gene suppression. The off-targets caused by siRNAs originate from base-complementarity of the seed regions of the RISC-loaded antisense strand of siRNA with one or more mRNA. The miRNA-like off-target effects in siRNAs have been reported in several studies, and affect expression of multitude of genes depending on sequences of the seed regions and are serious enough to cause up to 30% of the positive hits in siRNA based phenotype screening. Additionally, in the case of miRNAs, they are also reported to silence target genes through compensatory pairings within their 3' end regions (3'-compensatory pairing) when the interactions between seed region and targets become weak, implicating that the miRNA-like off-target effects are likely to be mediated by such mechanism.

[0005] There is thus an ongoing effort to eliminate or reduce miRNA-like off-target effects of siRNAs by modulating siRNA design by judicious application of chemical modifications without compromising the gene silencing efficacy of siRNA gene therapeutics. This invention is directed to that effort.

SUMMARY

[0006] This disclosure describes effective nucleotide or chemical motifs for dsRNA molecules, which are advantageous for inhibition of target gene expression, while having reduced off-target gene silencing effects, as well as RNAi compositions suitable for therapeutic use.

[0007] The inventors have discovered *inter alia* that dsRNA molecules where the antisense strand comprises at least one thermally destabilizing modification of the duplex within the seed region (*i.e.*, at position 2-9 of the 5'-end of the antisense strand, counting from the 5'-end) and the dsRNA molecule has a melting temperature in the range of from about 40°C to about 80°C can be more effective in mediating RNA interference than the parent dsRNA molecule lacking the destabilizing modification.

[0007a] In one aspect, the present invention refers to a double-stranded RNA (dsRNA) molecule capable of inhibiting the expression of a target gene, comprising a sense strand and an antisense strand, each strand having 14 to 40 nucleotides, wherein the antisense strand has sufficient complementarity to the target sequence to mediate RNA interference, wherein said antisense strand comprises at least one thermally destabilizing modification of the duplex within the first 9 nucleotide positions of the 5' region or a precursor thereof, wherein said sense strand

comprises an ASGPR ligand. In another aspect, the present invention refers to a double-stranded RNA molecule capable of inhibiting the expression of a target gene, comprising a sense strand and an antisense strand, each strand having 14 to 40 nucleotides, wherein the antisense strand has sufficient complementarity to the target sequence to mediate RNA interference, wherein the antisense strand comprises at least one thermally destabilizing modification of the duplex within the first 9 nucleotide positions of the 5′ region, and the dsRNA has a melting temperature of from about 40°C to about 80°C. In yet another aspect, the present invention refers to a pharmaceutical composition comprising the dsRNA agent of the present invention alone or in combination with a pharmaceutically acceptable carrier or excipient. In yet another aspect, the present invention refers to a gene silencing kit containing the dsRNA molecule of the present invention. In yet another aspect, the present invention refers to the dsRNA molecule of the present invention for use in therapy.

[0008] Also described herein is a dsRNA molecule capable of inhibiting the expression of a target gene, comprising a sense strand and an antisense strand, each strand having 14 to 40 nucleotides, wherein the antisense strand has sufficient complementarity to the target sequence to mediate RNA interference and wherein the antisense strand comprises at least one thermally destabilizing modification of the duplex within the seed region (*i.e.*, at position 2-9 of the 5'-end of the antisense strand, counting from the 5′-end), and the dsRNA further has at least one (e.g., one, two, three, four, five, six seven, eight or all nine) of the following characteristics: (i) a melting temperature (T_m) of from about 40°C to about 80°C; (ii) the antisense comprises 2, 3, 4, 5 or 6 2'-fluoro modifications; (iii) the antisense comprises 1, 2, 3 or 4 phosphorothioate internucleotide linkages; (iv) the sense strand is conjugated with a ligand; (v) the sense strand comprises 2, 3, 4 or 5 2'-fluoro modifications; (vi) the sense strand comprises 1, 2, 3 or 4 phosphorothioate internucleotide linkages; (vii) the dsRNA comprises at least four 2'-fluoro modifications; (viii) the dsRNA comprises a duplex region of 12-40 nucleotide pairs in length; and (ix) a blunt end at 5'end of the antisense strand.

[0009] In some examples, the disclosure provides a dsRNA molecule capable of inhibiting the expression of a target gene, comprising a sense strand and an antisense strand, each strand having 14 to 40 nucleotides, wherein the antisense strand has sufficient complementarity to the target sequence to mediate RNA interference and wherein the antisense strand comprises at least one thermally destabilizing modification of the duplex within the seed region (*i.e.*, at position 2-

9, preferably 3-8, of the 5'-end of the antisense strand, counting from the 5'-end), and the dsRNA further has at least one (e.g., one, two, three, four, five, six seven, eight or all nine) of the following characteristics: (i) a melting temperature (T_m) of from about 40°C to about 80°C; (ii) the antisense comprises 6, 7, 8, 9, 10, 11 or 12 2'-OMe modifications; (iii) the antisense comprises 1, 2, 3 or 4 phosphorothioate internucleotide linkages; (iv) the sense strand is conjugated with a ligand; (v) the sense strand comprises 6, 7, 8, 9, 10, 11 or 12 2'-OMe modifications; (vi) the sense strand comprises 1, 2, 3 or 4 phosphorothioate internucleotide linkages; (vii) the dsRNA comprises at least 1, 2, 3, 4 or 5 2'-deoxy modification(s); (viii) the dsRNA comprises a duplex region of 12-40 nucleotide pairs in length; and (ix) a blunt end at 5'end of the antisense strand.

