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(54) Title: IMPROVING THE BIOLOGICAL ACTIVITY OF SIRNA THROUGH MODULATION OF ITS THERMODYNAMIC PROFILE

(57) Abstract: The present invention describes the combination of thermally stabilizing and destabilizing chemical modifications and sequence alterations incorporated in specific regions of the duplex to optimize the thermodynamic profile of a given siRNA duplex and to further improve its silencing activity.



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***Improving the biological activity of siRNA through
modulation of its thermodynamic profile***

PRIORITY CLAIM

This application claims priority to U.S. Provisional Application No. 61/309,311, filed March 1, 2010.

BACKGROUND

Double-stranded RNA molecules (dsRNA) can block gene expression by virtue of a highly conserved regulatory mechanism known as RNA interference (RNAi). Briefly, RNA III Dicer, an enzyme, processes dsRNA into small interfering RNA (also sometimes called short interfering RNA or siRNA) of approximately 22 nucleotides. One strand of the siRNA (the “antisense strand”) then serves as a guide sequence to induce cleavage by an RNA-induced silencing complex, RISC, of messenger RNAs (mRNAs) including a nucleotide sequence which is at least partially complementary to the antisense strand. The antisense strand is not cleaved or otherwise degraded in this process, and the RISC including the antisense strand can subsequently affect the cleavage of further mRNAs.

During the RISC assembly process the passenger (or sense) strand is generally cleaved between positions 9 and 10, and subsequently separated from the complementary guide (or antisense) strand to generate the active RISC complex. (See Matranga, C. et al. (2005) Passenger-Strand Cleavage Facilitates Assembly of siRNA into Ago2-Containing RNAi Enzyme Complexes. *Cell* **123**, 607-620.) The passenger strand is cleaved during the course of RISC assembly, and certain chemical modifications at this putative cleavage site in the passenger strand can severely impair silencing activity. (See Leuschner P.J.F., Ameres S.L., et al. (2006). Cleavage of the siRNA passenger strand during RISC assembly in human cells. *EMBO reports* **7**, 314-20.) .

In a previous study Khvorova et al. calculated the average internal thermodynamic profiles for 180 siRNAs targeting firefly luciferase and human cyclophilin, which were divided into functional, non-functional and remaining (intermediate) siRNAs (Khvorova, A., Reynolds, A. and Jayasena, S.D. (2003) Functional siRNAs and miRNAs exhibit strand bias. *Cell*, 115, 209-216). The profiles were calculated according to a nearest neighbor method reported by

Freier et al. (Freier, S.M., Kierzek, R., Jaeger, J.A., Sugimoto, N., Caruthers, M.H., Neilson, T. and Turner, D.H. (1986) Improved free-energy parameters for predictions of RNA duplex stability. *Proc Natl Acad Sci U S A*, 83, 9373-9377). The results indicated that the profiles of functional and non-functional siRNAs differed significantly across different regions of the duplex and highlighted the importance of the internal thermal stability profile for functional activity of siRNA.

Thus, it is greatly desired that oligonucleotides be able to be synthesized to have enhanced properties based on the overall thermodynamic profile.

SUMMARY

This invention relates to the placement of nucleotides bearing certain chemical modifications at the most effective position of the double stranded oligonucleotide based on the thermodynamic profile calculated. Thermally destabilizing chemical modifications such as abasic residues, universal bases with reduced or abolished capability to form hydrogen bonds with popposing bases (e.g. 2,4-difluorotoluy, 5-nitroindole) or unlocked nucleic acids in the low thermal stability region of the duplex (e.g., region around the cleavage site or within the 5'-half of the antisense strand) or the placement of one or more mismatches in this low thermal stability region or both, in addition to chemical modifications, such as locked nucleic acid, 2'-modified RNA, to further stabilize the high thermal stability region at or near the 3'-end of the antisense strand will improve the potency of siRNA, including the silencing activity of siRNA.

DESCRIPTION OF THE DRAWINGS

FIG. 1 is various schematic representations of the specific regions of a 19mer siRNA duplex and their thermal stability requirements for optimal functional activity.

FIG. 2 is a ΔG graph of an energetic profiles of effective ($KD \geq 80\%$) and ineffective ($KD \geq 20\%$) duplexes.

FIG. 3 is a $\Delta\Delta G$ graph of an energetic profiles of effective ($KD \geq 80\%$) and ineffective ($KD \geq 20\%$) duplexes.

DETAILED DESCRIPTION

Previous in-house experiments showed that the potency of a given siRNA can be significantly improved by thermal destabilization of the central region of the duplex. This was achieved by placing base pair mismatches, universal bases or other modifications, designed to decrease the thermodynamic stability of a given duplex, in the central region (positions 9-12) of the sense strand (WO 20100011895, which is hereby incorporated by its entirety). The present invention describes a more comprehensive approach of combining thermally stabilizing and destabilizing chemical modifications in different regions of the siRNA duplex to modulate the thermodynamic profile and improve the biological activity.

The present invention describes the combination of thermally stabilizing and destabilizing chemical modifications and sequence alterations incorporated in specific regions of the duplex to optimize the thermodynamic profile of a given siRNA duplex and to further improve its silencing activity.

Figure 2 shows that the average internal stability profile of highly active siRNAs is highly specific and clearly distinguishable from non-functional ones. With large numbers of possible siRNAs against a given target, it is generally possible to identify potent siRNAs with a close-to-optimal thermodynamic profile. However in many cases, the number of possible siRNAs against a given target RNA is limited due to short target RNA sequence or poor sequence homology across species (for cross-reactive siRNAs). In these cases, modulating the thermal stability of the duplex by selectively placing thermally stabilizing and/or destabilizing modifications could be particularly useful to generate active siRNAs. The choice of modification and their position will depend on the thermodynamic profile of the siRNA to be modified.

Furthermore, many of modifications used to modulate thermal stability described in this invention are non-natural nucleotide modifications, which can be expected to enhance the stability of the siRNA against nuclease attack thereby further improving the drug-like properties of the molecule.

In one embodiment, there are least 2, at least 3, at least 4, at least 5, at least 6, at least 7, at least 8, at least 9, at least 10, at least 15, at least 20, or at least 25 regions of thermal stability of a 10-mer to a 30-mer duplex, where thermally destabilizing chemical modifications are placed at the low

thermal stability regions and stabilizing chemical modifications are placed at the high thermal stability regions.

In one embodiment, where there are at least 2 regions of thermal stability of a 19-mer duplex, destabilizing chemical modifications are placed in the low thermal stability region at positions -19 from the 5'-end of the sense strand and stabilizing chemical modifications are placed at the high thermal stability at positions 1-7 from the 5'-end of the sense strand.

In one embodiment, where there are at least 3 regions of thermal stability of a 19-mer duplex, destabilizing chemical modifications are placed in the low thermal stability region at positions 9-12 and 18-19 from the 5'-end of the sense strand and stabilizing chemical modifications are placed at the high thermal stability region at positions 1-4 from the 5'-end of the sense strand.

In one embodiment, where there are at least 3 regions of thermal stability of a 19-mer duplex, destabilizing chemical modifications are placed in the low thermal stability region at positions 8 from the 5'-end of the sense strand and stabilizing chemical modifications are placed at the high thermal stability at positions 1-6 from the 5'-end of the sense strand.

In one embodiment, where there are at least 3 regions of thermal stability of a 19-mer duplex, destabilizing chemical modifications are placed in the low thermal stability region at positions 9-12 and 18-19 from the 5'-end of the sense strand and stabilizing chemical modifications are placed at the high thermal stability region at positions 1-4 from the 5'-end of the sense strand.

In one embodiment, where there are at least 2 regions of thermal stability fo a 10-mer to a15-mer duplex, destabilized chemical modifications are placed at the low thermal stability at positions 8-14 from the 5'-end of the sense strand.and stabilized chemical modifications are placed at the high thermal stability at positions 1-4 from the 5'-end of the sense strand.

In one embodiment, where there are at least 3 regions of thermal stability of a 25-mer to a 30-mer duplex, destabilizinged chemical modifications are placed in the low thermal stability region at positions 8-14, 6-19, 23-26 from the 5'-end of the sense strand and stabilizing chemical modifications are placed at the high thermal stability at positions 1-6 and 19-22 from the 5'-end of the sense strand.

In one example, the invention provides a double-stranded agent with increased RNAi silencing activity, comprising:

- a. a first strand that is complementary to a target gene; and
- b. a second strand that is complementary to said first strand and comprises
 - i. at least one destabilized chemical modification placed at at least one of the low-thermal-stability positions in the second strand; and
 - ii. at least one stabilized chemical modification placed at at least one of the high-thermal-stability positions in the second strand;

wherein said increased RNAi silencing activity is relative to a corresponding unmodified agent, as determined by comparing their respective IC₅₀ values in a RNAi silencing assay.

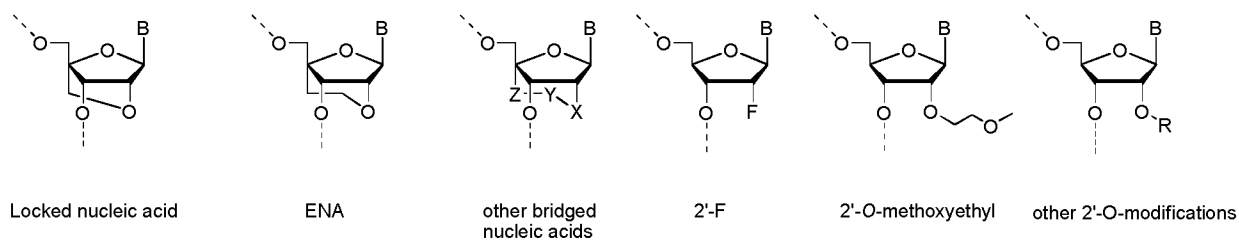
In one embodiment, the invention provides a double-stranded agent with increased RNAi silencing activity, comprising:

- c. a first strand that is complementary to a target gene; and a second strand that is complementary to said first strand and comprising
 - i. at least one thermally destabilizing chemical modification placed within atleast one of the low-thermal-stability regions in the doubled-stranded agent or
 - ii. at least one thermally stabilizing chemical modification placed at at least one of the high-thermal-stability regions within the doubled-stranded agent
 - iii. a combination of i. and ii.

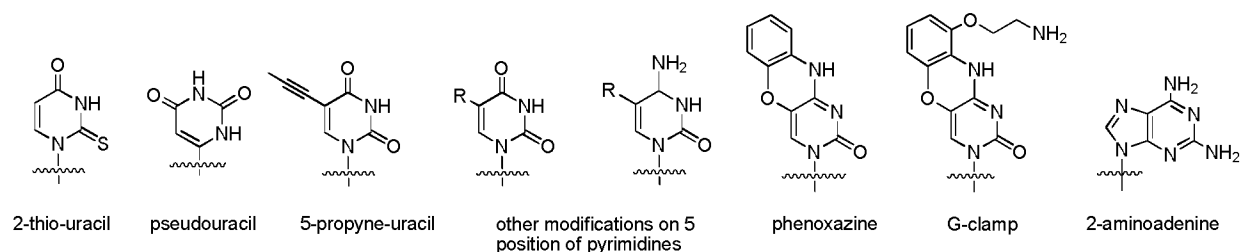
wherein said increased RNAi silencing activity is relative to a corresponding unmodified agent, as determined by comparing their respective IC₅₀ values in a RNAi silencing assay.

In one embodiment, the thermally stabilizing chemical modifications include, but are not limited to,

Sugar-Modifications:

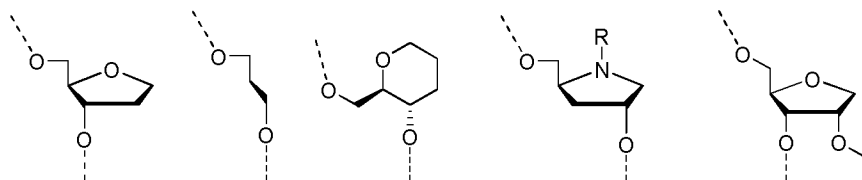


Nucleobase Modifications:



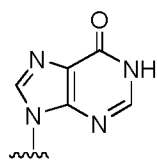
In one embodiment, the thermally destabilizing chemical modifications include, but are not limited to,

Abasic Modifications:

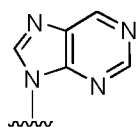


Nucleobase Modifications:

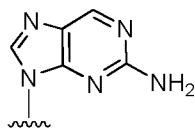
Shown are examples of nucleobase modifications with impaired or completely abolished capability to form hydrogen bonds with bases in the opposite strand, some of which have been evaluated for destabilization of the central region of the duplex as part of WO 20100011895.



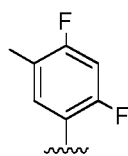
inosine



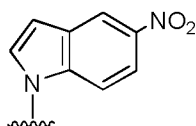
nebularine



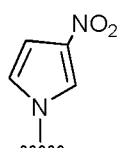
2-aminopurine



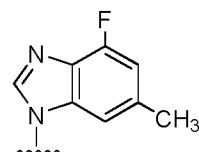
2,4-difluorotoluene



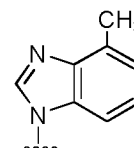
5-nitroindole



3-nitropyrrole

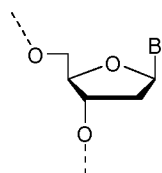


4-Fluoro-6-methylbenzimidazole

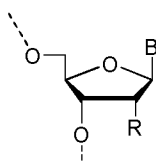
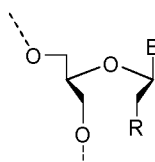
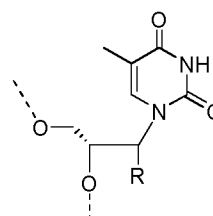


4-Methylbenzimidazole

Sugar Modifications:

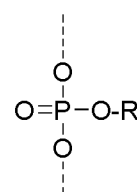
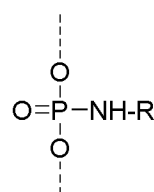
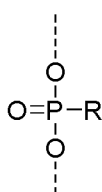
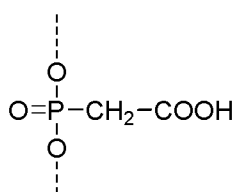
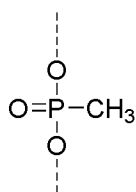
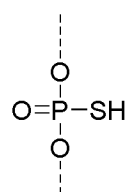


2'-deoxy

2'-alkyl
R = CH₃, CH₂F, CF₃,
CH₂-CH₃, etc.)unlocked nucleic acid
R = H, OH, O-alkylglycol nucleic acid
R = H, OH, O-alkyl

Phosphate Modifications:

Shown below are some examples of phosphate modifications known to decrease the thermal stability of nucleic acid duplexes compared to natural phosphodiester linkages



R = alkyl

In one embodiment, the UA base pair at position 13 and/or 14 is unmodified.

In one embodiment, the double-stranded short interfering ribonucleic acid (siRNA) molecule further comprises at least one, at least two, at least three, or at least four of the criteria selected from the group consisting of having A at position 1 of the 5'-end of antisense, a GC content of about 25% to about 50%, asymmetry at ends of duplex, having AU pair at position 13 and/or 14, and at least 1, at least 2, at least 3, at least 4, at least 5 UAs in the seed region.

In one embodiment, the double-stranded short interfering ribonucleic acid (siRNA) molecule of the invention is a 19 bp duplex and comprises at least one, at least two, at least three, or at least four of the criteria selected from the group consisting of having A at position 1 of the 5'-end of antisense, a GC content of about 25% to about 50%, asymmetry at ends of duplex, having AU pair at position 13 and/or 14, and at least 1, at least 2, at least 3, at least 4, at least 5 UAs in the seed region.

One aspect of the invention relates to a double-stranded short interfering ribonucleic acid (siRNA) molecule, where each strand is, for example, about 10 to about 30 nucleotides in length, such as 19 to 29 nucleotides in length, and wherein at least one nucleotide comprises a modified or non-natural base. The invention provides inhibitory RNA agents (iRNA agents) that generally provide increased RNAi silencing activity. In one embodiment, the iRNA agent contains a first strand which is complementary to a target gene, and a second strand which is complementary to the first strand and contains at least one destabilized chemical modified nucleobase at positions 9-12 and 18-19 from the 5'-end of the second strand and at least one stabilized chemical modified nucleobase at positions 1-4 from the 5'-end of the second strand. The first strand may represent the antisense strand and the second strand may represent the sense strand. The increased RNAi silencing activity is generally determined relative to a corresponding iRNA agent not containing the modified nucleobase, and is determined by measuring IC₅₀. The destabilized modified nucleobase is, *e.g.*, a non-natural nucleobase, a universal nucleobase, or the nucleobase is absent, *i.e.* an abasic nucleoside/nucleotide. For example, the destabilized modified nucleobase may be a 2'-deoxy nucleobase. In some embodiments, the destabilized modified nucleobase is an optionally substituted difluorotolyl (*e.g.*, 2,4-difluorotolyl), an optionally substituted indolyl (*e.g.*, 5-nitroindole), an optionally substituted pyrrolyl, or an optionally substituted benzimidazolyl. The iRNA agent containing a destabilized modified nucleobase at positions 9 -12 and 18-19 from the 5'-end of the sense strand and at least one stabilized chemical modified nucleobase at positions 1-4 from the 5'-end of the

sense strand exhibits an IC_{50} value less than or equal to about 90%, 80%, 70%, 60%, 50%, 40%, 30%, 20%, 10%, or 5% of the IC_{50} value of the corresponding unmodified iRNA agent or the corresponding iRNA agent with one fewer mismatch base pairings. IC_{50} is measured in an *in vitro* system. Alternatively, the IC_{50} is measured in an *in vivo* system. The destabilized modified nucleobase may be in position 9, 10, 11, 12, 18 or 19 from the 5'-end of the sense strand. The stabilized modified nucleobase may be at position 1, 2, 3 or 4 from the 5'-end of the sense strand.

Another aspect of the invention relates to a double-stranded short interfering ribonucleic acid (siRNA) molecule, where each strand is, for example, about 12 to about 30 nucleotides in length, such as 19 to 29 nucleotides in length, and wherein contained within the double-stranded siRNA molecule is at least one base pairing mismatch. The invention provides iRNA agents containing one or more mismatches that generally provide increased RNAi silencing activity. In one embodiment the invention provides a double stranded iRNA agent with increased RNAi silencing activity, which contains an antisense strand which is complementary to a target gene, and a sense strand which is complementary to the antisense strand and contains one, two or more than two mismatch base pairings with the antisense strand at positions 9 to 12 from the 5'-end of the sense strand, where the increased RNAi silencing activity is relative to an iRNA agent with fewer mismatch base pairings or no mismatch base pairings with the antisense strand, as determined by measuring IC_{50} . In embodiments, the iRNA agent contains a mismatch at position 9, 10, 11 or 12, or a combination thereof, from the 5'-end of the sense strand. Mismatch basepairs can be G:G, G:A, G:U, G:T, A:A, A:C, C:C, C:U, C:T, U:U, T:T or U:T, or a combination thereof. Other mismatch base pairings known in the art are also amenable to the present invention. In certain embodiments, the iRNA agent contains at least one nucleobase in the mismatch pairing that is a 2'-deoxy nucleobase; preferably, the 2'-deoxy nucleobase is in the sense strand.

Each strand of the iRNA agents described herein is independently at least about 15 nucleobases in length, and no more than about 30 nucleobases in length. For example, each strand is between 19 to 29 nucleotides in length, 19 to 25 nucleobases in length, or 21 to 23 nucleobases in length. In some embodiments, the sense and antisense strands are each 21 nucleobases in length. Also provided are iRNA agents containing a single stranded overhang on

at least one terminal end. The single stranded overhang contains, *e.g.*, 1, 2, 3 or more than 3 nucleobases.

Another aspect of the present invention relates to a single-stranded oligonucleotide comprising at least strand of the double-stranded iRNA agents described herein.

In another aspect, the invention provides a method of reducing the expression of a target gene in a cell, by contacting the cell with an iRNA agent disclosed herein.

Another aspect of the invention relates to a method of gene silencing, comprising administering to a mammal in need thereof a therapeutically effective amount of a double-stranded iRNA agent. Another aspect of the invention relates to compositions and methods for delivery of an siNA, *e.g.*, an siRNA or other nucleic acid that contains one or more modified nucleosides comprising a non-natural nucleobase, or contains one or more mismatch base pairings. Another aspect of the invention relates to a method of suppressing the endogenous expression of a gene, comprising contacting a cell with an effective amount of the composition or iRNA agent of the invention, wherein the effective amount is an amount that partially or substantially suppresses the endogenous expression of said gene.

One aspect of the invention provides siRNA and siNA compositions containing modified nucleotides or mismatched base pairings, particularly in the sense strand of a double stranded siRNA, as well as methods for inhibiting the expression of a target gene in a cell, tissue or mammal using these compositions. The invention also provides compositions and methods for treating diseases in a mammal caused by the aberrant expression of a target gene, or a mutant form thereof, using siRNA compositions.

Described herein are nucleic acid-containing compositions that target specific mRNA sequences for removal by RISC. In particular are compositions containing double stranded iRNA agents that contain either a nucleobase modification (such as a universal base) or a mismatched base pairing. Without being limited by theory, it is believed that the presence of the nucleobase modifications or mismatches facilitates sense strand removal during RISC assembly, possibly through local destabilization of the duplex at the putative cleavage site.

In one aspect the, the invention provides a double stranded iRNA agent with increased RNAi silencing activity comprising (a) an antisense strand which is complimentary to a target

gene; (b) a sense strand which is complimentary to said antisense strand and comprises at least one modified nucleobase in the region corresponding to the target cleavage site region; and wherein said increased RNAi silencing activity is relative the corresponding unmodified RNAi agent as determined by comparing their respective IC₅₀ values measured either *in vitro* or *in vivo*.

In one embodiment, the modified nucleobase comprising nucleotide further comprises at least one modification chosen from a group of sugar modifications and backbone modifications described herein. In one embodiment, the nucleotide comprising the modified nucleobase is a 2'-deoxy nucleotide.

In one embodiment, the destabilized modified nucleobase is at the first position of the cleavage site region from the 5'-end of the sense strand.

In one embodiment, the destabilized modified nucleobase is at the second position of the cleavage site region from the 5'-end of the sense strand.

In one embodiment, the destabilized modified nucleobase is at the third position of the cleavage site region from the 5'-end of the sense strand.

In one embodiment, the destabilized modified nucleobase is at the fourth position of the cleavage site region from the 5'-end of the sense strand.

In another aspect the, the invention provides a double stranded iRNA agent with increased RNAi silencing activity comprising (a) an antisense strand which is complimentary to a target gene; (b) a sense strand which is complimentary to said antisense strand and comprises one or two or more mismatch base pairings with the antisense strand in the region corresponding to the target cleavage site region; and wherein said increased RNAi silencing activity is relative the corresponding unmodified RNAi agent as determined by comparing their respective IC₅₀ values measured either *in vitro* or *in vivo*.

In one embodiment, the modified nucleobase comprising nucleotide further comprises at least one modification chosen from a group of sugar modification and backbone modification described herein. In one embodiment, the nucleotide comprising the modified nucleobase is a 2'-deoxy nucleotide.

In one embodiment, the mismatch is at the first position of the cleavage site region from the 5'-end of the sense strand.

In one embodiment, the mismatch is at the second position of the cleavage site region from the 5'-end of the sense strand.

In one embodiment, the mismatch is at the third position of the cleavage site region from the 5'-end of the sense strand.

In one embodiment, the mismatch is at the fourth position of the cleavage site region from the 5'-end of the sense strand.

The "target cleavage site" herein means the backbone linkage in the target gene or the sense strand that is cleaved by the RISC mechanism by utilizing the iRNA agent. And the target cleavage site region comprises at least one or at least two nucleotides on both side of the cleavage site. For the sense strand, the target cleavage site is the backbone linkage in the sense strand that would get cleaved if the sense strand itself was the target to be cleaved by the RNAi mechanism. The target cleavage site can be determined using methods known in the art, for example the 5'-RACE assay as detailed in Soutschek *et al.*, *Nature* (2004) 432, 173-178. As is well understood in the art, the cleavage site region for a conical double stranded RNAi agent comprising two 21-nucleotides long strands (wherein the strands form a double stranded region of 19 consecutive basepairs having 2-nucleotide single stranded overhangs at the 3'-ends), the cleavage site region corresponds to positions 9-12 from the 5'-end of the sense strand.

In another aspect the, the invention provides a double stranded iRNA agent with increased RNAi silencing activity comprising (a) an antisense strand which is complementary to a target gene; (b) a sense strand which is complementary to said antisense strand and comprises at least one modification in the region corresponding to the target cleavage site region; and wherein said increased RNAi silencing activity is relative the corresponding unmodified RNAi agent as determined by comparing their respective IC₅₀ values measured either *in vitro* or *in vivo* and said modification locally destabilizes the duplex.

In one embodiment, the modified nucleobase comprising nucleotide further comprises at least one modification chosen from a group of sugar modification and backbone modification described herein. In one embodiment, the nucleotide comprising the modified nucleobase is a 2'-deoxy nucleotide.

Definitions

Unless stated otherwise, or implicit from context, the following terms and phrases include the meanings provided below. Unless explicitly stated otherwise, or apparent from context, the terms and phrases below do not exclude the meaning that the term or phrase has acquired in the art to which it pertains. The definitions are provided to aid in describing particular embodiments, and are not intended to limit the claimed invention, because the scope of the invention is limited only by the claims.

The phrase “antisense strand” as used herein, refers to a polynucleotide that is substantially or 100% complementary to a target nucleic acid of interest. An antisense strand may comprise a polynucleotide that is RNA, DNA or chimeric RNA/DNA. For example, an antisense strand may be complementary, in whole or in part, to a molecule of messenger RNA, an RNA sequence that is not mRNA (e.g., microRNA, piwiRNA, tRNA, rRNA and hnRNA) or a sequence of DNA that is either coding or non-coding. The phrase “antisense strand” includes the antisense region of both polynucleotides that are formed from two separate strands, as well as unimolecular polynucleotides that are capable of forming hairpin structures. The terms “antisense strand” and “guide strand” are used interchangeably herein.

The phrase “sense strand” refers to a polynucleotide that has the same nucleotide sequence, in whole or in part, as a target nucleic acid such as a messenger RNA or a sequence of DNA. The sense strand is not incorporated into the functional riboprotein RISC. The terms “sense strand” and “passenger strand” are used interchangeably herein.

The term “duplex” includes a region of complementarity between two regions of two or more polynucleotides that comprise separate strands, such as a sense strand and an antisense strand, or between two regions of a single contiguous polynucleotide.

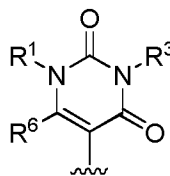
The term “complementary” refers to the ability of polynucleotides to form base pairs with one another. Base pairs are typically formed by hydrogen bonds between nucleotide units in antiparallel polynucleotide strands. Complementary polynucleotide strands can base pair in the Watson-Crick manner (e.g., a to t, a to u, c to g), or in any other manner that allows for the formation of stable duplexes. “Perfect complementarity” or 100% complementarity refers to the situation in which each nucleotide unit of one polynucleotide strand can hydrogen bond with each nucleotide unit of a second polynucleotide strand. Less than perfect complementarity refers

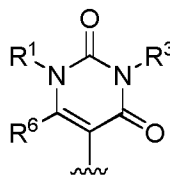
to the situation in which some, but not all, nucleotide units of two strands can hydrogen bond with each other. “Substantial complementarity” refers to polynucleotide strands exhibiting 90% or greater complementarity, excluding regions of the polynucleotide strands, such as overhangs, that are selected so as to be noncomplementary.

The phrase “from the 5’-end of the sense strand” includes the relative position of a given nucleotide in the sense strand with respect to the nucleotide of the sense strand that is located at the 5’ most position of that strand.

The phrase “first 5’ terminal nucleotide” includes first 5’ terminal antisense nucleotides and first 5’ terminal antisense nucleotides. “First 5’ terminal antisense nucleotide” refers to the nucleotide of the antisense strand that is located at the 5’ most position of that strand with respect to the bases of the antisense strand that have corresponding complementary bases on the sense strand. Thus, in a double stranded polynucleotide that is made of two separate strands, it refers to the 5’ most base other than bases that are part of any 5’ overhang on the antisense strand. When the first 5’ terminal antisense nucleotide is part of a hairpin molecule, the term “terminal” refers to the 5’ most relative position within the antisense region and thus is the 5’ most nucleotide of the antisense region. The phrase “first 5’ terminal sense nucleotide” is defined in reference to the antisense nucleotide. In molecules comprising two separate strands, it refers to the nucleotide of the sense strand that is located at the 5’ most position of that strand with respect to the bases of the sense strand that have corresponding complementary bases on the antisense strand. Thus, in a double stranded polynucleotide that is made of two separate strands, it is the 5’ most base other than bases that are part of any 5’ overhang on the sense strand.

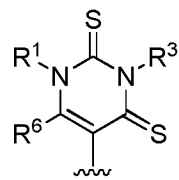
The term “nucleotide” includes a ribonucleotide or a deoxyribonucleotide or modified form thereof, as well as an analog thereof. Nucleotides include species that comprise purines, *e.g.*, adenine, hypoxanthine, guanine, and their derivatives and analogs, as well as pyrimidines, *e.g.*, cytosine, uracil, thymine, and their derivatives and analogs.



The term “pseudouracil” or “5-uracil” refers to  when R¹, R³ and R⁶ are hydrogen. Substituted pseudouracils are defined as follows: when R¹ is not hydrogen, it is a

1-substituted pseudouracil; when R^3 is not hydrogen, it is a 3-substituted pseudouracil; and when R^6 is not hydrogen, it is a 6-substituted uracil. The terms “2-(thio)pseudouracil”,

“4-(thio)pseudouracil” and “2,4-(dithio)pseudouracil” refer to

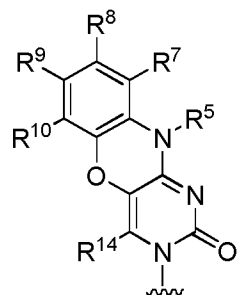


, respectively. Suitable R^1 , R^3 and R^6 include, without limitation, halo, hydroxy, oxo, nitro, haloalkyl, alkyl, alkaryl, aryl, aralkyl, alkoxy, aryloxy, amino, acylamino, alkylcarbamoyl, arylcarbamoyl, aminoalkyl, alkoxycarbonyl, carboxy, hydroxyalkyl, alkanesulfonyl, arenesulfonyl, alkanesulfonamido, arenesulfonamido, aralkylsulfonamido, alkylcarbonyl, acyloxy, cyano, ureido or conjugate groups.

