unmodified or chemically modified;

each B is a terminal cap moiety that is present or absent;

(N) represents overhanging nucleotides, each of which is independently unmodified or a 2'-O-methyl nucleotide, 2'-deoxy-2'-fluoro nucleotide, or 2'-deoxyribonucleotide;

[N] represents nucleotides that are ribonucleotides;

X1 and X2 are independently integers from 0 to 4;

X3 is an integer from 17 to 36;

X4 is an integer from 11 to 35, provided that the sum of X4 and X5 is 17-36;

X5 is an integer from 1 to 6; and wherein

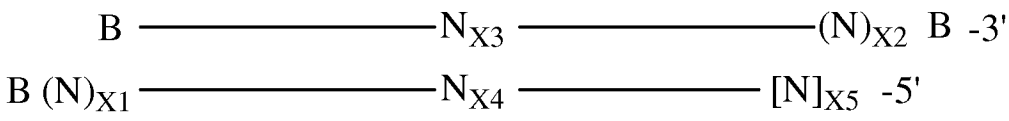
(a) each pyridmidine nucleotide in N_{X4} positions is independently a 2'-deoxy-2'-fluoro nucleotide or a 2'-O-methyl nucleotide;

each purine nucleotide in N_{X4} positions is a 2'-O-methyl nucleotide;

(b) each pyrimidine nucleotide in N_{X3} positions is a 2'-deoxy-2'-fluoro nucleotide;

each purine nucleotide in N_{X3} positions is a 2'-deoxyribonucleotide.

19. A double stranded nucleic acid (siNA) molecule comprising structure SXIII' having a sense strand and an antisense strand:



SXIII'

wherein

the upper strand is the sense strand and the lower strand is the antisense strand of the double stranded nucleic acid molecule; said antisense strand comprises a sequence having complementarity to SEQ ID NO: 10, SEQ ID NO: 13, SEQ ID NO: 16, or SEQ ID NO: 21, and said sense strand comprises a sequence having complementarity to the antisense strand;

each N is independently a nucleotide which is unmodified or chemically modified;

each B is a terminal cap moiety that is present or absent;

(N) represents overhanging nucleotides, each of which is independently unmodified or a 2'-O-methyl nucleotide, 2'-deoxy-2'-fluoro nucleotide, or 2'-deoxyribonucleotide;

[N] represents nucleotides that are ribonucleotides;

X1 and X2 are independently integers from 0 to 4;

X3 is an integer from 17 to 36;

X4 is an integer from 11 to 35, provided that the sum of X4 and X5 is 17-36;

X5 is an integer from 1 to 6; and wherein

(a) each pyridmidine nucleotide in N_{X4} positions is a nucleotide having a ribo-like, Northern or A-form helix configuration;

each purine nucleotide in N_{X4} positions is a 2'-O-methyl nucleotide;

(b) each pyrimidine nucleotide in N_{X3} positions is a nucleotide having a ribo-like, Northern or A-form helix configuration;

each purine nucleotide in N_{X3} positions is a 2'-O-methyl nucleotide.

20. A pharmaceutical composition comprising the double stranded nucleic acid (siNA) of claim 1 in a pharmaceutically acceptable carrier or diluent.

21. A pharmaceutical composition comprising the double stranded nucleic acid (siNA) molecule of claim 3 in a pharmaceutically acceptable carrier or diluent.

22. A pharmaceutical composition comprising the double stranded nucleic acid (siNA) molecule of claim 8 in a pharmaceutically acceptable carrier or diluent.

23. A pharmaceutical composition comprising the double stranded nucleic acid (siNA) molecule of claim 10 in a pharmaceutically acceptable carrier or diluent.

24. A pharmaceutical composition comprising the double stranded nucleic acid (siNA) molecule of claim 12 in a pharmaceutically acceptable carrier or diluent.

25. A pharmaceutical composition comprising the double stranded nucleic acid (siNA) molecule of claim 14 in a pharmaceutically acceptable carrier or diluent.

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

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

28. A pharmaceutical composition comprising the double stranded nucleic acid (siNA) molecule of claim 18 in a pharmaceutically acceptable carrier or diluent.