[0010] In some examples, the dsRNA has a melting temperature with a lower end of the range from about 40°C, 45°C, 50°C, 55°C, 60°C or 65°C, and upper end of the range from about 70°C, 75°C or 80°C. In some examples, the dsRNA has a melting temperature in the range from about 55°C to about 70°C. In some examples, the dsRNA has a melting temperature in the range from about 57°C to about 67°C. In some particular examples, the dsRNA has a melting temperature in the range from about 60°C to about 67°C. In some additional examples, the dsRNA has a melting temperature in the range from about 62°C to about 66°C.

[0011] The inventors have also discovered that dsRNA molecules having a melting temperature of at least 60°C are more effective *in vivo* and *in vitro*. Thus, in some examples, the dsRNA has a melting temperature of at least 60°C.

[0012] The inventors also discovered that for the dsRNA molecules to be more effective *in vivo*, there must be at least 40-50% of the antisense strand present at day 7 *in vivo*, for example in the mouse liver, after administration.

[0013] In another example, the disclosure further provides a method for delivering the dsRNA molecule of the disclosure to a specific target in a subject by subcutaneous or intravenous administration. The disclosure further provides the dsRNA molecules of the disclosure for use in a method for delivering said agents to a specific target in a subject by subcutaneous or intravenous administration.

BRIEF DESCRIPTION OF THE DRAWINGS

[0014] This patent or application file contains at least one drawing executed in color. Copies of this patent or patent application publication with color drawing(s) will be provided by the Office upon request and payment of the necessary fee.

[0015] **Fig. 1** shows some exemplary destabilizing modifications of the invention.

[0016] **Fig. 2** shows positional effect of a single (*S*)-glycol nucleic acid (GNA) modification in the antisense strand on *in vitro* conjugate activity. Single substitution with the (*S*)-GNA is well tolerated at or opposite antisense seed region (positions 5-8 of antisense strand) but not tolerated in sensitive positions (positions 1 and 2 of antisense strand, and positions 11 and 12 of sense strand)

[0017] **Fig. 3** shows that an exemplary dsRNA according to the invention had equivalent on target activity relative to the parent dsRNA.

[0018] **Fig. 4** shows that an exemplary dsRNA of the invention had no off-target activity at high doses.

[0019] **Fig. 5** shows that exemplary dsRNAs of the invention have comparable gene (GO1 and TTR) knockdown in rat hepatocytes.

[0020] **Fig. 6** shows that exemplary dsRNAs of the invention mitigate endogenous off-target effects. As seen, both dsRNAs significantly reduced the number of genes that were down- or up-regulated by their respective parent dsRNAs.

We claim:

1. A double-stranded RNA (dsRNA) molecule capable of inhibiting the expression of a target gene, comprising a sense strand and an antisense strand, each strand having 14 to 40 nucleotides, wherein the antisense strand has sufficient complementarity to the target sequence to mediate RNA interference, wherein said antisense strand comprises at least one thermally destabilizing modification of the duplex within the first 9 nucleotide positions of the 5′ region or a precursor thereof, wherein said sense strand comprises an ASGPR ligand.

2. The dsRNA molecule according to claim 1, wherein the dsRNA comprises at least four 2'-fluoro.

3. The ds RNA molecule according to claim 2, wherein there are no 2'-fluoro modifications at nucleotide positions 3-9 of the antisense strand.

4. The dsRNA molecule according to claim 1, having the following characteristics:

a) the thermally destabilizing modification of the duplex is located in position 4-8 of the 5′ region of the antisense strand;

b) and each of the sense and antisense strands comprise at least two 2'-fluoro modifications; and

c) an ASGPR ligand attached to either end of the sense strand.

5. The dsRNA molecule according to claim 4, wherein there are no 2'-fluoro modifications at nucleotide positions 3-9 of the antisense strand.

6. The dsRNA molecule according to claim 1, wherein the antisense strand has at least two of the following characteristics:

a) the thermally destabilizing modification of the duplex modification is located in position 4 to 8 of the antisense strand;

b) at least two 2'-fluoro modifications;

c) phosphorothioate internucleotide linkages between nucleotide positions 1 and 2 (counting from the 5' end);

d) it has a length of 18 to 35 nucleotides.

7. The dsRNA molecule according to claim 6, wherein there are no 2'-fluoro modifications at nucleotide positions 3-9 of the antisense strand.