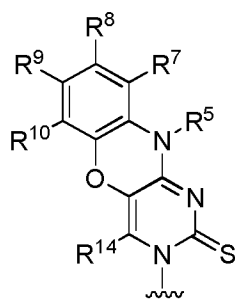
The phrases “2’-modification” and “2’-modified nucleotide” refer to a nucleotide unit having a sugar moiety, for example a ribosyl moiety, that is modified at the 2’-position such that the hydroxyl group (2’-OH) is replaced by, for example, -F, -H, -CH₃, -CH₂CH₃, -OCH₃, -OCH₂CH₃, -OCH₂CH₂OMe, -OCH₂C(=O)NHMe, -OCH₂-(4’-C) (a so-called “LNA sugar modification”), or -OCH₂CH₂-(4’-C) (a so-called “ENA sugar modification”). For example, the phrases “2’-fluoro modification” and “2’-fluoro modified nucleotide” refer to a nucleotide unit having a sugar moiety, for example a ribosyl moiety, that is modified at the 2’-position such that the hydroxyl group (2’-OH) is replaced by a fluoro group (2’-F). U.S. Pat. Nos. 6,262,241, and 5,459,255 (both of which are incorporated by reference), are drawn to, *inter alia*, methods of synthesizing 2’-fluoro modified nucleotides and oligonucleotides.

The phrase “phosphorothioate internucleotide linkage” refers to the replacement of a P=O group with a P=S group, and includes phosphorodithioate internucleoside linkages. One, some or all of the internucleotide linkages that are present in the oligonucleotide can be phosphorothioate internucleotide linkages. U.S. Pat. Nos. 6,143,881, 5,587,361 and 5,599,797 (all of which are incorporated by reference), are drawn to, *inter alia*, oligonucleotides having phosphorothioate linkages.

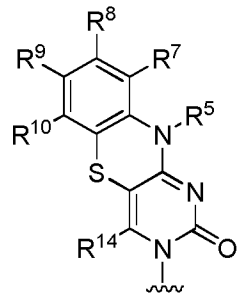
The term “1,3-(diaz)-2-(oxo)-phenoxazin-1-yl” as used herein refers to



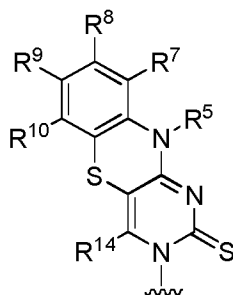
when R^3 , R^7 , R^8 , R^9 , R^{10} , and R^{14} are hydrogen. Substituted 1,3-(diaz)-2-(oxo)-phenoxazin-1-yl are numbered as are the R groups (e.g., a 7-substituted 1,3-(diaz)-2-(oxo)-phenoxazin-1-yl is a compound wherein R^7 is not hydrogen). The term “1-(aza)-2-(thio)-



3-(aza)-phenoxazin-1-yl” as used herein refers to when R^3 , R^7 , R^8 , R^9 , R^{10} , and R^{14} are hydrogen. The term “1,3-(diaz)-2-(oxo)-phenthiazin-1-yl” as used herein refers to



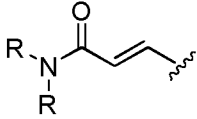
when R^3 , R^7 , R^8 , R^9 , R^{10} , and R^{14} are hydrogen. The term “1-(aza)-2-(thio)-3-

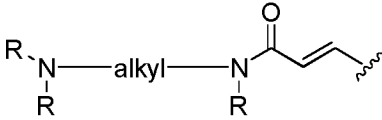


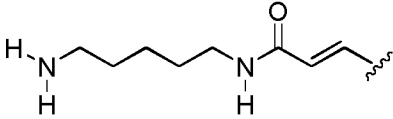
(aza)-phenthiazin-1-yl” as used herein refers to when R^3 , R^7 , R^8 , R^9 , R^{10} , and R^{14} are hydrogen. Suitable R^5 , R^7 to R^{10} , and R^{14} include, without limitation, halo, hydroxy, oxo, nitro, haloalkyl, alkyl, alkaryl, aryl, aralkyl, alkoxy, aryloxy, amino, acylamino, alkylcarbamoyl, arylcarbamoyl, aminoalkyl, alkoxycarbonyl, carboxy, hydroxyalkyl, alkanesulfonyl,

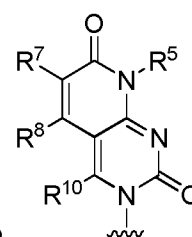
arenesulfonyl, alkanesulfonamido, arenesulfonamido, aralkylsulfonamido, alkylcarbonyl, acyloxy, cyano, ureido or conjugate groups.

As used herein, “aminoalkylhydroxy” refers to -O-alkyl-amino (*e.g.*, -OCH₂CH₂NH₂). As used herein, “guanidiniumalkylhydroxy” refers to -O-alkyl-guanidinium (*e.g.*, -OCH₂CH₂N(H)C(=NH)NH₂).

As used herein, “aminocarbonylethylenyl” refers to  (*e.g.*, when both R are hydrogen, H₂N-C(=O)-CH=CH₂). As used herein, “aminoalkylaminocarbonylethylenyl” refers to

 (*e.g.*, when all three R are hydrogen,

). Suitable R include, without limitation, halo, hydroxy, oxo, nitro, haloalkyl, alkyl, alkaryl, aryl, aralkyl, alkoxy, aryloxy, amino, acylamino, alkylcarbamoyl, arylcarbamoyl, aminoalkyl, alkoxy carbonyl, carboxy, hydroxyalkyl, alkanesulfonyl, arenesulfonyl, alkanesulfonamido, arenesulfonamido, aralkylsulfonamido, alkylcarbonyl, acyloxy, cyano, ureido or conjugate groups.



The term “1,3,5-(triazine)-2,6-(dioxo)-naphthalene” as used herein refers to when R⁵, R⁷, R⁸ and R¹⁰ are hydrogen. Suitable R⁵, R⁷, R⁸ and R¹⁰ include, without limitation, halo, hydroxy, oxo, nitro, haloalkyl, alkyl, alkaryl, aryl, aralkyl, alkoxy, aryloxy, amino, acylamino, alkylcarbamoyl, arylcarbamoyl, aminoalkyl, alkoxy carbonyl, carboxy, hydroxyalkyl, alkanesulfonyl, arenesulfonyl, alkanesulfonamido, arenesulfonamido, aralkylsulfonamido, alkylcarbonyl, acyloxy, cyano, ureido or conjugate groups.

The term “off-target” and the phrase “off-target effects” refer to any instance in which an siRNA or shRNA directed against a given target causes an unintended affect by interacting either directly or indirectly with another mRNA sequence, a DNA sequence or a cellular protein or other moiety. For example, an “off-target effect” may occur when there is a simultaneous degradation of other transcripts due to partial homology or complementarity between that other transcript and the sense and/or antisense strand of the siRNA or shRNA

The phrase “pharmaceutically acceptable carrier or diluent” includes compositions that facilitate the introduction of nucleic acid therapeutics such as siRNA, dsRNA, dsDNA, shRNA, microRNA, antimicroRNA, antagomir, antimir, antisense, aptamer or dsRNA/DNA hybrids into a cell and includes but is not limited to solvents or dispersants, coatings, anti-infective agents, isotonic agents, and agents that mediate absorption time or release of the inventive polynucleotides and double stranded polynucleotides. The phrase “pharmaceutically acceptable” includes approval by a regulatory agency of a government, for example, the U.S. federal government, a non-U.S. government, or a U.S. state government, or inclusion in a listing in the U.S. Pharmacopeia or any other generally recognized pharmacopeia for use in animals, including in humans.

The terms “silence” and “inhibit the expression of” and related terms and phrases, refer to the at least partial suppression of the expression of a gene targeted by an siRNA or siNA, as manifested by a reduction of the amount of mRNA transcribed from the target gene which may be isolated from a first cell or group of cells in which the target gene is transcribed and which has or have been treated such that the expression of the target gene is inhibited, as compared to a second cell or group of cells substantially identical to the first cell or group of cells but which has or have not been so treated (*i.e.*, control cells).

The term “halo” refers to any radical of fluorine, chlorine, bromine or iodine. The term “alkyl” refers to saturated and unsaturated non-aromatic hydrocarbon chains that may be a straight chain or branched chain, containing the indicated number of carbon atoms (these include without limitation propyl, allyl, or propargyl), which may be optionally inserted with N, O, or S. For example, C₁-C₁₀ indicates that the group may have from 1 to 10 (inclusive) carbon atoms in it. The term “alkoxy” refers to an -O-alkyl radical. The term “alkylene” refers to a divalent alkyl (*i.e.*, -R-). The term “alkylenedioxo” refers to a divalent species of the structure -O-R-O-,

in which R represents an alkylene. The term “aminoalkyl” refers to an alkyl substituted with an amino. The term “mercapto” refers to an -SH radical. The term “thioalkoxy” refers to an -S-alkyl radical.

The term “aryl” refers to a 6-carbon monocyclic or 10-carbon bicyclic aromatic ring system wherein 0, 1, 2, 3, or 4 atoms of each ring may be substituted by a substituent. Examples of aryl groups include phenyl, naphthyl and the like. The term “arylalkyl” or the term “aralkyl” refers to alkyl substituted with an aryl. The term “arylalkoxy” refers to an alkoxy substituted with aryl.

The term “cycloalkyl” as employed herein includes saturated and partially unsaturated cyclic hydrocarbon groups having 3 to 12 carbons, for example, 3 to 8 carbons, and, for example, 3 to 6 carbons, wherein the cycloalkyl group additionally may be optionally substituted. Cycloalkyl groups include, without limitation, cyclopropyl, cyclobutyl, cyclopentyl, cyclopentenyl, cyclohexyl, cyclohexenyl, cycloheptyl, and cyclooctyl.

The term “heteroaryl” refers to an aromatic 5-8 membered monocyclic, 8-12 membered bicyclic, or 11-14 membered tricyclic ring system having 1-3 heteroatoms if monocyclic, 1-6 heteroatoms if bicyclic, or 1-9 heteroatoms if tricyclic, said heteroatoms selected from O, N, or S (*e.g.*, carbon atoms and 1-3, 1-6, or 1-9 heteroatoms of N, O, or S if monocyclic, bicyclic, or tricyclic, respectively), wherein 0, 1, 2, 3, or 4 atoms of each ring may be substituted by a substituent. Examples of heteroaryl groups include pyridyl, furyl or furanyl, imidazolyl, benzimidazolyl, pyrimidinyl, thiophenyl or thienyl, quinoliny, indolyl, thiazolyl, and the like. The term “heteroarylalkyl” or the term “heteroaralkyl” refers to an alkyl substituted with a heteroaryl. The term “heteroarylalkoxy” refers to an alkoxy substituted with heteroaryl.

The term “heterocyclyl” refers to a nonaromatic 5-8 membered monocyclic, 8-12 membered bicyclic, or 11-14 membered tricyclic ring system having 1-3 heteroatoms if monocyclic, 1-6 heteroatoms if bicyclic, or 1-9 heteroatoms if tricyclic, said heteroatoms selected from O, N, or S (*e.g.*, carbon atoms and 1-3, 1-6, or 1-9 heteroatoms of N, O, or S if monocyclic, bicyclic, or tricyclic, respectively), wherein 0, 1, 2 or 3 atoms of each ring may be substituted by a substituent. Examples of heterocyclyl groups include piperazinyl, pyrrolidinyl, dioxanyl, morpholinyl, tetrahydrofuranyl, and the like.

The term “oxo” refers to an oxygen atom, which forms a carbonyl when attached to carbon, an N-oxide when attached to nitrogen, and a sulfoxide or sulfone when attached to sulfur. The term “thio” refers to a sulfur atom, which forms a thiocarbonyl when attached to a carbon.

The term “acyl” refers to an alkylcarbonyl, cycloalkylcarbonyl, arylcarbonyl, heterocyclylcarbonyl, or heteroarylcarbonyl substituent, any of which may be further substituted by substituents.

The term "substituents" refers to a group “substituted” on an alkyl, cycloalkyl, aryl, heterocyclyl, or heteroaryl group at any atom of that group. Suitable substituents include, without limitation, halo, hydroxy, oxo, nitro, haloalkyl, alkyl, alkaryl, aryl, aralkyl, alkoxy, aryloxy, amino, acylamino, alkylcarbamoyl, arylcarbamoyl, aminoalkyl, alkoxycarbonyl, carboxy, hydroxyalkyl, alkanesulfonyl, arenesulfonyl, alkanesulfonamido, arenesulfonamido, aralkylsulfonamido, alkylcarbonyl, acyloxy, cyano, ureido or conjugate groups.

In many cases, protecting groups are used during preparation of the compounds of the invention. As used herein, the term “protected” means that the indicated moiety has a protecting group appended thereon. In some certain embodiments of the invention, compounds contain one or more protecting groups. A wide variety of protecting groups can be employed in the methods of the invention. In general, protecting groups render chemical functionalities inert to specific reaction conditions, and can be appended to and removed from such functionalities in a molecule without substantially damaging the remainder of the molecule.

Representative hydroxyl protecting groups, for example, are disclosed by Beaucage et al. (*Tetrahedron* **1992**, 48, 2223-2311). Further hydroxyl protecting groups, as well as other representative protecting groups, are disclosed in Greene and Wuts, *Protective Groups in Organic Synthesis*, Chapter 2, 2d ed., John Wiley & Sons, New York, 1991, and *Oligonucleotides And Analogues A Practical Approach*, Ekstein, F. Ed., IRL Press, N.Y, 1991.

Examples of hydroxyl protecting groups include, but are not limited to, t-butyl, t-butoxymethyl, methoxymethyl, tetrahydropyranyl, 1-ethoxyethyl, 1-(2-chloroethoxy)ethyl, 2-trimethylsilylethyl, p-chlorophenyl, 2,4-dinitrophenyl, benzyl, 2,6-dichlorobenzyl, diphenylmethyl, p,p'-dinitrobenzhydryl, p-nitrobenzyl, triphenylmethyl, trimethylsilyl, triethylsilyl, t-butyldimethylsilyl, t-butyldiphenylsilyl, triphenylsilyl, benzoylformate, acetate,

chloroacetate, trichloroacetate, trifluoroacetate, pivaloate, benzoate, p-phenylbenzoate, 9-fluorenylmethyl carbonate, mesylate and tosylate.

Amino-protecting groups stable to acid treatment are selectively removed with base treatment, and are used to make reactive amino groups selectively available for substitution. Examples of such groups are the Fmoc (E. Atherton and R. C. Sheppard in *The Peptides*, S. Udenfriend, J. Meienhofer, Eds., Academic Press, Orlando, 1987, volume 9, p.1) and various substituted sulfonylethyl carbamates exemplified by the Nsc group (Samukov et al., *Tetrahedron Lett.* **1994**, 35, 7821; Verhart and Tesser, *Rec. Trav. Chim. Pays-Bas* **1987**, 107, 621).

Additional amino-protecting groups include, but are not limited to, carbamate protecting groups, such as 2-trimethylsilylethoxycarbonyl (Teoc), 1-methyl-1-(4-biphenyl)ethoxycarbonyl (Bpoc), t-butoxycarbonyl (BOC), allyloxycarbonyl (Alloc), 9-fluorenylmethyloxycarbonyl (Fmoc), and benzyloxycarbonyl (Cbz); amide protecting groups, such as formyl, acetyl, trihaloacetyl, benzoyl, and nitrophenylacetyl; sulfonamide protecting groups, such as 2-nitrobenzenesulfonyl; and imine and cyclic imide protecting groups, such as phthalimido and dithiasuccinoyl. Equivalents of these amino-protecting groups are also encompassed by the compounds and methods of the present invention.

siRNA Compositions

Provided herein are siRNA compositions containing one or more short interfering ribonucleic acid (siRNA) molecules. These siRNAs can be single stranded or double stranded. Generally, each siRNA strand will be from about 10 in length (*e.g.*, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24 25 or more) to about 35 nucleotides in length (*e.g.*, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36, 37, 38, 39, 40 or more). Preferably, each strand is from about 19 to about 29 nucleotides in length.

Double stranded siRNA ("dsiRNA") compositions contain two single strands with at least substantial complementarity. For example, the first and second strands are each about 19 to about 29 nucleotides in length, and are capable of forming a duplex of between 17 and 25 base pairs. Regions of the strands, such as overhangs, are generally selected so as to be noncomplementary, and are not included in the formed duplex. siRNA compositions may contain one or two strands that have one or more terminal deoxythymidine (dT) nucleotide bases.

Generally, these dT nucleotides are included in the overhang region and do not form or contribute to a duplex structure.

Described herein are single stranded RNA molecules bearing certain base modifications, such as the universal bases 2,4-difluorotoluyil or 5-nitroindole. Additional non-limiting base modifications are provided herein, such as in Examples 1 and 2. These modifications are generally in the central region of the sense strand (*e.g.*, region corresponding to the target cleavage site, *e.g.*, nucleotides 9 to 12). Also described are single stranded RNA molecules bearing one or more mismatched nucleotides, such that binding of the single stranded RNA molecule to a second single stranded RNA molecule occurs at less than 100% of the nucleotides.

A “single strand siRNA compound” or a “single stranded siRNA compound” as used herein, is an siRNA compound which is made up of a single molecule. It may include a duplexed region, formed by intra-strand pairing, *e.g.*, it may be, or include, a hairpin or pan-handle structure. Single strand siRNA compounds may be antisense with regard to the target molecule. In certain embodiments single strand siRNA compounds are 5' phosphorylated or include a phosphoryl analog at the 5' prime terminus. 5'-phosphate modifications include those which are compatible with RISC mediated gene silencing. Suitable modifications include: 5'-monophosphate ((HO)₂(O)P-O-5'); 5'-diphosphate ((HO)₂(O)P-O-P(HO)(O)-O-5'); 5'-triphosphate ((HO)₂(O)P-O-(HO)(O)P-O-P(HO)(O)-O-5'); 5'-guanosine cap (7-methylated or non-methylated) (7m-G-O-5'-(HO)(O)P-O-(HO)(O)P-O-P(HO)(O)-O-5'); 5'-adenosine cap (Appp), and any modified or unmodified nucleotide cap structure (N-O-5'-(HO)(O)P-O-(HO)(O)P-O-P(HO)(O)-O-5'); 5'-monothiophosphate (phosphorothioate; (HO)₂(S)P-O-5'); 5'-monodithiophosphate (phosphorodithioate; (HO)(HS)(S)P-O-5'), 5'-phosphorothiolate ((HO)₂(O)P-S-5'); any additional combination of oxygen/sulfur replaced monophosphate, diphosphate and triphosphates (*e.g.*, 5'-alpha-thiotriphosphate, 5'-gamma-thiotriphosphate, etc.), 5'-phosphoramidates ((HO)₂(O)P-NH-5', (HO)(NH₂)(O)P-O-5'), 5'-alkylphosphonates (R = alkyl = methyl, ethyl, isopropyl, propyl, etc., *e.g.*, RP(OH)(O)-O-5', (HO)₂(O)P-5'-CH₂-), 5'-alkyletherphosphonates (R = alkylether = methoxymethyl (MeOCH₂-), ethoxymethyl, etc., *e.g.*, RP(OH)(O)-O-5'-). (These modifications can also be used with the antisense strand of a double stranded iRNA.)

A single strand siRNA compound may be sufficiently long that it can enter the RISC and participate in RISC mediated cleavage of a target mRNA. A single strand siRNA compound is at least 14, and in other embodiments at least 15, 20, 25, 29, 35, 40, or 50 nucleotides in length. In certain embodiments, it is less than 200, 100, or 60 nucleotides in length.

Hairpin siRNA compounds will have a duplex region equal to or at least 17, 18, 19, 29, 21, 22, 23, 24, or 25 nucleotide pairs. The duplex region will may be equal to or less than 200, 100, or 50, in length. In certain embodiments, ranges for the duplex region are 15-30, 17 to 23, 19 to 23, and 19 to 21 nucleotides pairs in length. The hairpin may have a single strand overhang or terminal unpaired region, in some embodiments at the 3', and in certain embodiments on the antisense side of the hairpin. In some embodiments, the overhangs are 2-3 nucleotides in length.

A "double stranded siRNA compound" as used herein, is an siRNA compound which includes more than one, and in some cases two, strands in which interchain hybridization can form a region of duplex structure.

The antisense strand of a double stranded siRNA compound may be equal to or at least, 10, 11, 12, 13, 14, 15, 16 17, 18, 19, 25, 29, 40, or 60 nucleotides in length. It may be equal to or less than 200, 100, or 50, nucleotides in length. Ranges may be 10 to 15, 17 to 25, 19 to 23, 19 to 21 and 25-30 nucleotides in length.

The sense strand of a double stranded siRNA compound may be equal to or at least 10, 11, 12, 13, 14, 15, 16 17, 18, 19, 25, 29, 40, or 60 nucleotides in length. It may be equal to or less than 200, 100, or 50, nucleotides in length. Ranges may be 10 to 15, 17 to 25, 19 to 23, 19 to 21 and 25-30 nucleotides in length.

The double strand portion of a double stranded siRNA compound may be equal to or at least, 10, 11, 12, 13, 14, 15, 16 17, 18, 19, 25, 29, 40, or 60 nucleotide pairs in length. It may be equal to or less than 200, 100, or 50, nucleotides pairs in length. Ranges may be 10 to 15, 17 to 25, 19 to 23, 19 to 21 and 25-30 nucleotides pairs in length.

In many embodiments, the ds siRNA compound is sufficiently large that it can be cleaved by an endogenous molecule, *e.g.*, by Dicer, to produce smaller ds siRNA compounds, *e.g.*, siRNAs agents

It may be desirable to modify one or both of the antisense and sense strands of a double strand siRNA compound. In some cases they will have the same modification or the same class of modification but in other cases the sense and antisense strand will have different modifications, *e.g.*, in some cases it is desirable to modify only the sense strand. It may be desirable to modify only the sense strand, *e.g.*, to inactivate it, *e.g.*, the sense strand can be modified in order to inactivate the sense strand and prevent formation of an active siRNA/protein or RISC. This can be accomplished by a modification which prevents 5'-phosphorylation of the sense strand, *e.g.*, by modification with a 5'-O-methyl ribonucleotide (see Nykänen *et al.*, (2001) ATP requirements and small interfering RNA structure in the RNA interference pathway. Cell 107, 309-321.) Other modifications which prevent phosphorylation can also be used, *e.g.*, simply substituting the 5'-OH by H rather than O-Me. Alternatively, a large bulky group may be added to the 5'-phosphate turning it into a phosphodiester linkage, though this may be less desirable as phosphodiesterases can cleave such a linkage and release a functional siRNA 5'-end. Antisense strand modifications include 5' phosphorylation as well as any of the other 5' modifications discussed herein, particularly the 5' modifications discussed above in the section on single stranded iRNA molecules.

The sense and antisense strands may be chosen such that the ds siRNA compound includes a single strand or unpaired region at one or both ends of the molecule. Thus, a ds siRNA compound may contain sense and antisense strands, paired to contain an overhang, *e.g.*, one or two 5' or 3' overhangs, or a 3' overhang of 2-3 nucleotides. Many embodiments will have a 3' overhang. Certain ssiRNA compounds will have single-stranded overhangs, in some embodiments 3' overhangs, of 1 or 2 or 3 nucleotides in length at each end. The overhangs can be the result of one strand being longer than the other, or the result of two strands of the same length being staggered. 5' ends may be phosphorylated.

In some embodiments, the length for the duplexed region is between 10 and 30, or 12, 15, 18, 19, 20, 21, 22, 23, 25, 27, or 29 nucleotides in length, *e.g.*, in the ssiRNA compound range discussed above. ssiRNA compounds can resemble in length and structure the natural Dicer processed products from long dsRNAs. Embodiments in which the two strands of the ssiRNA compound are linked, *e.g.*, covalently linked are also included. Hairpin, or other single strand structures which provide the required double stranded region, and a 3' overhang are also within the invention.

The isolated siRNA compounds described herein, including ds siRNA compounds and ssiRNA compounds can mediate silencing of a target RNA, *e.g.*, mRNA, *e.g.*, a transcript of a gene that encodes a protein. For convenience, such mRNA is also referred to herein as mRNA to be silenced. Such a gene is also referred to as a target gene. In general, the RNA to be silenced is an endogenous gene or a pathogen gene. In addition, RNAs other than mRNA, *e.g.*, tRNAs, and viral RNAs, can also be targeted.

As used herein, the phrase “mediates RNAi” refers to the ability to silence, in a sequence specific manner, a target RNA. While not wishing to be bound by theory, it is believed that silencing uses the RNAi machinery or process and a guide RNA, *e.g.*, an ssiRNA compound of 21 to 23 nucleotides.

As used herein, “specifically hybridizable” and “complementary” are terms which are used to indicate a sufficient degree of complementarity such that stable and specific binding occurs between a compound of the invention and a target RNA molecule. Specific binding requires a sufficient degree of complementarity to avoid non-specific binding of the oligomeric compound to non-target sequences under conditions in which specific binding is desired, *i.e.*, under physiological conditions in the case of *in vivo* assays or therapeutic treatment, or in the case of *in vitro* assays, under conditions in which the assays are performed. The non-target sequences typically differ by at least 5 nucleotides.

In one embodiment, an siRNA compound is “sufficiently complementary” to a target RNA, *e.g.*, a target mRNA, such that the siRNA compound silences production of protein encoded by the target mRNA. In another embodiment, the siRNA compound is “exactly complementary” to a target RNA, *e.g.*, the target RNA and the siRNA compound anneal, for example to form a hybrid made exclusively of Watson-Crick base pairs in the region of exact complementarity. A “sufficiently complementary” target RNA can include an internal region (*e.g.*, of at least 10 nucleotides) that is exactly complementary to a target RNA. Moreover, in some embodiments, the siRNA compound specifically discriminates a single-nucleotide difference. In this case, the siRNA compound only mediates RNAi if exact complementarity is found in the region (*e.g.*, within 7 nucleotides of) the single-nucleotide difference.

As used herein, the term “oligonucleotide” refers to a nucleic acid molecule (RNA or DNA) for example of length less than 100, 200, 300, or 400 nucleotides.

RNA agents discussed herein include unmodified RNA as well as RNA which have been modified, *e.g.*, to improve efficacy, and polymers of nucleoside surrogates. Unmodified RNA refers to a molecule in which the components of the nucleic acid, namely sugars, bases, and phosphate moieties, are the same or essentially the same as that which occur in nature, for example as occur naturally in the human body. The art has often referred to rare or unusual, but naturally occurring, RNAs as modified RNAs, see, *e.g.*, Limbach *et al.*, (1994) Summary: the modified nucleosides of RNA, *Nucleic Acids Res.* 22: 2183-2196. Such rare or unusual RNAs, often termed modified RNAs (apparently because they are typically the result of a post transcriptionally modification) are within the term unmodified RNA, as used herein. Modified RNA refers to a molecule in which one or more of the components of the nucleic acid, namely sugars, bases, and phosphate moieties, are different from that which occur in nature, for example, different from that which occurs in the human body. While they are referred to as modified “RNAs,” they will of course, because of the modification, include molecules which are not RNAs. Nucleoside surrogates are molecules in which the ribophosphate backbone is replaced with a non-ribophosphate construct that allows the bases to be presented in the correct spatial relationship such that hybridization is substantially similar to what is seen with a ribophosphate backbone, *e.g.*, non-charged mimics of the ribophosphate backbone. Examples of all of the above are discussed herein.

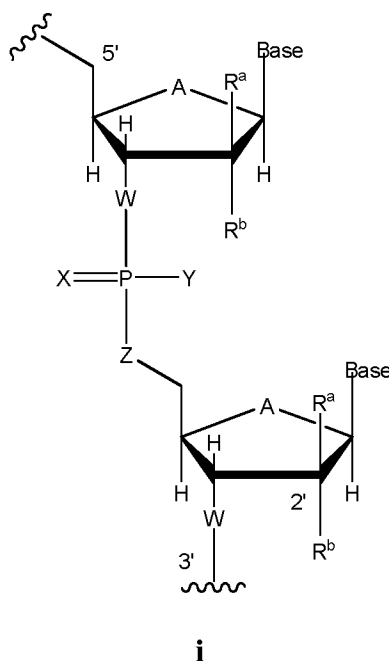
Much of the discussion below refers to single strand molecules. In many embodiments of the invention a double stranded siRNA compound, *e.g.*, a partially double stranded siRNA compound, is envisioned. Thus, it is understood that that double stranded structures (*e.g.*, where two separate molecules are contacted to form the double stranded region or where the double stranded region is formed by intramolecular pairing (*e.g.*, a hairpin structure)) made of the single stranded structures described below are within the invention. Lengths are described elsewhere herein.

As nucleic acids are polymers of subunits, many of the modifications described below occur at a position which is repeated within a nucleic acid, *e.g.*, a modification of a base, or a phosphate moiety, or the a non-linking O of a phosphate moiety. In some cases the modification will occur at all of the subject positions in the nucleic acid but in many cases it will not. By way of example, a modification may only occur at a 3' or 5' terminal position, may only occur in a terminal regions, *e.g.*, at a position on a terminal nucleotide or in the last 2, 3, 4, 5, or 10

nucleotides of a strand. A modification may occur in a double strand region, a single strand region, or in both the first strand and the second strand. A modification may occur only in the double strand region of an RNA or may only occur in a single strand region of an RNA. *E.g.*, a phosphorothioate modification at a non-linking O position may only occur at one or both termini, may only occur in a terminal regions, *e.g.*, at a position on a terminal nucleotide or in the last 2, 3, 4, 5, or 10 nucleotides of a strand, or may occur in double strand and single strand regions, particularly at termini. The 5' end or ends can be phosphorylated.

In some embodiments it is possible, *e.g.*, to enhance stability, to include particular bases in overhangs, or to include modified nucleotides or nucleotide surrogates, in single strand overhangs, *e.g.*, in a 5' or 3' overhang, or in both the first strand and the second strand. *E.g.*, it can be desirable to include purine nucleotides in overhangs. In some embodiments all or some of the bases in a 3' or 5' overhang will be modified, *e.g.*, with a modification described herein. Modifications can include, *e.g.*, the use of modifications at the 2' OH group of the ribose sugar, *e.g.*, the use of deoxyribonucleotides, *e.g.*, deoxythymidine, instead of ribonucleotides, and modifications in the phosphate group, *e.g.*, phosphorothioate modifications. Overhangs need not be homologous with the target sequence.