29. A pharmaceutical composition comprising the double stranded nucleic acid (siNA) molecule of claim 19 in a pharmaceutically acceptable carrier or diluent.

30. A pharmaceutical composition comprising the double stranded nucleic acid (siNA) molecule of claim 3 which is adapted for inhaled delivery.

31. A pharmaceutical composition comprising the double stranded nucleic acid (siNA) molecule of claim 8 which is adapted for inhaled delivery.

32. A pharmaceutical composition comprising the double stranded nucleic acid (siNA) molecule of claim 10 which is adapted for inhaled delivery.

33. A pharmaceutical composition comprising the double stranded nucleic acid (siNA) molecule of claim12 which is adapted for inhaled delivery.

33. A pharmaceutical composition comprising the double stranded nucleic acid (siNA) molecule of claim 14 which is adapted for inhaled delivery.

34. A method of treating a human subject suffering from a condition which is mediated by the action, or by loss of action, of ENaC which comprises administering to said subject an effective amount of the double stranded nucleic acid (siNA) molecule of claim 3.

35. A method of treating a human subject suffering from a condition which is mediated by the action, or by loss of action, of ENaC which comprises administering to said subject an effective amount of the double stranded nucleic acid (siNA) molecule of claim 8.

36. A method of treating a human subject suffering from a condition which is mediated by the action, or by loss of action, of ENaC which comprises administering to said subject an effective amount of the double stranded nucleic acid (siNA) molecule of claim 10.

37. A method of treating a human subject suffering from a condition which is mediated by the action, or by loss of action, of ENaC which comprises administering to said subject an effective amount of the double stranded nucleic acid (siNA) molecule of claim 12.

38. A method of treating a human subject suffering from a condition which is mediated by the action, or by loss of action, of ENaC which comprises administering to said subject an effective amount of the double stranded nucleic acid (siNA) molecule of claim 14.

39. The method according to claim 34 wherein the condition is a respiratory disease.

40. The method according to claim 35 wherein the condition is a respiratory disease.

41. The method according to claim 36 wherein the condition is a respiratory disease.

42. The method according to claim 37 wherein the condition is a respiratory disease.

43. The method according to claim 38 wherein the condition is a respiratory disease.

44. The method according to claim 39 wherein the respiratory disease is selected from the group consisting of COPD, asthma, cystic fibrosis, eosinophilic cough, bronchitis, sarcoidosis, pulmonary fibrosis, rhinitis, sinusitis and bronchiectasis.

45. The method according to claim 40 wherein the respiratory disease is selected from the group consisting of COPD, asthma, cystic fibrosis, eosinophilic cough, bronchitis, sarcoidosis, pulmonary fibrosis, rhinitis, sinusitis and bronchiectasis.

46. The method according to claim 41 wherein the respiratory disease is selected from the group consisting of COPD, asthma, cystic fibrosis, eosinophilic cough, bronchitis, sarcoidosis, pulmonary fibrosis, rhinitis, sinusitis and bronchiectasis.

47. The method according to claim 42 wherein the respiratory disease is selected from the group consisting of COPD, asthma, cystic fibrosis, eosinophilic cough, bronchitis, sarcoidosis, pulmonary fibrosis, rhinitis, sinusitis and bronchiectasis.

48. The method according to claim 43 wherein the respiratory disease is selected from the group consisting of COPD, asthma, cystic fibrosis, eosinophilic cough, bronchitis, sarcoidosis, pulmonary fibrosis, rhinitis, sinusitis and bronchiectasis.

49. The method according to claim 44 wherein the respiratory disease is selected from the group consisting of COPD, cystic fibrosis, bronchiectasis, and asthma.

50. The method according to claim 45 wherein the respiratory disease is selected from the group consisting of COPD, cystic fibrosis, bronchiectasis, and asthma.

51. The method according to claim 46 wherein the respiratory disease is selected from the group consisting of COPD, cystic fibrosis, bronchiectasis, and asthma.

52. The method according to claim 47 wherein the respiratory disease is selected from the group consisting of COPD, cystic fibrosis, bronchiectasis, and asthma.

53. The method according to claim 48 wherein the respiratory disease is selected from the group consisting of COPD, cystic fibrosis, bronchiectasis, and asthma.