8. The dsRNA molecule according to claim 1, wherein the sense strand has at least one of the following characteristics:

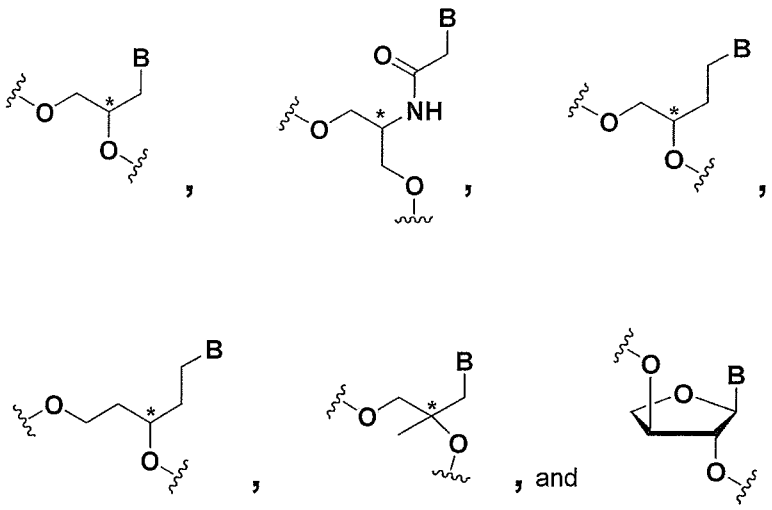
a) the ASGPR ligand attached to either end of the sense strand;

b) at least two 2'-fluoro modifications;

c) the sense strand and the antisense strand show sufficient complementarity to form a double stranded region spanning at least 19 nucleotide positions and wherein the thermally destabilizing modification of the duplex is located within said double-stranded region.

9. The dsRNA molecule according to claim 8, wherein there are no 2'-fluoro modifications at nucleotide positions 3-9 of the antisense strand.

10. The dsRNA molecule according to claim 1, wherein the thermally destabilizing modification of the duplex is selected from the group consisting of

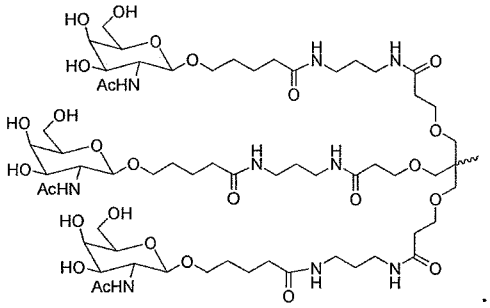


wherein B is nucleobase.

11. The dsRNA molecule according to 1, wherein the stabilized modification is located in position 7 of the antisense strand.

12. The dsRNA molecule according to claim 1, wherein the ASGPR ligand is one or more GalNAc derivatives attached through a bivalent or trivalent branched linker.

13. The dsRNA molecule of claim 8, wherein the ASGPR ligand is:



14. A double-stranded RNA molecule capable of inhibiting the expression of a target gene, comprising a sense strand and an antisense strand, each strand having 14 to 40 nucleotides, wherein the antisense strand has sufficient complementarity to the target sequence to mediate RNA interference, wherein the antisense strand comprises at least one thermally destabilizing

modification of the duplex within the first 9 nucleotide positions of the 5′ region, and the dsRNA has a melting temperature of from about 40°C to about 80°C.

15. The dsRNA molecule of claim 14, wherein the dsRNA has a melting temperature of from about 55°C to about 67°C.

16. The dsRNA molecule of claim 1, wherein at least 50% of the antisense strand is present in liver at day 7 after administration.

17. The dsRNA of claim 16, wherein the dsRNA further has at least one of the following characteristics: (i) the antisense comprises 2, 3, 4, 5 or 6 2'-fluoro modifications; (ii) the antisense comprises 1, 2, 3 or 4 phosphorothioate internucleotide linkages; (iii) the sense strand is conjugated with a ligand; (iv) the sense strand comprises 2, 3, 4 or 5 2'-fluoro modifications; (v) the sense strand comprises 1, 2, 3 or 4 phosphorothioate internucleotide linkages; (vi) the dsRNA comprises at least four 2'-fluoro modifications; (vii) the dsRNA comprises a duplex region of 12-40 nucleotide pairs in length; (viii) a blunt end at 5'end of the antisense strand; and (ix) the sense strand comprises one or more LNA modifications.

18. The dsRNA of claims 17, wherein there are no 2'-fluoro modifications at positions 3-9 of the antisense strand.

19. The dsRNA agent according to any one of the preceding claims wherein the sense strand has 21 nucleotides, and the antisense strand has 23 nucleotides.

20. A pharmaceutical composition comprising the dsRNA agent according to any one of the preceding claims alone or in combination with a pharmaceutically acceptable carrier or excipient.

21. A gene silencing kit containing the dsRNA molecule of any one of the preceding claims.

22. The dsRNA molecule of any one of claims 1 to 19 for use in therapy.