Modifications and nucleotide surrogates are discussed below, with reference to scaffold **i** shown below.



The scaffold **i** presented above represents a portion of a ribonucleic acid. The basic components are the ribose sugar, the base, the terminal phosphates, and phosphate internucleotide linkers. Where the bases are naturally occurring bases, *e.g.*, adenine, uracil, guanine or cytosine, the sugars are the unmodified 2' hydroxyl ribose sugar (A is O, R^a is H, and R^b is OH) and W, X, Y, and Z are all O, formula **i** represents a naturally occurring unmodified oligoribonucleotide.

Unmodified oligoribonucleotides may be less than optimal in some applications, *e.g.*, unmodified oligoribonucleotides can be prone to degradation by *e.g.*, cellular nucleases. Nucleases can hydrolyze nucleic acid phosphodiester bonds. However, chemical modifications to one or more of the above RNA components can confer improved properties, and, *e.g.*, can render oligoribonucleotides more stable to nucleases.

Modified nucleic acids and nucleotide surrogates can include one or more of:

(i) alteration, *e.g.*, replacement, of one or both of the non-linking (X and Y) phosphate oxygens and/or of one or more of the linking (W and Z) phosphate oxygens (When the phosphate is in the terminal position, one of the positions W or Z will not link the phosphate to an additional element in a naturally occurring ribonucleic acid. However, for simplicity of terminology, except where otherwise noted, the W position at the 5' end of a nucleic acid and the terminal Z position at the 3' end of a nucleic acid, are within the term "linking phosphate oxygens" as used herein);

(ii) alteration, *e.g.*, replacement, of a constituent of the ribose sugar, *e.g.*, of the 2' hydroxyl on the ribose sugar (i.e., R^b to -F and/or A to S);

(iii) wholesale replacement of the phosphate moiety with "dephospho" linkers;

(iv) modification or replacement of a naturally occurring base with a non-natural base;

(v) replacement or modification of the ribose-phosphate backbone;

(vi) modification of the 3' end or 5' end of the RNA, *e.g.*, removal, modification or replacement of a terminal phosphate group or conjugation of a moiety, *e.g.*, a fluorescently labeled moiety, to either the 3' or 5' end of RNA; and

(vii) modification of the sugar (*e.g.*, six membered rings).

The terms replacement, modification, alteration, and the like, as used in this context, do not imply any process limitation, *e.g.*, modification does not mean that one must start with a reference or naturally occurring ribonucleic acid and modify it to produce a modified ribonucleic acid but rather modified simply indicates a difference from a naturally occurring molecule.

It is understood that the actual electronic structure of some chemical entities cannot be adequately represented by only one canonical form (*i.e.*, Lewis structure). While not wishing to be bound by theory, the actual structure can instead be some hybrid or weighted average of two or more canonical forms, known collectively as resonance forms or structures. Resonance structures are not discrete chemical entities and exist only on paper. They differ from one another only in the placement or “localization” of the bonding and nonbonding electrons for a particular chemical entity. It can be possible for one resonance structure to contribute to a greater extent to the hybrid than the others. Thus, the written and graphical descriptions of the embodiments of the present invention are made in terms of what the art recognizes as the predominant resonance form for a particular species. For example, any phosphoroamidate (replacement of a nonlinking oxygen with nitrogen) would be represented by $X = O$ and $Y = N$.

Specific modifications are discussed in more detail below.

The Phosphate Group

The phosphate group is a negatively charged species. The charge is distributed equally over the two non-linking oxygen atoms (*i.e.*, X and Y in Formula 1 above). However, the phosphate group can be modified by replacing one of the oxygens with a different substituent. One result of this modification to RNA phosphate backbones can be increased resistance of the oligoribonucleotide to nucleolytic breakdown. Thus while not wishing to be bound by theory, it can be desirable in some embodiments to introduce alterations which result in either an uncharged linker or a charged linker with unsymmetrical charge distribution.

Examples of modified phosphate groups include phosphorothioate, phosphoroselenates, borano phosphates, borano phosphate esters, hydrogen phosphonates, phosphoroamidates, alkyl or aryl phosphonates and phosphotriesters. Phosphorodithioates have both non-linking oxygens replaced by sulfur. Unlike the situation where only one of X or Y is altered, the phosphorus center in the phosphorodithioates is achiral which precludes the formation of oligoribonucleotides diastereomers. Diastereomer formation can result in a preparation in which

the individual diastereomers exhibit varying resistance to nucleases. Further, the hybridization affinity of RNA containing chiral phosphate groups can be lower relative to the corresponding unmodified RNA species. Thus, while not wishing to be bound by theory, modifications to both X and Y which eliminate the chiral center, *e.g.*, phosphorodithioate formation, may be desirable in that they cannot produce diastereomer mixtures. Thus, X can be any one of S, Se, B, C, H, N, or OR (R is alkyl or aryl). Thus Y can be any one of S, Se, B, C, H, N, or OR (R is alkyl or aryl). Replacement of X and/or Y with sulfur is possible.

The phosphate linker can also be modified by replacement of a linking oxygen (*i.e.*, W or Z in Formula 1) with nitrogen (bridged phosphoroamidates), sulfur (bridged phosphorothioates) and carbon (bridged methylenephosphonates). The replacement can occur at a terminal oxygen (position W (3') or position Z (5')). Replacement of W with carbon or Z with nitrogen is possible.

Candidate agents can be evaluated for suitability as described below.

The Sugar Group

A modified RNA can include modification of all or some of the sugar groups of the ribonucleic acid. For example, the 2' hydroxyl group (OH) can be modified or replaced with a number of different "oxy" or "deoxy" substituents. While not being bound by theory, enhanced stability is expected since the hydroxyl can no longer be deprotonated to form a 2' alkoxide ion. The 2' alkoxide can catalyze degradation by intramolecular nucleophilic attack on the linker phosphorus atom. Again, while not wishing to be bound by theory, it can be desirable to some embodiments to introduce alterations in which alkoxide formation at the 2' position is not possible.

Examples of "oxy"-2' hydroxyl group modifications include alkoxy or aryloxy (OR, *e.g.*, R = H, alkyl, cycloalkyl, aryl, aralkyl, heteroaryl or sugar); polyethyleneglycols (PEG), $O(CH_2CH_2O)_nCH_2CH_2OR$; "locked" nucleic acids (LNA) in which the 2' hydroxyl is connected, *e.g.*, by a methylene bridge, to the 4' carbon of the same ribose sugar; O-amine (amine = NH_2 ; alkylamino, dialkylamino, heterocyclyl, arylamino, diaryl amino, heteroaryl amino, or diheteroaryl amino, ethylene diamine, polyamino) and aminoalkoxy, $O(CH_2)_n$ amine, (*e.g.*, amine = NH_2 ; alkylamino, dialkylamino, heterocyclyl, arylamino, diaryl amino, heteroaryl amino, or diheteroaryl amino, ethylene diamine, polyamino). It is noteworthy that oligonucleotides

containing only the methoxyethyl group (MOE), (OCH₂CH₂OCH₃, a PEG derivative), exhibit nuclease stabilities comparable to those modified with the robust phosphorothioate modification.

“Deoxy” modifications include hydrogen (*i.e.*, deoxyribose sugars, which are of particular relevance to the overhang portions of partially ds RNA); halo (*e.g.*, fluoro); amino (*e.g.*, NH₂; alkylamino, dialkylamino, heterocyclyl, arylamino, diaryl amino, heteroaryl amino, diheteroaryl amino, or amino acid); NH(CH₂CH₂NH)_nCH₂CH₂-amine (amine = NH₂; alkylamino, dialkylamino, heterocyclyl, arylamino, diaryl amino, heteroaryl amino, or diheteroaryl amino), NHC(O)R (R = alkyl, cycloalkyl, aryl, aralkyl, heteroaryl or sugar), cyano; mercapto; alkyl-thio-alkyl; thioalkoxy; and alkyl, cycloalkyl, aryl, alkenyl and alkynyl, which may be optionally substituted with *e.g.*, an amino functionality. Other substituents of certain embodiments include 2'-methoxyethyl, 2'-OCH₃, 2'-O-allyl, 2'-C-allyl, and 2'-fluoro.

The sugar group can also contain one or more carbons that possess the opposite stereochemical configuration than that of the corresponding carbon in ribose. Thus, a modified RNA can include nucleotides containing *e.g.*, arabinose, as the sugar.

Modified RNA's can also include “abasic” sugars, which lack a nucleobase at C-1'. These abasic sugars can also be further contain modifications at one or more of the constituent sugar atoms.

To maximize nuclease resistance, the 2' modifications can be used in combination with one or more phosphate linker modifications (*e.g.*, phosphorothioate). The so-called “chimeric” oligonucleotides are those that contain two or more different modifications.

The natural sugar ring may also be expanded to a six-membered ring. In addition, the oxygen in the sugar may be replaced with a sulfur.

Candidate modifications can be evaluated as described below.

Replacement of the Phosphate Group

The phosphate group can be replaced by non-phosphorus containing connectors. While not wishing to be bound by theory, it is believed that since the charged phosphodiester group is the reaction center in nucleolytic degradation, its replacement with neutral structural mimics should impart enhanced nuclease stability. Again, while not wishing to be bound by theory, it

can be desirable, in some embodiment, to introduce alterations in which the charged phosphate group is replaced by a neutral moiety.

Examples of moieties which can replace the phosphate group include siloxane, carbonate, carboxymethyl, carbamate, amide, thioether, ethylene oxide linker, sulfonate, sulfonamide, thioformacetal, formacetal, oxime, methyleneimino, methylenemethylimino, methylenehydrazo, methylenedimethylhydrazo and methyleneoxymethylimino. In certain embodiments, replacements may include the methylenecarbonylamino and methylenemethylimino groups.

Candidate modifications can be evaluated as described below.

Replacement of Ribophosphate Backbone

Oligonucleotide- mimicking scaffolds can also be constructed wherein the phosphate linker and ribose sugar are replaced by nuclease resistant nucleoside or nucleotide surrogates. While not wishing to be bound by theory, it is believed that the absence of a repetitively charged backbone diminishes binding to proteins that recognize polyanions (*e.g.*, nucleases). Again, while not wishing to be bound by theory, it can be desirable in some embodiment, to introduce alterations in which the bases are tethered by a neutral surrogate backbone.

Examples include the morpholino, cyclobutyl, pyrrolidine and peptide nucleic acid (PNA) nucleoside surrogates. In certain embodiments, PNA surrogates may be used.

Candidate modifications can be evaluated as described below.

Terminal Modifications

The 3' and 5' ends of an oligonucleotide can be modified. Such modifications can be at the 3' end, 5' end or both ends of the molecule. They can include modification or replacement of an entire terminal phosphate or of one or more of the atoms of the phosphate group. For example, the 3' and 5' ends of an oligonucleotide can be conjugated to other functional molecular entities such as labeling moieties, *e.g.*, fluorophores (*e.g.*, pyrene, TAMRA, fluorescein, Cy3 or Cy5 dyes) or protecting groups (based *e.g.*, on sulfur, silicon, boron or ester). The functional molecular entities can be attached to the sugar through a phosphate group and/or a spacer. The terminal atom of the spacer can connect to or replace the linking atom of the phosphate group or the C-3' or C-5' O, N, S or C group of the sugar. Alternatively, the spacer can connect to or replace the terminal atom of a nucleotide surrogate (*e.g.*, PNAs). These

spacers or linkers can include *e.g.*, $-(\text{CH}_2)_n-$, $-(\text{CH}_2)_n\text{N}-$, $-(\text{CH}_2)_n\text{O}-$, $-(\text{CH}_2)_n\text{S}-$, $-\text{O}(\text{CH}_2\text{CH}_2\text{O})_n\text{CH}_2\text{CH}_2\text{O}-$ (*e.g.*, $n = 3$ or 6), abasic sugars, amide, carboxy, amine, oxyamine, oxyimine, thioether, disulfide, thiourea, sulfonamide, or morpholino, or biotin and fluorescein reagents. When a spacer/phosphate-functional molecular entity-spacer/phosphate array is interposed between two strands of siRNA compounds, this array can substitute for a hairpin RNA loop in a hairpin-type RNA agent. The 3' end can be an -OH group. While not wishing to be bound by theory, it is believed that conjugation of certain moieties can improve transport, hybridization, and specificity properties. Again, while not wishing to be bound by theory, it may be desirable to introduce terminal alterations that improve nuclease resistance. Other examples of terminal modifications include dyes, intercalating agents (*e.g.*, acridines), cross-linkers (*e.g.*, psoralene, mitomycin C), porphyrins (TPPC4, texaphyrin, Sapphyrin), polycyclic aromatic hydrocarbons (*e.g.*, phenazine, dihydrophenazine), artificial endonucleases (*e.g.*, EDTA), lipophilic carriers (*e.g.*, cholesterol, cholic acid, adamantane acetic acid, 1-pyrene butyric acid, dihydrotestosterone, 1,3-bis-O(hexadecyl)glycerol, geranyloxyhexyl group, hexadecylglycerol, borneol, menthol, 1,3-propanediol, heptadecyl group, palmitic acid, myristic acid, O3-(oleoyl)lithocholic acid, O3-(oleoyl)cholenic acid, dimethoxytrityl, or phenoxazin-1-yl) and peptide conjugates (*e.g.*, antennapedia peptide, Tat peptide), alkylating agents, phosphate, amino, mercapto, PEG (*e.g.*, PEG-40K), MPEG, [MPEG]₂, polyamino, alkyl, substituted alkyl, radiolabeled markers, enzymes, haptens (*e.g.*, biotin), transport/absorption facilitators (*e.g.*, aspirin, vitamin E, folic acid), synthetic ribonucleases (*e.g.*, imidazole, bisimidazole, histamine, imidazole clusters, acridine-imidazole conjugates, Eu^{3+} complexes of tetraazamacrocycles).

Terminal modifications can be added for a number of reasons, including as discussed elsewhere herein to modulate activity or to modulate resistance to degradation. Terminal modifications useful for modulating activity include modification of the 5' end with phosphate or phosphate analogs. For example, in certain embodiments siRNA compounds, especially antisense strands, are 5' phosphorylated or include a phosphoryl analog at the 5' prime terminus. 5'-phosphate modifications include those which are compatible with RISC mediated gene silencing. Suitable modifications include: 5'-monophosphate $((\text{HO})_2(\text{O})\text{P}-\text{O}-5')$; 5'-diphosphate $((\text{HO})_2(\text{O})\text{P}-\text{O}-\text{P}(\text{HO})(\text{O})-\text{O}-5')$; 5'-triphosphate $((\text{HO})_2(\text{O})\text{P}-\text{O}-(\text{HO})(\text{O})\text{P}-\text{O}-\text{P}(\text{HO})(\text{O})-\text{O}-5')$; 5'-guanosine cap (7-methylated or non-methylated) $(7\text{m-G}-\text{O}-5'-(\text{HO})(\text{O})\text{P}-\text{O}-(\text{HO})(\text{O})\text{P}-\text{O}-\text{P}(\text{HO})(\text{O})-\text{O}-5')$; 5'-adenosine cap (A_{ppp}), and any modified or unmodified nucleotide cap

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

Terminal modifications can also be useful for monitoring distribution, and in such cases the groups to be added may include fluorophores, *e.g.*, fluorescein or an Alexa dye, *e.g.*, Alexa 488. Terminal modifications can also be useful for enhancing uptake, useful modifications for this include cholesterol. Terminal modifications can also be useful for cross-linking an RNA agent to another moiety; modifications useful for this include mitomycin C.

Candidate modifications can be evaluated as described below.

The Bases

Adenine, guanine, cytosine and uracil are the most common bases found in RNA. These bases can be modified or replaced to provide RNAs having improved properties. For example, nuclease resistant oligoribonucleotides can be prepared with these bases or with synthetic and natural nucleobases (*e.g.*, inosine, thymine, xanthine, hypoxanthine, nubularine, isoguanisine, or tubercidine) and any one of the above modifications. Alternatively, substituted or modified analogs of any of the above bases and "universal bases" can be employed. Examples include 2-(halo)adenine, 2-(alkyl)adenine, 2-(propyl)adenine, 2-(amino)adenine, 2-(aminoalkyl)adenine, 2-(aminopropyl)adenine, 2-(methylthio)-N⁶-(isopentenyl)adenine, 6-(alkyl)adenine, 6-(methyl)adenine, 7-(deaza)adenine, 8-(alkenyl)adenine, 8-(alkyl)adenine, 8-(alkynyl)adenine, 8-(amino)adenine, 8-(halo)adenine, 8-(hydroxyl)adenine, 8-(thioalkyl)adenine, 8-(thiol)adenine, N⁶-(isopentyl)adenine, N⁶-(methyl)adenine, N⁶, N⁶-(dimethyl)adenine, 2-(alkyl)guanine, 2-(propyl)guanine, 6-(alkyl)guanine, 6-(methyl)guanine, 7-(alkyl)guanine, 7-(methyl)guanine, 7-(deaza)guanine, 8-(alkyl)guanine, 8-(alkenyl)guanine, 8-(alkynyl)guanine, 8-(amino)guanine, 8-(halo)guanine, 8-(hydroxyl)guanine, 8-(thioalkyl)guanine, 8-(thiol)guanine, N-(methyl)guanine, 2-(thio)cytosine, 3-(deaza)-5-(aza)cytosine, 3-(alkyl)cytosine,

3-(methyl)cytosine, 5-(alkyl)cytosine, 5-(alkynyl)cytosine, 5-(halo)cytosine, 5-(methyl)cytosine, 5-(propynyl)cytosine, 5-(propynyl)cytosine, 5-(trifluoromethyl)cytosine, 6-(azo)cytosine, N⁴-(acetyl)cytosine, 3-(3-amino-3-carboxypropyl)uracil, 2-(thio)uracil, 5-(methyl)-2-(thio)uracil, 5-(methylaminomethyl)-2-(thio)uracil, 4-(thio)uracil, 5-(methyl)-4-(thio)uracil, 5-(methylaminomethyl)-4-(thio)uracil, 5-(methyl)-2,4-(dithio)uracil, 5-(methylaminomethyl)-2,4-(dithio)uracil, 5-(2-aminopropyl)uracil, 5-(alkyl)uracil, 5-(alkynyl)uracil, 5-(allylamino)uracil, 5-(aminoallyl)uracil, 5-(aminoalkyl)uracil, 5-(guanidiniumalkyl)uracil, 5-(1,3-diazole-1-alkyl)uracil, 5-(cyanoalkyl)uracil, 5-(dialkylaminoalkyl)uracil, 5-(dimethylaminoalkyl)uracil, 5-(halo)uracil, 5-(methoxy)uracil, uracil-5-oxyacetic acid, 5-(methoxycarbonylmethyl)-2-(thio)uracil, 5-(methoxycarbonyl-methyl)uracil, 5-(propynyl)uracil, 5-(propynyl)uracil, 5-(trifluoromethyl)uracil, 6-(azo)uracil, dihydrouracil, N³-(methyl)uracil, 5-uracil (*i.e.*, pseudouracil), 2-(thio)pseudouracil, 4-(thio)pseudouracil, 2,4-(dithio)pseudouracil, 5-(alkyl)pseudouracil, 5-(methyl)pseudouracil, 5-(alkyl)-2-(thio)pseudouracil, 5-(methyl)-2-(thio)pseudouracil, 5-(alkyl)-4-(thio)pseudouracil, 5-(methyl)-4-(thio)pseudouracil, 5-(alkyl)-2,4-(dithio)pseudouracil, 5-(methyl)-2,4-(dithio)pseudouracil, 1-substituted pseudouracil, 1-substituted 2(thio)-pseudouracil, 1-substituted 4-(thio)pseudouracil, 1-substituted 2,4-(dithio)pseudouracil, 1-(aminocarbonylethylenyl)-pseudouracil, 1-(aminocarbonylethylenyl)-2(thio)-pseudouracil, 1-(aminocarbonylethylenyl)-4-(thio)pseudouracil, 1-(aminocarbonylethylenyl)-2,4-(dithio)pseudouracil, 1-(aminoalkylaminocarbonylethylenyl)-pseudouracil, 1-(aminoalkylamino-carbonylethylenyl)-2(thio)-pseudouracil, 1-(aminoalkylaminocarbonylethylenyl)-4-(thio)pseudouracil, 1-(aminoalkylaminocarbonylethylenyl)-2,4-(dithio)pseudouracil, 1,3-(diaz)-2-(oxo)-phenoxazin-1-yl, 1-(aza)-2-(thio)-3-(aza)-phenoxazin-1-yl, 1,3-(diaz)-2-(oxo)-phenthiazin-1-yl, 1-(aza)-2-(thio)-3-(aza)-phenthiazin-1-yl, 7-substituted 1,3-(diaz)-2-(oxo)-phenoxazin-1-yl, 7-substituted 1-(aza)-2-(thio)-3-(aza)-phenoxazin-1-yl, 7-substituted 1,3-(diaz)-2-(oxo)-phenthiazin-1-yl, 7-substituted 1-(aza)-2-(thio)-3-(aza)-phenthiazin-1-yl, 7-(aminoalkylhydroxy)-1,3-(diaz)-2-(oxo)-phenoxazin-1-yl, 7-(aminoalkylhydroxy)-1-(aza)-2-(thio)-3-(aza)-phenoxazin-1-yl, 7-(aminoalkylhydroxy)-1,3-(diaz)-2-(oxo)-phenthiazin-1-yl, 7-(aminoalkylhydroxy)-1-(aza)-2-(thio)-3-(aza)-phenthiazin-1-yl, 7-(guanidiniumalkylhydroxy)-1,3-(diaz)-2-(oxo)-phenoxazin-1-yl, 7-(guanidiniumalkylhydroxy)-1-(aza)-2-(thio)-3-(aza)-phenoxazin-1-yl, 7-(guanidiniumalkylhydroxy)-1,3-(diaz)-2-(oxo)-phenthiazin-1-yl, 7-

(guanidiniumalkylhydroxy)-1-(aza)-2-(thio)-3-(aza)-phenthiazin-1-yl, 1,3,5-(triaz)-2,6-(diox)-naphthalene, inosine, xanthine, hypoxanthine, nubarine, tubercidine, isoguanisine, inosinyl, 2-aza-inosinyl, 7-deaza-inosinyl, nitroimidazolyl, nitropyrazolyl, nitrobenzimidazolyl, nitroindazolyl, aminoindolyl, pyrrolopyrimidinyl, 3-(methyl)isocarbostyryl, 5-(methyl)isocarbostyryl, 3-(methyl)-7-(propynyl)isocarbostyryl, 7-(aza)indolyl, 6-(methyl)-7-(aza)indolyl, imidizopyridinyl, 9-(methyl)-imidizopyridinyl, pyrrolopyridinyl, isocarbostyryl, 7-(propynyl)isocarbostyryl, propynyl-7-(aza)indolyl, 2,4,5-(trimethyl)phenyl, 4-(methyl)indolyl, 4,6-(dimethyl)indolyl, phenyl, naphthalenyl, anthracenyl, phenanthracenyl, pyrenyl, stilbenyl, tetracenyl, pentacenyl, difluorotolyl, 4-(fluoro)-6-(methyl)benzimidazole, 4-(methyl)benzimidazole, 6-(azo)thymine, 2-pyridinone, 5-nitroindole, 3-nitropyrrole, 6-(aza)pyrimidine, 2-(amino)purine, 2,6-(diamino)purine, 5-substituted pyrimidines, N²-substituted purines, N⁶-substituted purines, O⁶-substituted purines, substituted 1,2,4-triazoles, or any O-alkylated or N-alkylated derivatives thereof;

Further purines and pyrimidines include those disclosed in U.S. Pat. No. 3,687,808, hereby incorporated by reference, those disclosed in the Concise Encyclopedia Of Polymer Science And Engineering, pages 858-859, Kroschwitz, J. I., ed. John Wiley & Sons, 1990, and those disclosed by Englisch *et al.*, Angewandte Chemie, International Edition, 1991, 30, 613.

Generally, base changes are not used for promoting stability, but they can be useful for other reasons, *e.g.*, some, *e.g.*, 2,6-diaminopurine and 2 amino purine, are fluorescent. Modified bases can reduce target specificity. This may be taken into consideration in the design of siRNA compounds.

Candidate modifications can be evaluated as described below.

Cationic Groups

Modifications can also include attachment of one or more cationic groups to the sugar, base, and/or the phosphorus atom of a phosphate or modified phosphate backbone moiety. A cationic group can be attached to any atom capable of substitution on a natural, unusual or universal base. A preferred position is one that does not interfere with hybridization, *i.e.*, does not interfere with the hydrogen bonding interactions needed for base pairing. A cationic group can be attached *e.g.*, through the C2' position of a sugar or analogous position in a cyclic or acyclic sugar surrogate. Cationic groups can include *e.g.*, protonated amino groups, derived

from e.g., O-AMINE (AMINE = NH_2 ; alkylamino, dialkylamino, heterocyclyl, arylamino, diaryl amino, heteroaryl amino, or diheteroaryl amino, ethylene diamine, polyamino); aminoalkoxy, e.g., $\text{O}(\text{CH}_2)_n\text{AMINE}$, (e.g., AMINE = NH_2 ; alkylamino, dialkylamino, heterocyclyl, arylamino, diaryl amino, heteroaryl amino, or diheteroaryl amino, ethylene diamine, polyamino); amino (e.g. NH_2 ; alkylamino, dialkylamino, heterocyclyl, arylamino, diaryl amino, heteroaryl amino, diheteroaryl amino, or amino acid); or $\text{NH}(\text{CH}_2\text{CH}_2\text{NH})_n\text{CH}_2\text{CH}_2\text{-AMINE}$ (AMINE = NH_2 ; alkylamino, dialkylamino, heterocyclyl, arylamino, diaryl amino, heteroaryl amino, or diheteroaryl amino).

Exemplary Modifications and Placement within an iRNA agent

Some modifications may preferably be included on an iRNA agent at a particular location, e.g., at an internal position of a strand, or on the 5' or 3' end of a strand of an iRNA agent. A preferred location of a modification on an iRNA agent, may confer preferred properties on the agent. For example, preferred locations of particular modifications may confer optimum gene silencing properties, or increased resistance to endonuclease or exonuclease activity. A modification described herein and below may be the sole modification, or the sole type of modification included on multiple ribonucleotides, or a modification can be combined with one or more other modifications described herein and below. For example, a modification on one strand of a multi-strand iRNA agent can be different than a modification on another strand of the multi-strand iRNA agent. Similarly, two different modifications on one strand can differ from a modification on a different strand of the iRNA agent. Other additional unique modifications, without limitation, can be incorporated into strands of the iRNA agent.

An iRNA agent may include a backbone modification to any nucleotide on an iRNA strand. For example, an iRNA agent may include a phosphorothioate linkage or P-alkyl modification in the linkages between one or more nucleotides of an iRNA agent. The nucleotides can be terminal nucleotides, e.g., nucleotides at the last position of a sense or antisense strand, or internal nucleotides.

An iRNA agent can include a sugar modification, e.g., a 2' or 3' sugar modification. Exemplary sugar modifications include, for example, a 2'-O-methylated nucleotide, a 2'-deoxy nucleotide, (e.g., a 2'-deoxyfluoro nucleotide), a 2'-O-methoxyethyl nucleotide, a 2'-O-NMA, a 2'-DMAEOE, a 2'-aminopropyl, 2'-hydroxy, or a 2'-ara-fluoro or a locked nucleic acid (LNA),

extended nucleic acid (ENA), hexose nucleic acid (HNA), or cyclohexene nucleic acid (CeNA). A 2' modification is preferably 2'-OMe, and more preferably, 2'-deoxyfluoro. When the modification is 2'-OMe, the modification is preferably on the sense strands. When the modification is a 2'-fluoro, and the modification may be on any strand of the iRNA agent. A 2'-ara-fluoro modification will preferably be on the sense strands of the iRNA agent. An iRNA agent may include a 3' sugar modification, e.g., a 3'-OMe modification. Preferably a 3'-OMe modification is on the sense strand of the iRNA agent.

An iRNA agent may include a 5'-methyl-pyrimidine (e.g., a 5'-methyl-uridine modification or a 5'-methyl-cytidine) modification.

The modifications described herein can be combined onto a single iRNA agent. For example, an iRNA agent may have a phosphorothioate linkage and a 2' sugar modification, e.g., a 2'-OMe or 2'-F modification. In another example, an iRNA agent may include at least one 5-Me-pyrimidine and a 2'-sugar modification, e.g., a 2'-F or 2'-OMe modification.

An iRNA agent may include a nucleobase modification, such as a cationic modification, such as a 3'-abasic cationic modification. The cationic modification can be e.g., an alkylamino-dT (e.g., a C6 amino-dT), an allylamino conjugate, a pyrrolidine conjugate, a pthalamido, a porphyrin, or a hydroxyprolinol conjugate, on one or more of the terminal nucleotides of the iRNA agent. When an alkylamino-dT conjugate is attached to the terminal nucleotide of an iRNA agent, the conjugate is preferably attached to the 3' end of the sense or antisense strand of an iRNA agent. When a pyrrolidine linker is attached to the terminal nucleotide of an iRNA agent, the linker is preferably attached to the 3'- or 5'-end of the sense strand, or the 3'-end of the antisense strand. When a pyrrolidine linker is attached to the terminal nucleotide of an iRNA agent, the linker is preferably on the 3'- or 5'-end of the sense strand, and not on the 5'-end of the antisense strand.

An iRNA agent may include at least one conjugate, such as a lipophile, a terpene, a protein binding agent, a vitamin, a carbohydrate, or a peptide. For example, the conjugate can be naproxen, nitroindole (or another conjugate that contributes to stacking interactions), folate, ibuprofen, or a C5 pyrimidine linker. The conjugate can also be a glyceride lipid conjugate (e.g., a dialkyl glyceride derivatives), vitamin E conjugate, or a thio-cholesterol. In generally, and except where noted to the contrary below, when a conjugate is on the terminal nucleotide of a sense or antisense strand, the conjugate is preferably on the 5' or 3' end of the sense strand or on

the 5' end of the antisense strand, and preferably the conjugate is not on the 3' end of the antisense strand.

When the conjugate is naproxen, and the conjugate is on the terminal nucleotide of a sense or antisense strand, the conjugate is preferably on the 5' or 3' end of the sense or antisense strands. When the conjugate is cholesterol, and the conjugate is on the terminal nucleotide of a sense or antisense strand, the cholesterol conjugate is preferably on the 5' or 3' end of the sense strand and preferably not present on the antisense strand. Cholesterol may be conjugated to the iRNA agent by a pyrrolidine linker, serinol linker, hydroxyprolinol linker, or disulfide linkage. A dU-cholesterol conjugate may also be conjugated to the iRNA agent by a disulfide linkage. When the conjugate is cholic acid, and the conjugate is on the terminal nucleotide of a sense or antisense strand, the cholic acid is preferably attached to the 5' or 3' end of the sense strand, or the 3' end of the antisense strand. In one embodiment, the cholic acid is attached to the 3' end of the sense strand and the 3' end of the antisense strand.

One or more nucleotides of an iRNA agent may have a 2'-5' linkage. Preferably, the 2'-5' linkage is on the sense strand. When the 2'-5' linkage is on the terminal nucleotide of an iRNA agent, the 2'-5' linkage occurs on the 5' end of the sense strand. The iRNA agent may include an L-sugar, preferably on the sense strand, and not on the antisense strand.

The iRNA agent may include a methylphosphonate modification. When the methylphosphonate is on the terminal nucleotide of an iRNA agent, the methylphosphonate is at the 3' end of the sense or antisense strands of the iRNA agent.

An iRNA agent may be modified by replacing one or more ribonucleotides with deoxyribonucleotides. Preferably, adjacent deoxyribonucleotides are joined by phosphorothioate linkages, and the iRNA agent does not include more than four consecutive deoxyribonucleotides on the sense or the antisense strands.

An iRNA agent may include a difluorotoluidyl (DFT) modification, e.g., 2,4-difluorotoluidyl uracil, or a guanine to inosine substitution.

The iRNA agent may include at least one 5'-uridine-adenine-3' (5'-UA-3') dinucleotide wherein the uridine is a 2'-modified nucleotide, or a terminal 5'-uridine-guanine-3' (5'-UG-3') dinucleotide, wherein the 5'-uridine is a 2'-modified nucleotide, or a terminal 5'-cytidine-adenine-3' (5'-CA-3') dinucleotide, wherein the 5'-cytidine is a 2'-modified nucleotide, or a

terminal 5'-uridine-uridine-3' (5'-UU-3') dinucleotide, wherein the 5'-uridine is a 2'-modified nucleotide, or a terminal 5'-cytidine-cytidine-3' (5'-CC-3') dinucleotide, wherein the 5'-cytidine is a 2'-modified nucleotide, or a terminal 5'-cytidine-uridine-3' (5'-CU-3') dinucleotide, wherein the 5'-cytidine is a 2'-modified nucleotide, or a terminal 5'-uridine-cytidine-3' (5'-UC-3') dinucleotide, wherein the 5'-uridine is a 2'-modified nucleotide. The chemically modified nucleotide in the iRNA agent may be a 2'-O-methylated nucleotide. In some embodiments, the modified nucleotide can be a 2'-deoxy nucleotide, a 2'-deoxyfluoro nucleotide, a 2'-O-methoxyethyl nucleotide, a 2'-O-NMA, a 2'-DMAEOE, a 2'-aminopropyl, 2'-hydroxy, or a 2'-ara-fluoro, or a locked nucleic acid (LNA), extended nucleic acid (ENA), hexose nucleic acid (HNA), or cyclohexene nucleic acid (CeNA). The iRNA agents including these modifications are particularly stabilized against exonuclease activity, when the modified dinucleotide occurs on a terminal end of the sense or antisense strand of an iRNA agent, and are otherwise particularly stabilized against endonuclease activity.

An iRNA agent may have a single overhang, e.g., one end of the iRNA agent has a 3' or 5' overhang and the other end of the iRNA agent is a blunt end, or the iRNA agent may have a double overhang, e.g., both ends of the iRNA agent have a 3' or 5' overhang, such as a dinucleotide overhang. In another alternative, both ends of the iRNA agent may have blunt ends. The unpaired nucleotides may have at least one phosphorothioate dinucleotide linkage, and at least one of the unpaired nucleotides may be chemically modified in the 2'-position. The doublestrand region of the iRNA agent may include phosphorothioate dinucleotide linkages on one or both of the sense and antisense strands. Various strands of the multi-strand iRNA agent may be connected with a linker, e.g., a chemical linker such as hexaethylene glycol linker, a poly-(oxyphosphinico-oxy-1,3-propanediol) linker, an allyl linker, or a polyethylene glycol linker.

Nuclease resistant monomers

An iRNA agent can include monomers which have been modified so as to inhibit degradation, e.g., by nucleases, e.g., endonucleases or exonucleases, found in the body of a subject. These monomers are referred to herein as NRMs, or nuclease resistance promoting monomers or modifications. In many cases these modifications will modulate other properties of the iRNA agent as well, e.g., the ability to interact with a protein, e.g., a transport protein, e.g., serum albumin, or a member of the RISC (RNA-induced Silencing Complex), or the ability of

the first and second sequences to form a duplex with one another or to form a duplex with another sequence, e.g., a target molecule.

While not wishing to be bound by theory, it is believed that modifications of the sugar, base, and/or phosphate backbone in an iRNA agent can enhance endonuclease and exonuclease resistance, and can enhance interactions with transporter proteins and one or more of the functional components of the RISC complex. Preferred modifications are those that increase exonuclease and endonuclease resistance and thus prolong the half-life of the iRNA agent prior to interaction with the RISC complex, but at the same time do not render the iRNA agent resistant to endonuclease activity in the RISC complex. Again, while not wishing to be bound by any theory, it is believed that placement of the modifications at or near the 3' and/or 5' end of antisense strands can result in iRNA agents that meet the preferred nuclease resistance criteria delineated above. Again, still while not wishing to be bound by any theory, it is believed that placement of the modifications at e.g., the middle of a sense strand can result in iRNA agents that are relatively less likely to undergo off-targeting.

Modifications described herein can be incorporated into any RNA and RNA-like molecule described herein, e.g., an iRNA agent, a carrier oligonucleotide. An iRNA agent may include a duplex comprising a hybridized sense and antisense strand, in which the antisense strand and/or the sense strand may include one or more of the modifications described herein. The anti sense strand may include modifications at the 3' end and/or the 5' end and/or at one or more positions that occur 1-6 (e.g., 1-5, 1-4, 1-3, 1-2) nucleotides from either end of the strand. The sense strand may include modifications at the 3' end and/or the 5' end and/or at any one of the intervening positions between the two ends of the strand. The iRNA agent may also include a duplex comprising two hybridized antisense strands. The first and/or the second antisense strand may include one or more of the modifications described herein. Thus, one and/or both antisense strands may include modifications at the 3' end and/or the 5' end and/or at one or more positions that occur 1-6 (e.g., 1-5, 1-4, 1-3, 1-2) nucleotides from either end of the strand. Particular configurations are discussed below.

Modifications that can be useful for producing iRNA agents that meet the preferred nuclease resistance criteria delineated above can include one or more of the following chemical and/or stereochemical modifications of the sugar, base, and/or phosphate backbone:

- (i) chiral (S_P) thioates. Thus, preferred NRMs include nucleotide dimers with an enriched or pure for a particular chiral form of a modified phosphate group containing a heteroatom at the nonbridging position, e.g., S_P or R_P , where this is the position normally occupied by the oxygen. The heteroatom can be S, Se, Nr_2 , or Br_3 . When the heteroatom is S, enriched or chirally pure S_P linkage is preferred. Enriched means at least 70, 80, 90, 95, or 99% of the preferred form. Such NRMs are discussed in more detail below;
- (ii) attachment of one or more cationic groups to the sugar, base, and/or the phosphorus atom of a phosphate or modified phosphate backbone moiety. Thus, preferred NRMs include monomers at the terminal position derivatized at a cationic group. As the 5' end of an antisense sequence should have a terminal $-OH$ or phosphate group this NRM is preferably not used at the 5' end of an anti-sense sequence. The group should be attached at a position on the base which minimizes interference with H bond formation and hybridization, e.g., away from the face which interacts with the complementary base on the other strand, e.g., at the 5' position of a pyrimidine or a 7-position of a purine. These are discussed in more detail below;
- (iii) nonphosphate linkages at the termini. Thus, preferred NRMs include Non-phosphate linkages, e.g., a linkage of 4 atoms which confers greater resistance to cleavage than does a phosphate bond. Examples include 3' $CH_2-NCH_3-O-CH_2-5'$ and 3' $CH_2-NH-(O=)-CH_2-5'$;
- (iv) 3'-bridging thiophosphates and 5'-bridging thiophosphates. Thus, preferred NRM's can include these structures;
- (v) L-RNA, 2'-5' linkages, inverted linkages, a-nucleosides. Thus, other preferred NRM's include: L nucleosides and dimeric nucleotides derived from L-nucleosides; 2'-5' phosphate, non-phosphate and modified phosphate linkages (e.g., thiophosphates, phosphoramidates and boronophosphates); dimers having inverted linkages, e.g., 3'-3' or 5'-5' linkages; monomers having an alpha linkage at the 1' site on the sugar, e.g., the structures described herein having an alpha linkage;
- (vi) conjugate groups. Thus, preferred NRM's can include e.g., a targeting moiety or a conjugated ligand described herein conjugated with the monomer, e.g., through the sugar, base, or backbone;

- (vii) abasic linkages. Thus, preferred NRM's can include an abasic monomer, e.g., an abasic monomer as described herein (e.g., a nucleobaseless monomer); an aromatic or heterocyclic or polyheterocyclic aromatic monomer as described herein.; and
- (viii) 5'-phosphonates and 5'-phosphate prodrugs. Thus, preferred NRM's include monomers, preferably at the terminal position, e.g., the 5' position, in which one or more atoms of the phosphate group is derivatized with a protecting group, which protecting group or groups, are removed as a result of the action of a component in the subject's body, e.g, a carboxyesterase or an enzyme present in the subject's body. E.g., a phosphate prodrug in which a carboxy esterase cleaves the protected molecule resulting in the production of a thioate anion which attacks a carbon adjacent to the O of a phosphate and resulting in the production of an unprotected phosphate.

One or more different NRM modifications can be introduced into an iRNA agent or into a sequence of an iRNA agent. An NRM modification can be used more than once in a sequence or in an iRNA agent. As some NRM's interfere with hybridization the total number incorporated, should be such that acceptable levels of iRNA agent duplex formation are maintained.

In some embodiments NRM modifications are introduced into the terminal the cleavage site or in the cleavage region of a sequence (a sense strand or sequence) which does not target a desired sequence or gene in the subject. This can reduce off-target silencing.

Evaluation of Candidate RNAs

One can evaluate a candidate RNA agent, *e.g.*, a modified RNA, for a selected property by exposing the agent or modified molecule and a control molecule to the appropriate conditions and evaluating for the presence of the selected property. For example, resistance to a degradant can be evaluated as follows. A candidate modified RNA (and a control molecule, usually the unmodified form) can be exposed to degradative conditions, *e.g.*, exposed to a milieu, which includes a degradative agent, *e.g.*, a nuclease. *E.g.*, one can use a biological sample, *e.g.*, one that is similar to a milieu, which might be encountered, in therapeutic use, *e.g.*, blood or a cellular fraction, *e.g.*, a cell-free homogenate or disrupted cells. The candidate and control could then be evaluated for resistance to degradation by any of a number of approaches. For example, the candidate and control could be labeled prior to exposure, with, *e.g.*, a radioactive or

enzymatic label, or a fluorescent label, such as Cy3 or Cy5. Control and modified RNA's can be incubated with the degradative agent, and optionally a control, *e.g.*, an inactivated, *e.g.*, heat inactivated, degradative agent. A physical parameter, *e.g.*, size, of the modified and control molecules are then determined. They can be determined by a physical method, *e.g.*, by polyacrylamide gel electrophoresis or a sizing column, to assess whether the molecule has maintained its original length, or assessed functionally. Alternatively, Northern blot analysis can be used to assay the length of an unlabeled modified molecule.

A functional assay can also be used to evaluate the candidate agent. A functional assay can be applied initially or after an earlier non-functional assay, (*e.g.*, assay for resistance to degradation) to determine if the modification alters the ability of the molecule to silence gene expression. For example, a cell, *e.g.*, a mammalian cell, such as a mouse or human cell, can be co-transfected with a plasmid expressing a fluorescent protein, *e.g.*, GFP, and a candidate RNA agent homologous to the transcript encoding the fluorescent protein (see, *e.g.*, WO 00/44914). For example, a modified dsRNA homologous to the GFP mRNA can be assayed for the ability to inhibit GFP expression by monitoring for a decrease in cell fluorescence, as compared to a control cell, in which the transfection did not include the candidate dsRNA, *e.g.*, controls with no agent added and/or controls with a non-modified RNA added. Efficacy of the candidate agent on gene expression can be assessed by comparing cell fluorescence in the presence of the modified and unmodified dsRNA compounds.

In an alternative functional assay, a candidate dsRNA compound homologous to an endogenous mouse gene, for example, a maternally expressed gene, such as *c-mos*, can be injected into an immature mouse oocyte to assess the ability of the agent to inhibit gene expression *in vivo* (see, *e.g.*, WO 01/36646). A phenotype of the oocyte, *e.g.*, the ability to maintain arrest in metaphase II, can be monitored as an indicator that the agent is inhibiting expression. For example, cleavage of *c-mos* mRNA by a dsRNA compound would cause the oocyte to exit metaphase arrest and initiate parthenogenetic development (Colledge *et al.* Nature 370: 65-68, 1994; Hashimoto *et al.* Nature, 370:68-71, 1994). The effect of the modified agent on target RNA levels can be verified by Northern blot to assay for a decrease in the level of target mRNA, or by Western blot to assay for a decrease in the level of target protein, as compared to a negative control. Controls can include cells in which with no agent is added and/or cells in which a non-modified RNA is added.

Ligands

A wide variety of entities, such as targeting moieties, endosomolytic agents and PK modulating entities, can be coupled to the iRNA agents at various places, for example at the 3'-end, 5'-end, both the 3'- and 5'-end, internally or a combination of them. Only one or both strands of an iRNA agent can comprise one or more ligand in addition to the modifications described herein. Preferred methods of conjugation, preferred monomers for conjugation and preferred ligands are described in copending United States Patent applications #10/916,185, filed August 10, 2004; #10/946,873, filed September 21, 2004; #10/985,426, filed November 9, 2004; #11/833,934, filed August 3, 2007; #11/115,989, filed April 27, 2005; #11/119,533, filed April 29, 2005 and #11/197,753, filed August 4, 2005. Further preferred ligands and ligand conjugated monomers are described in the United States provisional application #60/992,309 filed December 4, 2007 and #61/013,597 filed December 13, 2007.

Physiological Effects

The siRNA compounds described herein can be designed such that determining therapeutic toxicity is made easier by the complementarity of the siRNA with both a human and a non-human animal sequence. By these methods, an siRNA can consist of a sequence that is fully complementary to a nucleic acid sequence from a human *and* a nucleic acid sequence from at least one non-human animal, *e.g.*, a non-human mammal, such as a rodent, ruminant or primate. For example, the non-human mammal can be a mouse, rat, dog, pig, goat, sheep, cow, monkey, Pan paniscus, Pan troglodytes, Macaca mulatto, or Cynomolgus monkey. The sequence of the siRNA compound could be complementary to sequences within homologous genes, *e.g.*, oncogenes or tumor suppressor genes, of the non-human mammal and the human. By determining the toxicity of the siRNA compound in the non-human mammal, one can extrapolate the toxicity of the siRNA compound in a human. For a more strenuous toxicity test, the siRNA can be complementary to a human and more than one, *e.g.*, two or three or more, non-human animals.

The methods described herein can be used to correlate any physiological effect of an siRNA compound on a human, *e.g.*, any unwanted effect, such as a toxic effect, or any positive, or desired effect.

Increasing Cellular Uptake of siRNAs

Described herein are various siRNA compositions that contain covalently attached conjugates that increase cellular uptake and/or intracellular targeting of the siRNAs.

Additionally provided are methods of the invention that include administering an siRNA compound and a drug that affects the uptake of the siRNA into the cell. The drug can be administered before, after, or at the same time that the siRNA compound is administered. The drug can be covalently or non-covalently linked to the siRNA compound. The drug can be, for example, a lipopolysaccharide, an activator of p38 MAP kinase, or an activator of NF- κ B. The drug can have a transient effect on the cell. The drug can increase the uptake of the siRNA compound into the cell, for example, by disrupting the cell's cytoskeleton, *e.g.*, by disrupting the cell's microtubules, microfilaments, and/or intermediate filaments. The drug can be, for example, taxon, vincristine, vinblastine, cytochalasin, nocodazole, japlakinolide, latrunculin A, phalloidin, swinholide A, indanocine, or myoservin. The drug can also increase the uptake of the siRNA compound into a given cell or tissue by activating an inflammatory response, for example. Exemplary drugs that would have such an effect include tumor necrosis factor alpha (TNF α), interleukin-1 beta, a CpG motif, gamma interferon or more generally an agent that activates a toll-like receptor.

siRNA Production

An siRNA can be produced, *e.g.*, in bulk, by a variety of methods. Exemplary methods include: organic synthesis and RNA cleavage, *e.g.*, *in vitro* cleavage.

Organic Synthesis. An siRNA can be made by separately synthesizing a single stranded RNA molecule, or each respective strand of a double-stranded RNA molecule, after which the component strands can then be annealed.

A large bioreactor, *e.g.*, the OligoPilot II from Pharmacia Biotec AB (Uppsala Sweden), can be used to produce a large amount of a particular RNA strand for a given siRNA. The OligoPilotII reactor can efficiently couple a nucleotide using only a 1.5 molar excess of a phosphoramidite nucleotide. To make an RNA strand, ribonucleotides amidites are used. Standard cycles of monomer addition can be used to synthesize the 21 to 23 nucleotide strand for

the siRNA. Typically, the two complementary strands are produced separately and then annealed, *e.g.*, after release from the solid support and deprotection.

Organic synthesis can be used to produce a discrete siRNA species. The complementary of the species to a particular target gene can be precisely specified. For example, the species may be complementary to a region that includes a polymorphism, *e.g.*, a single nucleotide polymorphism. Further the location of the polymorphism can be precisely defined. In some embodiments, the polymorphism is located in an internal region, *e.g.*, at least 4, 5, 7, or 9 nucleotides from one or both of the termini.

dsiRNA Cleavage. siRNAs can also be made by cleaving a larger siRNA. The cleavage can be mediated *in vitro* or *in vivo*. For example, to produce iRNAs by cleavage *in vitro*, the following method can be used:

In vitro transcription. dsiRNA is produced by transcribing a nucleic acid (DNA) segment in both directions. For example, the HiScribe™ RNAi transcription kit (New England Biolabs) provides a vector and a method for producing a dsiRNA for a nucleic acid segment that is cloned into the vector at a position flanked on either side by a T7 promoter. Separate templates are generated for T7 transcription of the two complementary strands for the dsiRNA. The templates are transcribed *in vitro* by addition of T7 RNA polymerase and dsiRNA is produced. Similar methods using PCR and/or other RNA polymerases (*e.g.*, T3 or SP6 polymerase) can also be used. Dtoxins that may contaminate preparations of the recombinant enzymes.

In Vitro Cleavage. In one embodiment, RNA generated by this method is carefully purified to remove endsiRNA is cleaved *in vitro* into siRNAs, for example, using a Dicer or comparable RNase III-based activity. For example, the dsiRNA can be incubated in an *in vitro* extract from *Drosophila* or using purified components, *e.g.*, a purified RNase or RISC complex (RNA-induced silencing complex). See, *e.g.*, Ketting *et al. Genes Dev* 2001 Oct 15;15(20):2654-9. and Hammond *Science* 2001 Aug 10;293(5532):1146-50.

dsiRNA cleavage generally produces a plurality of siRNA species, each being a particular 21 to 23 nt fragment of a source dsiRNA molecule. For example, siRNAs that include sequences complementary to overlapping regions and adjacent regions of a source dsiRNA molecule may be present.

Regardless of the method of synthesis, the siRNA preparation can be prepared in a solution (*e.g.*, an aqueous and/or organic solution) that is appropriate for formulation. For example, the siRNA preparation can be precipitated and redissolved in pure double-distilled water, and lyophilized. The dried siRNA can then be resuspended in a solution appropriate for the intended formulation process.

Formulations

The siRNA compounds described herein can be formulated for administration to a subject. It is understood that these formulations, compositions and methods can be practiced with modified siRNA compounds, and such practice is within the invention.

A formulated siRNA composition can assume a variety of states. In some examples, the composition is at least partially crystalline, uniformly crystalline, and/or anhydrous (*e.g.*, less than 80, 50, 30, 20, or 10% water). In another example, the siRNA is in an aqueous phase, *e.g.*, in a solution that includes water.

The aqueous phase or the crystalline compositions can, *e.g.*, be incorporated into a delivery vehicle, *e.g.*, a liposome (particularly for the aqueous phase) or a particle (*e.g.*, a microparticle as can be appropriate for a crystalline composition). Generally, the siRNA composition is formulated in a manner that is compatible with the intended method of administration, as described herein. For example, in particular embodiments the composition is prepared by at least one of the following methods: spray drying, lyophilization, vacuum drying, evaporation, fluid bed drying, or a combination of these techniques; or sonication with a lipid, freeze-drying, condensation and other self-assembly.

A siRNA preparation can be formulated in combination with another agent, *e.g.*, another therapeutic agent or an agent that stabilizes an siRNA, *e.g.*, a protein that complexes with siRNA to form an iRNP. Still other agents include chelators, *e.g.*, EDTA (*e.g.*, to remove divalent cations such as Mg^{2+}), salts, RNase inhibitors (*e.g.*, a broad specificity RNase inhibitor such as RNasin) and so forth.

In one embodiment, the siRNA preparation includes another siRNA compound, *e.g.*, a second siRNA that can mediate RNAi with respect to a second gene, or with respect to the same gene. Still other preparation can include at least 3, 5, ten, twenty, fifty, or a hundred or more

different siRNA species. Such siRNAs can mediate RNAi with respect to a similar number of different genes.

In one embodiment, the siRNA preparation includes at least a second therapeutic agent (*e.g.*, an agent other than an RNA or a DNA). For example, an siRNA composition for the treatment of a viral disease, *e.g.*, HIV, might include a known antiviral agent (*e.g.*, a protease inhibitor or reverse transcriptase inhibitor). In another example, an siRNA composition for the treatment of a cancer might further comprise a chemotherapeutic agent.

Exemplary formulations are discussed below.

Liposomes. For ease of exposition the formulations, compositions and methods in this section are discussed largely with regard to unmodified siRNA compounds. It may be understood, however, that these formulations, compositions and methods can be practiced with other siRNA compounds, *e.g.*, modified siRNAs, and such practice is within the invention. An siRNA compound, *e.g.*, a double-stranded siRNA compound, or ssiRNA compound, (*e.g.*, a precursor, *e.g.*, a larger siRNA compound which can be processed into a ssiRNA compound, or a DNA which encodes an siRNA compound, *e.g.*, a double-stranded siRNA compound, or ssiRNA compound, or precursor thereof) preparation can be formulated for delivery in a membranous molecular assembly, *e.g.*, a liposome or a micelle. As used herein, the term “liposome” refers to a vesicle composed of amphiphilic lipids arranged in at least one bilayer, *e.g.*, one bilayer or a plurality of bilayers. Liposomes include unilamellar and multilamellar vesicles that have a membrane formed from a lipophilic material and an aqueous interior. The aqueous portion contains the siRNA composition. The lipophilic material isolates the aqueous interior from an aqueous exterior, which typically does not include the siRNA composition, although in some examples, it may. Liposomes are useful for the transfer and delivery of active ingredients to the site of action. Because the liposomal membrane is structurally similar to biological membranes, when liposomes are applied to a tissue, the liposomal bilayer fuses with bilayer of the cellular membranes. As the merging of the liposome and cell progresses, the internal aqueous contents that include the siRNA are delivered into the cell where the siRNA can specifically bind to a target RNA and can mediate RNAi. In some cases the liposomes are also specifically targeted, *e.g.*, to direct the siRNA to particular cell types.

A liposome containing an siRNA can be prepared by a variety of methods. In one example, the lipid component of a liposome is dissolved in a detergent so that micelles are formed with the lipid component. For example, the lipid component can be an amphipathic cationic lipid or lipid conjugate. The detergent can have a high critical micelle concentration and may be nonionic. Exemplary detergents include cholate, CHAPS, octylglucoside, deoxycholate, and lauroyl sarcosine. The siRNA preparation is then added to the micelles that include the lipid component. The cationic groups on the lipid interact with the siRNA and condense around the siRNA to form a liposome. After condensation, the detergent is removed, *e.g.*, by dialysis, to yield a liposomal preparation of siRNA.

If necessary a carrier compound that assists in condensation can be added during the condensation reaction, *e.g.*, by controlled addition. For example, the carrier compound can be a polymer other than a nucleic acid (*e.g.*, spermine or spermidine). pH can also be adjusted to favor condensation.

Further description of methods for producing stable polynucleotide delivery vehicles, which incorporate a polynucleotide/cationic lipid complex as structural components of the delivery vehicle, are described in, *e.g.*, WO 96/37194. Liposome formation can also include one or more aspects of exemplary methods described in Felgner, P. L. *et al.*, *Proc. Natl. Acad. Sci.*, USA 8:7413-7417, 1987; U.S. Pat. No. 4,897,355; U.S. Pat. No. 5,171,678; Bangham, *et al. M. Mol. Biol.* 23:238, 1965; Olson, *et al. Biochim. Biophys. Acta* 557:9, 1979; Szoka, *et al. Proc. Natl. Acad. Sci.* 75: 4194, 1978; Mayhew, *et al. Biochim. Biophys. Acta* 775:169, 1984; Kim, *et al. Biochim. Biophys. Acta* 728:339, 1983; and Fukunaga, *et al. Endocrinol.* 115:757, 1984. Commonly used techniques for preparing lipid aggregates of appropriate size for use as delivery vehicles include sonication and freeze-thaw plus extrusion (see, *e.g.*, Mayer, *et al. Biochim. Biophys. Acta* 858:161, 1986). Microfluidization can be used when consistently small (50 to 200 nm) and relatively uniform aggregates are desired (Mayhew, *et al. Biochim. Biophys. Acta* 775:169, 1984). These methods are readily adapted to packaging siRNA preparations into liposomes.

Liposomes that are pH-sensitive or negatively-charged entrap nucleic acid molecules rather than complex with them. Since both the nucleic acid molecules and the lipid are similarly charged, repulsion rather than complex formation occurs. Nevertheless, some nucleic acid

molecules are entrapped within the aqueous interior of these liposomes. pH-sensitive liposomes have been used to deliver DNA encoding the thymidine kinase gene to cell monolayers in culture. Expression of the exogenous gene was detected in the target cells (Zhou *et al.*, *Journal of Controlled Release*, 19, (1992) 269-274).

One major type of liposomal composition includes phospholipids other than naturally-derived phosphatidylcholine. Neutral liposome compositions, for example, can be formed from dimyristoyl phosphatidylcholine (DMPC) or dipalmitoyl phosphatidylcholine (DPPC). Anionic liposome compositions generally are formed from dimyristoyl phosphatidylglycerol, while anionic fusogenic liposomes are formed primarily from dioleoyl phosphatidylethanolamine (DOPE). Another type of liposomal composition is formed from phosphatidylcholine (PC) such as, for example, soybean PC, and egg PC. Another type is formed from mixtures of phospholipid and/or phosphatidylcholine and/or cholesterol.

Examples of other methods to introduce liposomes into cells *in vitro* and *in vivo* include U.S. Pat. No. 5,283,185; U.S. Pat. No. 5,171,678; WO 94/00569; WO 93/24640; WO 91/16024; Felgner, *J. Biol. Chem.* 269:2550, 1994; Nabel, *Proc. Natl. Acad. Sci.* 90:11307, 1993; Nabel, *Human Gene Ther.* 3:649, 1992; Gershon, *Biochem.* 32:7143, 1993; and Strauss *EMBO J.* 11:417, 1992.

In one embodiment, cationic liposomes are used. Cationic liposomes possess the advantage of being able to fuse to the cell membrane. Non-cationic liposomes, although not able to fuse as efficiently with the plasma membrane, are taken up by macrophages *in vivo* and can be used to deliver siRNAs to macrophages.

Further advantages of liposomes include: liposomes obtained from natural phospholipids are biocompatible and biodegradable; liposomes can incorporate a wide range of water and lipid soluble drugs; liposomes can protect encapsulated siRNAs in their internal compartments from metabolism and degradation (Rosoff, in "Pharmaceutical Dosage Forms," Lieberman, Rieger and Banker (Eds.), 1988, volume 1, p. 245). Important considerations in the preparation of liposome formulations are the lipid surface charge, vesicle size and the aqueous volume of the liposomes.

A positively charged synthetic cationic lipid, N-[1-(2,3-dioleyloxy)propyl]-N,N,N-trimethylammonium chloride (DOTMA) can be used to form small liposomes that interact spontaneously with nucleic acid to form lipid-nucleic acid complexes which are capable of

fusing with the negatively charged lipids of the cell membranes of tissue culture cells, resulting in delivery of siRNA (see, *e.g.*, Felgner, P. L. *et al.*, Proc. Natl. Acad. Sci., USA 8:7413-7417, 1987 and U.S. Pat. No. 4,897,355 for a description of DOTMA and its use with DNA).

A DOTMA analogue, 1,2-bis(oleoyloxy)-3-(trimethylammonia)propane (DOTAP) can be used in combination with a phospholipid to form DNA-complexing vesicles. Lipofectin™ (Bethesda Research Laboratories, Gaithersburg, Md.) is an effective agent for the delivery of highly anionic nucleic acids into living tissue culture cells that comprise positively charged DOTMA liposomes which interact spontaneously with negatively charged polynucleotides to form complexes. When enough positively charged liposomes are used, the net charge on the resulting complexes is also positive. Positively charged complexes prepared in this way spontaneously attach to negatively charged cell surfaces, fuse with the plasma membrane, and efficiently deliver functional nucleic acids into, for example, tissue culture cells. Another commercially available cationic lipid, 1,2-bis(oleoyloxy)-3,3-(trimethylammonia)propane (“DOTAP”) (Boehringer Mannheim, Indianapolis, Indiana) differs from DOTMA in that the oleoyl moieties are linked by ester, rather than ether linkages.

Other reported cationic lipid compounds include those that have been conjugated to a variety of moieties including, for example, carboxyspermine which has been conjugated to one of two types of lipids and includes compounds such as 5-carboxyspermylglycine dioctaoyleamide (“DOGS”) (Transfectam™, Promega, Madison, Wisconsin) and dipalmitoylphosphatidylethanolamine 5-carboxyspermyl-amide (“DPPE”) (see, *e.g.*, U.S. Pat. No. 5,171,678).

Another cationic lipid conjugate includes derivatization of the lipid with cholesterol (“DC-Chol”) which has been formulated into liposomes in combination with DOPE (See, Gao, X. and Huang, L., Biochim. Biophys. Res. Commun. 179:280, 1991). Lipopolylysine, made by conjugating polylysine to DOPE, has been reported to be effective for transfection in the presence of serum (Zhou, X. *et al.*, Biochim. Biophys. Acta 1065:8, 1991). For certain cell lines, these liposomes containing conjugated cationic lipids, are said to exhibit lower toxicity and provide more efficient transfection than the DOTMA-containing compositions. Other commercially available cationic lipid products include DMRIE and DMRIE-HP (Vical, La Jolla, California) and Lipofectamine (DOSPA) (Life Technology, Inc., Gaithersburg, Maryland).

Other cationic lipids suitable for the delivery of oligonucleotides are described in WO 98/39359 and WO 96/37194.

Liposomal formulations are particularly suited for topical administration, liposomes present several advantages over other formulations. Such advantages include reduced side effects related to high systemic absorption of the administered drug, increased accumulation of the administered drug at the desired target, and the ability to administer siRNA, into the skin. In some implementations, liposomes are used for delivering siRNA to epidermal cells and also to enhance the penetration of siRNA into dermal tissues, *e.g.*, into skin. For example, the liposomes can be applied topically. Topical delivery of drugs formulated as liposomes to the skin has been documented (see, *e.g.*, Weiner *et al.*, *Journal of Drug Targeting*, 1992, vol. 2, 405-410 and du Plessis *et al.*, *Antiviral Research*, 18, 1992, 259-265; Mannino, R. J. and Fould-Fogerite, S., *Biotechniques* 6:682-690, 1988; Itani, T. *et al.* *Gene* 56:267-276, 1987; Nicolau, C. *et al.* *Meth. Enz.* 149:157-176, 1987; Straubinger, R. M. and Papahadjopoulos, D. *Meth. Enz.* 101:512-527, 1983; Wang, C. Y. and Huang, L., *Proc. Natl. Acad. Sci. USA* 84:7851-7855, 1987).

Non-ionic liposomal systems have also been examined to determine their utility in the delivery of drugs to the skin, in particular systems comprising non-ionic surfactant and cholesterol. Non-ionic liposomal formulations comprising Novasome I (glyceryl dilaurate/cholesterol/polyoxyethylene-10-stearyl ether) and Novasome II (glyceryl distearate/cholesterol/polyoxyethylene-10-stearyl ether) were used to deliver a drug into the dermis of mouse skin. Such formulations with siRNA are useful for treating a dermatological disorder.

Liposomes that include siRNA can be made highly deformable. Such deformability can enable the liposomes to penetrate through pore that are smaller than the average radius of the liposome. For example, transfersomes are a type of deformable liposomes. Transfersomes can be made by adding surface edge activators, usually surfactants, to a standard liposomal composition. Transfersomes that include siRNA can be delivered, for example, subcutaneously by infection in order to deliver siRNA to keratinocytes in the skin. In order to cross intact mammalian skin, lipid vesicles must pass through a series of fine pores, each with a diameter less than 50 nm, under the influence of a suitable transdermal gradient. In addition, due to the lipid properties, these transfersomes can be self-optimizing (adaptive to the shape of pores, *e.g.*, in

the skin), self-repairing, and can frequently reach their targets without fragmenting, and often self-loading.

Other formulations amenable to the present invention are described in United States provisional application serial nos. 61/018,616, filed January 2, 2008; 61/018,611, filed January 2, 2008; 61/039,748, filed March 26, 2008; 61/047,087, filed April 22, 2008 and 61/051,528, filed May 8, 2008. PCT application no PCT/US2007/080331, filed October 3, 2007 also describes formulations that are amenable to the present invention.

Surfactants. For ease of exposition the formulations, compositions and methods in this section are discussed largely with regard to unmodified siRNA compounds. It may be understood, however, that these formulations, compositions and methods can be practiced with other siRNA compounds, *e.g.*, modified siRNA compounds, and such practice is within the scope of the invention. Surfactants find wide application in formulations such as emulsions (including microemulsions) and liposomes (see above). siRNA (or a precursor, *e.g.*, a larger dsRNA which can be processed into a siRNA, or a DNA which encodes a siRNA or precursor) compositions can include a surfactant. In one embodiment, the siRNA is formulated as an emulsion that includes a surfactant. The most common way of classifying and ranking the properties of the many different types of surfactants, both natural and synthetic, is by the use of the hydrophile/lipophile balance (HLB). The nature of the hydrophilic group provides the most useful means for categorizing the different surfactants used in formulations (Rieger, in "Pharmaceutical Dosage Forms," Marcel Dekker, Inc., New York, NY, 1988, p. 285).

If the surfactant molecule is not ionized, it is classified as a nonionic surfactant. Nonionic surfactants find wide application in pharmaceutical products and are usable over a wide range of pH values. In general their HLB values range from 2 to about 18 depending on their structure. Nonionic surfactants include nonionic esters such as ethylene glycol esters, propylene glycol esters, glyceryl esters, polyglyceryl esters, sorbitan esters, sucrose esters, and ethoxylated esters. Nonionic alkanolamides and ethers such as fatty alcohol ethoxylates, propoxylated alcohols, and ethoxylated/propoxylated block polymers are also included in this class. The polyoxyethylene surfactants are the most popular members of the nonionic surfactant class.

If the surfactant molecule carries a negative charge when it is dissolved or dispersed in water, the surfactant is classified as anionic. Anionic surfactants include carboxylates such as soaps, acyl lactylates, acyl amides of amino acids, esters of sulfuric acid such as alkyl sulfates and ethoxylated alkyl sulfates, sulfonates such as alkyl benzene sulfonates, acyl isethionates, acyl taurates and sulfosuccinates, and phosphates. The most important members of the anionic surfactant class are the alkyl sulfates and the soaps.

If the surfactant molecule carries a positive charge when it is dissolved or dispersed in water, the surfactant is classified as cationic. Cationic surfactants include quaternary ammonium salts and ethoxylated amines. The quaternary ammonium salts are the most used members of this class.

If the surfactant molecule has the ability to carry either a positive or negative charge, the surfactant is classified as amphoteric. Amphoteric surfactants include acrylic acid derivatives, substituted alkylamides, N-alkylbetaines and phosphatides.

The use of surfactants in drug products, formulations and in emulsions has been reviewed (Rieger, in "Pharmaceutical Dosage Forms," Marcel Dekker, Inc., New York, NY, 1988, p. 285).

Micelles and other Membranous Formulations. For ease of exposition the micelles and other formulations, compositions and methods in this section are discussed largely with regard to unmodified siRNA compounds. It may be understood, however, that these micelles and other formulations, compositions and methods can be practiced with other siRNA compounds, *e.g.*, modified siRNA compounds, and such practice is within the invention. The siRNA compound, *e.g.*, a double-stranded siRNA compound, or ssiRNA compound, (*e.g.*, a precursor, *e.g.*, a larger siRNA compound which can be processed into a ssiRNA compound, or a DNA which encodes an siRNA compound, *e.g.*, a double-stranded siRNA compound, or ssiRNA compound, or precursor thereof)) composition can be provided as a micellar formulation. "Micelles" are defined herein as a particular type of molecular assembly in which amphipathic molecules are arranged in a spherical structure such that all the hydrophobic portions of the molecules are directed inward, leaving the hydrophilic portions in contact with the surrounding aqueous phase. The converse arrangement exists if the environment is hydrophobic.

A mixed micellar formulation suitable for delivery through transdermal membranes may be prepared by mixing an aqueous solution of the siRNA composition, an alkali metal C₈ to C₂₂

alkyl sulphate, and a micelle forming compounds. Exemplary micelle forming compounds include lecithin, hyaluronic acid, pharmaceutically acceptable salts of hyaluronic acid, glycolic acid, lactic acid, chamomile extract, cucumber extract, oleic acid, linoleic acid, linolenic acid, monoolein, monooleates, monolaurates, borage oil, evening of primrose oil, menthol, trihydroxy oxo cholanyl glycine and pharmaceutically acceptable salts thereof, glycerin, polyglycerin, lysine, polylysine, triolein, polyoxyethylene ethers and analogues thereof, polidocanol alkyl ethers and analogues thereof, chenodeoxycholate, deoxycholate, and mixtures thereof. The micelle forming compounds may be added at the same time or after addition of the alkali metal alkyl sulphate. Mixed micelles will form with substantially any kind of mixing of the ingredients but vigorous mixing in order to provide smaller size micelles.

In one method a first micellar composition is prepared which contains the siRNA composition and at least the alkali metal alkyl sulphate. The first micellar composition is then mixed with at least three micelle forming compounds to form a mixed micellar composition. In another method, the micellar composition is prepared by mixing the siRNA composition, the alkali metal alkyl sulphate and at least one of the micelle forming compounds, followed by addition of the remaining micelle forming compounds, with vigorous mixing.

Phenol and/or m-cresol may be added to the mixed micellar composition to stabilize the formulation and protect against bacterial growth. Alternatively, phenol and/or m-cresol may be added with the micelle forming ingredients. An isotonic agent such as glycerin may also be added after formation of the mixed micellar composition.

For delivery of the micellar formulation as a spray, the formulation can be put into an aerosol dispenser and the dispenser is charged with a propellant. The propellant, which is under pressure, is in liquid form in the dispenser. The ratios of the ingredients are adjusted so that the aqueous and propellant phases become one, *i.e.*, there is one phase. If there are two phases, it is necessary to shake the dispenser prior to dispensing a portion of the contents, *e.g.*, through a metered valve. The dispensed dose of pharmaceutical agent is propelled from the metered valve in a fine spray.

Propellants may include hydrogen-containing chlorofluorocarbons, hydrogen-containing fluorocarbons, dimethyl ether and diethyl ether. In certain embodiments, HFA 134a (1,1,1,2-tetrafluoroethane) may be used.

The specific concentrations of the essential ingredients can be determined by relatively straightforward experimentation. For absorption through the oral cavities, it is often desirable to increase, *e.g.*, at least double or triple, the dosage for through injection or administration through the gastrointestinal tract.

Particles. For ease of exposition the particles, formulations, compositions and methods in this section are discussed largely with regard to modified siRNA compounds. It may be understood, however, that these particles, formulations, compositions and methods can be practiced with other siRNA compounds, *e.g.*, unmodified siRNA compounds, and such practice is within the invention. In another embodiment, an siRNA compound, *e.g.*, a double-stranded siRNA compound, or ssiRNA compound, (*e.g.*, a precursor, *e.g.*, a larger siRNA compound which can be processed into a ssiRNA compound, or a DNA which encodes an siRNA compound, *e.g.*, a double-stranded siRNA compound, or ssiRNA compound, or precursor thereof) preparations may be incorporated into a particle, *e.g.*, a microparticle. Microparticles can be produced by spray-drying, but may also be produced by other methods including lyophilization, evaporation, fluid bed drying, vacuum drying, or a combination of these techniques. See below for further description.

Sustained-Release Formulations. An siRNA compound, *e.g.*, a double-stranded siRNA compound, or ssiRNA compound, (*e.g.*, a precursor, *e.g.*, a larger siRNA compound which can be processed into a ssiRNA compound, or a DNA which encodes an siRNA compound, *e.g.*, a double-stranded siRNA compound, or ssiRNA compound, or precursor thereof) described herein can be formulated for controlled, *e.g.*, slow release. Controlled release can be achieved by disposing the siRNA within a structure or substance which impedes its release. *E.g.*, siRNA can be disposed within a porous matrix or in an erodable matrix, either of which allow release of the siRNA over a period of time.

Polymeric particles, *e.g.*, polymeric in microparticles can be used as a sustained-release reservoir of siRNA that is taken up by cells only released from the microparticle through biodegradation. The polymeric particles in this embodiment should therefore be large enough to preclude phagocytosis (*e.g.*, larger than 10 μm or larger than 20 μm). Such particles can be produced by the same methods to make smaller particles, but with less vigorous mixing of the first and second emulsions. That is to say, a lower homogenization speed, vortex mixing speed,

or sonication setting can be used to obtain particles having a diameter around 100 μm rather than 10 μm . The time of mixing also can be altered.

Larger microparticles can be formulated as a suspension, a powder, or an implantable solid, to be delivered by intramuscular, subcutaneous, intradermal, intravenous, or intraperitoneal injection; via inhalation (intranasal or intrapulmonary); orally; or by implantation. These particles are useful for delivery of any siRNA when slow release over a relatively long term is desired. The rate of degradation, and consequently of release, varies with the polymeric formulation.

Microparticles may include pores, voids, hollows, defects or other interstitial spaces that allow the fluid suspension medium to freely permeate or perfuse the particulate boundary. For example, the perforated microstructures can be used to form hollow, porous spray dried microspheres.

Polymeric particles containing siRNA (*e.g.*, a siRNA) can be made using a double emulsion technique, for instance. First, the polymer is dissolved in an organic solvent. A polymer may be polylactic-co-glycolic acid (PLGA), with a lactic/glycolic acid weight ratio of 65:35, 50:50, or 75:25. Next, a sample of nucleic acid suspended in aqueous solution is added to the polymer solution and the two solutions are mixed to form a first emulsion. The solutions can be mixed by vortexing or shaking, and in the mixture can be sonicated. Any method by which the nucleic acid receives the least amount of damage in the form of nicking, shearing, or degradation, while still allowing the formation of an appropriate emulsion is possible. For example, acceptable results can be obtained with a Vibra-cell model VC-250 sonicator with a 1/8" microtip probe, at setting #3.

Spray Drying. An siRNA compound, *e.g.*, a double-stranded siRNA compound, or ssiRNA compound, (*e.g.*, a precursor, *e.g.*, a larger siRNA compound which can be processed into a ssiRNA compound, or a DNA which encodes an siRNA compound, *e.g.*, a double-stranded siRNA compound, or ssiRNA compound, or precursor thereof)) can be prepared by spray drying. Spray dried siRNA can be administered to a subject or be subjected to further formulation. A pharmaceutical composition of siRNA can be prepared by spray drying a homogeneous aqueous mixture that includes a siRNA under conditions sufficient to provide a dispersible powdered composition, *e.g.*, a pharmaceutical composition. The material for spray

drying can also include one or more of: a pharmaceutically acceptable excipient, or a dispersibility-enhancing amount of a physiologically acceptable, water-soluble protein. The spray-dried product can be a dispersible powder that includes the siRNA.

Spray drying is a process that converts a liquid or slurry material to a dried particulate form. Spray drying can be used to provide powdered material for various administrative routes including inhalation. See, for example, M. Sacchetti and M. M. Van Oort in: *Inhalation Aerosols: Physical and Biological Basis for Therapy*, A. J. Hickey, ed. Marcel Dekkar, New York, 1996.

Spray drying can include atomizing a solution, emulsion, or suspension to form a fine mist of droplets and drying the droplets. The mist can be projected into a drying chamber (*e.g.*, a vessel, tank, tubing, or coil) where it contacts a drying gas. The mist can include solid or liquid pore forming agents. The solvent and pore forming agents evaporate from the droplets into the drying gas to solidify the droplets, simultaneously forming pores throughout the solid. The solid (typically in a powder, particulate form) then is separated from the drying gas and collected.

Spray drying includes bringing together a highly dispersed liquid, and a sufficient volume of air (*e.g.*, hot air) to produce evaporation and drying of the liquid droplets. The preparation to be spray dried can be any solution, course suspension, slurry, colloidal dispersion, or paste that may be atomized using the selected spray drying apparatus. Typically, the feed is sprayed into a current of warm filtered air that evaporates the solvent and conveys the dried product to a collector. The spent air is then exhausted with the solvent. Several different types of apparatus may be used to provide the desired product. For example, commercial spray dryers manufactured by Buchi Ltd. or Niro Corp. can effectively produce particles of desired size.

Spray-dried powdered particles can be approximately spherical in shape, nearly uniform in size and frequently hollow. There may be some degree of irregularity in shape depending upon the incorporated medicament and the spray drying conditions. In many instances the dispersion stability of spray-dried microspheres appears to be more effective if an inflating agent (or blowing agent) is used in their production. Certain embodiments may comprise an emulsion with an inflating agent as the disperse or continuous phase (the other phase being aqueous in nature). An inflating agent may be dispersed with a surfactant solution, using, for instance, a commercially available microfluidizer at a pressure of about 5000 to 15,000 psi. This process forms an emulsion, which may be stabilized by an incorporated surfactant, typically comprising

submicron droplets of water immiscible blowing agent dispersed in an aqueous continuous phase. The formation of such dispersions using this and other techniques are common and well known to those in the art. The blowing agent may be a fluorinated compound (*e.g.*, perfluorohexane, perfluorooctyl bromide, perfluorodecalin, perfluorobutyl ethane) which vaporizes during the spray-drying process, leaving behind generally hollow, porous aerodynamically light microspheres. As will be discussed in more detail below, other suitable blowing agents include chloroform, freons, and hydrocarbons. Nitrogen gas and carbon dioxide are also contemplated as a suitable blowing agent.

Although the perforated microstructures may be formed using a blowing agent as described above, it will be appreciated that, in some instances, no blowing agent is required and an aqueous dispersion of the medicament and surfactant(s) are spray dried directly. In such cases, the formulation may be amenable to process conditions (*e.g.*, elevated temperatures) that generally lead to the formation of hollow, relatively porous microparticles. Moreover, the medicament may possess special physicochemical properties (*e.g.*, high crystallinity, elevated melting temperature, surface activity, etc.) that make it particularly suitable for use in such techniques.

The perforated microstructures may optionally be associated with, or comprise, one or more surfactants. Moreover, miscible surfactants may optionally be combined with the suspension medium liquid phase. It will be appreciated by those skilled in the art that the use of surfactants may further increase dispersion stability, simplify formulation procedures or increase bioavailability upon administration. Of course combinations of surfactants, including the use of one or more in the liquid phase and one or more associated with the perforated microstructures are contemplated as being within the scope of the invention. By “associated with or comprise” it is meant that the structural matrix or perforated microstructure may incorporate, adsorb, absorb, be coated with or be formed by the surfactant.

Surfactants suitable for use include any compound or composition that aids in the formation and maintenance of the stabilized respiratory dispersions by forming a layer at the interface between the structural matrix and the suspension medium. The surfactant may comprise a single compound or any combination of compounds, such as in the case of co-surfactants. Particularly certain surfactants are substantially insoluble in the propellant, nonfluorinated, and

selected from the group consisting of saturated and unsaturated lipids, nonionic detergents, nonionic block copolymers, ionic surfactants, and combinations of such agents. It may be emphasized that, in addition to the aforementioned surfactants, suitable (*i.e.*, biocompatible) fluorinated surfactants are compatible with the teachings herein and may be used to provide the desired stabilized preparations.

Lipids, including phospholipids, from both natural and synthetic sources may be used in varying concentrations to form a structural matrix. Generally, compatible lipids comprise those that have a gel to liquid crystal phase transition greater than about 40° C. In certain embodiments, the incorporated lipids are relatively long chain (*i.e.*, C₆-C₂₂) saturated lipids and may comprise phospholipids. Exemplary phospholipids useful in the disclosed stabilized preparations comprise egg phosphatidylcholine, dilauroylphosphatidylcholine, dioleoylphosphatidylcholine, dipalmitoylphosphatidylcholine, disteoylphosphatidylcholine, short-chain phosphatidylcholines, phosphatidylethanolamine, dioleoylphosphatidylethanolamine, phosphatidylserine, phosphatidylglycerol, phosphatidylinositol, glycolipids, ganglioside GM1, sphingomyelin, phosphatidic acid, cardiolipin; lipids bearing polymer chains such as, polyethylene glycol, chitin, hyaluronic acid, or polyvinylpyrrolidone; lipids bearing sulfonated mono-, di-, and polysaccharides; fatty acids such as palmitic acid, stearic acid, and oleic acid; cholesterol, cholesterol esters, and cholesterol hemisuccinate. Due to their excellent biocompatibility characteristics, phospholipids and combinations of phospholipids and poloxamers are particularly suitable for use in the stabilized dispersions disclosed herein.

Compatible nonionic detergents comprise: sorbitan esters including sorbitan trioleate (Spans™ 85), sorbitan sesquioleate, sorbitan monooleate, sorbitan monolaurate, polyoxyethylene (20) sorbitan monolaurate, and polyoxyethylene (20) sorbitan monooleate, oleyl polyoxyethylene (2) ether, stearyl polyoxyethylene (2) ether, lauryl polyoxyethylene (4) ether, glycerol esters, and sucrose esters. Other suitable nonionic detergents can be easily identified using McCutcheon's Emulsifiers and Detergents (McPublishing Co., Glen Rock, N.J.). Certain block copolymers include diblock and triblock copolymers of polyoxyethylene and polyoxypropylene, including poloxamer 188 (Pluronic F68), poloxamer 407 (Pluronic F-127), and poloxamer 338. Ionic surfactants such as sodium sulfosuccinate, and fatty acid soaps may also be utilized. In certain embodiments, the microstructures may comprise oleic acid or its alkali salt.

In addition to the aforementioned surfactants, cationic surfactants or lipids may be used, especially in the case of delivery of an siRNA compound, *e.g.*, a double-stranded siRNA compound, or ssiRNA compound, (*e.g.*, a precursor, *e.g.*, a larger siRNA compound which can be processed into a ssiRNA compound, or a DNA which encodes an siRNA compound, *e.g.*, a double-stranded siRNA compound, or ssiRNA compound, or precursor thereof). Examples of suitable cationic lipids include: DOTMA, N-[-(2,3-dioleyloxy)propyl]-N,N,N-trimethylammonium-chloride; DOTAP, 1,2-dioleyloxy-3-(trimethylammonio)propane; and DOTB, 1,2-dioleoyl-3-(4'-trimethylammonio)butanoyl-sn-glycerol. Polycationic amino acids such as polylysine, and polyarginine are also contemplated.

For the spraying process, such spraying methods as rotary atomization, pressure atomization and two-fluid atomization can be used. Examples of the devices used in these processes include "Parubisu [phonetic rendering] Mini-Spray GA-32" and "Parubisu Spray Drier DL-41", manufactured by Yamato Chemical Co., or "Spray Drier CL-8," "Spray Drier L-8," "Spray Drier FL-12," "Spray Drier FL-16" or "Spray Drier FL-20," manufactured by Okawara Kakoki Co., can be used for the method of spraying using rotary-disk atomizer.

While no particular restrictions are placed on the gas used to dry the sprayed material, it is recommended to use air, nitrogen gas or an inert gas. The temperature of the inlet of the gas used to dry the sprayed materials such that it does not cause heat deactivation of the sprayed material. The range of temperatures may vary between about 50°C to about 200°C, for example, between about 50°C and 100°C. The temperature of the outlet gas used to dry the sprayed material, may vary between about 0°C and about 150°C, for example, between 0°C and 90°C, and for example between 0°C and 60°C.

The spray drying is done under conditions that result in substantially amorphous powder of homogeneous constitution having a particle size that is respirable, a low moisture content and flow characteristics that allow for ready aerosolization. In some cases, the particle size of the resulting powder is such that more than about 98% of the mass is in particles having a diameter of about 10 μm or less with about 90% of the mass being in particles having a diameter less than 5 μm . Alternatively, about 95% of the mass will have particles with a diameter of less than 10 μm with about 80% of the mass of the particles having a diameter of less than 5 μm .

The dispersible pharmaceutical-based dry powders that include the siRNA preparation may optionally be combined with pharmaceutical carriers or excipients which are suitable for respiratory and pulmonary administration. Such carriers may serve simply as bulking agents when it is desired to reduce the siRNA concentration in the powder which is being delivered to a patient, but may also serve to enhance the stability of the siRNA compositions and to improve the dispersibility of the powder within a powder dispersion device in order to provide more efficient and reproducible delivery of the siRNA and to improve handling characteristics of the siRNA such as flowability and consistency to facilitate manufacturing and powder filling.

Such carrier materials may be combined with the drug prior to spray drying, *i.e.*, by adding the carrier material to the purified bulk solution. In that way, the carrier particles will be formed simultaneously with the drug particles to produce a homogeneous powder. Alternatively, the carriers may be separately prepared in a dry powder form and combined with the dry powder drug by blending. The powder carriers will usually be crystalline (to avoid water absorption), but might in some cases be amorphous or mixtures of crystalline and amorphous. The size of the carrier particles may be selected to improve the flowability of the drug powder, typically being in the range from 25 μm to 100 μm . A carrier material may be crystalline lactose having a size in the above-stated range.

Powders prepared by any of the above methods will be collected from the spray dryer in a conventional manner for subsequent use. For use as pharmaceuticals and other purposes, it will frequently be desirable to disrupt any agglomerates which may have formed by screening or other conventional techniques. For pharmaceutical uses, the dry powder formulations will usually be measured into a single dose, and the single dose sealed into a package. Such packages are particularly useful for dispersion in dry powder inhalers, as described in detail below. Alternatively, the powders may be packaged in multiple-dose containers.

Methods for spray drying hydrophobic and other drugs and components are described in U.S. Pat. Nos. 5,000,888; 5,026,550; 4,670,419, 4,540,602; and 4,486,435 (all of which are incorporated by reference). Bloch and Speison (1983) *Pharm. Acta Helv* 58:14-22 teaches spray drying of hydrochlorothiazide and chlorthalidone (lipophilic drugs) and a hydrophilic adjuvant (pentaerythritol) in azeotropic solvents of dioxane-water and 2-ethoxyethanol-water. A number of Japanese Patent application Abstracts relate to spray drying of hydrophilic-hydrophobic

product combinations, including JP 806766; JP 7242568; JP 7101884; JP 7101883; JP 71018982; JP 7101881; and JP 4036233. Other foreign patent publications relevant to spray drying hydrophilic-hydrophobic product combinations include FR 2594693; DE 2209477; and WO 88/07870.

In one aspect, the invention features a spray-dried siRNA compound, *e.g.*, a double-stranded siRNA compound, or ssiRNA compound, (*e.g.*, a precursor, *e.g.*, a larger siRNA compound which can be processed into a ssiRNA compound, or a DNA which encodes an siRNA compound, *e.g.*, a double-stranded siRNA compound, or ssiRNA compound, or precursor thereof) composition suitable for inhalation by a subject, including: (a) a therapeutically effective amount of a siRNA compound suitable for treating a condition in the subject by inhalation; (b) a pharmaceutically acceptable excipient selected from the group consisting of carbohydrates and amino acids; and (c) optionally, a dispersibility-enhancing amount of a physiologically-acceptable, water-soluble polypeptide.

In one embodiment, the excipient is a carbohydrate. The carbohydrate can be selected from the group consisting of monosaccharides, disaccharides, trisaccharides, and polysaccharides. In some embodiments the carbohydrate is a monosaccharide selected from the group consisting of dextrose, galactose, mannitol, D-mannose, sorbitol, and sorbose. In another embodiment the carbohydrate is a disaccharide selected from the group consisting of lactose, maltose, sucrose, and trehalose.

In another embodiment, the excipient is an amino acid. In one embodiment, the amino acid is a hydrophobic amino acid. In some embodiments the hydrophobic amino acid is selected from the group consisting of alanine, isoleucine, leucine, methionine, phenylalanine, proline, tryptophan, and valine. In yet another embodiment the amino acid is a polar amino acid. In some embodiments the amino acid is selected from the group consisting of arginine, histidine, lysine, cysteine, glycine, glutamine, serine, threonine, tyrosine, aspartic acid and glutamic acid.

In one embodiment, the dispersibility-enhancing polypeptide is selected from the group consisting of human serum albumin, α -lactalbumin, trypsinogen, and polyalanine.

In one embodiment, the spray-dried siRNA compound composition includes particles having a mass median diameter (MMD) of less than 10 microns. In another embodiment, the spray-dried siRNA compound composition includes particles having a mass median diameter of

less than 5 microns. In yet another embodiment the spray-dried siRNA compound composition includes particles having a mass median aerodynamic diameter (MMAD) of less than 5 microns.

Lyophilization. An siRNA compound, *e.g.*, a double-stranded siRNA compound, or ssiRNA compound, (*e.g.*, a precursor, *e.g.*, a larger siRNA compound which can be processed into a ssiRNA compound, or a DNA which encodes an siRNA compound, *e.g.*, a double-stranded siRNA compound, or ssiRNA compound, or precursor thereof) preparation can be made by lyophilization. Lyophilization is a freeze-drying process in which water is sublimed from the composition after it is frozen. The particular advantage associated with the lyophilization process is that biologicals and pharmaceuticals that are relatively unstable in an aqueous solution can be dried without elevated temperatures (thereby eliminating the adverse thermal effects), and then stored in a dry state where there are few stability problems. With respect to the instant invention such techniques are particularly compatible with the incorporation of nucleic acids in perforated microstructures without compromising physiological activity. Methods for providing lyophilized particulates are known to those of skill in the art and it would clearly not require undue experimentation to provide dispersion compatible microstructures in accordance with the teachings herein. Accordingly, to the extent that lyophilization processes may be used to provide microstructures having the desired porosity and size, they are conformance with the teachings herein and are expressly contemplated as being within the scope of the instant invention.

Pharmaceutical Compositons

In one aspect, the invention features a pharmaceutical composition that includes an siRNA compound, *e.g.*, a double-stranded siRNA compound, or ssiRNA compound, (*e.g.*, a precursor, *e.g.*, a larger siRNA compound which can be processed into a ssiRNA compound, or a DNA which encodes an siRNA compound, *e.g.*, a double-stranded siRNA compound, or ssiRNA compound, or precursor thereof) including a nucleotide sequence complementary to a target RNA, *e.g.*, substantially and/or exactly complementary. The target RNA can be a transcript of an endogenous human gene. In one embodiment, the siRNA compound (a) is 19-25 nucleotides long, for example, 21-23 nucleotides, (b) is complementary to an endogenous target RNA, and, optionally, (c) includes at least one 3' overhang 1-5 nt long. In one embodiment, the pharmaceutical composition can be an emulsion, microemulsion, cream, jelly, or liposome.

In one example the pharmaceutical composition includes an siRNA compound mixed with a topical delivery agent. The topical delivery agent can be a plurality of microscopic vesicles. The microscopic vesicles can be liposomes. In some embodiments the liposomes are cationic liposomes.

In another aspect, the pharmaceutical composition includes an siRNA compound, *e.g.*, a double-stranded siRNA compound, or ssiRNA compound (*e.g.*, a precursor, *e.g.*, a larger siRNA compound which can be processed into a ssiRNA compound, or a DNA which encodes an siRNA compound, *e.g.*, a double-stranded siRNA compound, or ssiRNA compound, or precursor thereof) admixed with a topical penetration enhancer. In one embodiment, the topical penetration enhancer is a fatty acid. The fatty acid can be arachidonic acid, oleic acid, lauric acid, caprylic acid, capric acid, myristic acid, palmitic acid, stearic acid, linoleic acid, linolenic acid, dicaprate, tricaprate, monolein, dilaurin, glyceryl 1-monocaprate, 1-dodecylazacycloheptan-2-one, an acylcarnitine, an acylcholine, or a C₁₋₁₀ alkyl ester, monoglyceride, diglyceride or pharmaceutically acceptable salt thereof.

In another embodiment, the topical penetration enhancer is a bile salt. The bile salt can be cholic acid, dehydrocholic acid, deoxycholic acid, glucolic acid, glycholic acid, glycodeoxycholic acid, taurocholic acid, taurodeoxycholic acid, chenodeoxycholic acid, ursodeoxycholic acid, sodium tauro-24,25-dihydro-fusidate, sodium glycodihydrofusidate, polyoxyethylene-9-lauryl ether or a pharmaceutically acceptable salt thereof.

In another embodiment, the penetration enhancer is a chelating agent. The chelating agent can be EDTA, citric acid, a salicylate, a N-acyl derivative of collagen, laureth-9, an N-amino acyl derivative of a beta-diketone or a mixture thereof.

In another embodiment, the penetration enhancer is a surfactant, *e.g.*, an ionic or nonionic surfactant. The surfactant can be sodium lauryl sulfate, polyoxyethylene-9-lauryl ether, polyoxyethylene-20-cetyl ether, a perfluorchemical emulsion or mixture thereof.

In another embodiment, the penetration enhancer can be selected from a group consisting of unsaturated cyclic ureas, 1-alkyl-alkones, 1-alkenylazacyclo-alkanones, steroidal anti-inflammatory agents and mixtures thereof. In yet another embodiment the penetration enhancer can be a glycol, a pyrrol, an azone, or a terpenes.

In one aspect, the invention features a pharmaceutical composition including an siRNA compound, *e.g.*, a double-stranded siRNA compound, or ssiRNA compound, (*e.g.*, a precursor, *e.g.*, a larger siRNA compound which can be processed into a ssiRNA compound, or a DNA which encodes an siRNA compound, *e.g.*, a double-stranded siRNA compound, or ssiRNA compound, or precursor thereof) in a form suitable for oral delivery. In one embodiment, oral delivery can be used to deliver an siRNA compound composition to a cell or a region of the gastro-intestinal tract, *e.g.*, small intestine, colon (*e.g.*, to treat a colon cancer), and so forth. The oral delivery form can be tablets, capsules or gel capsules. In one embodiment, the siRNA compound of the pharmaceutical composition modulates expression of a cellular adhesion protein, modulates a rate of cellular proliferation, or has biological activity against eukaryotic pathogens or retroviruses. In another embodiment, the pharmaceutical composition includes an enteric material that substantially prevents dissolution of the tablets, capsules or gel capsules in a mammalian stomach. In some embodiments the enteric material is a coating. The coating can be acetate phthalate, propylene glycol, sorbitan monoleate, cellulose acetate trimellitate, hydroxy propyl methylcellulose phthalate or cellulose acetate phthalate.

In another embodiment, the oral dosage form of the pharmaceutical composition includes a penetration enhancer. The penetration enhancer can be a bile salt or a fatty acid. The bile salt can be ursodeoxycholic acid, chenodeoxycholic acid, and salts thereof. The fatty acid can be capric acid, lauric acid, and salts thereof.

In another embodiment, the oral dosage form of the pharmaceutical composition includes an excipient. In one example the excipient is polyethyleneglycol. In another example the excipient is precirol.

In another embodiment, the oral dosage form of the pharmaceutical composition includes a plasticizer. The plasticizer can be diethyl phthalate, triacetin dibutyl sebacate, dibutyl phthalate or triethyl citrate.

In one aspect, the invention features a pharmaceutical composition including an siRNA compound and a delivery vehicle. In one embodiment, the siRNA compound is (a) is 19-25 nucleotides long, for example, 21-23 nucleotides, (b) is complementary to an endogenous target RNA, and, optionally, (c) includes at least one 3' overhang 1-5 nucleotides long.

In one embodiment, the delivery vehicle can deliver an siRNA compound, *e.g.*, a double-stranded siRNA compound, or ssiRNA compound, (*e.g.*, a precursor, *e.g.*, a larger siRNA compound which can be processed into a ssiRNA compound, or a DNA which encodes an siRNA compound, *e.g.*, a double-stranded siRNA compound, or ssiRNA compound, or precursor thereof) to a cell by a topical route of administration. The delivery vehicle can be microscopic vesicles. In one example the microscopic vesicles are liposomes. In some embodiments the liposomes are cationic liposomes. In another example the microscopic vesicles are micelles. In one aspect, the invention features a pharmaceutical composition including an siRNA compound, *e.g.*, a double-stranded siRNA compound, or ssiRNA compound, (*e.g.*, a precursor, *e.g.*, a larger siRNA compound which can be processed into a ssiRNA compound, or a DNA which encodes an siRNA compound, *e.g.*, a double-stranded siRNA compound, or ssiRNA compound, or precursor thereof) in an injectable dosage form. In one embodiment, the injectable dosage form of the pharmaceutical composition includes sterile aqueous solutions or dispersions and sterile powders. In some embodiments the sterile solution can include a diluent such as water; saline solution; fixed oils, polyethylene glycols, glycerin, or propylene glycol.

In one aspect, the invention features a pharmaceutical composition including an siRNA compound, *e.g.*, a double-stranded siRNA compound, or ssiRNA compound, (*e.g.*, a precursor, *e.g.*, a larger siRNA compound which can be processed into a ssiRNA compound, or a DNA which encodes an siRNA compound, *e.g.*, a double-stranded siRNA compound, or ssiRNA compound, or precursor thereof) in oral dosage form. In one embodiment, the oral dosage form is selected from the group consisting of tablets, capsules and gel capsules. In another embodiment, the pharmaceutical composition includes an enteric material that substantially prevents dissolution of the tablets, capsules or gel capsules in a mammalian stomach. In some embodiments the enteric material is a coating. The coating can be acetate phthalate, propylene glycol, sorbitan monoleate, cellulose acetate trimellitate, hydroxy propyl methyl cellulose phthalate or cellulose acetate phthalate. In one embodiment, the oral dosage form of the pharmaceutical composition includes a penetration enhancer, *e.g.*, a penetration enhancer described herein.

In another embodiment, the oral dosage form of the pharmaceutical composition includes an excipient. In one example the excipient is polyethyleneglycol. In another example the excipient is precirol.

In another embodiment, the oral dosage form of the pharmaceutical composition includes a plasticizer. The plasticizer can be diethyl phthalate, triacetin dibutyl sebacate, dibutyl phthalate or triethyl citrate.

In one aspect, the invention features a pharmaceutical composition including an siRNA compound, *e.g.*, a double-stranded siRNA compound, or ssiRNA compound, (*e.g.*, a precursor, *e.g.*, a larger siRNA compound which can be processed into a ssiRNA compound, or a DNA which encodes an siRNA compound, *e.g.*, a double-stranded siRNA compound, or ssiRNA compound, or precursor thereof) in a rectal dosage form. In one embodiment, the rectal dosage form is an enema. In another embodiment, the rectal dosage form is a suppository.

In one aspect, the invention features a pharmaceutical composition including an siRNA compound, *e.g.*, a double-stranded siRNA compound, or ssiRNA compound, (*e.g.*, a precursor, *e.g.*, a larger siRNA compound which can be processed into a ssiRNA compound, or a DNA which encodes an siRNA compound, *e.g.*, a double-stranded siRNA compound, or ssiRNA compound, or precursor thereof) in a vaginal dosage form. In one embodiment, the vaginal dosage form is a suppository. In another embodiment, the vaginal dosage form is a foam, cream, or gel.

In one aspect, the invention features a pharmaceutical composition including an siRNA compound, *e.g.*, a double-stranded siRNA compound, or ssiRNA compound, (*e.g.*, a precursor, *e.g.*, a larger siRNA compound which can be processed into a ssiRNA compound, or a DNA which encodes an siRNA compound, *e.g.*, a double-stranded siRNA compound, or ssiRNA compound, or precursor thereof) in a pulmonary or nasal dosage form. In one embodiment, the siRNA compound is incorporated into a particle, *e.g.*, a macroparticle, *e.g.*, a microsphere. The particle can be produced by spray drying, lyophilization, evaporation, fluid bed drying, vacuum drying, or a combination thereof. The microsphere can be formulated as a suspension, a powder, or an implantable solid.

Methods of Treatment of Conditions and Diseases

A subject can be treated by administering a defined amount of an siRNA compound, *e.g.*, a double-stranded siRNA compound, or ssiRNA compound, (*e.g.*, a precursor, *e.g.*, a larger siRNA compound which can be processed into a ssiRNA compound) composition that is in a powdered form, *e.g.*, a collection of microparticles, such as crystalline particles. The composition

can include a plurality of siRNA compounds, *e.g.*, specific for one or more different endogenous target RNAs. The method can include other features described herein.

A subject can be treated by administering a defined amount of an siRNA compound composition that is prepared by a method that includes spray-drying, *i.e.*, atomizing a liquid solution, emulsion, or suspension, immediately exposing the droplets to a drying gas, and collecting the resulting porous powder particles. The composition can include a plurality of siRNA compounds, *e.g.*, specific for one or more different endogenous target RNAs. The method can include other features described herein.

In one aspect, the invention provides a method of treating a subject at risk for or afflicted with a disease that may benefit from the administration of the siRNA of the invention. The method comprises administering the siRNA of the invention to a subject in need thereof, thereby treating the subject. The nucleic acid that is administered will depend on the condition or disease being treated.

Genes. Gene expression in a subject can be modulated by administering a pharmaceutical composition including an siRNA compound.

The transcriptional complex hypoxia inducible factor (HIF) is a key regulator of oxygen homeostasis. Hypoxia induces the expression of genes participating in many cellular and physiological processes, including oxygen transport and iron metabolism, erythropoiesis, angiogenesis, glycolysis and glucose uptake, transcription, metabolism, pH regulation, growth-factor signaling, response to stress and cell adhesion. These gene products participate in either increasing oxygen delivery to hypoxic tissues or activating an alternative metabolic pathway (glycolysis) which does not require oxygen. Hypoxia-induced pathways, in addition to being required for normal cellular processes, can also aid tumor growth by allowing or aiding angiogenesis, immortalization, genetic instability, tissue invasion and metastasis (Harris, *Nat. Rev. Cancer*, 2002, 2, 38-47; Maxwell et al., *Curr. Opin. Genet. Dev.*, 2001, 11, 293-299). The transcription factor hypoxia-inducible factor 1 (HIF-1) plays an essential role in homeostatic responses to hypoxia by binding to the DNA sequence 5'-TACGTGCT-3' and activating the transcription of dozens of genes in vivo under hypoxic conditions (Wang and Semenza, *J. Biol. Chem.*, 1995, 270, 1230-1237). Hypoxia-inducible factor-1 alpha is a heterodimer composed of a 120 kDa alpha subunit complexed with a 91 to 94 kDa beta subunit, both of which contain a

basic helix-loop-helix. The gene encoding hypoxia-inducible factor-1 alpha (HIF1 α also called HIF-1 alpha, HIF1A, HIF-1A, HIF1-A, and MOP1) was cloned in 1995 (Wang *et al.*, Proc. Natl. Acad. Sci. U.S.A., 1995, 92, 5510-5514). A nucleic acid sequence encoding HIF1 α is disclosed and claimed in U.S. Pat. No. 5,882,914, as are expression vectors expressing the recombinant DNA, and host cells containing said vectors (Semenza, 1999). US Patent 7,217,572 (the disclosure of which is incorporated herein by reference) discloses at SEQ ID NO: 189 the antisense oligonucleotides sequence: GTGCAGTATT GTAGCCAGGC (SEQ ID NO: 16), and discloses at SEQ ID NO: 446 the antisense oligonucleotide sequence: CCTCATGGTC ACATGGATGA (SEQ ID NO: 17).

Aberrant expression of or constitutive expression of STAT3 is associated with a number of disease processes. STAT3 has been shown to be involved in cell transformation. Constitutive activation and/or overexpression of STAT3 appears to be involved in several forms of cancer, including myeloma, breast carcinomas, prostate cancer, brain tumors, head and neck carcinomas, melanoma, leukemias and lymphomas, particularly chronic myelogenous leukemia and multiple myeloma. Niu *et al.*, Cancer Res., 1999, 59, 5059-5063. Breast cancer cell lines that overexpress EGFR constitutively express phosphorylated STAT3 (Sartor, C. I., *et al.*, Cancer Res., 1997, 57, 978-987; Garcia, R., *et al.*, Cell Growth and Differentiation, 1997, 8, 1267-1276). Activated STAT3 levels were also found to be elevated in low grade glioblastomas and medulloblastomas (Cattaneo, E., *et al.*, Anticancer Res., 1998, 18, 2381-2387). US Patent 7,307,069 (the disclosure of which is incorporated herein by reference) discloses at SEQ ID NO: 184 the antisense oligonucleotide sequence: TTGGCTTCTC AAGATACCTG (SEQ ID NO: 19), and discloses at SEQ ID NO: 342 the antisense oligonucleotides sequence: GACTCTTGCA GGAAGCGGCT (SEQ ID NO: 20).

Huntington's disease is a progressive neurodegenerative disorder characterized by motor disturbance, cognitive loss and psychiatric manifestations (Martin and Gusella, N. Engl. J. Med. 315:1267-1276 (1986). Although an actual mechanism for Huntington's disease remains elusive, Huntington's disease has been shown to be an autosomal dominant neurodegenerative disorder caused by an expanding glutamine repeat in a gene termed IT15 or Huntingtin (HD). Although this gene is widely expressed and is required for normal development, the pathology of Huntington's disease is restricted to the brain, for reasons that remain poorly understood. The

Huntingtin gene product is expressed at similar levels in patients and controls, and the genetics of the disorder suggest that the expansion of the polyglutamine repeat induces a toxic gain of function, perhaps through interactions with other cellular proteins. US Patent 7,320,965 (the disclosure of which is incorporated herein by reference) discloses an antisense strand for inhibiting the expression of a human Huntingtin gene at SEQ ID NO: 793: CUGCACGGUU CUUUGUGACT T (SEQ ID NO: 21).

The intracellular transport of proteins, lipids, and mRNA to specific locations within the cell, as well as the proper alignment and separation of chromosomes in dividing cells, is essential to the functioning of the cell. The superfamily of proteins called kinesins (KIF), along with the myosins and dyneins, function as molecular engines to bind and transport vesicles and organelles along microtubules with energy supplied by ATP. KIFs have been identified in many species ranging from yeast to humans. The amino acid sequences which comprise the motor domain are highly conserved among eukaryotic phyla, while the region outside of the motor domain serves to bind to the cargo and varies in amino acid sequence among KIFs. The movement of a kinesin along a microtubule can occur in either the plus or minus direction, but any given kinesin can only travel in one direction, an action that is mediated by the polarity of the motor and the microtubule. The KIFs have been grouped into three major types depending on the position of the motor domain: the amino-terminal domain, the middle motor domain, and the carboxyl-terminal domain, referred to respectively as N-kinesin, M-kinesin, and C-kinesins. These are further classified into 14 classes based on a phylogenetic analysis of the 45 known human and mouse kinesin genes (Miki et al., Proc. Natl. Acad. Sci. U.S.A., 2001, 98, 7004-7011). One such kinesin, kinesin-like 1, a member of the N-2 (also called bimC) family of kinesins and is involved in separating the chromosomes by directing their movement along microtubules in the bipolar spindle. During mitosis, the microtubule bipolar spindle functions to distribute the duplicated chromosomes equally to daughter cells. Kinesin-like 1 is first phosphorylated by the kinase p34^{cdc2} and is essential for centrosome separation and assembly of bipolar spindles at prophase (Blangy *et al.*, Cell, 1995, 83, 1159-1169). In rodent neurons, kinesin-like 1 is expressed well past their terminal mitotic division, and has been implicated in regulating microtubule behaviors within the developing axons and dendrites (Ferhat *et al.*, J. Neurosci., 1998, 18, 7822-7835). The gene encoding human kinesin-like 1 (also called KNSL1, Eg5, HsEg5, HKSP, KIF11, thyroid interacting protein 5, and TRIP5) was cloned in 1995 (Blangy *et*

al., Cell, 1995, 83, 1159-1169). Inhibition of kinesin-like 1 has been suggested as a target for arresting cellular proliferation in cancer because of the central role kinesin-like 1 holds in mitosis. Expression of kinesin-like 1 may also contribute to other disease states. A contribution of kinesin-like 1 to B-cell leukemia has been demonstrated in mice as a result of upregulated expression of kinesin-like 1 following a retroviral insertion mutation in the proximity of the kinesin-like 1 gene (Hansen and Justice, Oncogene, 1999, 18, 6531-6539). Autoantibodies to a set of proteins in the mitotic spindle assembly have been detected in human sera and these autoantibodies have been associated with autoimmune diseases including carpal tunnel syndrome, Raynaud's phenomenon, systemic sclerosis, Sjorgren's syndrome, rheumatoid arthritis, polymyositis, and polyarteritis. One of these autoantigens is kinesin-like 1 and has been identified in systemic lupus erythematosus (Whitehead *et al.*, Arthritis Rheum., 1996, 39, 1635-1642). US Patent 7,199,107 (the disclosure of which is incorporated herein by reference) discloses an antisense strand for inhibiting the expression of a human kinesin-1 gene at NO: 122: ACGTGGAATT ATACCAGCCA (SEQ ID NO: 22).

A number of therapeutic strategies exist for inhibiting aberrant angiogenesis, which attempt to reduce the production or effect of VEGF. For example, anti-VEGF or anti-VEGF receptor antibodies (Kim E S *et al.* (2002), PNAS USA 99: 11399-11404), and soluble VEGF "traps" which compete with endothelial cell receptors for VEGF binding (Holash J *et al.* (2002), PNAS USA 99: 11393-11398) have been developed. Classical VEGF "antisense" or aptamer therapies directed against VEGF gene expression have also been proposed (U.S. published application 2001/0021772 of Uhlmann *et al.*, the disclosure of which is incorporated herein by reference). However, the anti-angiogenic agents used in these therapies can produce only a stoichiometric reduction in VEGF or VEGF receptor, and the agents are typically overwhelmed by the abnormally high production of VEGF by the diseased tissue. The results achieved with available anti-angiogenic therapies have therefore been unsatisfactory. US Patent 7,345,027 (the disclosure of which is incorporated herein by reference) discloses an antisense strand for inhibiting the expression of a human VEGF gene at SEQ ID NO: 78: GUGCUGGCCUUGGUGAGGUTT (The terminal two Ts are overhangs; SEQ ID NO: 23).

The NF- κ B or nuclear factor κ B is a transcription factor that plays a critical role in inflammatory diseases by inducing the expression of a large number of proinflammatory and

anti-apoptotic genes. These include cytokines such as IL-1, IL-2, IL-11, TNF- α and IL-6, chemokines including IL-8, GRO1 and RANTES, as well as other proinflammatory molecules including COX-2 and cell adhesion molecules such as ICAM-1, VCAM-1, and E-selectin. Pahl H L, (1999) *Oncogene* 18, 6853-6866; Jobin et al, (2000) *Am. J. Physiol. Cell. Physiol.* 278: 451-462. Under resting conditions, NF- κ B is present in the cytosol of cells as a complex with I κ B. The I κ B family of proteins serve as inhibitors of NF- κ B, interfering with the function of its nuclear localization signal (see for example U. Siebenlist *et al*, (1994) *Ann. Rev. Cell Bio.*, 10: 405). Upon disruption of the I κ B-NF- κ B complex following cell activation, NF- κ B translocates to the nucleus and activates gene transcription. Disruption of the I κ B-NF- κ B complex and subsequent activation of NF- κ B is initiated by degradation of I κ B. Activators of NF- κ B mediate the site-specific phosphorylation of two amino terminal serines in each I κ B which makes nearby lysines targets for ubiquitination, thereby resulting in I κ B proteasomal destruction. NF- κ B is then free to translocate to the nucleus and bind DNA leading to the activation of a host of inflammatory response target genes. (Baldwin, A., Jr., (1996) *Annu Rev Immunol* 14: 649-683, Ghosh, S. *et al*, (1998) *Annu Rev Immunol* 16, 225-260.) Recent evidence has shown that NF- κ B subunits dynamically shuttle between the cytoplasm and the nucleus but a dominant acting nuclear export signal in I κ B α ensures their transport back to the cytoplasm. Even though NF- κ B is largely considered to be a transcriptional activator, under certain circumstances it can also be involved in directly repressing gene expression (reviewed in Ghosh, S. *et al*. (1998) *Annu. Rev. Immunol.*, 16: 225-260). US Patent 7,235,654 (the disclosure of which is incorporated herein by reference) discloses an siRNA at SEQ ID NO: 3: GUCUGUGUAU CACGUGACGN N (wherein N is a 2'-deoxy-thymidine; SEQ ID NO: 24).

Control of the risk factors involved in hypercholesterolemia and cardiovascular disease has been the focus of much research in academia and industry. Because an elevated level of circulating plasma low-density lipoprotein cholesterol has been identified as an independent risk factor in the development of hypercholesterolemia and cardiovascular disease, many strategies have been directed at lowering the levels of cholesterol carried in this atherogenic lipoprotein. AcylCoA cholesterol acyltransferase (ACAT) enzymes catalyze the synthesis of cholesterol esters from free cholesterol and fatty acyl-CoA. These enzymes are also involved in regulation of the concentration of cellular free sterols (Buhman *et al.*, *Biochim. Biophys. Acta*, 2000, 1529,

142-154; Burnett *et al.*, Clin. Chim. Acta, 1999, 286, 231-242; Chang *et al.*, Annu. Rev. Biochem., 1997, 66, 613-638; Rudel *et al.*, Curr. Opin. Lipidol., 2001, 12, 121-127; Rudel and Shelness, Nat. Med., 2000, 6, 1313-1314). Chang *et al.* cloned the first example of a human ACAT gene in 1993 (Chang *et al.*, J. Biol. Chem., 1993, 268, 20747-20755). This original ACAT enzyme is now known as ACAT-1. Subsequently, the work of Meiner *et al.* suggested the presence of more than one ACAT gene in mammals (Meiner *et al.*, J. Lipid Res., 1997, 38, 1928-1933). The cloning and expression of a second human ACAT isoform now known as acyl CoA cholesterol acyltransferase-2, was accomplished recently (Oelkers *et al.*, J. Biol. Chem., 1998, 273, 26765-26771). Murine acyl CoA cholesterol acyltransferase-2 has also been identified and cloned (Cases *et al.*, J. Biol. Chem., 1998, 273, 26755-26764). US Patent 7,335,764 (the disclosure of which is incorporated herein by reference) discloses siRNAs targeted to a nucleic acid molecule encoding acyl CoA cholesterol acyltransferase-2 at SEQ ID NOs: 25 (GCACGAAGGA TCCCAGGCAC (SEQ ID NO: 25)), 26 (GGATCCCCTC ACCTCGTCTG (SEQ ID NO: 26)) and 27 (GTTCTTGGCC ACATAATTCC (SEQ ID NO: 27)).

Lp(a) contains two disulfide-linked distinct proteins, apolipoprotein(a) (or ApoA) and apolipoprotein B (or ApoB) (Rainwater and Kammerer, J. Exp. Zool., 1998, 282, 54-61). Apolipoprotein(a) is a unique apolipoprotein encoded by the LPA gene which has been shown to exclusively control the physiological concentrations of Lp(a) (Rainwater and Kammerer, J. Exp. Zool., 1998, 282, 54-61). It varies in size due to interallelic differences in the number of tandemly repeated Kringle 4-encoding 5.5 kb sequences in the LPA gene (Rainwater and Kammerer, J. Exp. Zool., 1998, 282, 54-61). Elevated plasma levels of Lp(a), caused by increased expression of apolipoprotein(a), are associated with increased risk for atherosclerosis and its manifestations, which include hypercholesterolemia (Seed *et al.*, N. Engl. J. Med., 1990, 322, 1494-1499), myocardial infarction (Sandkamp *et al.*, Clin. Chem., 1990, 36, 20-23), and thrombosis (Nowak-Gottl *et al.*, Pediatrics, 1997, 99, E11). Moreover, the plasma concentration of Lp(a) is strongly influenced by heritable factors and is refractory to most drug and dietary manipulation (Katan and Beynen, Am. J. Epidemiol., 1987, 125, 387-399; Vessby *et al.*, Atherosclerosis, 1982, 44, 61-71.). Pharmacologic therapy of elevated Lp(a) levels has been only modestly successful and apheresis remains the most effective therapeutic modality (Hajjar and Nachman, Annu. Rev. Med., 1996, 47, 423-442). US Patent 7,259,150 (the disclosure of which is

incorporated herein by reference) discloses an siRNA for inhibiting the expression of apolipoprotein(a) at SEQ ID NO: 23 (ACCTGACACC GGGATCCCTC (SEQ ID NO: 28)).

Routes of Delivery

For ease of exposition the formulations, compositions and methods in this section are discussed largely with regard to modified siRNA compounds. It may be understood, however, that these formulations, compositions and methods can be practiced with other siRNA compounds, *e.g.*, unmodified siRNA compounds, and such practice is within the invention. A composition that includes a iRNA can be delivered to a subject by a variety of routes. Exemplary routes include: intravenous, topical, rectal, anal, vaginal, nasal, pulmonary, ocular.

The iRNA molecules of the invention can be incorporated into pharmaceutical compositions suitable for administration. Such compositions typically include one or more species of iRNA and a pharmaceutically acceptable carrier. As used herein the language “pharmaceutically acceptable carrier” is intended to include any and all solvents, dispersion media, coatings, antibacterial and antifungal agents, isotonic and absorption delaying agents, and the like, compatible with pharmaceutical administration. The use of such media and agents for pharmaceutically active substances is well known in the art. Except insofar as any conventional media or agent is incompatible with the active compound, use thereof in the compositions is contemplated. Supplementary active compounds can also be incorporated into the compositions.

The pharmaceutical compositions of the present invention may be administered in a number of ways depending upon whether local or systemic treatment is desired and upon the area to be treated. Administration may be topical (including ophthalmic, vaginal, rectal, intranasal, transdermal), oral or parenteral. Parenteral administration includes intravenous drip, subcutaneous, intraperitoneal or intramuscular injection, or intrathecal or intraventricular administration.

The route and site of administration may be chosen to enhance targeting. For example, to target muscle cells, intramuscular injection into the muscles of interest would be a logical choice. Lung cells might be targeted by administering the iRNA in aerosol form. The vascular endothelial cells could be targeted by coating a balloon catheter with the iRNA and mechanically introducing the DNA.

Formulations for topical administration may include transdermal patches, ointments, lotions, creams, gels, drops, suppositories, sprays, liquids and powders. Conventional pharmaceutical carriers, aqueous, powder or oily bases, thickeners and the like may be necessary or desirable. Coated condoms, gloves and the like may also be useful.

Compositions for oral administration include powders or granules, suspensions or solutions in water, syrups, elixirs or non-aqueous media, tablets, capsules, lozenges, or troches. In the case of tablets, carriers that can be used include lactose, sodium citrate and salts of phosphoric acid. Various disintegrants such as starch, and lubricating agents such as magnesium stearate, sodium lauryl sulfate and talc, are commonly used in tablets. For oral administration in capsule form, useful diluents are lactose and high molecular weight polyethylene glycols. When aqueous suspensions are required for oral use, the nucleic acid compositions can be combined with emulsifying and suspending agents. If desired, certain sweetening and/or flavoring agents can be added.

Compositions for intrathecal or intraventricular administration may include sterile aqueous solutions which may also contain buffers, diluents and other suitable additives.

Formulations for parenteral administration may include sterile aqueous solutions which may also contain buffers, diluents and other suitable additives. Intraventricular injection may be facilitated by an intraventricular catheter, for example, attached to a reservoir. For intravenous use, the total concentration of solutes may be controlled to render the preparation isotonic.

For ocular administration, ointments or droppable liquids may be delivered by ocular delivery systems known to the art such as applicators or eye droppers. Such compositions can include mucomimetics such as hyaluronic acid, chondroitin sulfate, hydroxypropyl methylcellulose or poly(vinyl alcohol), preservatives such as sorbic acid, EDTA or benzylchonium chloride, and the usual quantities of diluents and/or carriers.

In one embodiment, the administration of the siRNA compound, *e.g.*, a double-stranded siRNA compound, or ssiRNA compound, composition is parenteral, *e.g.*, intravenous (*e.g.*, as a bolus or as a diffusible infusion), intradermal, intraperitoneal, intramuscular, intrathecal, intraventricular, intracranial, subcutaneous, transmucosal, buccal, sublingual, endoscopic, rectal, oral, vaginal, topical, pulmonary, intranasal, urethral or ocular. Administration can be provided by the subject or by another person, *e.g.*, a health care provider. The medication can be provided

in measured doses or in a dispenser which delivers a metered dose. Selected modes of delivery are discussed in more detail below.

Rectal Administration. The invention also provides methods, compositions, and kits, for rectal administration or delivery of siRNA compounds described herein.

Accordingly, an siRNA compound, *e.g.*, a double-stranded siRNA compound, or ssiRNA compound, (*e.g.*, a precursor, *e.g.*, a larger siRNA compound which can be processed into a ssiRNA compound, or a DNA which encodes a an siRNA compound, *e.g.*, a double-stranded siRNA compound, or ssiRNA compound, or precursor thereof) described herein, *e.g.*, a therapeutically effective amount of a siRNA compound described herein, *e.g.*, a siRNA compound having a double stranded region of less than 40, and, for example, less than 30 nucleotides and having one or two 1-3 nucleotide single strand 3' overhangs can be administered rectally, *e.g.*, introduced through the rectum into the lower or upper colon. This approach is particularly useful in the treatment of, inflammatory disorders, disorders characterized by unwanted cell proliferation, *e.g.*, polyps, or colon cancer.

The medication can be delivered to a site in the colon by introducing a dispensing device, *e.g.*, a flexible, camera-guided device similar to that used for inspection of the colon or removal of polyps, which includes means for delivery of the medication.

The rectal administration of the siRNA compound is by means of an enema. The siRNA compound of the enema can be dissolved in a saline or buffered solution. The rectal administration can also by means of a suppository, which can include other ingredients, *e.g.*, an excipient, *e.g.*, cocoa butter or hydropropylmethylcellulose.

Ocular Deilvery. Any of the siRNA compounds described herein can be administered to ocular tissue. For example, the medications can be applied to the surface of the eye or nearby tissue, *e.g.*, the inside of the eyelid. They can be applied topically, *e.g.*, by spraying, in drops, as an eyewash, or an ointment. Administration can be provided by the subject or by another person, *e.g.*, a health care provider. The medication can be provided in measured doses or in a dispenser which delivers a metered dose. The medication can also be administered to the interior of the eye, and can be introduced by a needle or other delivery device which can introduce it to a selected area or structure. Ocular treatment is particularly desirable for treating inflammation of the eye or nearby tissue.

Topical Delivery. Any of the siRNA compounds described herein can be administered directly to the skin. For example, the medication can be applied topically or delivered in a layer of the skin, *e.g.*, by the use of a microneedle or a battery of microneedles which penetrate into the skin, but, for example, not into the underlying muscle tissue. Administration of the siRNA compound composition can be topical. Topical applications can, for example, deliver the composition to the dermis or epidermis of a subject. Topical administration can be in the form of transdermal patches, ointments, lotions, creams, gels, drops, suppositories, sprays, liquids or powders. A composition for topical administration can be formulated as a liposome, micelle, emulsion, or other lipophilic molecular assembly. The transdermal administration can be applied with at least one penetration enhancer, such as iontophoresis, phonophoresis, and sonophoresis.

For ease of exposition the formulations, compositions and methods in this section are discussed largely with regard to unmodified siRNA compounds. It may be understood, however, that these formulations, compositions and methods can be practiced with other siRNA compounds, *e.g.*, unmodified siRNA compounds, and such practice is within the invention. In some embodiments, an siRNA compound, *e.g.*, a double-stranded siRNA compound, or ssiRNA compound, (*e.g.*, a precursor, *e.g.*, a larger siRNA compound which can be processed into a ssiRNA compound, or a DNA which encodes an siRNA compound, *e.g.*, a double-stranded siRNA compound, or ssiRNA compound, or precursor thereof) is delivered to a subject via topical administration. "Topical administration" refers to the delivery to a subject by contacting the formulation directly to a surface of the subject. The most common form of topical delivery is to the skin, but a composition disclosed herein can also be directly applied to other surfaces of the body, *e.g.*, to the eye, a mucous membrane, to surfaces of a body cavity or to an internal surface. As mentioned above, the most common topical delivery is to the skin. The term encompasses several routes of administration including, but not limited to, topical and transdermal. These modes of administration typically include penetration of the skin's permeability barrier and efficient delivery to the target tissue or stratum. Topical administration can be used as a means to penetrate the epidermis and dermis and ultimately achieve systemic delivery of the composition. Topical administration can also be used as a means to selectively deliver oligonucleotides to the epidermis or dermis of a subject, or to specific strata thereof, or to an underlying tissue.

The term “skin,” as used herein, refers to the epidermis and/or dermis of an animal. Mammalian skin consists of two major, distinct layers. The outer layer of the skin is called the epidermis. The epidermis is comprised of the stratum corneum, the stratum granulosum, the stratum spinosum, and the stratum basale, with the stratum corneum being at the surface of the skin and the stratum basale being the deepest portion of the epidermis. The epidermis is between 50 μm and 0.2 mm thick, depending on its location on the body.

Beneath the epidermis is the dermis, which is significantly thicker than the epidermis. The dermis is primarily composed of collagen in the form of fibrous bundles. The collagenous bundles provide support for, inter alia, blood vessels, lymph capillaries, glands, nerve endings and immunologically active cells.

One of the major functions of the skin as an organ is to regulate the entry of substances into the body. The principal permeability barrier of the skin is provided by the stratum corneum, which is formed from many layers of cells in various states of differentiation. The spaces between cells in the stratum corneum is filled with different lipids arranged in lattice-like formations that provide seals to further enhance the skins permeability barrier.

The permeability barrier provided by the skin is such that it is largely impermeable to molecules having molecular weight greater than about 750 Da. For larger molecules to cross the skin's permeability barrier, mechanisms other than normal osmosis must be used.

Several factors determine the permeability of the skin to administered agents. These factors include the characteristics of the treated skin, the characteristics of the delivery agent, interactions between both the drug and delivery agent and the drug and skin, the dosage of the drug applied, the form of treatment, and the post treatment regimen. To selectively target the epidermis and dermis, it is sometimes possible to formulate a composition that comprises one or more penetration enhancers that will enable penetration of the drug to a preselected stratum.

Transdermal delivery is a valuable route for the administration of lipid soluble therapeutics. The dermis is more permeable than the epidermis and therefore absorption is much more rapid through abraded, burned or denuded skin. Inflammation and other physiologic conditions that increase blood flow to the skin also enhance transdermal adsorption. Absorption via this route may be enhanced by the use of an oily vehicle (inunction) or through the use of one or more penetration enhancers. Other effective ways to deliver a composition disclosed herein

via the transdermal route include hydration of the skin and the use of controlled release topical patches. The transdermal route provides a potentially effective means to deliver a composition disclosed herein for systemic and/or local therapy.

In addition, iontophoresis (transfer of ionic solutes through biological membranes under the influence of an electric field) (Lee *et al.*, Critical Reviews in Therapeutic Drug Carrier Systems, 1991, p. 163), phonophoresis or sonophoresis (use of ultrasound to enhance the absorption of various therapeutic agents across biological membranes, notably the skin and the cornea) (Lee *et al.*, Critical Reviews in Therapeutic Drug Carrier Systems, 1991, p. 166), and optimization of vehicle characteristics relative to dose position and retention at the site of administration (Lee *et al.*, Critical Reviews in Therapeutic Drug Carrier Systems, 1991, p. 168) may be useful methods for enhancing the transport of topically applied compositions across skin and mucosal sites.

The compositions and methods provided may also be used to examine the function of various proteins and genes *in vitro* in cultured or preserved dermal tissues and in animals. The invention can be thus applied to examine the function of any gene. The methods of the invention can also be used therapeutically or prophylactically. For example, for the treatment of animals that are known or suspected to suffer from diseases such as psoriasis, lichen planus, toxic epidermal necrolysis, erythema multiforme, basal cell carcinoma, squamous cell carcinoma, malignant melanoma, Paget's disease, Kaposi's sarcoma, pulmonary fibrosis, Lyme disease and viral, fungal and bacterial infections of the skin.

Pulmonary Delivery. Any of the siRNA compounds described herein can be administered to the pulmonary system. Pulmonary administration can be achieved by inhalation or by the introduction of a delivery device into the pulmonary system, *e.g.*, by introducing a delivery device which can dispense the medication. Certain embodiments may use a method of pulmonary delivery by inhalation. The medication can be provided in a dispenser which delivers the medication, *e.g.*, wet or dry, in a form sufficiently small such that it can be inhaled. The device can deliver a metered dose of medication. The subject, or another person, can administer the medication. Pulmonary delivery is effective not only for disorders which directly affect pulmonary tissue, but also for disorders which affect other tissue. siRNA compounds can be formulated as a liquid or nonliquid, *e.g.*, a powder, crystal, or aerosol for pulmonary delivery.

For ease of exposition the formulations, compositions and methods in this section are discussed largely with regard to modified siRNA compounds. It may be understood, however, that these formulations, compositions and methods can be practiced with other siRNA compounds, *e.g.*, unmodified siRNA compounds, and such practice is within the invention. A composition that includes an siRNA compound, *e.g.*, a double-stranded siRNA compound, or ssiRNA compound, (*e.g.*, a precursor, *e.g.*, a larger siRNA compound which can be processed into a ssiRNA compound, or a DNA which encodes an siRNA compound, *e.g.*, a double-stranded siRNA compound, or ssiRNA compound, or precursor thereof) can be administered to a subject by pulmonary delivery. Pulmonary delivery compositions can be delivered by inhalation by the patient of a dispersion so that the composition, for example, iRNA, within the dispersion can reach the lung where it can be readily absorbed through the alveolar region directly into blood circulation. Pulmonary delivery can be effective both for systemic delivery and for localized delivery to treat diseases of the lungs.

Pulmonary delivery can be achieved by different approaches, including the use of nebulized, aerosolized, micellular and dry powder-based formulations. Delivery can be achieved with liquid nebulizers, aerosol-based inhalers, and dry powder dispersion devices. Metered-dose devices are may be used. One of the benefits of using an atomizer or inhaler is that the potential for contamination is minimized because the devices are self contained. Dry powder dispersion devices, for example, deliver drugs that may be readily formulated as dry powders. A iRNA composition may be stably stored as lyophilized or spray-dried powders by itself or in combination with suitable powder carriers. The delivery of a composition for inhalation can be mediated by a dosing timing element which can include a timer, a dose counter, time measuring device, or a time indicator which when incorporated into the device enables dose tracking, compliance monitoring, and/or dose triggering to a patient during administration of the aerosol medicament.

The term “powder” means a composition that consists of finely dispersed solid particles that are free flowing and capable of being readily dispersed in an inhalation device and subsequently inhaled by a subject so that the particles reach the lungs to permit penetration into the alveoli. Thus, the powder is said to be “respirable.” For example, the average particle size is less than about 10 μm in diameter with a relatively uniform spheroidal shape distribution. In some embodiments, the diameter is less than about 7.5 μm and in some embodiments less than

about 5.0 μm . Usually the particle size distribution is between about 0.1 μm and about 5 μm in diameter, sometimes about 0.3 μm to about 5 μm .

The term “dry” means that the composition has a moisture content below about 10% by weight (% w) water, usually below about 5% w and in some cases less it than about 3% w. A dry composition can be such that the particles are readily dispersible in an inhalation device to form an aerosol.

The term “therapeutically effective amount” is the amount present in the composition that is needed to provide the desired level of drug in the subject to be treated to give the anticipated physiological response.

The term “physiologically effective amount” is that amount delivered to a subject to give the desired palliative or curative effect.

The term “pharmaceutically acceptable carrier” means that the carrier can be taken into the lungs with no significant adverse toxicological effects on the lungs.

The types of pharmaceutical excipients that are useful as carrier include stabilizers such as human serum albumin (HSA), bulking agents such as carbohydrates, amino acids and polypeptides; pH adjusters or buffers; salts such as sodium chloride; and the like. These carriers may be in a crystalline or amorphous form or may be a mixture of the two.

Bulking agents that are particularly valuable include compatible carbohydrates, polypeptides, amino acids or combinations thereof. Suitable carbohydrates include monosaccharides such as galactose, D-mannose, sorbose, and the like; disaccharides, such as lactose, trehalose, and the like; cyclodextrins, such as 2-hydroxypropyl-.beta.-cyclodextrin; and polysaccharides, such as raffinose, maltodextrins, dextrans, and the like; alditols, such as mannitol, xylitol, and the like. A group of carbohydrates may include lactose, threhalose, raffinose maltodextrins, and mannitol. Suitable polypeptides include aspartame. Amino acids include alanine and glycine, with glycine being used in some embodiments.

Additives, which are minor components of the composition of this invention, may be included for conformational stability during spray drying and for improving dispersibility of the powder. These additives include hydrophobic amino acids such as tryptophan, tyrosine, leucine, phenylalanine, and the like.

Suitable pH adjusters or buffers include organic salts prepared from organic acids and bases, such as sodium citrate, sodium ascorbate, and the like; sodium citrate may be used in some embodiments.

Pulmonary administration of a micellar iRNA formulation may be achieved through metered dose spray devices with propellants such as tetrafluoroethane, heptafluoroethane, dimethylfluoropropane, tetrafluoropropane, butane, isobutane, dimethyl ether and other non-CFC and CFC propellants.

Oral or Nasal Delivery. Any of the siRNA compounds described herein can be administered orally, e.g., in the form of tablets, capsules, gel capsules, lozenges, troches or liquid syrups. Further, the composition can be applied topically to a surface of the oral cavity.

Any of the siRNA compounds described herein can be administered nasally. Nasal administration can be achieved by introduction of a delivery device into the nose, e.g., by introducing a delivery device which can dispense the medication. Methods of nasal delivery include spray, aerosol, liquid, e.g., by drops, or by topical administration to a surface of the nasal cavity. The medication can be provided in a dispenser with delivery of the medication, e.g., wet or dry, in a form sufficiently small such that it can be inhaled. The device can deliver a metered dose of medication. The subject, or another person, can administer the medication.

Nasal delivery is effective not only for disorders which directly affect nasal tissue, but also for disorders which affect other tissue siRNA compounds can be formulated as a liquid or nonliquid, e.g., a powder, crystal, or for nasal delivery. As used herein, the term “crystalline” describes a solid having the structure or characteristics of a crystal, i.e., particles of three-dimensional structure in which the plane faces intersect at definite angles and in which there is a regular internal structure. The compositions of the invention may have different crystalline forms. Crystalline forms can be prepared by a variety of methods, including, for example, spray drying.

For ease of exposition the formulations, compositions and methods in this section are discussed largely with regard to modified siRNA compounds. It may be understood, however, that these formulations, compositions and methods can be practiced with other siRNA compounds, e.g., unmodified siRNA compounds, and such practice is within the invention. Both the oral and nasal membranes offer advantages over other routes of administration. For example,

drugs administered through these membranes have a rapid onset of action, provide therapeutic plasma levels, avoid first pass effect of hepatic metabolism, and avoid exposure of the drug to the hostile gastrointestinal (GI) environment. Additional advantages include easy access to the membrane sites so that the drug can be applied, localized and removed easily.

In oral delivery, compositions can be targeted to a surface of the oral cavity, *e.g.*, to sublingual mucosa which includes the membrane of ventral surface of the tongue and the floor of the mouth or the buccal mucosa which constitutes the lining of the cheek. The sublingual mucosa is relatively permeable thus giving rapid absorption and acceptable bioavailability of many drugs. Further, the sublingual mucosa is convenient, acceptable and easily accessible.

The ability of molecules to permeate through the oral mucosa appears to be related to molecular size, lipid solubility and peptide protein ionization. Small molecules, less than 1000 daltons appear to cross mucosa rapidly. As molecular size increases, the permeability decreases rapidly. Lipid soluble compounds are more permeable than non-lipid soluble molecules. Maximum absorption occurs when molecules are un-ionized or neutral in electrical charges. Therefore charged molecules present the biggest challenges to absorption through the oral mucosae.

A pharmaceutical composition of iRNA may also be administered to the buccal cavity of a human being by spraying into the cavity, without inhalation, from a metered dose spray dispenser, a mixed micellar pharmaceutical formulation as described above and a propellant. In one embodiment, the dispenser is first shaken prior to spraying the pharmaceutical formulation and propellant into the buccal cavity. For example, the medication can be sprayed into the buccal cavity or applied directly, *e.g.*, in a liquid, solid, or gel form to a surface in the buccal cavity. This administration is particularly desirable for the treatment of inflammations of the buccal cavity, *e.g.*, the gums or tongue, *e.g.*, in one embodiment, the buccal administration is by spraying into the cavity, *e.g.*, without inhalation, from a dispenser, *e.g.*, a metered dose spray dispenser that dispenses the pharmaceutical composition and a propellant.

Devices

In another aspect, the invention features a device, *e.g.*, an implantable device, wherein the device can dispense or administer a composition that includes an siRNA compound, *e.g.*, a double-stranded siRNA compound, or ssiRNA compound, (*e.g.*, a precursor, *e.g.*, a larger siRNA

compound which can be processed into a ssiRNA compound, or a DNA which encodes an siRNA compound, *e.g.*, a double-stranded siRNA compound, or ssiRNA compound, or precursor thereof), *e.g.*, a siRNA compound that silences an endogenous transcript. In one embodiment, the device is coated with the composition. In another embodiment the siRNA compound is disposed within the device. In another embodiment, the device includes a mechanism to dispense a unit dose of the composition. In other embodiments the device releases the composition continuously, *e.g.*, by diffusion. Exemplary devices include stents, catheters, pumps, artificial organs or organ components (*e.g.*, artificial heart, a heart valve, etc.), and sutures.

For ease of exposition the devices, formulations, compositions and methods in this section are discussed largely with regard to modified siRNA compounds. It may be understood, however, that these devices, formulations, compositions and methods can be practiced with other siRNA compounds, *e.g.*, unmodified siRNA compounds, and such practice is within the invention. An siRNA compound, *e.g.*, a double-stranded siRNA compound, or ssiRNA compound, (*e.g.*, a precursor, *e.g.*, a larger siRNA compound which can be processed into a ssiRNA compound, or a DNA which encodes an siRNA compound, *e.g.*, a double-stranded siRNA compound, or ssiRNA compound, or precursor thereof) can be disposed on or in a device, *e.g.*, a device which implanted or otherwise placed in a subject. Exemplary devices include devices which are introduced into the vasculature, *e.g.*, devices inserted into the lumen of a vascular tissue, or which devices themselves form a part of the vasculature, including stents, catheters, heart valves, and other vascular devices. These devices, *e.g.*, catheters or stents, can be placed in the vasculature of the lung, heart, or leg.

Other devices include non-vascular devices, *e.g.*, devices implanted in the peritoneum, or in organ or glandular tissue, *e.g.*, artificial organs. The device can release a therapeutic substance in addition to an siRNA, *e.g.*, a device can release insulin.

Other devices include artificial joints, *e.g.*, hip joints, and other orthopedic implants.

In one embodiment, unit doses or measured doses of a composition that includes iRNA are dispensed by an implanted device. The device can include a sensor that monitors a parameter within a subject. For example, the device can include pump, *e.g.*, and, optionally, associated electronics.

Tissue, *e.g.*, cells or organs can be treated with an siRNA compound, *e.g.*, a double-stranded siRNA compound, or ssiRNA compound, (*e.g.*, a precursor, *e.g.*, a larger siRNA compound which can be processed into a ssiRNA compound, or a DNA which encodes an siRNA compound, *e.g.*, a double-stranded siRNA compound, or ssiRNA compound, or precursor thereof), *ex vivo* and then administered or implanted in a subject.

The tissue can be autologous, allogeneic, or xenogeneic tissue. *E.g.*, tissue can be treated to reduce graft versus host disease. In other embodiments, the tissue is allogeneic and the tissue is treated to treat a disorder characterized by unwanted gene expression in that tissue. *E.g.*, tissue, *e.g.*, hematopoietic cells, *e.g.*, bone marrow hematopoietic cells, can be treated to inhibit unwanted cell proliferation.

Introduction of treated tissue, whether autologous or transplant, can be combined with other therapies.

In some implementations, the iRNA treated cells are insulated from other cells, *e.g.*, by a semi-permeable porous barrier that prevents the cells from leaving the implant, but enables molecules from the body to reach the cells and molecules produced by the cells to enter the body. In one embodiment, the porous barrier is formed from alginate.

In one embodiment, a contraceptive device is coated with or contains an siRNA compound, *e.g.*, a double-stranded siRNA compound, or ssiRNA compound, (*e.g.*, a precursor, *e.g.*, a larger siRNA compound which can be processed into a ssiRNA compound, or a DNA which encodes an siRNA compound, *e.g.*, a double-stranded siRNA compound, or ssiRNA compound, or precursor thereof). Exemplary devices include condoms, diaphragms, IUD (implantable uterine devices, sponges, vaginal sheaths, and birth control devices. In one embodiment, the iRNA is chosen to inactivate sperm or egg. In another embodiment, the iRNA is chosen to be complementary to a viral or pathogen RNA, *e.g.*, an RNA of an STD. In some instances, the iRNA composition can include a spermicide.

Dosage

The dosage of a pharmaceutical composition including a siRNA compound can be administered in order to alleviate the symptoms of a disease state, *e.g.*, cancer or a cardiovascular

disease. A subject can be treated with the pharmaceutical composition by any of the methods mentioned above.

In one aspect, the invention features a method of administering an siRNA compound, *e.g.*, a double-stranded siRNA compound, or ssiRNA compound, to a subject (*e.g.*, a human subject). The method includes administering a unit dose of the siRNA compound, *e.g.*, a ssiRNA compound, *e.g.*, double stranded ssiRNA compound that (a) the double-stranded part is 19-25 nucleotides (nt) long, for example, 21-23 nt, (b) is complementary to a target RNA (*e.g.*, an endogenous or pathogen target RNA), and, optionally, (c) includes at least one 3' overhang 1-5 nucleotide long. In one embodiment, the unit dose is less than 1.4 mg per kg of bodyweight, or less than 10, 5, 2, 1, 0.5, 0.1, 0.05, 0.01, 0.005, 0.001, 0.0005, 0.0001, 0.00005 or 0.00001 mg per kg of bodyweight, and less than 200 nmole of RNA agent (*e.g.*, about 4.4×10^{16} copies) per kg of bodyweight, or less than 1500, 750, 300, 150, 75, 15, 7.5, 1.5, 0.75, 0.15, 0.075, 0.015, 0.0075, 0.0015, 0.00075, 0.00015 nmole of RNA agent per kg of bodyweight.

The defined amount can be an amount effective to treat or prevent a disease or disorder, *e.g.*, a disease or disorder associated with the target RNA. The unit dose, for example, can be administered by injection (*e.g.*, intravenous or intramuscular), an inhaled dose, or a topical application. In some embodiments dosages may be less than 2, 1, or 0.1 mg/kg of body weight.

In some embodiments, the unit dose is administered less frequently than once a day, *e.g.*, less than every 2, 4, 8 or 30 days. In another embodiment, the unit dose is not administered with a frequency (*e.g.*, not a regular frequency). For example, the unit dose may be administered a single time.

RNAi silencing persists for several days after administering an siRNA or siNA composition so, in many instances, it is possible to administer the composition with a frequency of less than once per day, or, for some instances, only once for the entire therapeutic regimen. For example, treatment of some cancer cells may be mediated by a single bolus administration, whereas a chronic viral infection may require regular administration, *e.g.*, once or more per week or once or less per month.

In one embodiment, the effective dose is administered with other traditional therapeutic modalities. In one embodiment, the subject has a viral infection and the modality is an antiviral agent other than an siRNA compound, *e.g.*, other than a double-stranded siRNA compound, or

ssiRNA compound. In another embodiment, the subject has atherosclerosis and the effective dose of an siRNA compound, *e.g.*, a double-stranded siRNA compound, or ssiRNA compound, is administered in combination with, *e.g.*, after surgical intervention, *e.g.*, angioplasty.

In one embodiment, a subject is administered an initial dose and one or more maintenance doses of an siRNA compound, *e.g.*, a double-stranded siRNA compound, or ssiRNA compound, (*e.g.*, a precursor, *e.g.*, a larger siRNA compound which can be processed into a ssiRNA compound, or a DNA which encodes an siRNA compound, *e.g.*, a double-stranded siRNA compound, or ssiRNA compound, or precursor thereof). The maintenance dose or doses are generally lower than the initial dose, *e.g.*, one-half less of the initial dose. A maintenance regimen can include treating the subject with a dose or doses ranging from 0.01 μg to 1.4 mg/kg of body weight per day, *e.g.*, 10, 1, 0.1, 0.01, 0.001, or 0.00001 mg per kg of bodyweight per day. The maintenance doses are, for example, administered no more than once every 5, 10, or 30 days. Further, the treatment regimen may last for a period of time which will vary depending upon the nature of the particular disease, its severity and the overall condition of the patient. In certain embodiments the dosage may be delivered no more than once per day, *e.g.*, no more than once per 24, 36, 48, or more hours, *e.g.*, no more than once for every 5 or 8 days. Following treatment, the patient can be monitored for changes in his condition and for alleviation of the symptoms of the disease state. The dosage of the compound may either be increased in the event the patient does not respond significantly to current dosage levels, or the dose may be decreased if an alleviation of the symptoms of the disease state is observed, if the disease state has been ablated, or if undesired side-effects are observed.

The effective dose can be administered in a single dose or in two or more doses, as desired or considered appropriate under the specific circumstances. If desired to facilitate repeated or frequent infusions, implantation of a delivery device, *e.g.*, a pump, semi-permanent stent (*e.g.*, intravenous, intraperitoneal, intracisternal or intracapsular), or reservoir may be advisable.

In one embodiment, the siRNA compound pharmaceutical composition includes a plurality of siRNA compound species. In another embodiment, the siRNA compound species has sequences that are non-overlapping and non-adjacent to another species with respect to a naturally occurring target sequence. In another embodiment, the plurality of siRNA compound

species is specific for different naturally occurring target genes. In another embodiment, the siRNA compound is allele specific.

In some cases, a patient is treated with a siRNA compound in conjunction with other therapeutic modalities. For example, a patient being treated for a viral disease, e.g., an HIV associated disease (e.g., AIDS), may be administered a siRNA compound specific for a target gene essential to the virus in conjunction with a known antiviral agent (e.g., a protease inhibitor or reverse transcriptase inhibitor). In another example, a patient being treated for cancer may be administered a siRNA compound specific for a target essential for tumor cell proliferation in conjunction with a chemotherapy.

Following successful treatment, it may be desirable to have the patient undergo maintenance therapy to prevent the recurrence of the disease state, wherein the compound of the invention is administered in maintenance doses, ranging from 0.01 μg to 100 g per kg of body weight (see US 6,107,094).

The concentration of the siRNA compound composition is an amount sufficient to be effective in treating or preventing a disorder or to regulate a physiological condition in humans. The concentration or amount of siRNA compound administered will depend on the parameters determined for the agent and the method of administration, e.g., nasal, buccal, pulmonary. For example, nasal formulations tend to require much lower concentrations of some ingredients in order to avoid irritation or burning of the nasal passages. It is sometimes desirable to dilute an oral formulation up to 10-100 times in order to provide a suitable nasal formulation.

Certain factors may influence the dosage required to effectively treat a subject, including but not limited to the severity of the disease or disorder, previous treatments, the general health and/or age of the subject, and other diseases present. Moreover, treatment of a subject with a therapeutically effective amount of an siRNA compound, e.g., a double-stranded siRNA compound, or ssiRNA compound, (e.g., a precursor, e.g., a larger siRNA compound which can be processed into a ssiRNA compound, or a DNA which encodes an siRNA compound, e.g., a double-stranded siRNA compound, or ssiRNA compound, or precursor thereof) can include a single treatment or, for example, can include a series of treatments. It will also be appreciated that the effective dosage of a siRNA compound such as a ssiRNA compound used for treatment may increase or decrease over the course of a particular treatment. Changes in dosage may result

and become apparent from the results of diagnostic assays as described herein. For example, the subject can be monitored after administering a siRNA compound composition. Based on information from the monitoring, an additional amount of the siRNA compound composition can be administered.

Dosing is dependent on severity and responsiveness of the disease condition to be treated, with the course of treatment lasting from several days to several months, or until a cure is effected or a diminution of disease state is achieved. Optimal dosing schedules can be calculated from measurements of drug accumulation in the body of the patient. Persons of ordinary skill can easily determine optimum dosages, dosing methodologies and repetition rates. Optimum dosages may vary depending on the relative potency of individual compounds, and can generally be estimated based on EC50s found to be effective in in vitro and in vivo animal models. In some embodiments, the animal models include transgenic animals that express a human gene, e.g., a gene that produces a target RNA. The transgenic animal can be deficient for the corresponding endogenous RNA. In another embodiment, the composition for testing includes a siRNA compound that is complementary, at least in an internal region, to a sequence that is conserved between the target RNA in the animal model and the target RNA in a human.

The inventors have discovered that siRNA compounds described herein can be administered to mammals, particularly large mammals such as nonhuman primates or humans in a number of ways.

Kits

In certain other aspects, the invention provides kits that include a suitable container containing a pharmaceutical formulation of an siRNA compound, e.g., a double-stranded siRNA compound, or ssiRNA compound, (e.g., a precursor, e.g., a larger siRNA compound which can be processed into a ssiRNA compound, or a DNA which encodes an siRNA compound, e.g., a double-stranded siRNA compound, or ssiRNA compound, or precursor thereof). In certain embodiments the individual components of the pharmaceutical formulation may be provided in one container. Alternatively, it may be desirable to provide the components of the pharmaceutical formulation separately in two or more containers, e.g., one container for an siRNA compound preparation, and at least another for a carrier compound. The kit may be packaged in a number of different configurations such as one or more containers in a single box.

The different components can be combined, *e.g.*, according to instructions provided with the kit. The components can be combined according to a method described herein, *e.g.*, to prepare and administer a pharmaceutical composition. The kit can also include a delivery device.

The invention is further illustrated by the following examples, which should not be construed as further limiting. The contents of all references, pending patent applications and published patents, cited throughout this application are hereby expressly incorporated by reference.

EXEMPLIFICATION

The invention now being generally described, it will be more readily understood by reference to the following examples which are included merely for purposes of illustration of certain aspects and embodiments of the present invention, and are not intended to limit the invention.

Example 1 – Calculation of thermodynamic profile of effective and ineffective duplexes

Single-dose screening data for 8337 duplexes across 63 targets assayed via qPCR or bDNA at doses of 1 nM or higher (typically, 10 nM). For each duplex, we calculated delta-G in 5-nucleotide windows of the antisense strand according to Xia et al. 1998 (*Biochemistry* **37**:14719-14735). Windows extending beyond position 19 utilized the average delta-G of all possible random nucleotide words of the appropriate lengths; for example, the window beginning at position 19 of a particular duplex would contain nucleotide #19 plus all possible random tetramers. Duplexes were then divided into “Effective”, “Nondescript”, and “Ineffective” categories according to whether they knocked down their target mRNAs by $\geq 80\%$, between 20 and 80%, or $\leq 20\%$, respectively (N = 2028, 4411, 1898). Mean delta-delta-G for Effective and Ineffective duplexes was then calculated as the difference for each 5-nt window between mean delta-G of Effective (Ineffective) duplexes and mean delta-G of Nondescript duplexes. The graph (FIG. 2) indicates these mean delta-delta-G “profiles”; the thick lines chart the means, and the thin dashed lines chart the standard errors.

Example 2 – siRNAs targeting firefly luciferase and containing duplexes designed to evaluate combination of thermally destabilizing and stabilizing chemical modifications

Table 1. Duplexes containing DFT in the central region of the sense strand and LNA or 2'-F at the 3'-end of antisense strand or 5'-end of sense strand.

ssstrand #	Duplex	Strand	Sequence 5' to 3'	Modifications
A-1000	AD-1000	S	CUU ACG CUG AGU ACU UCG AdTdT	none
A-1001		AS	UCG AAG UAC UCA GCG UAA GdTdT	none
A-30133	AD-3202	S	CUU ACG CUG Y1GU ACU UCG AdTdT	A10 -> DFT
A-1001		AS	UCG AAG UAC UCA GCG UAA GdTdT	none
A-30133		S	CUU ACG CUG Y1GU ACU UCG AdTdT	A10 -> DFT
A-64035		AS	UCG AAG UAC UCA GCG UA(Aln) (Gln)dTdT	LNA @ pos.18,19
A-30133		S	CUU ACG CUG Y1GU ACU UCG AdTdT	A10 -> DFT
A-64036		AS	UCG AAG UAC UCA GCG U(Aln)A (Gln)dTdT	LNA @ pos.17,19
A-1000		S	CUU ACG CUG AGU ACU UCG AdTdT	none
A-64035		AS	UCG AAG UAC UCA GCG UA(Aln) (Gln)dTdT	LNA @ pos.18,19
A-1000		S	CUU ACG CUG AGU ACU UCG AdTdT	none
A-64036		AS	UCG AAG UAC UCA GCG U(Aln)A (Gln)dTdT	LNA @ pos.17,19
A-64031		S	CUU ACG CUG AGU ACU UCG Y32dTdT	A19 -> C3 spacer
A-64035		AS	UCG AAG UAC UCA GCG UA(Aln) (Gln)dTdT	LNA @ pos.18,19
A-64031		S	CUU ACG CUG AGU ACU UCG Y32dTdT	A19 -> C3 spacer
A-64036		AS	UCG AAG UAC UCA GCG U(Aln)A (Gln)dTdT	LNA @ pos.17,19
A-64032		S	CUU ACG CUG Y1GU ACU UCG Y32dTdT	A10->DFT, A19-> C3-spacer
A-64035		AS	UCG AAG UAC UCA GCG UA(Aln) (Gln)dTdT	LNA @ pos.18,19
A-64032		S	CUU ACG CUG Y1GU ACU UCG Y32dTdT	A10->DFT, A19-> C3-spacer
A-64036		AS	UCG AAG UAC UCA GCG U(Aln)A (Gln)dTdT	LNA @ pos.17,19
A-30133		S	CUU ACG CUG Y1GU ACU UCG AdTdT	A10 -> DFT
A-65068		AS	UCG AAG UAC UCA GCG UAfAfGfdTdT	2'-F @ pos. 17-19 / dTdT oh
A-64032		S	CUU ACG CUG Y1GU ACU UCG Y32dTdT	A10->DFT, A19-> C3-spacer
A-65068		AS	UCG AAG UAC UCA GCG UAfAfGfdTdT	2'-F @ pos. 17-19 / dTdT oh
A-65065		S	CfUfUf ACG CUG Y1GU ACU UCG AdTdT	2'-F @ pos. 1-3, A10 -> DFT
A-1001		AS	UCG AAG UAC UCA GCG UAA GdTdT	none
A-65066		S	(m5Cln)U(Tln) ACG CUG Y1GU ACU UCG AdTdT	LNA @ pos. 1, 3, A10-> DFT
A-1001		AS	UCG AAG UAC UCA GCG UAA GdTdT	none
A-65067		S	(Y28)UU ACG CUG Y1GU ACU UCG AdTdT	G-clamp @ pos. 1, A10_> DFT
A-1001		AS	UCG AAG UAC UCA GCG UAA GdTdT	none
A-65820		S	CfUfUf ACG CUG AGU ACU UCG AdTdT	2'-F @ pos. 1-3, A10 -> DFT
A-1001		AS	UCG AAG UAC UCA GCG UAA GdTdT	none
A-65819		S	(m5Cln)U(Tln) ACG CUG AGU ACU UCG AdTdT	LNA @ pos. 1, 3, A10-> DFT
A-1001		AS	UCG AAG UAC UCA GCG UAA GdTdT	none
A-65821		S	(Y28)UU ACG CUG AGU ACU UCG AdTdT	G-clamp @ pos. 1, A10_> DFT
A-1001		AS	UCG AAG UAC UCA GCG UAA GdTdT	none

PTEN Assay Procedure

HeLa cells were cultured in DMEM (Invitrogen) supplemented with 10% FBS and 1x Glutamax and plated in 96-well plates (opaque walls), 10K cells/well in DMEM / 10% FBS w/o antibiotics.

Transfection was performed using Lipofectamine 24 hours after plating the cells and after another 24 h of incubation, PTEN assay was performed as described briefly below: Standard PTEN probe sets for use in QuantiGene 1.0 Reagent Systems were obtained from Panomics, Inc. The probe set for a target gene consists of three types of oligonucleotide probes that capture the target RNA to the surface of a plate well and then hybridize to DNA signal amplification molecules. For each target sequence, the software algorithm that designs the probe, identifies one or more continuous regions that can serve as annealing templates for CEs (capture extenders, 5–10 per gene), LEs (label extenders, 10–20 per gene), or BL (blocking probes). QuantiGene 1.0 Reagent System was performed according to manufacturer's recommended protocols (Panomics, Inc.). Briefly, the probe set oligonucleotides (250 fmol CE, 500 fmol BL, and 1000 fmol LE) were mixed with the sample, and the mixture was added to an assay well in a 96-well plate covalently coated with capture probe Oligonucleotide. Target RNA was captured during an overnight incubation at 53°C. Unbound material was removed on day 2 by three washes with 200 to 300 µl of wash buffer (0.1x standard saline citrate containing 0.3 g/L lithium lauryl sulfate) followed by sequential hybridization of DNA amplifier molecules, then 3'-alkaline phosphatase-conjugated Label Probe oligonucleotides, with three washes after each incubation. After the final wash, luminescent substrate Dioxetane was added to the wells, and following a short incubation the luminescent signal was measured by a luminometer. The "no template" background values were subtracted from each probe set signal. Values were normalized to the GAPDH (Glyceraldehyde-3-phosphate dehydrogenase) values, which is a housekeeping gene. Additionally, a ratio of normalized values was calculated for evaluating mRNA levels which correlates to the extent of gene silencing.

Results

Tabulated below are IC₅₀ values derived using results from HeLa cell based PTEN assay, as described above. Figure 17 depicts graphically the primary data obtained from the assay. The results confirm the findings obtained for the siRNA sequence targeting firefly luciferase described in Examples 1 and 2 in that local destabilization of the central region of the sense strand with mismatch base pairings or modified nucleobases can lead to substantial potency enhancements. For this particular sequence, mismatched base pairings or introduction of

2,4 difluorotoluyyl ribonucleotide in positions 9 and 10 were found to significantly enhance potency over the parent unmodified compound.

EQUIVALENTS

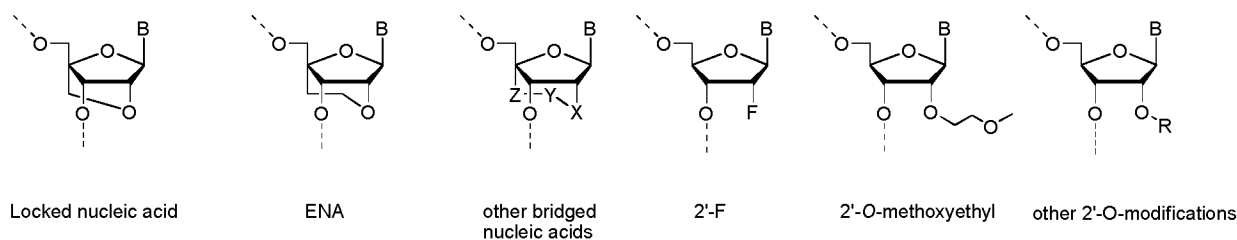
Those skilled in the art will recognize, or be able to ascertain using no more than routine experimentation, many equivalents to the specific embodiments of the invention described herein. It is, therefore, to be understood that the foregoing embodiments are presented by way of example only and that, within the scope of the appended claims and equivalents thereto, the invention may be practiced otherwise than as specifically described and claimed.

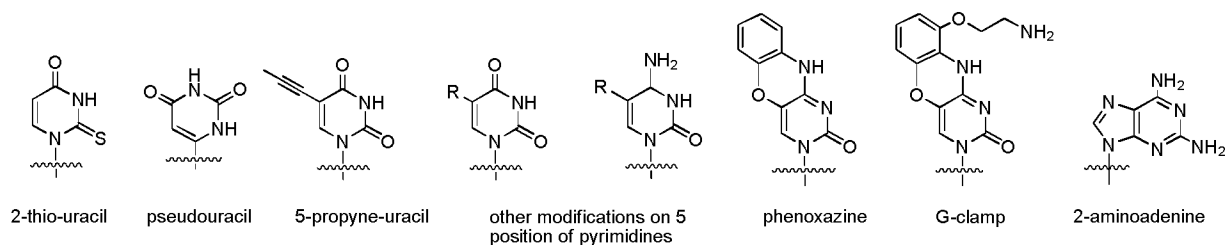
We claim:

1. A double-stranded agent with increased RNAi silencing activity, comprising:
 - a. a first strand that is complementary to a target gene; and
 - b. a second strand that is complementary to said first strand;
 wherein said double-stranded agent comprises:
 - i. at least one thermally destabilizing chemical modification placed within at least one of the low-thermal-stability regions in the doubled-stranded agent;
 - ii. at least one thermally stabilizing chemical modification placed within at least one of the high-thermal-stability regions in the doubled-stranded agent; or
 - iii. a combination of (i) and (ii);

wherein said increased RNAi silencing activity is relative to a corresponding unmodified agent, as determined by comparing their respective IC_{50} values in a RNAi silencing assay.

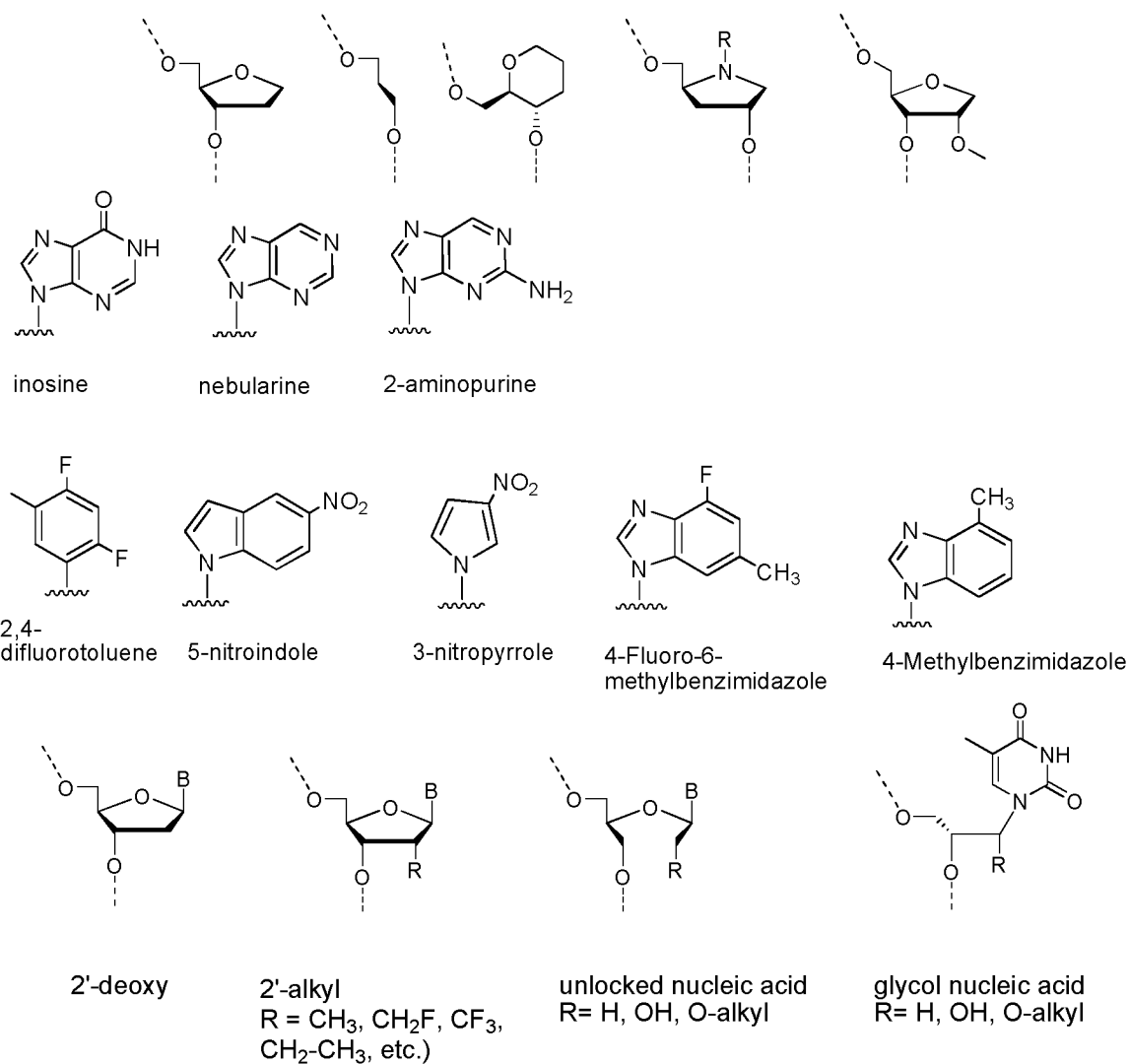
2. The agent of claim 1, wherein at least one of the thermally stabilizing chemical modifications is selected from the following:

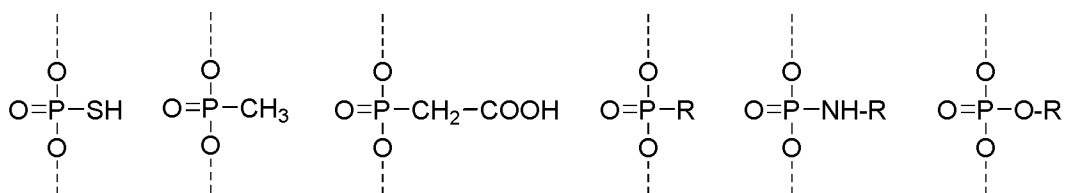




wherein B is a nucleobase; R is alkyl, H, OH, or O-alkyl; and X, Y, and Z are independently O, S, or CH₂.

3. The agent of claim 1, wherein at least one of the thermally destabilizing chemical modifications is selected from the following:





R = alkyl

wherein, unless specified above, B is nucleobase, and R is alkyl, H, OH, or O-alkyl.

4. The agent of any one of claims 1-3, wherein said agent exhibits an IC_{50} value less than or equal to about 50% of the IC_{50} value of the corresponding unmodified agent.
5. The agent of any one of claims 1-3, wherein said agent exhibits an IC_{50} value less than or equal to about 33% of the IC_{50} value of the corresponding unmodified agent.
6. The agent of any one of claims 1-3, wherein said agent exhibits an IC_{50} value less than or equal to about 20% of the IC_{50} value of the corresponding unmodified agent.
7. The agent of claim 4, 5 or 6, wherein said IC_{50} value is measured in an *in vitro* system.
8. The agent of claim 4, 5 or 6, wherein said IC_{50} value is measured in an *in vivo* system.
9. The agent of any of the above claims, wherein said stabilizing chemical modification is at position N1-N7 from the 5'-end of the sense strand.
10. The agent of any of the above claims, wherein said destabilizing chemical modification is at position N8-N19 from the 5'-end of the sense strand.
11. The agent of any of the above claims, wherein said stabilized chemical modification is at the third position of the cleavage site region from the 5'-end of the second strand.

12. The agent of any of the above claims, wherein said stabilized chemical modification is at the fourth position of the cleavage site region from the 5'-end of the second strand.
13. The agent of any of the above claims, wherein the agent comprises at least one mismatch selected from the group consisting of G:G, G:A, G:U, G:T, A:A, A:C, C:C, C:U, C:T, U:U, and U:T.
14. The agent of any of the above claims, wherein said first and second strands are each 15 to 30 nucleobases in length.
15. The agent of any of the above claims, wherein said first and second strands are each 19 to 25 nucleobases in length.
16. The agent of any of the above claims, wherein said first and second strands are each 21 to 23 nucleobases in length.
17. The agent of any of the above claims, wherein said first and second strands are each 21 nucleobases in length.
18. The agent of any of the above claims, wherein said agent comprises a single-stranded overhang on at least one terminal end.
19. The agent of claim 18, wherein said single-stranded overhang consists of 1, 2 or 3 nucleobases.
20. A method of reducing the expression of a target gene in a cell, comprising contacting said cell with an agent of any of the above claims.

21. The agent of claim 1, wherein the agent comprises two 21-nucleotide-long strands, wherein the strands form a double-stranded region of 19 consecutive base pairs having a two-nucleotide overhang at the 3'-end, and wherein the agent contains a cleavage site region corresponding to positions 9-12 from the 5'-end of the second strand.
22. The agent of claim 21, wherein the cleavage site region corresponds to position 10 or 11 from the 5'-end of the second strand.
23. The agent of claim 21, wherein the cleavage site region corresponds to position 10 from the 5'-end of the second strand.
24. The agent of claim 1, wherein the agent is a double-stranded short interfering ribonucleic acid (siRNA) molecule that comprises at least one, at least two, at least three, or at least four of the following criteria:
 - a. having an A at position 1 of the 5'-end of the first strand,
 - b. having a GC content of about 25% to about 50%,
 - c. having asymmetry at the ends of the duplex,
 - d. having AU pair at position 13 and/or 14 of the 5'-end of the first strand, and
 - e. having at least 1, at least 2, at least 3, at least 4, or at least 5 UAs in the seed region.
25. The agent of claim 24, wherein one of the criteria is having an AU pair at position 13 and/or 14 of the 5'-end of the first strand, and wherein the AU base pair at position 13 and/or 14 is unmodified.
26. A double-stranded agent with increased RNAi silencing activity, comprising:

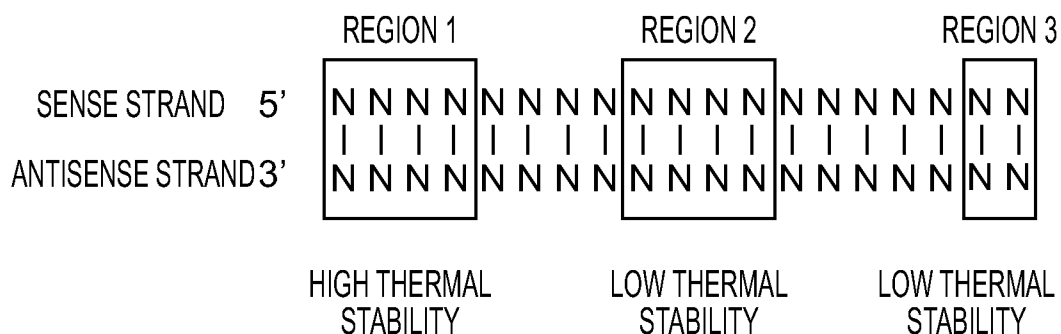
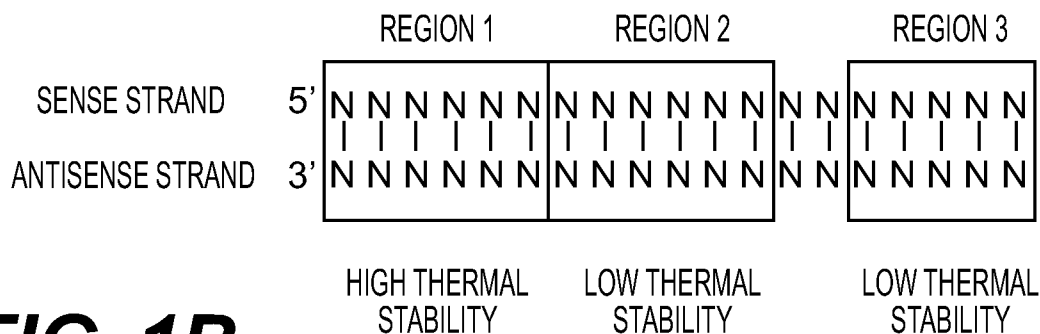
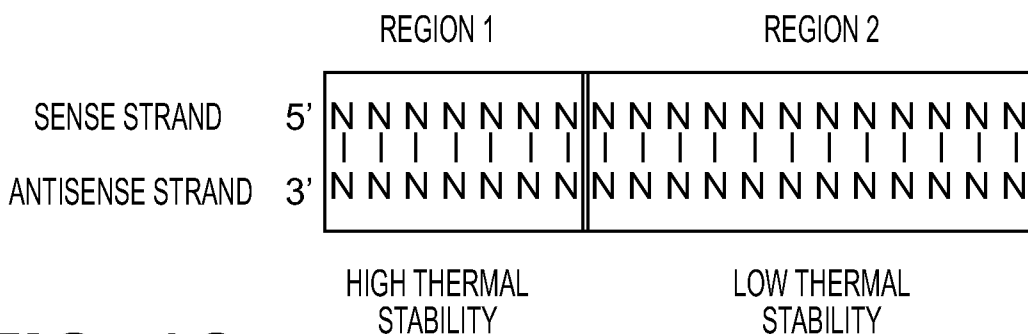
- a. a first strand that is complementary to a target gene;
- b. a second strand that is complementary to said first strand and comprises
 - i. at least one destabilized chemical modification placed at at least one of positions 9-12 and 18-19 from the 5'-end of the second strand; and
 - ii. at least one stabilized chemical modification placed at at least one of positions 1-4 from the 5'-end of the second strand;

wherein said increased RNAi silencing activity is relative to a corresponding unmodified agent, as determined by comparing their respective IC_{50} values in a RNAi silencing assay.

27. A double-stranded agent with increased RNAi silencing activity, comprising:
- a. an antisense strand that is complementary to a target gene;
 - b. a sense strand that is complementary to said antisense strand and comprises
 - i. at least one destabilized chemical modification placed at the low thermal stability at positions 9-12 and 18-19 from the 5'-end of the sense strand;
and
 - ii. at least one stabilized chemical modification placed at the high thermal stability at positions 1-4 from the 5'-end of the sense strand;

wherein said increased RNAi silencing activity is relative to a corresponding unmodified agent, as determined by comparing their respective IC_{50} values in a RNAi silencing assay.

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**FIG. 1A****FIG. 1B****FIG. 1C**

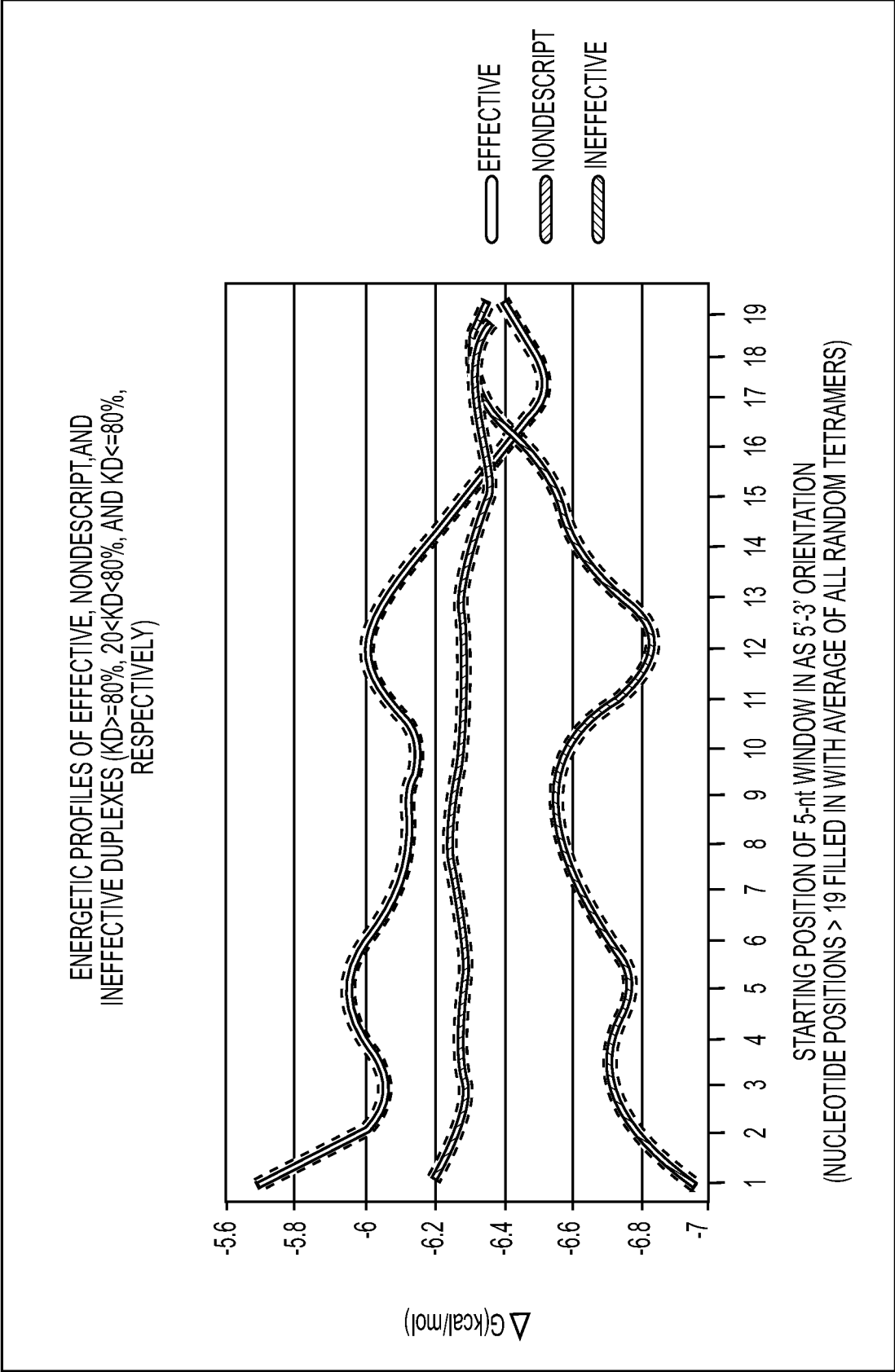


FIG. 2

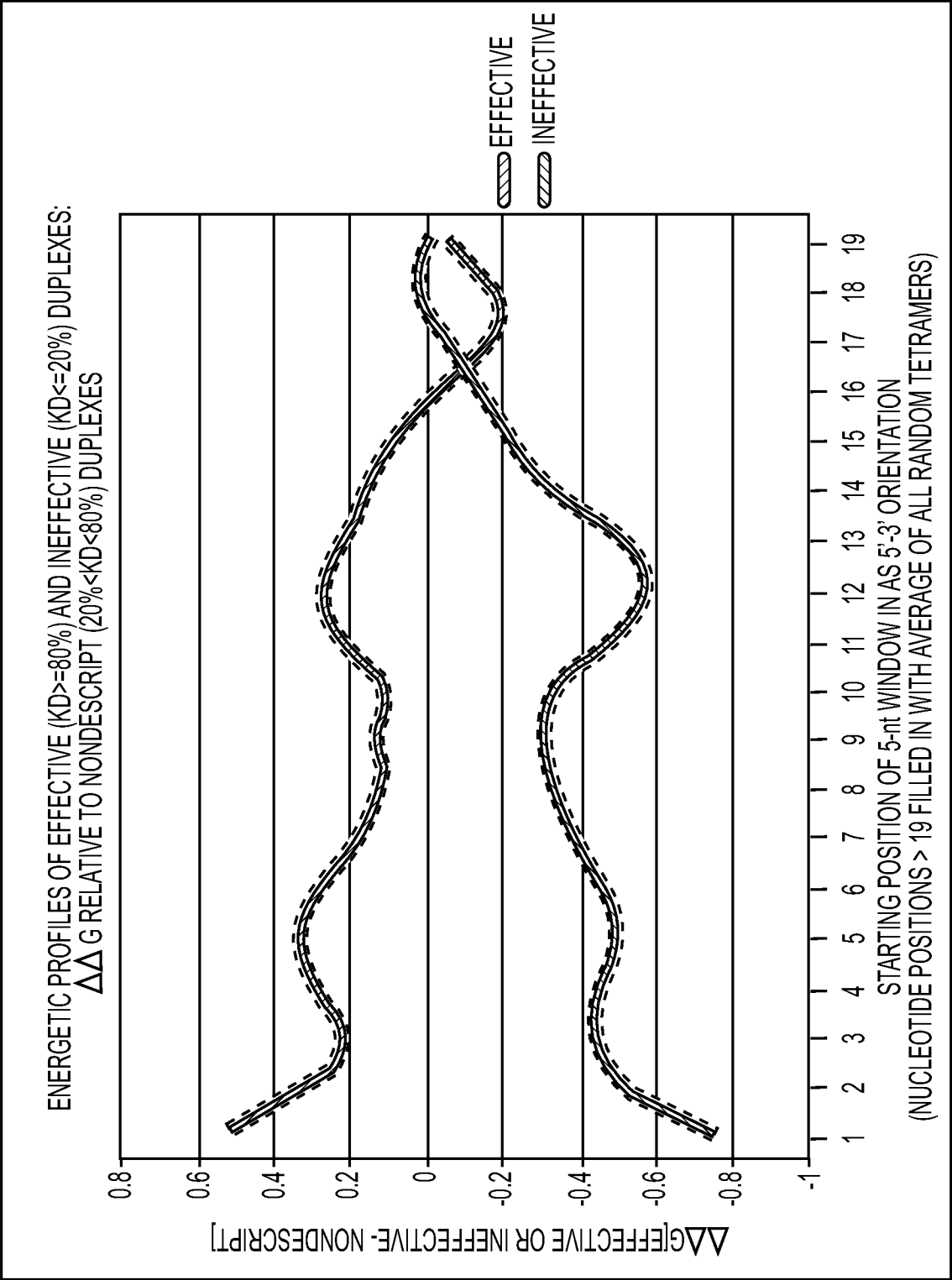


FIG. 3